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IN VITRO EFFECT OF ANTITUMOR DRUGS ON LYMPHOCYTIC BLASTOGENESIS IN CHILDHOOD ACUTE LYMPHOBLASTIC LEUKEMIA (ALL) AND NON-HODGKIN'S LYMPHOMA (NHL)

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"In vitro" sensitivity of lectin (PHA, Con A)-stimulated lymphocytes to antitumor drugs (ARA-C, ADR, VM_{26}, MTX, CP, VCR, Vepesid, ACLA) and the clinical efficiency of the complex therapy was compared in 7 patients with ALL and 2 patients with NHL. H^3-thymidin incorporation of lymphocytes labelled prior to the drug exposure was used as "in vitro" method. A fairly good correlation was found between the "in vitro" test and the clinical response to the drug administered. These results suggest that this "in vitro" test is useful in choosing the drugs to be administered in case of malignancies of children.

INTRODUCTION

In the last 20 years several institutions and researchers have developed new treatment protocols for childhood lymphomas. The best results have been achieved with patients suffering from stage I and II disease /34/. Curability for these early stages is in the range of 90%, except when immunosupression develops due to complications of chemotherapy or an onset of an unrelated disease, which can cause death. In selecting the optimal individual drug combination with special care to avoiding drug resistancy during relapse, a determination of drug sensitivity in vitro would be helpful.

Morphological, cytogenetic /14, 17/ and immunological /7, 11, 26/ findings urged us to develop a new classification and therapeutic strategies /13/ for childhood malignancies. This approach was promoted by widening the methodologies using in vitro human cell cultures /18, 37, 38/. However, the

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antineoplastic effects of cytostatic drugs are not the same "in vitro", as "in vivo" /37/, therefore this technique has certain limitations to the preclinical screening of chemotherapeutics. The valuation of their antiproliferative effect on the lectin [photohaemagglutinine (PHA) and concavalin-A (con-A)] - stimulated cells /6, 9, 10, 15, 23, 35/ during the established treatment regimen has some advantage in a modified approach of treatment with regard to the patient's individual situation and drug sensitivity.

When the lectin-binding ability (response to PHA and con-A) of leukemic lymphocytes, which may play an important part in changing the surface receptors, is altered it can also influence the humoral and cellular immune-responses. The multiplication of tumor cells depends on the lectin-binding spots and the success of therapy may be influenced by the behavior of blastic or normal (intact) lymphocytes. This method can obviously not cover all aspects of cellular responses. Only local reactions i.e. drug-cell interactions are determined independent of the host, giving some important information about the effect of drugs on the cells' proliferative response. This kind of approach in selecting the optimum treatment may allow for avoiding the cumulative toxic effect of drugs in effective on the growthcontrol of the malignant cell-proliferation. In our former study we found that the incidence of infections in ALL patients increased in the case of lower helper and Pan-T cell levels during the consolidation and maintenance stage of the therapy /2/.

In this paper we have measured the 3H-thymidine incorporation rate after the lectin (PHA and conA) stimulatory effect on proliferating lymphocytes in childhood malignancies of the lymphoid system (ALL, NHL), in the presence and absence of different chemotherapeutic agents, especially before the stage of attempting to treat relapses /8/.

MATERIALS AND METHODS

Whole blood was obtained by venipuncture into heparinized tubes (0.01 ml heparin/10 ml syringes). The blood was then diluted with Hank's balanced solution to a ratio of 3:1. The lymphocytes were isolated by Ficoll-Hypaque density gradient centrifugation /4/. At first, the donor sera was heat inactivated and filtered, prior to use in self-culture at a concentration of 30%. Parallel to the self-cultured samples we used fetal calf serum (GIBCO) to optimise culture conditions. After establishing the clinical status, the surface antigen phenotype of the lymphocytes in the separated cells was determined by monoclonal antibodies (Behring) /26/, and then a specimen was prepared by the modified Matutes' method /20/ for electron-microscopic investigation.

To stimulate blastogenesis of T-lymphocytes, phyto-hemagglutinin-M (PHA) was used in each culture [0.03 ml/10 ml RPMI-1640 medium (GIBCO)], without antibiotics. The lymphocyte suspension was adjusted to one million cells/ml of medium. Cultures from each sample were divided into subgroups:

- Controls, 2 tubes containing RPMI-1640 medium supplemented with 30% fetal calf serum and no PHA,
- PHA stimulated groups, cells cultured in the presence of PHA (0.025 ml/ml) dissolved in RPMI-1640 medium,
- PHA (0.025 ml/ml RPMI-1640 + different cytostatics agents; detailed protocol see below.
- Concavalin-A (Con-A) group 2 tubes containing Con-A/100 µg/ml dissolved in RPMI medium /6, 10, 32, 36/.
- The experiments were repeated in two parallels with doses of cytostatic agents included in treatment protocol, the doses were calculated to body surface and serum volume, and adjusted in a volume of 100 µl. Controls were applied in the same way.

The cells were incubated for 42 hours at 37°C in humidified CO_2 (7.5% in air), using a LABOR-MIM incubator (Hungary).

In the final 6-12 hours of incubation, 3H-thymidine (Amersham), diluted to a final concentration of 1 µCi/ml, was added to each culture. Then cultivation was stopped by adding 0.1 M cold citric acid. Thereafter 50 µl of the cell suspension was pipetted into 5 ml of the scintillation cocktail and radioactivity was assayed by scintillation counting (Hewlett-Packard) to determine (3H)-thymidine incorporation /33/. The cell suspension treated with the cytostatic agents were incubated for 60 minutes in a CO_2 incubator, then the cells in each tube were washed twice in Hank's buffer solution resuspended in a mixture of 0.7 ml of PHA and 0.3 ml of fetal calf serum Phylaxia, Hungary, and placed back in the incubator for 36 h. Then the above-described procedure was performed. The cells were fixed with a mixture of methanol-acetic acid-formaldehyde and centrifuged with 600 g. 50 µl of fixed cell nuclei, then they were air dried to the surface of a slide where the scintillation value for a single cell was calculated with the help of the cell count measured in a Buerker chamber to 50 µl. Labelling indexes were obtained on the basis of the measurements of radioactivity in the DNA after treatment with

various drugs and were calculated for a single cell, according to the next formulas:

In group one: $\frac{\text{(contr.)cpm}}{\text{(PHA)cpm}}$

In group two: $\frac{\text{(contr.) cpm}}{\text{(Con-A)cpm}}$

In group three: $\frac{\text{(Con-A)cpm}}{\text{(PHA)cpm}}$

and in the groups of investigated $\frac{\text{(PHA+Cytostatic agent}^{*}\text{)cpm}}{\text{(PHA)cpm}}$

Patients

The study was carried out on five children with newly diagnosed malignancy and four children with relapse before restarting therapy. The patient's data were compared to those of healthy children of the same age (Table I). During the study the patients were referred to by codes. Some major features of the history were presented to facilitate the evaluation of the results obtained.

Case Nº B_3: The histopathological investigation of the right axillary lymph node confirmed a lymphoblastic lymphoma at clinical stage I in 1988, consequently NHL stage I. treatment was applied. Remission ensued, at present maintenance treatment is being performed.

Case Nº B_4: An enlarged right lateral jugular lymph node was observed in 1988. Toxoplasmosis was suspected and corresponding treatment was performed, however, the node enlarged. Histopathology revealed lymphoblastic lymphoma. As there were also alterations detectable in the kidneys and a proliferation of cells in the CSF, the child was classified as a stage III patient and treated according to the LSA_2L_2 protocol. Treatment was well tolerated, at present the child is symptom-free and maintenance therapy is being pursued.

Case Nº B_9: The high malignancy ALL patient (girl, time of diagnosis: 1987.) was treated according to the "HPOG-MMI-86" protocol. After the CNS relapse the Induction Protocol E was applied.

* (according to treatment protocols: see Table I and list of abbreviations)

TABLE I

Data of patients and healthy children of the same age and sex. They were referred by codes numbers.

Patients	Characterisation of the investigated groups							
Healthy controls	age (years)	sex	dg	risk factors			treatment	present state
				BFM	FAB	Imm.		
B_1	4	♂						
B_2	14	♀						
B_4	9	♂						
B_{11}	10	♂						
B_{13}	5	♂						
B_{16}	7	♂						
B_{18}	3	♂						
Newly diagnosed cases								
B_3	9	♂	NHL	St. I.			Murphy prot.	compl. rem.
B_5	14	♂	NHL	St. III.			Wollner prot.	compl. rem.
B_9	4	♀	ALL	RF>1,7	L_2	O	HPOG-86	nonresponder
B_{12}	7	♂	ALL	RF>1,7	L_1	CALLA	ALL-88	compl. rem.
B_{14}	4	♂	ALL	RF<1,7	L_1	CALLA	ALL-88	compl. rem.
Isolated CNS and medullary relapses								
B_8	6	♀	ALL CNS	RF<1,7	L_1	CALLA	ALL-83 Block R1+R2	nonresponder
B_{10}	11	♂	ALL	RF<1,7	L_2	CALLA	ALL-83	remission
B_{15}	11	♂	ALL CNS	RF<1,7	L_1	CALLA	ALL-83 Block R1+R2	nonresponder
B_{17}	3	♀	ALL	RF>1,7	L_1	O	ALL-83 ("E")	remission

Case N^{o} B_{12}: This ALL case had large lymph nodes in the right lateral submandibular region. During ALL-88 Prot.I.subtotal lymphadenoctomy was performed, and maintenance therapy, prescribed for high risk cases continued.

Case N^{o} B_{14}: In the case of this child with C-ALL, the ALL'88 treatment was initiated. Maintenance therapy was pursued. Complete remission was recorded.

Case N^{o} B_{8}: In 1988 CNS relapse was observed in the ALL patient of average malignancy. The child was treated according to the BFM ALL meningeal relapse protocol of 1983. In 1989 recurrent meningeal relapse, after several weeks a systemic relapse was recorded.

Case N^{o} B_{10}: In 1982 ALL -a late systemic relapse-was diagnosed in this boy, so the BMF protocol of 1983 was applied. His present condition is statisfactory.

Case N^{o} B_{15}: In a boy under treatment for ALL of average malignancy, CNS relapse was diagnosed in May 1988, thus the BMF meningeal relapse protocol of 1983 was attempted. After 6 months, decerebration symptoms, paraplegy and sensormotor aphasy developed.

Case N^{o} B_{17}: ALL systemic relapse was diagnosed in an infant in the 52nd week of "HPOG-MMI-86" therapy, and BFM'83 ALL-relapse induction protocol ("E") was initiated. At the beginning of the treatment his overall condition improved then high fever developed and he died due to pancytopenia and cardiovascular-respiratory failure.

RESULTS

The characterisation of the groups investigated is illustrated in Table I. Results (Fig. 1) are related to the mathematical means (dotted line) of healthy children.

The RPMI/PHA index* (Fig. 1) is higher than the mathematical means of healthy children.

The change in the RPMI/Con-A ratio (Fig. 1) shows a similar trend, while the ratios of the two lectins, Con-A and PHA were practically identical or lower than the mathematical means of the corresponding healthy values or controls.

* of ALL, NHL patients

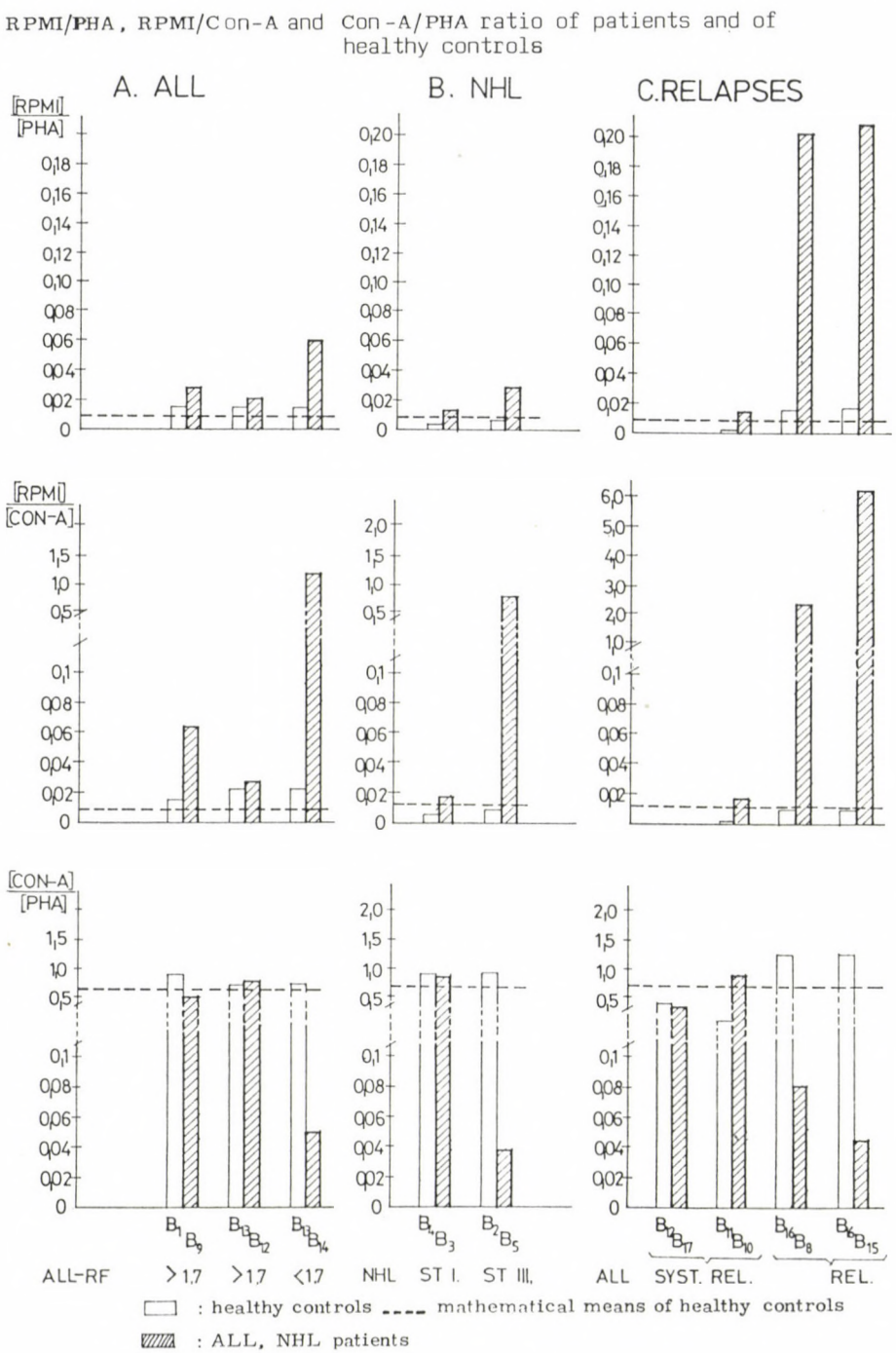

Fig. 1. RPMI/PHA, RPMI/Con-A index and Con-A/PHA ratio patients related to the matemathical means of healthy children (dotted line). There are matched controls.

The results obtained with the cytostatic agents mentioned in the protocol, as well as the data of healthy children were corrected to the excepted level of PHA stimulation rate with the PHA values (Figs. 2, 3, 4).

ALL (Fig. 2):

B_9: From the drugs listed in the "HPOG-MMI-86" protocol, reduction was induced by C-ARA, VM_{26} and CP.

B_{12}, B_{14}: From the agents of the "ALL'88" protocol C-ARA, CP and ASP caused reduced levels compared to the control in these two children. In case B_{14}, VCR also resulted reduced level.

NHL (Fig. 3):

B_3: From the agents of treatment NHL stage I., the suppressive effect of MTX, C-ARA and ASP was distinct also at in vitro conditions.

B_5: Studying the agents of the LSA_2L_2 therapy, except for CP, the scintillation activity of the cell culture, corrected with the value assayed in PHA medium, showed a decrease in each case.

Isolated CNS and medullary relapse:

B_8, B_{15}: Fig. 4 demonstrates that treatment constituents VM-26, MTX and CP C-ARA, IFO and VCR induced reductions in the scintillation activity of lymphocytes compared to the controls in children submitted to ALL'83 CNS relapse therapy.

B_{10}, B_{17}: Fig. 5. Summing up the effect of therapy on the systemic ALL relapse in B_{17}, each constituent of the BFM'83 protocol based on induction, inhibited the blastic transformation of the lymphocytes of the patient, while in the case of patient B_{10}, reduction in the scintillation activity was induced only by MTX.

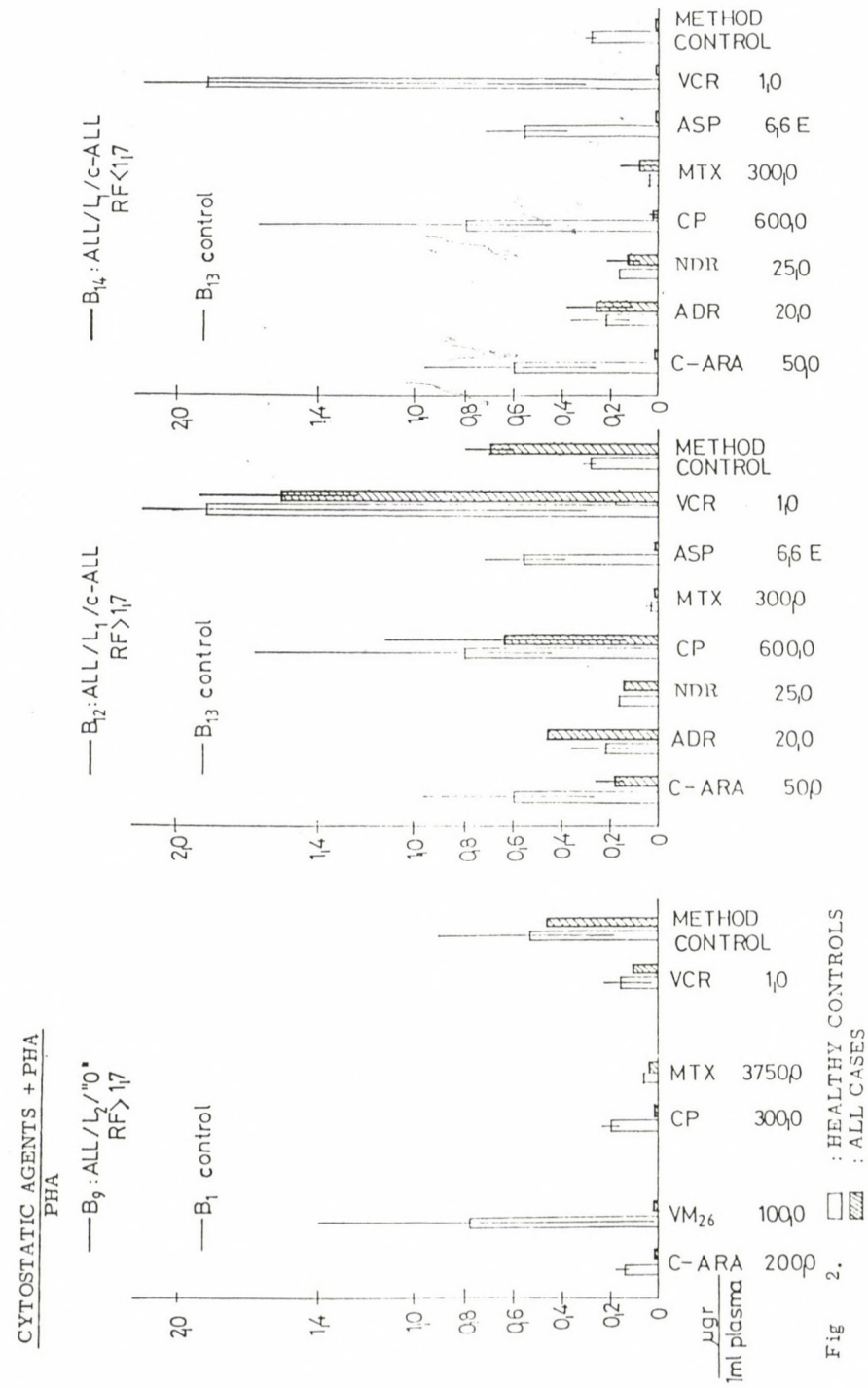

Fig 2. Results obtained with the cytostatic agents-data of patients and controls, corrected to the basic PHA values

Results obtained with the cytostatic agents-data of patients and controls, corrected to the basic PHA values

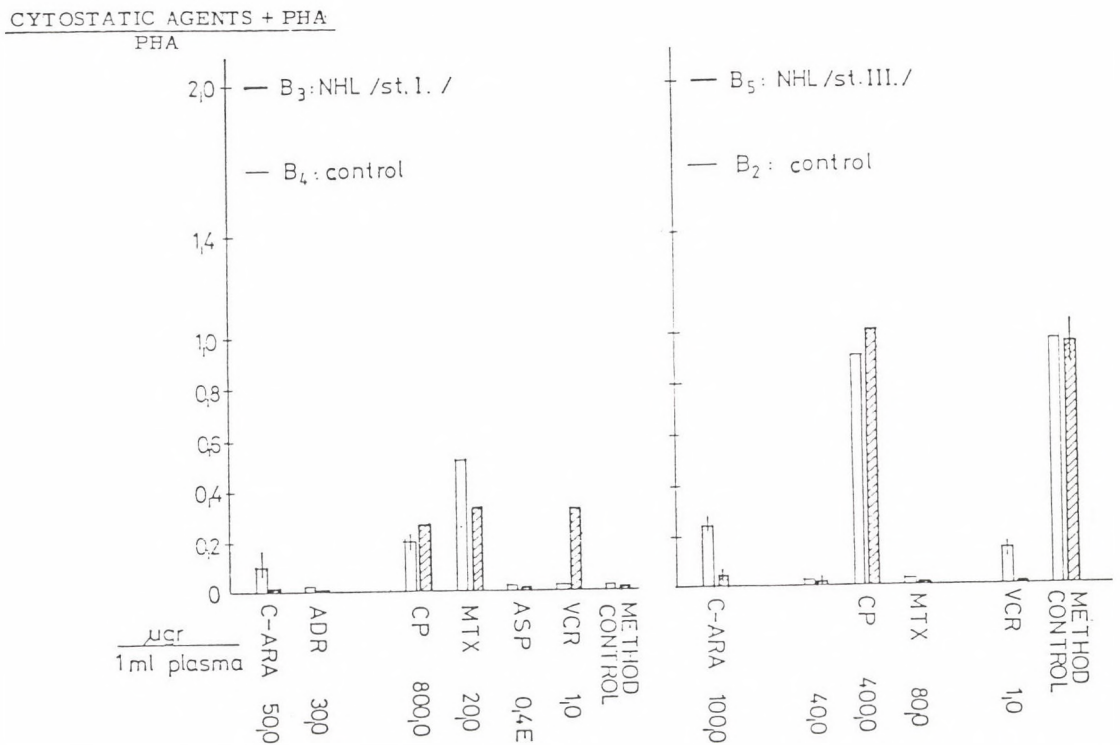

Fig 3. ☐ : HEALTHY CONTROLS
▨ : NHL CASES

Results obtained with the cytostatic agents-data of CNS relapses and controls, corrected to the basic PHA values

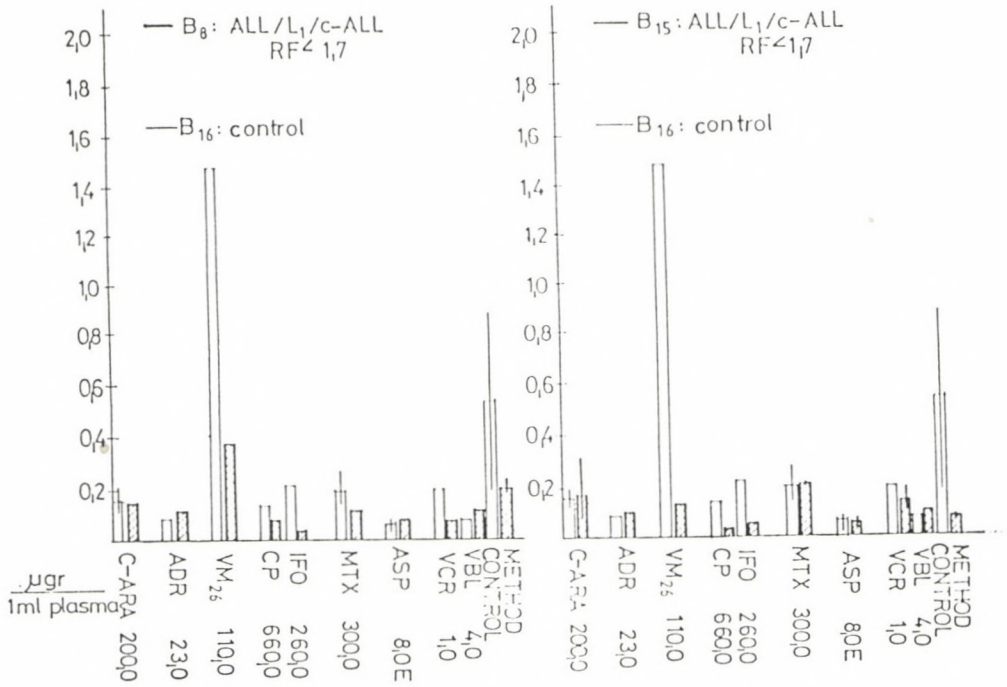

Fig 4. ☐ : HEALTHY CONTROLS
▨ : ALL, NHL PATIENTS

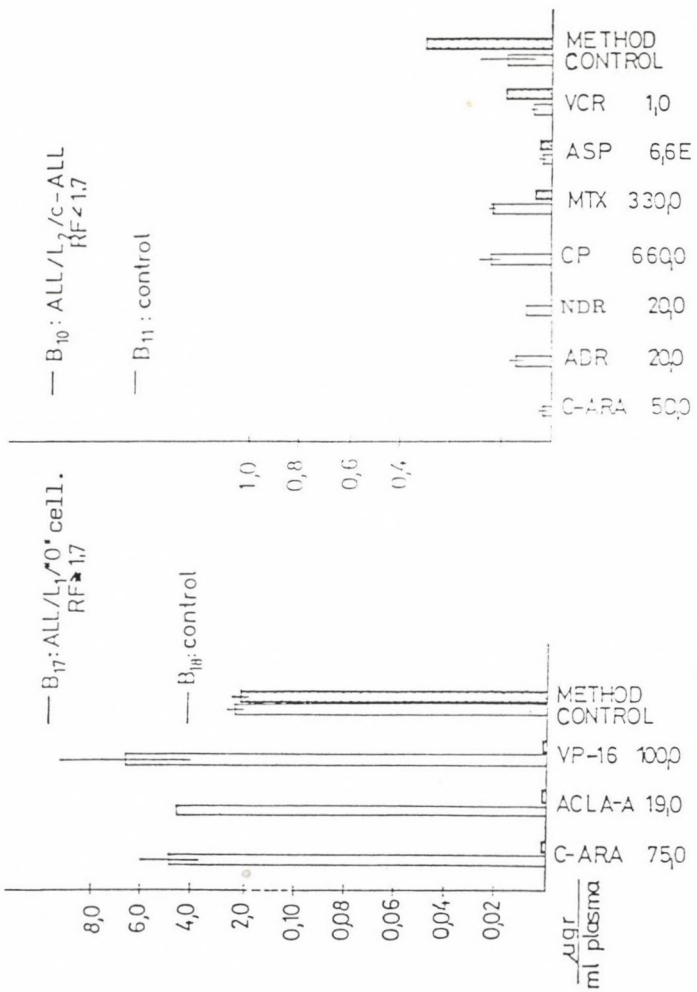
Results obtained with the cytostatic agents-data of CNS relapses and controls, corrected to the basic PHA values
— B17: ALL/L1"O" cell. RF≥1.7
— B18: control
— B10: ALL/L2/c-ALL RF<1.7
— B11: control
8,0
6,0
4,0
2,0
0,10
0,08
0,06
0,04
0,02
1,0
0,8
0,6
0,4
µgr / 1ml plasma
METHOD CONTROL
VP-16 100,0
ACLA-A 19,0
C-ARA 75,0
METHOD CONTROL
VCR 1,0
ASP 6,6E
MTX 330,0
CP 660,0
NDR 20,0
ADR 20,0
C-ARA 50,0
: HEALTHY CONTROLS
: ALL, NHL PATIENTS

Fig 5.

DISCUSSION

Several studies confirm the significance of the immune system in patients with ALL and NHL, proving a correlation between clinical therapeutical effects and in vitro prediction of tumor cell chemosensitivity /15, 22/. In this study in vitro reactivity of T-lymphocytes to PHA /22/. Con-A /6, 32, 36/ and some cytostatical drugs /24, 25, 28/ was investigated in ALL, NHL patients and healthy control children.

Attention should be drawn to the relationship between the change recorded in PHA medium and the basic activity (RPMI). We have concluded that in ALL patients /27/ lymphocytes showed a higher initial blastogenic activity assayed in RPMI medium with a higher RPMI/PHA ratio compared to the healthy control, while in ALL of high malignancy (RF > 1.7) the rate of blastic transformation induced by PHA is higher, the RPMI/PHA ratio being consequently lower, compared to the corresponding parameters of patients of average malignancy (RF < 1.7).

Similar observations have been made when data of NHL patients were compared to those of a healthy child of the same age /30/. In the present study the increased RPMI/PHA ratio was considered as a sign of relapse in patients with meningeal symptoms /8/, suggesting the presence of the increased number of lymphoblasts in the pheripheral blood. In the case of systemic relapse /3/, the rate of PHA stimulation rate was higher than those of ALL-patients with high malignancy (RF > 1.7), resulting a lower RPMI/PHA ratio.

In conclusion, our study showed the usefulness of "in vitro" determination of blastogenic response of T-lymphocytes in forecasting the responsiveness of the leukemic cells to the cytostatic drugs /25/ in the stage of relapses /8/. Although the CP and VCR treatment required metabolic activations to be active in the cells /28/, the resistance to this agent /12, 16, 31, 39/ can be achieved by a previous treatment of metabolizing cells or using the sera of already treated patients. An other possibility to estimate the changes in the response to these

drugs, is to compare the PHA stimulation rate of the first treatment time with the response measured relapse. This method has the advantage of being able to monitor the T-cell ability to blastogenic transformation in the presence of different lectins (PHA and conA) and to compare it to the changes of stimulation rate in the presence of different cytotoxic drugs. The protocol makes it possible to prevent the undesirable toxic side effects of the drug combination, /21, 28, 29/ and to peel up the ineffective component of the treatment trial. In the future we shall also attempt to perform the necessary metabolic activation of certain drugs, needed in the case /1/ prior to commencing the treatment. By exploiting the method of using in vitro systems /37/ to model the pathomechanisms of various differences /32/ in the cell-drug relationship /19/, the result can trigger an efficient antileukemic therapy with a consideration for the individual differences in the response /13, 35/.

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