Disease–Free Remission Exceeding 37 Years in Patients Treated as Children for Acute Leukemia (AL) with Immunotherapy Using Viable (Cryopreserved) Allogeneic Leukemic Cells

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Abstract: At present time in spite of great achievements in modern chemotherapy of acute leukemia (AL) the issue of eradication of residual leukemic cells (MRD) is still relevant. Since 1971 we included specific immunotherapy in the treatment of children with acute lymphoblastic leukemia in remission using viable cryopreserved allogeneic leukemic cells. 67 children in remission were divided into 2 groups: 27 constituted the control group (only continued standard-for-that-time chemotherapy) and 40 children – the treatment which received immunotherapy in addition to standard chemotherapy. In 3 years all children in the control group relapsed. The median length of remission was 15 months. In the treatment group we observed stabilization of remission only in children over 7 years of age when immunization was initiated after 6 or more months of remission and in children younger than 7 if it was initiated after 1-1,5 years of remission. The median length of remission was 60 months which significantly exceeded (4 times) that parameter in the control group of children. Cytotoxic antibodies against leukemic cells appeared in the serum of effectively immunized children at a higher titer than against donor lymphocytes. Intrathecal administration of this hyperimmune serum to patients with neuroleukemia resistant to chemotherapy led to a sharp decrease in the amount of leukemic cells in the spinal fluid. After 5 years of remission (and 3-5 years of immunotherapy) all treatment in these patients was stopped. Out of 19 patients who received immunotherapy on time, 8 patients (42%) have been in event-free remission for 37 to 41 years (median – 38 years) through the present time and enjoy high quality of life. Our results indicate that immunotherapy initiated during remission period of AL can lead to creation of anti-leukemic immunity with subsequent eradication of MRD and complete recovery.

Keywords: Acute leukemia, Immunotherapy, Cryopreserved leukemic cells, Prolongation of remission, Immunological indices.

INTRODUCTION

In spite of great achievements in chemotherapy and bone marrow transplantation in the treatment of AL in recent times the problem of achieving complete recovery hasn't been solved yet. It's very difficult to eradicate minimal residual leukemic cells (MRD) during the period of remission using modern modalities of therapy. Therefore, a constant threat of fatal relapse is always present [1-2].

Forty five years ago, i.e., in 1967, when we started our investigations, the efficacy of chemotherapy in children with AL using 6-Mercaptopurine (6-MP), Vincristine (V), Methotrexate (MTX) and Prednisolone (P) was significantly lower than now. Length of remission usually didn't exceed 1.5 to 2 years and survival - 3 years [3-4]. This prompted many researches in those years to look for ways to immunologically affect selective elimination of leukemic cells from the patient's body.

Unfortunately, the first use of adjuvant, adoptive or passive immunotherapy in acute phase and in remission of AL (BCG, *C. parvum*, interferon, serum, and others) weren't very successful. That served as grounds for the search of more effective methods of specific immunotherapy using leukemic cells.

It was a logical step since even in those years there were studies pointing at the possibility of discovering leukemia-associated antigens (LAA) present on leukemic cells and the ability of the human and animal organisms to respond to them with an immunological reaction [5-6].

First attempts to use native viable allogeneic leukemic cells were made by us in the acute period of AL in 1967. However, significant therapeutic effect was not observed either in children or adults [7,8]. Therefore, we considered it more prudent to apply this method in the period of remission when the quantity of leukemic cells in the peripheral blood is significantly lower.

We supposed that the effect of specific immunotherapy will depend on three main conditions:

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1. maximal immunogenicity and specificity of the injected antigen,

2. minimal mass of the tumor

3. the ability of the patient's organism to mount an adequate response to an injected antigen.

That is why we decided that the best effect could be expected from the use of the whole viable (not irradiated or formalinized which lowers immunogenicity) allogeneic (to avoid their engraftment since strong transplant antigens of MHC system are present) leukemic cells to elicit anti-leukemic response during remission when the number of leukemic cells is minimal and the immunocompetent system is recovering.

The final goal of the immunotherapy was to create strong anti-leukemic immunity caused by injecting leukemic cells that have common LAA with autologous leukemic cells that would lead to eradication of MRD in patients.

MATERIAL AND METHODS

Patients

67 children with AL (65 ALL and 2 AML), ages 3-14 years, in remission of different lengths that was achieved by using standard-for-that-time induction chemotherapy: P+6-MP+V+MTX are presented. In remission they received maintenance chemotherapy with 6-MP+MTX and once every 3 months re-induction (P+BK) chemotherapy.

Immunotherapy

40 patients out of 67 at different times of remission (from 1 to 48 months) receiving the above mentioned chemotherapy were also given immunotherapy. It consisted of regular (once every 2 weeks) intramuscular injections of allogeneic ABO and Rh factor compatible leukemic cells at a dose of $2x\ 10^7$ cells /year of life of a child. To increase the scope of different specimens of LAA in the course of the treatment we interchanged leukemic cells from various patients.

For ethical considerations immunotherapy was administered without canceling standard chemotherapy (except for 1 child who was 10 years old with AML who received only immunotherapy after 8 months of remission based on parents' wishes).

Bank of Cryopreserved Leukemic Cells

In order to administer long-term immunotherapy in 1967 we created a bank of different samples of leukemic cells cryopreserved (at $-196^{O}C$) with 30% glycerol or 10% DMSO suitable for clinical application [9,10]. Viability of cells after thawing was from 40 to 95 % based on supravital staining. Cells were extracted using methods of leukacytopheresis from peripheral blood of patients with AL and hyperleukocytosis and percentage of blast cells exceeding 75% in acute phase before treatment. Special studies showed preservation of immunological characteristics and properties of leukemic cells. Calculation of the dose was performed taking into account the total quantity of viable (unstained) leukemic cells.

Methods

The state of humoral immunity of immunized patients was determined according to the concentration of immunoglobulins A, G, and M in the serum and presence of C'- dependent cytotoxic antibodies against allogeneic leukemic cells and donor lymphocytes. Cell immunity was assessed in the reaction of PHA stimulation of lymphocytes using incorporation of 3H thymidin and cytotoxicity of lymphocytes with Cr^{51}-labeled allogeneic leukemic cells. The investigations were conducted once every 3 months. For comparison we examined also 25 children in the control group who received only chemotherapy during remission.

RESULTS

In 1971 we divided 54 children with AL who were in remission in that year into 2 equal groups with 27 patients in each group. These groups were formed purely on the desire or refusal of the parents either to participate in the new treatment modality or not with the consent to bring children for immunization once every 2 weeks for the foreseeable future (because at that time we couldn't estimate the duration of immunization period). Both groups received the same chemotherapy and children in both groups were similar in age, duration of remission until separating them into 2 groups (from 1-26 months in 1971), clinical and hematological indices in acute period, and detection of full remission prior to the beginning of the study in all patients.

Unfortunately, in those years acute lymphoblastic leukemia was considered to be one disease without any immunophetotypic variants, risk groups and so

forth. All patients were prescribed the same kind of chemotherapy.

The first comparative analysis of the state of the disease in both groups was performed in 1974, i.e., 3 years later. It turned out that all 27 patients from the control group experienced relapses at different times (median length of remission - 9 months). In the treatment group by 1974 remission continued in 6 out of 27 children (Figure **1**). An analysis showed that all children younger than 7 years of age (Figure **1A**) relapsed (the median length of remission was 8 months), but remission continued only in children older than 7 years of age who were in remission for 6 or more months prior to the start of immunotherapy. The median length of remission in those children was 38 months (28 – 54 months) and out of those on immunotherapy -26 months (23 - 30 months). Among those children there was a child with AML who after 2 months of chemoimmunotherapy (which included rubimycin) received starting with the 8th month of remission only immunotherapy.

In the control group out of 27 patients happened to be 14 similar children (aged more than 7 years with the duration of remission for chemotherapy exceeding 6 months before separation) (Figure **1C**). Median duration of remission was 15 months in contrast to 38 months in 6 similar children from the treatment group (Figure **1D**).

With this success of immunotherapy we continued the administration of immunotherapy to those 6 patients and included in 1974 additional 13 patients: 9

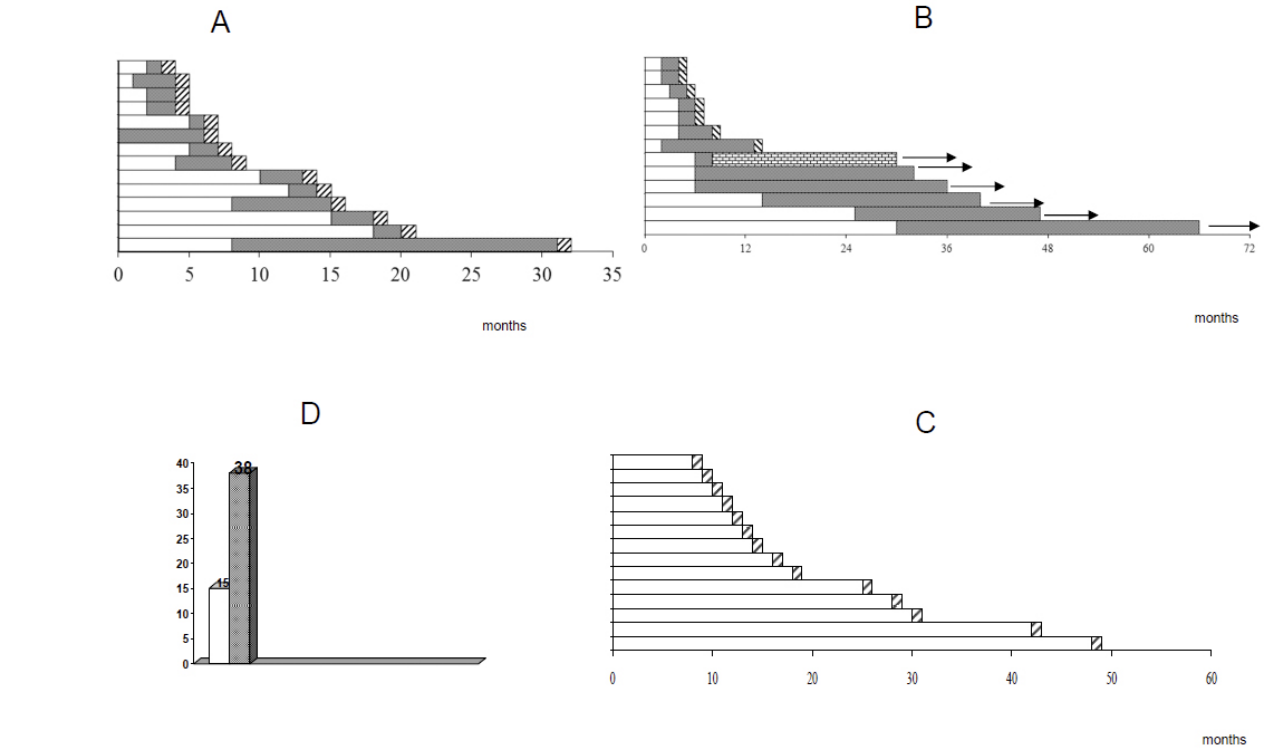


Figure 1: The first stage of research (1971-1974 yrs). The duration of remission in children with AL depending on age and the time of initiation of immunochemotherapy (in months).

A - children younger than 7 years of age who receive additional immunotherapy (treatment group).

B - children older than 7 years of age who receive additional immunotherapy (treatment group).

C - children older than 7 years of age with duration of remission above 6 months who receive only chemotherapy (control group).

D - medianas length of remission from control group B and treatment group C (in months).

children out of them over 7 years of age into the treatment group as well as 4 children younger than 7 years of age (older than 4 years of age) with the duration of remission exceeding one year. Therefore, beginning in 1974 in the treatment group we now had 19 patients whose duration of remission we compared with the same control group (Figure **1C**), since the chemotherapy regimen remained the same.

Analysis of the results of the second stage of the study was conducted in 1979, that is, 5 years later (Figure **2**). It was found that the median length remission in the whole treatment group was 60 months. 10 children during that period have relapsed and, as a result, the median duration of the remission for them was 52 months (22 -84 months). However, 9 patients (4 from the first stage of the study and 5 from the second) showed continued stabilization of remission that has lasted 72 months (58 – 108 months) that was considerably longer (P<0,01) than the median of remission in the control group (15 months.) All therapy in those 9 patients was stopped after 3 - 5 years of immunotherapy and more than 5 years of remission (Figure **2**). During that period patients received from 68 to 114 injections of leukemic cells.

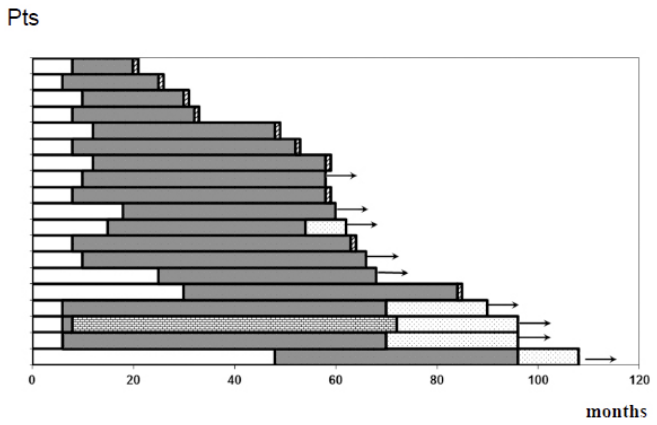


Figure 2: The second stage of research (1971-1974-1979 yrs). The duration of remission in AL children with additional patients included in the treatment group.

Immunological studies of children before the initiation of immunotherapy did not reveal any signs of specific anti-leukemic immunity. However, the majority of them showed lower levels of immunoglobulins in the serum (particularly IgG) and index PHA stimulation of lymphocytes. In the process of immunization we observed stimulation of humoral and cell-specific immune response that manifested itself in the appearance of cytotoxic C'dependent antibodies and cytotoxic lymphocytes against Cr^{51}-labeled allogeneic leukemic cells that were used for immunization. The maximal indicators of lymphocytotoxicity were observed after 3 - 4 immunizations which then decreased to the initial negative levels despite continued immunizations [11,12].

In contrast to cytotoxicity of lymphocytes cytotoxic antibodies against a wide panel of allogeneic cells used for immunization appeared and gradually increased in the majority of immunized patients in the serum after 2 - 6 months and sometimes after 2-3 years of immunotherapy (Figure **3**). The titer of antibodies was determined in the number of positive reactions out of 10 performed with various samples of leukemic cells or donor lymphocytes with the titer of anti-leukemic antibodies always exceeding the titer of antibodies to donor lymphocytes and having no reactions with the their own lymphocytes. We have noted higher cytotoxic activity in the sera of children with the positive effect from the immunotherapy. In patients with the subsequent relapse the titer of cytotoxic anti-leukemic antibodies was either low or totally absent despite immunization.

Anti-leukemic cytotoxic action of sera was confirmed by us *in vivo* in clinical studies. 2 children with 3 and 4 relapses, respectively, of neuroleukemia resistant to chemotherapy were injected intrathecally with 1 - 2 ml of sterile serum containing anti-leukemic antibodies with a titer of 8/10 – 10/10 against allogeneic leukemic cells 4 times in 1 -2 days (Figure **4**). As a result we obtained a sharp decrease in pleocytosis from 488 and 90 leukemic cells in spinal fluid to subnormal counts (22 and 10 cells, respectively).

These results indicate that anti-leukemic antibodies play a major role in elimination of leukemic cells (for prevention of relapse) and the predominance of humoral immunity over cell immunity. Unfortunately, we did not have an opportunity to check anti-leukemic cytotoxic activity of patients' sera against autologous leukemic cells from immunized patients in continued remission because we did not have their cryopreserved leukemic cells to that time.

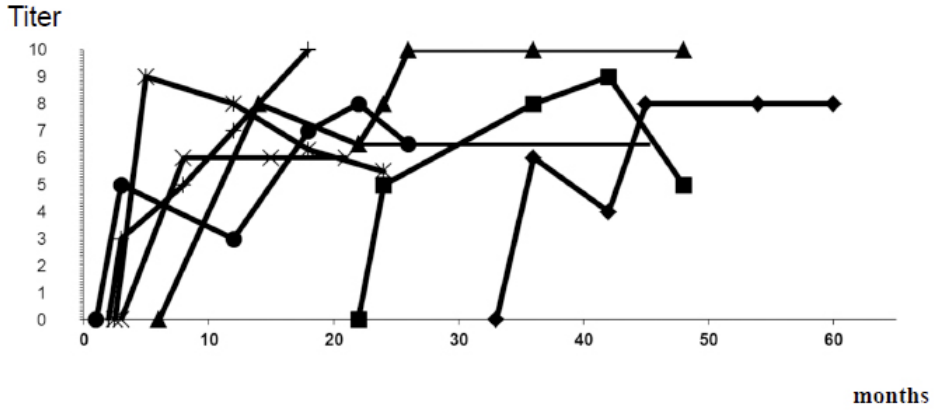
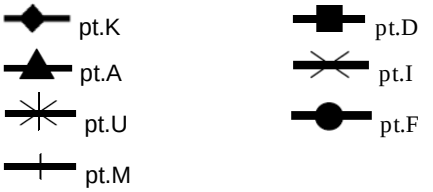


Figure 3: Time of appearance of cytotoxic antibodies to allogeneic leukemic cells in different immunized patients



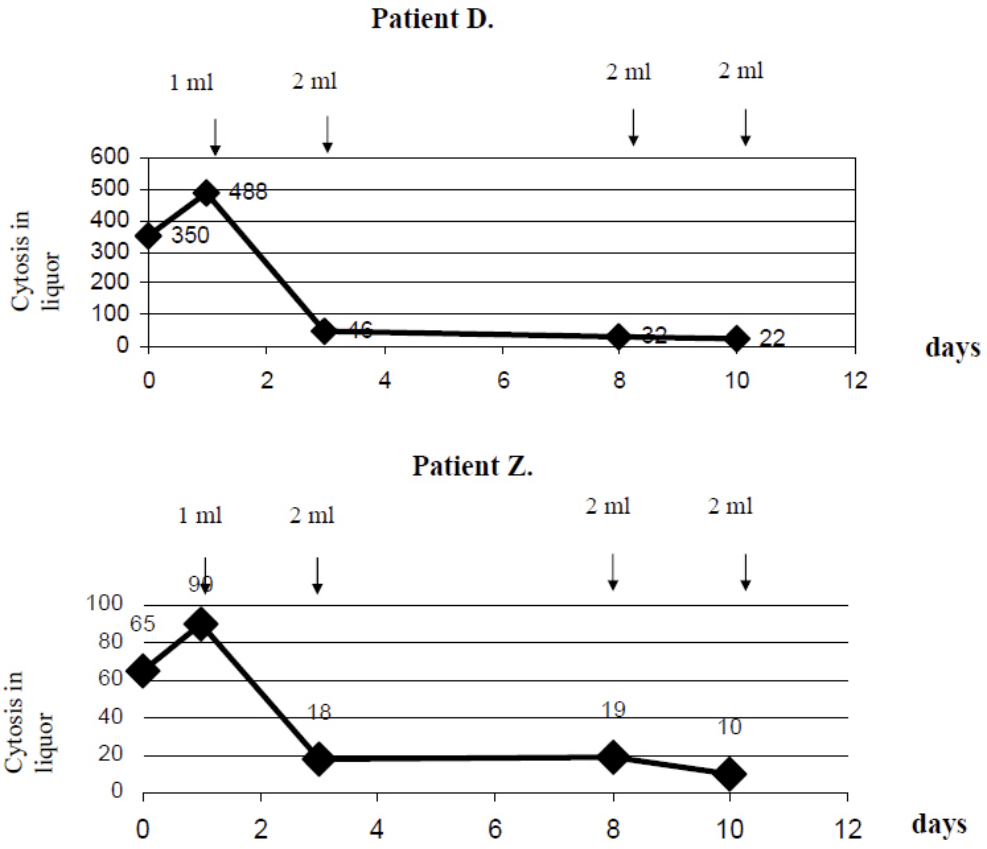


Figure 4: The results of intrathecal infusion of cytotoxic antileukemic serum into patients with resistance neuroleukimia.
→ - intrathecal injection of hyperimmune antileukemic serum
♦ - cytosis of blast cells in spinal liquor

After cessation of treatment all 9 patients remained under the close observation of the hematologist and all stayed in remission. However, one child (with AML) has relapsed in 1981 after 10 years of remission (out of those who received 5 years of immunotherapy only). Up to the present time (July 2013) the rest 8 out of 9 patients are alive and well with a median of event-free remission of 38 years (37 – 41 years) (Figure **5**). They

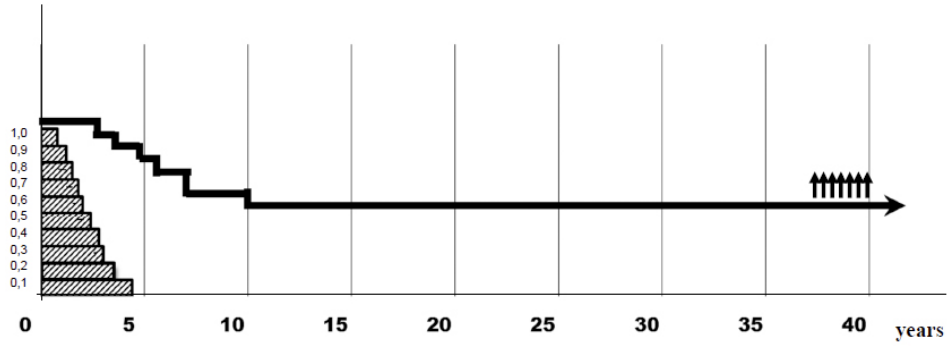


Figure 5: Duration of remission in children with ALL receiving chemotherapy or immunochemotherapy from 1971-1979 (data for the 2013 year).

Control group (only chemotherapy- stage 1, Figure **1C**)

Treatment group (immunochemotherapy – stage 2, and on 2013 year)

Patients from treatment group in a continued remission

became adults leading normal social and family lives, they have had healthy children and some – grandchildren.

All the consecutive stages of the conducted immunotherapy in those children could be tracked in the studies annually published from 1974 to 1979 in Russian and English language literature (13-20). In 1980 due to the circumstances beyond our control we were forced to hold off on publishing our results until present time. However all patients remained under observation of the haematologist.

DISCUSSION

Our data indicates that immunotherapy of AL with whole viable (cryopreserved at – 196^{O} C) allogeneic leukemic cells added to chemotherapy after 6 or more months of remission led to the prolongation of the event-free remission in 9 out of 19 children for 10 years and its stabilization in 8 of them (42%) for more than 37 years. At the time of submission of this article all patients were in a state of full clinical and hematological health and led active social and family lives. This duration of event-free remission enables us to talk about full recovery of immunized patients from AL.

What made these results possible? We are sure that success of immunotherapy is dependent on the adherence to three major rules:

1. The use of an optimal immunogen (live allogeneic leukemic cells)
2. The initiation of immunotherapy in the presence of only a minimal amount of residual leukemic cells in the body (e.g., during full remission achieved through and maintained by cytostatic chemotherapy)
3. Restoration of immunocompetency of the organism to create adequate immune response against the injected antigen (after 6 or more months of remission and cessation of intensive induction chemotherapy needed for restoration of immunity.)

Use of leukemic cells for immunotherapy was aimed at the creation of specific (e.g., anti-leukemic) immunity in the organism of the patient. Though the existence of specific antigens on leukemic cells has not been confirmed until present time there was indirect evidence that suggested the presence of LAAs on human leukemic cells and the possibility of their detection through an immunologic response towards them had appeared already in the 1960s and 70s . In 1983 we have also confirmed the existence of LAA judging by the presence of residual cytotoxic activity of anti-leukemic sera against leukemic cells after its adsorption by autologous lymphocytes in remission [21].

At present time we're seeing a renewed interest around the world towards experimental and clinical immunotherapy [22-28] and identification of LAA [26,29]. In particular, RHAMM/receptor for the hyaluronan acid had been listed among possible candidates for LAA in AML [30,31] and used for immunotherapy [31,32]. (Still earlier the difference

between LAA during AML and ALL had been demonstrated [33-35]). Among the methods of immunotherapy there are great hopes from the use of peptide vaccines and derivates and modifications of dendritic cells [2,36-41], however only few of those studies are concerned with AML [42-45]. All that serves as evidence of the justifiability of searching for rational methods of immunotherapy for recovery from AL.

For preservation of maximal immunogenicity of weak LAA we used whole viable leukemic cells as opposed to irradiated or destroyed cells because this treatment would lead to further lowering of the already weak immunogenicity of leukemic cells [46,47]. To increase immunogenicity some authors have used leukemic cells treated with neuromenidase or interferon [48-52].

The lack of engraftment of injected viable leukemic cells had been confirmed by us using examination of Y chromosomes in blood cells and in bone marrow. None of the immunized patients have displayed leukocytes of the opposite sex either during continued remission or during relapse, whereas leukemic cells during relapse had a phenotype and genotype of the host in the acute period of AL.

As incontrovertible evidence of the presence of the specific anti-leukemic immune response can serve the appearance of anti-leukemic antibodies in the blood of effectively immunized patients and a sharp decline in the number of leukemic cells in the cerebro-spinal fluid after intrathecal administration of the hyperimmune anti-leukemic serum to two patients with neuroleukemia (see Figure **4**).

Also of crucial importance is the time of the inclusion of immunotherapy into the treatment protocol. The time of effective application of immunotherapy (after 6 months of remission in children older than 7 years of age and after 1 to 1.5 years of remission in children of younger age) that we have established appears to be connected to both the maximal decline in the mass of leukemic cells (MRD) and the restoration of immunocompetent system. By that time patients would have received (after the induction) at least 2 courses of re-inductive chemotherapy and as a result the number of leukemic cells would have dropped to a minimum with a gradual restoration of normal hemo- and immunopoesis which have strengthened the effects of immunotherapy. Later other authors have also noted that restoration of immuncompetecy takes place only after 6 months of remission [53-55]. That explains the more favorable results obtained by other authors who used immunotherapy after 9 - 19 months and even after 2 years of chemotherapy in remission [56].

Our finding that in younger children it took a significantly longer remission before initiation of immunotherapy for it to be effective could be explained by insufficient maturation of the immunocompetent system at that age that was additionally suppressed by the administered of cytostatic (immunosuppressive) therapy that was necessary during treatment of AL. It can't be excluded that some immunocorrective but not stimulating therapy would facilitate the recovery of immunity in some patients (e.g., interferon, immunoglobulins and others). A low level of immunocompetency in all patients in remission was observed by us judging by a decrease in the quantity of T and B lymphocytes, immunoglobulin levels in the serum and the index of PHA stimulation of lymphocytes, that were normal only in one patient (with AML receiving only immunotherapy without chemotherapy) Even though it is known that cytostatic chemotherapy has a suppressive effect on the indices of immunity it leads to a decrease in the mass of leukemic cells which in turn increases the effectiveness of immunotherapy [11,12, 57,58].

Additional evidence for the decrease in immunocompetency of patients who receive immunotherapy in remission is the delay in the appearance of anti-leukemic and anti-lymphocytic cytotoxic antibodies against the injected leukemic cells (4-6 months and sometimes 2 to 3 years or after 10 or more injections) (see Figure **3**). In contrast, in healthy people anti-leukocytic antibodies appear already after 2-3 injections of allogeneic leukocytes. In our research anti-leukemic antibodies in the serum were absent in all patients who relapsed. Anti-leukemic antibodies were not only present in patients who were in remission that continued all through 1979 but they also exceeded the titer of anti-lymphocytic antibodies. That was facilitated by the long (more than 3-5 years) immunization with constant interchanging of the samples of leukemic cells from various patients. Unfortunately, we were unable to study the sera of successfully immunized patients with autologous leukemic cells because we didn't possess their cryopreserved leukemic cells obtained in acute phase of AL. However, in future immunological studies it will be necessary to cryopreserve leukemic cells from each patient in the initial acute period even in the absence of hyper-leukocytosis. At the same time performing leukapheresis prior to the initiation of

treatment has two advantages: not only for the cryopreserved bank of leukemic cells, but also for the patient by decreasing the nephrotoxic effect of the cytolitic action of chemotherapy. That prompts some authors to perform leukapheresis prior to treatment that leads to a decrease in the amount of leukemic cells in the patient's body and strengthens the effect of chemotherapy [59,60].

The analysis of our data allows us to conclude that the immunotherapy is effective not only in terms of stabilization of remission but is also safe if administered according to the revealed conditions. As a result the disease-free remission in 8 out 19 immunized patients was longer than 37 years that by far exceeds that of patients who were treated exclusively with chemotherapy not only in our control group but also as shown by other authors according to whom the average survival in ALL children for chemotherapy did not exceed 5-10 years [61-66]. We suppose that our results could be improved if we performed immunization with leukemic cells of the same form and immunophenotype of AL that was present in the patient. Unfortunately, at that time nobody knew about it.

The lack of positive results of immunotherapy widely used by other authors in 1970s could be explained by the fact that most authors used only adjuvants [67-74] and/or irradiated or destroyed leukemic cells [47,75-78]. At the same time all authors began immunotherapy within the first month of remission [47,70,74-84], sometimes stopping chemotherapy [73,85,86], while others administered small doses of leukemic cells and used short courses of immunization [78,81,83,84]. It seems that for those reasons none of the authors obtained significant therapeutic results which lead to decreased interest in using specific immunotherapy with leukemic cells in the complex treatment of AL. Unfortunately until present time we were unable to find later publications by those authors and any others that used allogeneic leukemic cells for immunotherapy of AL.

Since chemotherapy of ALL is more effective than of AML more studies should be devoted to a search of effective methods of immunotherapy of non-lymphoblastic (myeloblastic) leukemias. Taking into account the fact that the immune system in AML is preserved better than in ALL [47,50,87], where malignant transformation occurs in the cells of immmuno - hematopoesis we suppose that application of immunotherapy, devised by us, in AML would prove even more effective than in children with ALL.

We suppose that immunotherapy could be further improved if the selection of leukemic cells for immunization is performed correctly as well as if the protocol of immunization with leukemic cells is supplemented with immune-corrective and immune-reconstructive means as well as with administration of dendritic cells for a better recognition of antigenic stimuli and initiation of immune response. We think that active specific immunotherapy could be included in the treatment protocol at the last stage of chemotherapy or bone marrow transplantation for the final elimination of MRD that would lead to recovery of patients from AL.

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