NK cells in children with acute lymphoblastic leukemia and non-Hodgkin lymphoma after cessation of intensive chemotherapy

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Abstract

Intensive, combination chemotherapy for malignant diseases causes a profound immunosuppression, which persists for the whole treatment period and after its completion. Impairment of the NK cells status may increase the risk of severe, disseminated infections and cancer. The aim of the study was the investigation of recovery of NK cells after cessation of intensive chemotherapy in children with acute lymphoblastic leukemia (ALL) and non-Hodgkin lymphoma (NHL). The number of CD3–CD16+CD56+ cells in peripheral blood and NK cell cytotoxic activity were assessed in 23 children with ALL and 7 children with NHL at 2 weeks and 12 months after the cessation of intensive chemotherapy and in 15 healthy subjects. Absolute leukocyte, lymphocyte and NK cell counts and the percentage of NK cells in children with ALL were significantly lower than in control subjects both at 2 weeks and 12 months after intensive treatment. Additionally, the absolute numbers of leukocytes and lymphocytes decreased significantly after 12 months of observations in comparison to the initial time-point. In children with non-Hodgkin lymphoma at 2 weeks and 12 months after intensive treatment only absolute lymphocyte counts were significantly lower than values in healthy children. The absolute number, the percentage and cytotoxic activity of NK cells were comparable with values in the control group both at the initial and at the last time-point. The occurrence of infections during the 12 months of observations in patients with ALL were higher than in children with NHL and as many as eight of them were hospitalized because of severe infections. The differences between the ALL and NHL patients may be connected with the milder immunosuppressive effect of chemotherapy in the non-Hodgkin lymphoma since the children recovered from acute lymphoblastic leukemia remain with persistent defect of NK cells. It is then recommended that ALL children should be supervised with respect to an increased susceptibility to infections.

Key words: *NK cells, acute lymphoblastic leukemia, non-Hodgkin lymphoma, chemotherapy, infections, children.*

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Introduction

Nowadays malignancies are civilizational diseases with still high mortality. Among them acute leukemias and non-Hodgkin lymphoma are the most common in the young population comprising 30% of all neoplastic diseases in childhood [1, 2]. Intensification of an aggressive, multidrug, cytostatic treatment in these diseases resulted in an increase of total remissions but it is also associated with numerous complications related to toxicity of treatment, including its negative influence on the immunological system [3, 4].

Natural killer (NK) cells provide an early non-specific defense against viral infections, particularly in the early phase before the adaptive immune system has been activated [5]. They are also described as cells providing a crucial initial defense against different pathological microorganisms [6] and in tumor cell detection and elimination [7, 8]. As a part of the

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innate immune system, NK cells are critical both in host defense and immune regulation [9]. Lack of NK cells and impairment of their cytotoxic function increases the risk of severe, disseminated infections and cancer [10-12].

Chemotherapy-related immunosuppression affects also the innate immunity and population of NK cells [13]. Depressed number and function of NK cells in the peripheral blood have been also reported after chemotherapies for acute leukemias and solid tumors with an early or delayed reconstitution after cessation of therapy [13-15]. An impairment of the NK cells status, persisting after completion of the intensive chemotherapy, may increase the risk of severe, life-threatening infections.

The aim of the study was the investigation of the influence of chemotherapy on the status of NK cells and its recovery in children with acute lymphoblastic leukemia (ALL) and non-Hodgkin lymphoma (NHL) after cessation of intensive anti-neoplastic treatment.

Materials and Methods

Patients

Twenty three patients with ALL and seven patients with NHL, aged 3 to 16 years (median age 8 years), were enrolled into the study. All patients underwent the treatment in the Department of Pediatrics, Hematology, Oncology and Endocrinology at the Medical University of Gdansk and were in remission at cessation of intensive chemotherapy. The ALL patients were treated according to risk group with a protocol ALL IC 2002. Briefly, induction of remission (identical for all patients) was given with prednisone, vincristine, daunorubicin, asparaginase, cyclophosphamide, cytarabine and 6-mercaptopurine. Consolidation of standard and intermediate risk patients included intermediate dose methotrexate (2000 mg/m^2) infusions and peroral 6-mercaptopurine and, after randomization, reinductions were given with dexamethasone, vincristine, doxorubicin, asparaginase, cyclophosphamide, cytarabine and 6-thioguanine. High risk patients had more intensive consolidation therapy consisted of high dose methotrexate (5000 mg/m^2) and high dose cytarabine (2000 mg/m^2) infusions, dexamethasone, cyclophosphamide, iphosphamide, daunorubicin, vindesine, ethoposide, and asparaginase. All patients received prophylactic intrathecal methotrexate, high risk patients additionally intrathecal prednisone and cytarabine. Regardless of risk group and differing intensity of the intensive phase of treatment, all patients were treated with identical maintenance therapy (MT) consisting of daily oral 6-mercaptopurine and weekly oral methotrexate for at least one year after intensive chemotherapy. Four patients received prophylactic cranial irradiation of 12 or 18 Gy in the first month of MT. All patients with acute lymphoblastic leukemia were receiving antimicrobial prophylaxis against *Pneumocystis jiroveci* – trimethoprim-sulphametoxazole (3 times a week) during MT.

Five patients with B-NHL were treated with B-NHL 93 protocol (prednisone, dexamethasone, doxorubicin, vincristine, etoposide, cyclophosphamide, methotrexate, cytarabine and intrathecal methotrexate, hydrocortisone and cytarabine) and two patients with T-NHL – with the NB-NHL 93 (prednisone, dexamethasone, daunorubicin, vincristine, asparaginase, cyclophosphamide, methotrexate, cytarabine, 6-mercaptopurine, 6-thioguanine and intrathecal methotrexate). None of NHL patients received maintenance therapy nor cranial irradiation.

Peripheral blood samples were collected from each patient two weeks and 12 months after the last dose of the consolidation therapy.

Control blood samples were obtained from 15 healthy children, aged 3 to 16 years (median age 10 years), who had been free of clinical signs of infection during the preceeding 4 weeks.

The local ethics committee at the Medical University of Gdansk approved the study. Informed consent was obtained from all study participants and/or their parents.

Analysis of leukocyte and lymphocyte counts in the peripheral blood

The blood counts, including leukocyte differential, were measured with the use of an automatic blood cell analyzer (LH 750 Analyzer; Coulter, USA).

Natural killer cell cytotoxicity assay

Evaluation of NK-cell activity was performed by the lactic acid dehydrogenase (LDH) release assay (Roche Diagnostics GmbH, Germany). Briefly, peripheral blood mononuclear cells (PBMC) were isolated from heparinised venous blood samples on Fycoll-Hypaque gradient (Amersham Pharmacia, Sweden). After double washing, PBMC were incubated in RPMI 1640 (BioMed, Poland) containing 5% fetal calf serum (Gibco BRL, USA) in Petrischales placed at 37°C humidified 5% CO_2 incubator for 30 minutes. After that, peripheral blood lymphocytes (PBL), as cells non-adherent to plates, were transferred to a tube and resuspended in RPMI 1640. Then 100 µl PBL at a concentration of 2×10^6 cells per 1 ml of culture medium RPMI 1640 and 100 µl of the tumour cell line K-562 (from the Institute of Immunology and Experimental Therapy PAN, Wroclaw, Poland) in concentration 2×10^5, as an effector-target cell mixture, were added to 6 wells of an entire 96-microwell tissue culture plate (Medlab, Poland) and incubated for 4 hours at 37°C humid atmosphere containing 5% CO_2. The control for spontaneous LDH release (low control) and maximum LDH release by the addition of 5% tyloxapol (TRITON X- 100; Sigma, Germany) (high control) were carried out in triplicates. After that, the plates were centrifuged for 10 minutes at 200 g. Then 100 µl of supernatants from each well were transferred into 96 flat-bottom microwell plates and 100 µl of LDH substrate mixture was added. The change of colour took place over

a period of 30 minutes in the dark at +25°C and after that a Microtitre plate reader (Labsystem Multiscan MCC/340) was used at 492 nm for the evaluation of changes in the absorbance. The mean percentage of specific cytotoxicity was calculated according to the following formula:

$$\% \text{ cytotoxicity} = \frac{Ex - Sp}{MR - Sp} \times 100$$

where: Ex – experimental LDH release (from coculture of PBL and K-562 tumour cell line), Sp – spontaneous LDH release (from tumour cells in culture medium alone), MR – maximum LDH release (from culture of tumour cells with TRITON X).

Surface marker staining of NK cells in the peripheral blood

The heparinized whole blood samples were aliquoted into plastic tubes, 50 µl per tube. Then, cells were stained with combinations of monoclonal antibodies (mAbs) (BD Pharmingen, USA): anti-CD3, anti-CD16 and anti-CD56 labeled with appropriate fluorochromes (20 µg each mAb per test). Cells were stained also with an appropriate isotype control antibodies. The samples were incubated at room temperature in the darkness for 30 min and thereafter red blood cells were lysed and probes were fixed using Immuno-prep reagents (Immunotech, USA) with Q-prep Immunology Workstation (Coulter, USA). Then cells were analyzed within 2 hours.

Flow cytometric analysis

Listmodes were acquired on the Epics XL flow cytometer (Coulter, USA) and analyzed using WinMDI 2.8 software. The region containing lymphocytes was generated for surface antigen analysis on FSC and SS parameters and the population of CD3–CD16+CD56+ NK cells was gated. The threshold level for the fluorescence of positive cells was set for each sample at a interception between the histogram curves obtained from fluorescence with isotype control mAb and the specific mAb. The results were expressed as the percentage of marker-positive cells in sample preparations and the absolute number of NK cells was calculated [% positive cells x absolute lymphocyte count determined as described above].

Clinical assessment

The clinical assessment included: the physical examination, occurrence of infections during the period of observations, such as upper respiratory tract infection, otitis media, urinary tract infection, oral mucositis and occurrence of a severe infection, which might be life-threatening, i.e. severe pneumonia, sepsis, meningitis. The infections were clinically and, in several cases, microbiologically documented (with clinical signs and symptoms of infection and optionally also with pathogen isolated from a blood sample or an infection site). Severe, life-threatening infections were defined as infections needed hospitalization.

Statistical analysis

Statistica 8.0 software (Statsoft, Poland) was used for statistical analysis. Due to small samples and skew distributions, all data were analyzed with nonparametric tests, including Mann-Whitney *U*-test for comparison between control group (CG) and groups of patients (ALL and NHL) and Wilcoxon's signed rank-test to compare data at time-points 2 weeks and 12 months after intensive chemotherapy. The significance level was determined as $p<0.05$.

Results

Patients

Characteristics of the patients is presented in Table 1.

Leukocytes, lymphocytes and NK cells after the intensive therapy

Absolute leukocyte, lymphocyte and NK cell (CD3–CD16+CD56+) counts and the percentage of NK cells at 2 weeks and 12 months after intensive treatment in the children with ALL were significantly lower than in control subjects (Table 2). At the initial time-point of observations the cytotoxic activity of NK cells in patients with ALL was comparable to values of healthy children and was even higher when checked at 12 months after cessation of intensive chemotherapy. Additionally, in the ALL patients the absolute numbers of leukocytes and lymphocytes decreased signi-

Table 1. Patient characteristics

	Control group (CG)	ALL	NHL	p ALL vs. CG	p NHL vs. CG
Total (n)	15	23	7	–	–
Male (n, %)	6 (40%)	13 (56.5%)	4 (57%)	0.23	0.37
Age[#] (years)	10 (6-13)	4 (3-8)	11 (7.5-12.5)	0.003	0.77
Duration of intensive chemotherapy[#] (months)	–	8.5 (7-11)	3.5 (3-7)	0.004**	

ALL – patients with acute lymphoblastic leukemia; NHL – patients with non-Hodgkin lymphoma; CG – control group; ** a comparison between ALL and NHL group; [#] data analyzed with Mann-Whitney *U*-test and presented as median and range between lower and upper quartile.

Table 2. Leukocytes, lymphocytes and NK cells in children with ALL and NHL after cessation of intensive therapy compared to values in healthy children

2 weeks after cessation of chemotherapy	Control group (n = 15)	ALL (n = 23)	p ALL vs. CG	NHL (n = 7)	p NHL vs. CG
Leukocytes (cells/µl)	6500 (5650-6820)	3710 (2400-4750)	**0.00004**	4130 (2500-6710)	0.062
Lymphocytes (cells/µl)	3061 (2080-3590)	1501 (870-2470)	**0.0002**	939 (785-1340)	**0.001**
CD3–CD16+CD56+ cells (%)*	5 (3-10)	2.7 (0.9-3.7)	**0.020**	5.95 (3.6-9)	0.93
CD3–CD16+CD56+ cells (cells/µl)	193 (62-323)	26 (6-57)	**0.0005**	80 (14-95)	0.065
NK cells cytotoxic activity (%)	14.5 (10.0-21.0)	16.86 (8.5-24.22)	0.35	20.52 (16.25-22.96)	0.084
12 months after cessation of chemotherapy					
Leukocytes (cells/µl)	6500 (5650-6820)	3340 (2620-3650)	**0.00001**	4590 (3010-6185)	0.35
Lymphocytes (cells/µl)	3061 (2080-3590)	967 (663-1160)	**0.00001**	1984 (1331-2457)	**0.018**
CD3–CD16+CD56+ cells (%)*	5 (3-10)	0.5 (0.2-3.9)	**0.029**	7.6 (6.5-9)	0.39
CD3–CD16+CD56+ cells (cells/µl)	193 (62-323)	7 (2-32)	**0.003**	96 (50-256)	0.73
NK cells cytotoxic activity (%)	14.5 (10.0-21.0)	28.41 (15.89-37.51)	**0.042**	13.72 (8.76-18.67)	0.94

*The percentage of CD3–CD16+CD56+ cells among lymphocytes; ALL – acute lymphoblastic leukemia; NHL – non-Hodgkin lymphoma; p (ALL vs. CG) – differences between ALL and control group; p (NHL vs. CG) – differences between NHL and control group; data analyzed with Mann-Whitney *U*-test and presented as median and range between lower and upper quartile.

ficantly after 12 months of observations (respectively p = 0.008 and p = 0.006).

In the group with non-Hodgkin lymphoma only absolute lymphocyte counts were significantly lower than values in healthy children – both at 2 weeks and 12 months after intensive phase of treatment (Table 2), and the median of lymphocyte count increased, but insignificantly, after 12 months of observations (p = 0.067). However, the absolute number of NK cells increased significantly at 12 months after cessation of chemotherapy in NHL patients (p = 0.043), but the percentage and cytotoxic activity of these cells were comparable at the initial and the last time-point (respectively p = 0.34 and p = 0.28).

Infections in children after the intensive chemotherapy

During the first year after intensive therapy completion the total occurrence of infections in ALL patients was almost twice higher than in children with non-Hodgkin lymphoma (Fig. 1). Eight patients in this group developed severe infections, such a sepsis, severe pneumonia or both, and were hospitalized due to them. On the contrary – children with NHL had a small rate of infectious diseases and only one patient was hospitalized due to pneumonia and sepsis during the first three months after chemotherapy cessation.

Moreover, there was the negative correlation between the NK cells count (CD3–CD16+CD56+) at the initial time-point of observations and the number of hospitalizations due to severe infections during the twelve months after cessation of intensive chemotherapy in children with ALL (Fig. 2).

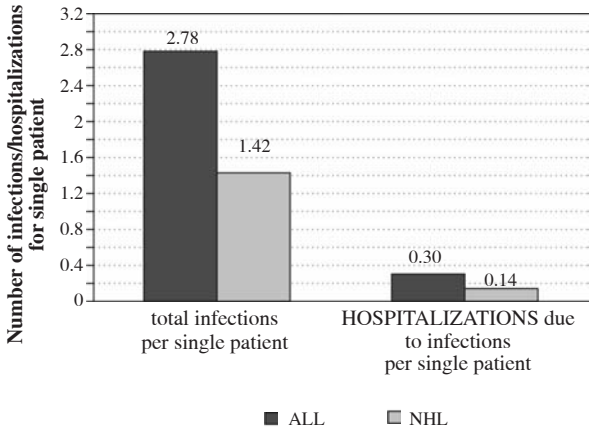

Fig. 1. The occurrence of total infections and hospitalizations due to severe infections (per single patient) in children with ALL and NHL during the twelve months after cessation of intensive chemotherapy

Discussion

Immunosuppression is a major side effect of chemotherapy. Previous studies have shown that young patients are predisposed to persistent immunological disturbances after completion of antineoplastic treatment [3, 4, 13-16]. Some other studies revealed that treatment intensity might be an important factor for the degree and duration of immunosuppression in children recovered from ALL [17]. Chemotherapy

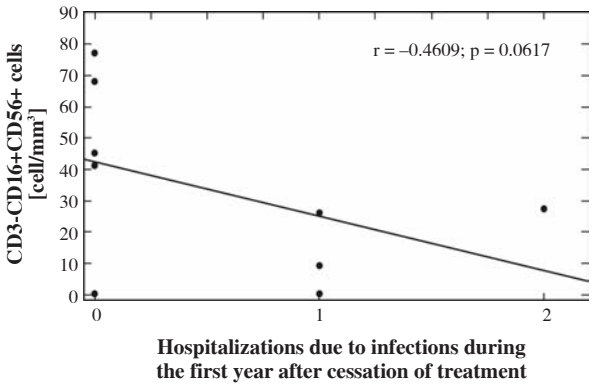

Fig. 2. Correlation between the NK cells count (CD3-CD16+ CD56+) at the initial time-point of observations and the number of hospitalizations due to severe infections in children with ALL during the twelve months after cessation of intensive chemotherapy

in childhood ALL differs from treatment of NHL especially with regard to total of cumulative doses of chemotherapeutic agents and duration of the treatment [18, 19]. Our study demonstrated that the status of immunosuppression, presented as decreased number and percentage of NK cells, even in the presence of normal cytotoxic activity of these cells, persisted during a year after cessation of intensive chemotherapy in children with ALL while NHL patients had no quantitative and qualitative disturbances of these cells both at 2 weeks and 12 months after treatment. Different findings were published by Mazur et al. [3] and Komada et al. [20], who observed an increase of NK cell counts to values statistically higher than normal after cessation of treatment. Such differences in their findings might result from the different end time-point of observations at the cessation of maintenance therapy. Interestingly, in our study, children with non-Hodgkin lymphoma, similar to ALL patients, had decreased absolute lymphocyte counts still after a year of observations in comparison to healthy subjects. Persistent lymphopenia in ALL patients was previously reported by Rytwinski et al. [21], while different results were obtained by Mazur et al. [3] and Nash et al. [22], where lymphocyte and leukocyte counts normalized within 12 months after cessation of chemotherapy. There were only a few studies showing such an early immunological reconstitution, concerning the count and activity of NK cells, in NHL patients after chemotherapy [13, 23], as we observed. Our findings of an early immune system recovery in NHL children may be connected with the improvement of therapy during the last years and its higher effectiveness in relation to lower toxicity [19].

Patients after chemotherapy had an increased susceptibility toward infections due to delayed reconstitution of the immune system [3, 24-26] and these findings are comparable to our results. In our study patients with acute lymphoblastic leukemia, in relation to children with NHL, had more infectious diseases and were more frequent hospitalized during a year after cessation of intensive treatment. It might be connected with lower values of NK cells, which correlate with a high predisposition to severe infections, as described also by Ogata et al. [27].

We conclude that children recovered from acute lymphoblastic leukemia have persistent quantitative defect of NK cells and therefore they should remain under special medical care.

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