

Late Immune Recovery in Children Treated for Malignant Diseases

Gabor T. Kovacs · Olga Barany · Barbara Schlick ·
Monika Csoka · Judit Gado · Andrea Ponyi ·
Judit Müller · Julia Nemeth · Peter Hauser ·
Daniel J. Erdelyi

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Abstract In this study we analyzed the recovery of the immune system in children after completion of the therapy. We analysed 88 children (51 boys, 37 girls, mean age at diagnosis: 7.8 years) receiving chemotherapy for malignant diseases (43 acute lymphoblastic leukemia, 15 lymphoma, 20 bone tumor, ten other solid tumors). Serum immunoglobulin levels (Ig), natural killer activity (NK), antibody-dependent cellular cytotoxicity (ADCC) and T and B cell proliferation were determined 1 year after cessation of therapy. The mean levels of Ig were in the normal range at a mean of 13 months after chemotherapy (IgG: 11.2 ± 3.3 , IgA: 1.6 ± 0.9 , IgM: 1.0 ± 0.5 g/l), however in the leukemic patients serum IgG was below the lower limit of the normal range in 3/43 (7.0%) cases, serum IgA was low in 5/43 (11.6%) and serum IgM was decreased in 4/43 (9.3%) cases. In the solid tumor patients IgG values were within the normal range and only 2–2/45 children had lower values for IgA and IgM (4.4%). NK activity decreased in 7/43 (16.3%) leukemic patients, and in 3/45 (6.7%) solid tumor patients, ADCC decreased in 8/43 (18.6%) and 3/45 (6.7%), respectively ($p < 0.001$). B-cell blastic transformation was decreased in 3/43 (7%) leukemic patients and in 4/45 (8.9%) solid tumor patients. At the same time T-cell blastic transformation was altered in 5/43 (11.6%) and in 4/45 (8.9%) cases, respectively. Leukemic patients had significantly more infections during the first year after chemotherapy than solid tumor patients (1.60 ± 1.18 vs 0.96 ± 1.14 ; $p = 0.011$). No significant correlations could be found between the investigated immune parameters and the number

and severity of infections. It is concluded, that cytotoxic therapy can lead to long-term depression of the immune system, first of all in leukemic patients.

Keywords Humoral immunity · Cellular immunity · Late effects · Malignant diseases · Children

Abbreviations

ADCC	antibody-dependent cellular cytotoxicity
ALL	acute lymphoblastic leukemia
AL	acute leukemia
AML	acute myeloblastic leukemia
BFM	Berlin-Frankfurt-Münster Study Group
BMT	bone marrow transplantation
CI	cytotoxicity index
Con-A	concanavalin A
DNA	desoxy-ribonucleic acid
HD	Hodgkin's disease
Ig	immunoglobulin
IL	interleukin
NHL	non-Hodgkin lymphoma
NK	natural killer
OSC	osteosarcoma
PHA	phytohemagglutinin
PWM	pokeweed mitogen
Th	T-helper cell

Introduction

The incidence of malignant diseases is 135 per 1,000,000 children at the age of 1 to 14 years. Leukemia and cancer of the central nervous system appear most frequently (29% and 27% of the cases, respectively) [1]. In the last decades

G. T. Kovacs (✉) · O. Barany · B. Schlick · M. Csoka · J. Gado ·
A. Ponyi · J. Müller · J. Nemeth · P. Hauser · D. J. Erdelyi
Second Department of Pediatrics, Semmelweis University,
Budapest, Hungary
e-mail: kovi@gyer2.sote.hu

the chance of long-term survival improved dramatically first of all due to the intensive chemotherapeutic protocols. Generally, at least 70% of the cases can be cured [2–5]. Long-term results, quality of life, prevention of late side effects of the therapy are getting more and more important for the physicians. Radiotherapy might cause the most serious side effects in children, so most of the protocols avoid or minimize its use for pediatric patients. The intensive chemotherapy also may have several side effects, and can affect the endocrine system, growth rate, the gonads, the kidney, the liver, the heart, the lung, the gastrointestinal tract etc. Neurological, late psychological and social problems and the increased risk of secondary malignancies can danger this patient population, too [6–10].

The immunosuppressive properties of cytotoxic drugs were first recognized in 1921. Mustard gas was shown to impair the ability of rabbits to form antibodies against sheep red blood cells [11, 12]. Almost all cytotoxic drugs can affect the immune system and might lead to bone marrow suppression [13]. Corticosteroids—used for the therapy of lymphoid malignancies—produce significant decrease in the number of lymphocytes, monocytes, eosinophiles and basophiles, due to the change of distribution of leukocytes in the human body. Steroids also block the chemotaxis of the phagocytes [13].

The recovery of the immune system after chemotherapy takes at least 1 year, according to the data in the medical literature [14–28]. Most of the immune functions normalize 12 months after the completion of the therapy; however, some parameters can remain pathologic in some cases [16, 20, 21, 24, 26, 28]. After bone marrow transplantation the regeneration of the immune system can last for 2–4 years [29–34]. The data are rather confusing, in most of the studies the number of the patients reported is only limited [10–30].

The aim of our study was to demonstrate the immune status of a relatively large population of children 1 year after intensive chemotherapy.

Patients and Methods

Eighty eight patients were studied, who had been treated for malignancy between May 1990 and April 2004 at the Second Department of Paediatrics, Semmelweis University, Budapest, Hungary. Fifty-one boys and 37 girls were enrolled. Their mean age was 7.8 years at the time of diagnosis (1 month to 17.7 years), and 9.3 years (0.8–20.0 years) after the cessation of therapy. The primary diagnosis of the investigated children were: 43 acute lymphoblastic leukemia (ALL), ten Hodgkin's disease (HD), five non-Hodgkin lymphoma (NHL), 16 osteosarcoma (OSC), four Ewing-sarcoma, four neuroblastoma and six other solid tumors. Intensive chemotherapy, surgery, radiotherapy and their combination were used in the treatment according to the international protocols used in Hungary. The main protocols are listed in Table 1. At the time of the immunological evaluation all children completed the chemotherapy and were in complete remission.

Quantisation of serum immunoglobulins IgG, IgM, IgA was performed by nephelometry. Age-matched reference values were taken as normal for the patients below 12 years of age. For patients >12 years the following lower cut-off values were used: 6.0 g/l for IgG, 0.6 g/l for IgA and 0.4 g/l for IgM.

Cellular immunity was investigated by the mitogen-induced blastogenesis. In this method mononuclear cells (lymphocytes) are exposed to plant's lectins and therefore they undergo activation and differentiation. Activated lymphocytes can be stained by isotope method and scintillation can be measured. Phytohemagglutinin (PHA) is present in kidney beans, concavalin-A (Con-A) is derived from Jack beans, pokeweed mitogen (PWM) is taken from the plant *Phytolacca Americana*. PHA and soluble Con-A are mitogenic for T-cells and surface-attached Con-A is mitogenic for B-cells. PWM has a stimulating effect on B-lymphocytes in the presence of T-cells.

Table 1 The chemotherapeutic protocols used in Hungary between 1990 and 2004

Diagnosis	Protocols	Cytostatic drugs
ALL	ALL-BFM-90, ALL-BFM-95, ALLIC-BFM-2002	Vincristine/vinblastine, prednisolone, dexamethasone, adriablastine/daunorubicine, cyclophosphamide/ifosfamid, asparaginase, etoposide, cytosin-arabinoside, 6-mercaptopurine, 6-thioguanine, methotrexate
HD	DAL-HD-90, GPOH-95	Prednisolone, procarbazine, etoposide, cyclophosphamide, vincristine, adriablastine
NHL	NHL-BFM-90, NHL-BFM-95	Vincristine/vinblastine, prednisolone, dexamethasone, adriablastine/daunorubicine, cyclophosphamide/ifosfamid, asparaginase, etoposide, cytosin-arabinoside, 6-mercaptopurine, 6-thioguanine, methotrexate
OSC	COSS-86, COSS-96	Methotrexate, adriablastine, ifosfamid, cisplatin, etoposide, carboplatin
Ewing-sarcoma	EICESS, CWS-96, Euro-Ewing-99	Vincristine, ifosfamid, adriablastine, actinomycine, etoposide
Neuroblastoma	OPEC/OJEC, HR-NBL	Vincristine, cisplatin, cyclophosphamide, adriablastine, etoposide

First, lymphocyte suspension was separated by Hank's solution and Ficoll-iodamide. The lymphocyte (effector) cell concentration was $1 \times 10^6/\text{ml}$.

Then mitogens were added to purified lymphocytes derived from peripheral blood (Con-A given in the dose of 25, 10 and 2.5 $\mu\text{g}/\text{ml}$, PHA in the dose of 5, 2 and 0.2 $\mu\text{g}/\text{ml}$ and PWM in a dilution of $\times 1200$, $\times 2400$ and $\times 12000$). The samples were incubated at 37°C in an atmosphere of 5% $\text{CO}_2/95\% \text{O}_2$ for 3 (ConA, PHA) or 5 days (PWM). In the last 6 h 1 $\mu\text{Ci}/20 \mu\text{l}$ of H3 thymidine was added. Then excessive DNA was removed, and H3 thymidine incorporated into newly synthesized DNA was measured by scintillation counter (Beckmann LS 6000SE) [35].

Normal values

PHA 5 $\mu\text{g}/\text{ml}$:	85.7–170.3
PHA 2 $\mu\text{g}/\text{ml}$:	64.8–143.0
PHA 0.2 $\mu\text{g}/\text{ml}$:	1.8–14.8
ConA 25 $\mu\text{g}/\text{ml}$:	20.6–51.6
ConA 10 $\mu\text{g}/\text{ml}$:	9.9–24.3
ConA 2.5 $\mu\text{g}/\text{ml}$:	3.2–10.8
PWM 1:1200:	1.1–22.2
PWM 1:2400:	6.4–14.4
PWM 1:12000:	0.8–6.1

The activity of NK and ADCC cells were detected by 51-Cr-Release Assay.

For NK testing erythroleukemia K 562 cell-line was used as target cells. The NK cells kill the tumor cells marked with an isotope. 5×10^6 K562 cells and 200 μCi $\text{Na}^{51}\text{CrO}_4$ isotopes were incubated for 30 min at 37°C . Then effector cells and target cells were incubated for 4 h at 37°C in an atmosphere of 5% $\text{CO}_2/95\% \text{O}_2$. The NK effector-target dilution titers were 50:1, 25:1, 12.5:5 and 6.25:1. Thereafter 100 μl samples were taken the amount of the isotopes relieved from the tumor cells was measured by gamma counter (Beckmann Gamma 5500).

Spontaneous cytotoxicity was expected to be <15 [36, 37].

Normal values

(CI %)

Effector/target cell

50/1	23.9–52.9
25/1	13.6–41.6
12.5/1	6.4–28.6
6.25/1	3.0–16.4

For the ADCC test washed, “O” Rh positive human red cells were incubated with 300 μCi $\text{Na}^{51}\text{CrO}_4$ isotope for

90 min at 37°C . In the last 30 min anti-D human IgG was added. The final cell concentration was $5 \times 10^5/\text{ml}$. Then effector cells and target cells were incubated for 4 h at 37°C in an atmosphere of 5% $\text{CO}_2/95\% \text{O}_2$. The ADCC effector-target titers were 10:1, 5:1, 2.5:1 and 1.25:1.

Finally, 100 μl samples were taken and the amount of the isotopes relieved from the tumor cells was measured by scintillography (Beckmann Gamma 5500).

Spontaneous cytotoxicity was expected to be $<5\%$ [36, 37].

Normal values

(CI %)

Effector/target

10/1	49.9–68.5
5/1	40.7–61.3
2.5/1	26.2–49.6
1.25/1	13.8–35.8

Fisher's exact test was used for comparison of the immunologic alterations present in leukemia and solid tumor patients. Student *t*-test was used to analyse the differences in the number of infections occurring in the investigated study groups.

Results

In the in vitro tests totally 23/43 patients of the leukemia group (53.5%) and 13/45 children in the group of solid tumor patients (28.9%) had any kind of abnormal immunological parameter while in the other 52 cases normal values were measured. This means that the leukemia patients suffer from more alterations of the immune system 1 year after completion of the therapy ($p < 0.001$).

The white blood cell counts were within the normal range or slightly lower 1 year after chemotherapy (Table 2). No severe granulocytopenia ($<1.0 \text{ G/l}$) or severe lymphopenia ($<1.5 \text{ G/l}$) could be detected. No significant difference could be seen between leukemia and solid tumor patients.

Table 2 Serum immunoglobulin levels and leukocyte counts 1 year after chemotherapy

	All patients	Leukemia	Solid tumor
IgG (g/l)	11.2 ± 3.3	10.4 ± 3.1	11.8 ± 3.5
IgA (g/l)	1.6 ± 0.9	1.4 ± 0.7	1.7 ± 1.0
IgM (g/l)	1.0 ± 0.5	0.9 ± 0.4	1.0 ± 0.6
Leukocyte (G/l)	5.8 ± 1.9	6.0 ± 2.4	5.5 ± 1.4
Granulocyte (G/l)	3.2 ± 1.6	3.4 ± 2.2	3.0 ± 1.0
Lymphocyte (G/l)	2.3 ± 1.0	2.1 ± 1.0	2.5 ± 0.9

The mean level of the serum immunoglobulins was within the normal range at a mean of 13 ± 3.3 months (Table 2). However, in 12/88 cases (13.6%) low Ig values could be detected (Table 3). Among leukemic patients only 35 children had all of their Ig values in the normal range, and eight patients (18.6%) had at least one type of Ig value in the abnormal range. IgG was below the lower limit of the normal range in 3/43 cases (7.0%), serum IgA was low in 5/43 cases (11.6%) and serum IgM was decreased in 4/43 cases (9.3%; some patients had more than one pathologic Ig value). Among the solid tumor patients only 4/45 children (8.9%) had any kind of abnormal Ig value. All IgG values were within the normal range and only 2–2/45 children had lower values for IgA and IgM (4.4–4.4%, respectively). The difference between leukemia and solid tumor patients was significant ($p < 0.001$).

Parameters of the cellular immunity were measured at a mean of 15.0 ± 4.4 months after the end of chemotherapy. Altogether 31 children (35.2%) had any parameter below the normal range in the in vitro tests (Table 4). Among the children with leukemia in 18/43 cases (41.9%) some pathologic parameters could be detected regarding the cellular immunity. In 23.3% of the cases ($n=10$) only one of the investigated parameters showed abnormal value, in 16.3% of the cases ($n=7$) two parameters were out of the reference range and in one case (2.3%) three tests results were abnormal.

On the other hand among the solid tumor patients in 13/45 cases (28.9%) could be any kind of abnormal values detected in the in vitro cellular immunity tests. Only one parameter was pathologic in 11 patients (24.4%), and two tests showed abnormal values in two more patients (4.4%).

The difference between leukemic and solid tumor patients was significant ($p < 0.001$), which means that the leukemia treatment leads to a more pronounced immune suppression.

The decrease in natural immunity (NK and ADCC) present after chemotherapy was slightly more expressed in

Table 4 Incidence of pathologic in vitro tests at about 1 year after chemotherapy

	Leukemia ($n=43$)	Solid tumor ($n=45$)
ADCC	6↓ 2↓↓	1↓ 2↓↓
NK	3↓ 2↓↓ 2↓↓↓	2↓↓ 1↓↓↓
ConA	2↓ 3↓↓	2↓
T-lymphocyte		
PHA	3↓	3↓
T-lymphocyte		
PWM	2↓ 1↓↓↓	2↓ 2↓↓
B-lymphocyte		
Patients with at least one abnormal value	18 (42%)	13 (29%)

↓ Slight decrease (0–20%) ↓↓ strong decrease (20–50%) ↓↓↓ very strong decrease (>50%)

leukemic patients. NK activity was decreased in 7/43 children with leukemia (16.3%) and in 3/45 patients with solid tumor (6.7%; $p=0.002$). ADCC was decreased in 8/43 (18.6%) and 3/45 (6.7%) cases, respectively ($p=0.001$).

The mitogen-induced activation of the mononuclear cells was equally suppressed in both groups. B-cell blastic transformation was decreased in 3/43 leukemic patients (7%) and in 4/45 solid tumor patients (8.9%). At the same time T-cell blastic transformation was altered in 5/43 (11.6%) and in 4/45 (8.9%) cases, respectively. The difference between the investigated groups was not significant.

We also analyzed the number of febrile episodes and the need for antibiotic treatment.

Leukemic patients suffered from more infections than those with solid tumors in the first year after chemotherapy. We registered 1.60 ± 1.18 infectious episodes in patients with leukemia and 0.96 ± 1.14 febrile episodes in children with solid tumors ($p=0.011$). No significant correlation could be found between the number of infectious complications and the measured immunological parameters (Fig. 1). In the group with normal in vitro immune test results less patients had two or more infectious complications (15/52, 29%) than those in the group with abnormal immunological parameters (15/36, 42%), but this difference was not statistically significant. Altogether, 36 patients had some alteration in the measured immunological parameters, and among these patients in 56 cases were infectious complications detected (1.5 ± 1.3 /patient). Otherwise, 52 patients had all of their immunological parameters in the normal range and in this patient population 57 infections could be seen (1.1 ± 1.1 /patient). However this difference was not statistically significant ($p=0.12$).

Among the patients with solid tumors, in the subgroup of children with any abnormal immunological parameters the

Table 3 Number of patients in the investigated groups with decreased serum Ig levels (related to the age-matched reference values)

Pathological values	Leukemia (%) $n=43$	Solid tumor (%) $n=45$
IgG	3 (7.0)	0 (0)
IgA	5 (11.6)	2 (4.4)
IgM	4 (9.3)	2 (4.4)
Two Ig levels	3	0
Three Ig levels	1	0
Total number of patients with altered Ig levels	8 (18.6)*	4 (8.9)

* $p < 0.001$

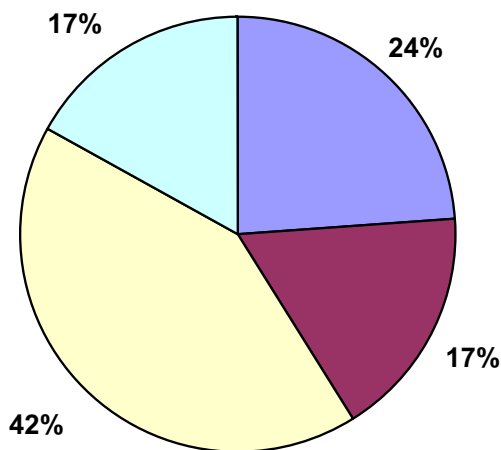


Fig. 1 Relationship between laboratory parameters and infections. ■ Any pathological in vitro immune test +0–1 infection in the first year (21/88 = 24%); ■ any pathological in vitro immune test +2 or more infections in the first year (15/88 = 17%); ■ normal in vitro immune test +0–1 infection in the first year (37/88 = 42%); ■ normal in vitro immune test +2 or more infections in the first year (15/88 = 17%)

number of infections per patient was 1.4 ± 1.5 while in the subgroup of children with normal immune status it was 0.7 ± 0.8 . However this difference did not reach statistical significance neither ($p=0.14$).

Discussion

Increasing number of children with malignancies can be cured with intensive chemotherapeutic protocols (currently about 70%) [1–5]. However the high-dose chemotherapeutic regimens may have several potential side effects. The long-term complications may influence the quality of life of the survivors.

Late side effects can involve almost all organs of the human body (heart, kidney, liver, lung, nervous system, endocrine organs etc.). Damage of the bone marrow and the immune system can be caused by most of the cytostatic drugs. Immune recovery is quite slow after finishing the treatment. There are only limited and confusing data in the medical literature regarding the reconstitution of the immune mechanisms after malignancy treatments [14–23]. For this reason in this present study we aimed to collect some data about the immunological parameters of a larger patient population in Hungary.

Our results showed that many children with cancer (36/88=41%) still have alterations in the different immune functions 1 year after the cessation of the therapy.

Similar to the data in the literature, white blood cell counts were within the normal range 1 year after chemotherapy. Alanko et al have noted, that patients with solid tumors had normal granulocyte and monocyte count at the cessation and their lymphocyte count also recovered within 12 months after therapy. Patients treated with irradiation

experienced a prolonged period of leucopenia proving the long-lasting immunosuppressive effect of radiotherapy [14]. Craenendonk et al when following 131 children with different solid tumors found that white blood cell and lymphocyte counts normalized between 1 and 12 months after the therapy, depending on the intensity of the treatment [17].

In our study humoral immunity (immunoglobulins) was less affected: the mean Ig values were within the normal range after 1 year of therapy. However, a significant number of patients (8/43 leukemic and 4/45 solid tumor patients) had lower Ig values.

Alanko et al. found slow immunologic recovery in childhood ALL: the normalization of the Ig values required 6 months while that of the T-cells needed more time [14, 25].

However it should be mentioned that the total serum Ig levels are not specific enough to describe humoral immunity. Ig subclasses and specific antibody responses for different Ag stimuli are also important. Some studies showed that the specific immune response can be diminished even years after chemotherapy [20, 21, 38].

Smith et al. investigated leukemic children who had completed the BFM protocol. They suggested a long-term follow-up and reimmunisation of the cured children, because in spite of the normal immunoglobulin levels these patients may have at least one type of antibodies specific to common childhood diseases at a non-protective level [20]. Scandinavian authors reported that a decline in the number of antibodies for measles and mumps had been observed in 6% of the treated children, for polio types in 18%, 12% and 25% of the patients and for diphtheria in 21% of the study group [21]. Others found similar results [15].

In contrast to the investigations of Smith et al [20] but similarly to other study groups [16, 22, 24] cellular immunity was depressed in our patients as well.

Lymphocytes responded poorly to mitogens in one-third of the treated children.

Studies of Mustafa et al. [15] reported that CD8+ and NK cells had recovered soon after chemotherapy, however 24% of patients with ALL, 20% of patients with solid tumor, and 55% of patients with HD still had low CD4 counts 1 year after the therapy. Patients responded poorly to mitogens during the first year.

A Japanese research group found reduced CD4+ and NK counts with elevated number of monocyte and CD8+ cells [16].

Mackall et al. [18] were looking for a correlation between the age of the patients and the Th cell number: they found a negative correlation. The ability of reconstitution of CD4+ cells also correlated with the appearance of CD4+ cells bearing CD45RA surface marker. An enlargement of the thymus could also be recognized in patients possessing a large quantity of CD45RA isoform. These results show that the thymus plays a central role in the regeneration of Th cells after chemotherapy [18, 26].

Others [15] also found more immunological alterations among the younger patient population than in older children.

In contrast to this Austrian colleagues did not find any significant correlation between the age of the patients and the Th count [19].

In this cohort study we were not able to follow the patients with regular flow cytometry however our data shows that both B and T cell functions are impaired in a significant number of patients.

In our investigated population a relatively low number of infections could be detected. Leukemic patients had more infectious episodes than the solid tumor patients. Others [14, 15] also found half as many infections in patients with solid tumors than those with leukemia.

We did not found any significant correlation between the frequency and severity of infections in the first year after chemotherapy and the investigated immunological parameters. However, a slightly increased number of infections could be detected in solid tumor patients with altered immunological parameters. This means that sensitive in vitro tests might help to find the most vulnerable patient population.

In summary, in about half of the leukemic patients and in one-third of the solid tumor patients immunological alterations could be detected about 1 year after the completion of the anti-tumor therapy. This means that the treatment of pediatric malignancies can lead to long-term immunosuppression, so careful monitoring of this patient population is mandatory. Further studies are needed to detect the most vulnerable cases regarding late infectious complications.

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