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EFFECTS OF A MODIFIED CMF TREATMENT (CYCLOPHOSPHAMIDE, METHOTREXATE AND 5-FLUOROURACIL) ON HEMATOPOIETIC TISSUES AND YOSHIDA SARCOMA IN RATS

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Effects of a modified CMF treatment on hematopoietic tissue and an implanted tumor were studied in rats. The modification of the treatment refers to the application of cyclophosphamide 24 h after methotrexate and 5-fluorouracil. The study was done on Wistar rats bearing Yoshida sarcoma in the ascites form. The controls were a) untreated animals bearing the tumor or b) treated conventionally with the 3 cytostatics and c) tumor-free animals under either conventional treatment or d) modified treatment. We examined survival, the appearance of metastases, and the regeneration of hematopoietic tissues. Improved survival, the absence of metastases, and improved regeneration of hematopoietic tissues was observed when modified CMF treatment was applied. These results support the importance of sequencing cytostatic protocols for basic hematological determinants and anti-tumor activity.

Combination chemotherapy is one of the proven strategies against malignant tumors (1). One major limitation of its effectiveness is, however, the damage to the hematopoietic system which often precludes application of doses necessary for eradication of the malignant tissue (2, 3). Usually these drugs cause a reduction of the blood cell count and depletion of the stem cell pool in the bone marrow. In a dose-dependent manner, tumor burden reduction and bone marrow aplasia, roughly parallel each other and limit the chemotherapy (4). This necessitates procedures that can protect the hematopoietic tissues during the therapy and without reducing the antineoplastic potential (5–8).

Protective effects of small priming doses of cyclophosphamide (CY) in chemotherapy and radiotherapy of experimental leukemia have been demonstrated (9). It has been reported that CY causes considerable reduction of the stem cell pool followed by a fast regeneration (10). CY, being a strong alkylating agent, has a stronger effect on mature hematopoietic progenitor cells than on pluripotent stem cells and different application protocols can have either immunosuppressing or immunostimulating effects (11, 12). Defined dosage of CY induces humoral stimulating activity (HSA) in sera of treated animals with evident mitogenic properties in various hematopoietic tissues (13, 14). The human lymphocyte most susceptible to derivatives of CY is the Con A-induced suppressor T cell (15). The ability of CY to break established immune tolerance and even facilitate the rejection of autologous tissue is of great relevance for cancer immunotherapy (16). Such overlapping immuno- and myelosuppressing effects of anticancer drugs may be important during cancer chemotherapy (12).

Following the working hypothesis that endogenously CY-induced HSA can be mitogenic for hematopoietic tissues, we modified the standard CMF protocol, which includes cyclophosphamide, methotrexate (MTX) and 5-fluorouracil (5-FU). The basic logic was to overlap, on the time scale, the nadir of hematopoietic tissue cellularity and the peak of CY-induced HSA. This was accomplished by application of CY 24 h after the injection of 5-FU and MTX. This modification resulted in significant protection of the hematopoietic tissues, compared with the protocol

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where all 3 cytostatics were applied simultaneously. In this report, we also describe the potential of the modified treatment for reduction of transplantable tumor burden.

Material and Methods

Tumors and rats. Transplantable Yoshida sarcoma was maintained in vivo by weekly i.p. injection of 10^5 viable cells in female Wistar rats 10 weeks old. Animals were maintained under standard laboratory conditions with 4 in each cage. They were fed laboratory chow and water ad libitum and 12 h light/dark cycles were used (light from 07.00–19.00 h). There were 70 animals in the two groups of tumor-bearing animals undergoing treatment and 50 animals in the two groups of tumor-free animals undergoing conventional or modified therapy. Survival was determined in groups of tumor-bearing or tumor-free animals (30 animals in each group) subjected to different treatment protocols or no treatment. There were 30 animals in the untreated tumor-bearing group of animals that was used for other analyses than survival. Three days after the inoculation with 6×10^7 viable cells of ascites of Yoshida sarcoma, the chemotherapy was initiated.

Treatment. The cytostatics were injected i.p. For the conventional treatment, cytostatics were applied simultaneously at the following dosage: MTX, 6.5 mg/kg, 5-FU, 75 mg/kg, and CY, 50 mg/kg. The modified protocol was identical except that the CY was administered 24 h after the application of 5-FU and MTX. To evaluate the modified protocol we regarded as controls animals bearing YAS that received either conventional therapy (A) or no treatment (receiving saline only) (B) and tumor-free animals under conventional treatment (C) or tumor-free animals under modified treatment (D). Normal values were obtained from untreated animals of the same age maintained under the same conditions.

Monitoring of therapy. In the course of the chemotherapy the following parameters were followed: mortality, tumor growth, appearance of metastases, and regeneration kinetics of the hematopoietic tissues. The appearance of solid tumors at the site of the needle penetration was also recorded. Peripheral blood, bone marrow, spleen, and ascites fluid were examined. At each specified time point 10 animals were sacrificed by cervical dislocation. a) Hematopoietic tissue: 1) For the peripheral blood analysis, blood was drawn from tail veins at specified time intervals prior to sacrifice. The count of erythrocytes and leukocytes was determined and expressed as cells/L $\pm$ standard error of the mean (SEM). 2) For the bone marrow analysis, the marrow was harvested from tibias of each rat by removing the tibias, stripping the muscle from the bone, sectioning the bone at each end, and aspirating the marrow by a 25-gauge needle and a syringe containing iced phosphate buffered saline (PBS). Erythroid, granuloid, and lymphoid elements were counted and expressed along with the SEM. Statistical significance was determined by the t-test and indicated in the text. 3) For the spleen analysis, the weight was determined, and the tissue forced through 50 μm stainless steel mesh. The resultant cell suspension was rinsed in PBS and total cellularity and counts of lymphoid, granuloid and erythroid elements were determined. The basic statistics were evaluated as described above. b) Tumor growth was evaluated by measuring the volume and cellularity of ascites fluid aspirated from the peritoneum of sacrificed animals. The appearance of solid tumors at the site of a needle penetration was recorded. Also, metastases in the spleen were evaluated microscopically as foci of Yoshida cells, on mutually perpendicular slices. c) Survival of animals was expressed as percentage at each time point with the actual number of animals specified in the text.

Results

Survival (Fig. 1). The survival in the control group B (animals bearing YAS and receiving no therapy), 8 days after the inoculation of the tumor, was 0% i.e. (no animal survived). The survival time was considerably prolonged in both groups treated with cytostatics. After 33 days, the survival of the animals receiving conventional treatment was 33% (10 animals out of 30). Animals treated with the modified protocol had a survival of 83% (25 out of 30). The survival of tumor-free animals was not affected by any of the two types of the treatment.

Tumor growth (Table 1). Three days after the inoculation, prior to any treatment, the growth of the ascites averaged 8.7 ml per animal with 9.2×10^7 cells/ml. In the untreated YAS-bearing (control B) the amount of ascites, 5 days following the inoculation of the tumor, averaged

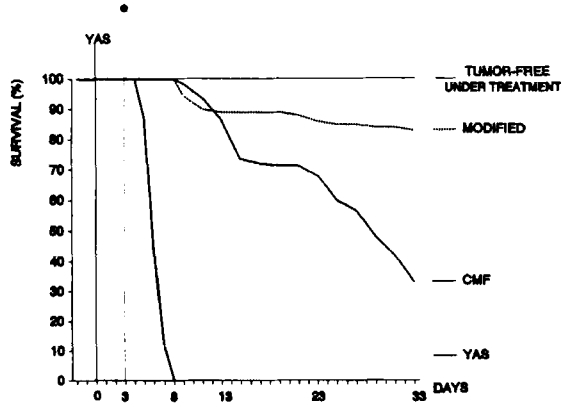

Fig. 1. Survival determined as described in 'Material and Methods'. Vertical line marked with 'YAS' stands for the time of the inoculation of Yoshida sarcoma cells, vertical line marked with '*' stands for the time of the initiation of therapy, curve marked with YAS represent survival of animals with YAS without treatment, CMF stands for animals with tumor under the conventional (CY, MTX and 5-FU) treatment and curve marked with 'modified' stands for animals with tumor under the modified treatment.

Table 1

Body weight, peripheral blood and tumor growth. Methodology is described under 'Material and Methods'. 'Er' and 'Lu' stand for erythrocytes and leukocytes respectively. PBC count stands for peripheral blood cell count. In experiments with tumor-free animals, time in 'Days' refers to the time after the initiation of the therapy and with tumor-bearing animals, time in 'Days' refers to the time after the tumor inoculation. Since the therapy was initiated 3 days after the inoculation, experiments with treated tumor-bearing animals should be compared with analogous experiments with tumor-free animals with 3 more days on the time scale (i.e. day 5 is comparable with day 8, day 10 is comparable with day 13, etc.)

Days	Body weight	PBC count		Ascites		
		Er $\times 10^{12}$/l	Lu $\times 10^{9}$/l	Number of animals with ascites	Vol. (ml)	YAS cells $\times 10^{7}$/ml
Tumor-free animals under conventional treatment						
5	202 ± 21	3.4 ± 0.3	2.3 ± 0.1	0	0	0
10	206 ± 12	3.8 ± 0.1	9.7 ± 1.0	0	0	0
20	220 ± 27	3.8 ± 0.2	8.8 ± 0.9	0	0	0
30	259 ± 15	4.3 ± 0.1	2.4 ± 0.2	0	0	0
Tumor-free animals under modified treatment						
5	196 ± 55	4.5 ± 0.3	2.6 ± 0.1	0	0	0
10	210 ± 18	5.0 ± 0.2	7.7 ± 0.9	0	0	0
20	220 ± 19	5.2 ± 3.0	6.9 ± 0.7	0	0	
30	215 ± 7	5.3 ± 0.2	5.5 ± 0.4	0	0	0
Tumor-bearing animals under conventional treatment						
8	190 ± 27	2.3 ± 0.1	0.6 ± 0.3	0	0	0
13	200 ± 9	3.6 ± 0.2	9.5 ± 0.7	0	0	0
23	210 ± 11	4.7 ± 0.1	9.1 ± 0.8	2	4 ± 0.5	3 ± 0.6
33	215 ± 7	5.0 ± 0.2	13.9 ± 1.0	5	11.6 ± 1	14 ± 1.3
Tumor-bearing animals under modified treatment						
8	173 ± 8	2.9 ± 0.2	1.3 ± 0.1	0	0	0
13	210 ± 6	3.3 ± 0.2	19.5 ± 2.1	0	0	0
23	250 ± 11	4.5 ± 0.3	11.5 ± 1.7	1	2 ± 0.5	1.8 ± 0.4
33	240 ± 11	4.7 ± 0.1	11.0 ± 1.0	2	4 ± 0.7	5.3 ± 0.5
Tumor-bearing animals without any treatment						
3	200 ± 7	4.6 ± 0.3	8.9 ± 0.6	100	8.7 ± 1	9.2 ± 2.1
5	215 ± 6	3.9 ± 0.2	12.0 ± 1.0	100	23 ± 1.4	12 ± 4.0
7	187 ± 4	2.9 ± 0.2	35.0 ± 2.9	100	42 ± 2.6	8.3 ± 1.7
Normal values						
	208 ± 10	4.5 ± 0.3	9.4 ± 0.5	0	0	0

23 ml per animal with 12×10^7 cells/ml. After 7 days, the volume of ascites was 42 ml per animal with a decrease of cellularity to 8.3×10^7 cells/ml. In this group, all animals had ascites. In both treated tumor-bearing groups, the appearance of ascites was significantly delayed and the amount of ascites reduced. In these groups, we detected ascites first 23 days after the inoculation. The cellularity was significantly lower ($p < 0.001$) in case of modified treatment. Solid tumors appeared at the site of the inoculating needle penetration in 100% of controls B and in only 20% of controls A. In the group receiving the modified treatment no such solid tumors were observed within 33 days after the inoculation.

Metastases in the spleen. Metastases in the spleen of animals from the control B, 3 days after the inoculation, was observed in 10% of animals (1 animal out of the 10 sacrificed for the analysis), after 5 days in 50% of animals (5 of the 10 sacrificed) and after 7 days in the spleen of all animals. In both groups of YAS-bearing animals that received chemotherapy, no metastases were observed 23 days after the inoculation, while after 33 days they appeared in 33% (3 animals of the 10 sacrificed) only in the control A group.

Blood cell counts. We observed no dramatic blood count difference between the controls and the experimental group (Table 1 and Fig. 2). An expected cytotoxic effect was observed 8 days after the inoculation. An exception was in the YAS-bearing group undergoing modified treatment where the leukocyte count, 13 days after the inoculation, was more than 100% higher than normal values. In the same group, erythropenia was avoided.

Bone marrow analysis (Table 2). In all animals treated with cytostatics, there was a significant reduction ($p < 0.001$) in the number of marrow cells 5 days after

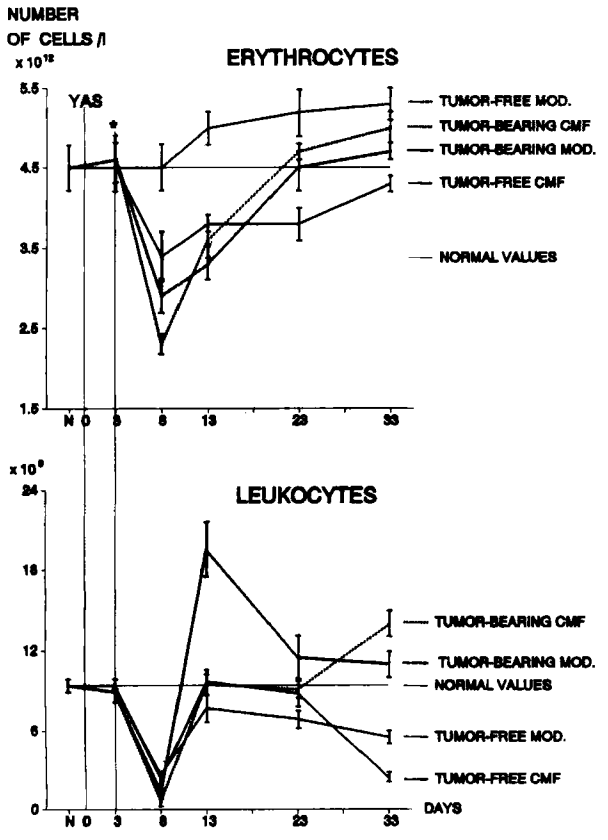

Fig. 2. Analysis of the peripheral blood performed as described in 'Material and Methods'. 'CMF' stands for the conventional therapy and 'MOD'. for the modified treatment. Error bars represent standard error of the mean.

treatment. This was more pronounced in tumor-bearing animals. In tumor-bearing animals without any treatment there was a small but statistically significant increase in total cellularity and in the count of erythroid elements. In all animals treated with cytostatics, 13 days after the inoculation and 10 days after the initiation of the therapy, the cellularity of the bone marrow approached normal values. In tumor-free animals under the modified treatment, total bone marrow cellularity (mostly due to granuloid elements) was significantly higher ($p < 0.001$) than the normal values at this time. Percentage-wise, all cell lineages contributed equally to this change. The same was true, but less pronounced, 33 days after the inoculaton.

Spleen analysis (Table 3). In all cases except the tumor-free animals under modified treatment, there was significant increase of the spleen weight. This was exceptionally large in case of tumor-bearing animals under the two types of cytostatic therapy, of which the modified treatment induced the highest increase of the spleen weight. Tumor-bearing animals without any treatment displayed significant increase of the spleen weight. The increase of the cellularity of the spleen did not parallel the increase of the weight of the spleen. In all animals, under cytostatic treatment, 8 days after the inoculation, there was a reduction of the spleen cellularity ($p < 0.001$). Animals under modified treatment displayed significant increase ($p < 0.001$) in the spleen cellularity 13 days after the inoculation. This increase was especially pronounced in tumor-bearing animals under modified treatment ($p < 0.001$). Increase of spleen cellularity was not observed in tumor-free animals under conventional treatment. Tumor-bearing animals under this treatment displayed significant increase, albeit significantly lower than that induced by the modified treatment. This increase, when observed, was mostly due to an increase of lymphoid elements.

Table 2

Bone marrow. Methodology is described under 'Material and Methods'. 'Er' and 'Gr' stand for erythroid and granuloid elements. Concerning 'Days', see text to Table 1

Days	Cellularity $\times 10^6$	Total number $\times 10^6$	
		Er	Gr
Tumor-free animals under conventional treatment			
5	6.0 ± 0.4	1.02 ± 0.94	2.02 ± 0.21
10	21.8 ± 2.0	2.02 ± 0.16	16.97 ± 1.48
20	18.7 ± 1.5	3.66 ± 0.39	9.84 ± 1.00
30	17.7 ± 1.1	4.36 ± 0.19	8.77 ± 0.7
Tumor-free animals under modified treatment			
5	3.7 ± 0.3	0.51 ± 0.04	2.02 ± 0.16
10	33.2 ± 3.1	6.41 ± 0.12	23.21 ± 2.10
20	22.2 ± 1.5	8.12 ± 0.75	11.24 ± 1.06
30	28.8 ± 2.2	8.07 ± 0.11	15.22 ± 1.31
Tumor-bearing animals under conventional treatment			
8	1.5 ± 0.3	0.19 ± 0.01	0.94 ± 0.01
13	17.1 ± 0.09	1.03 ± 0.11	14.33 ± 1.12
23	23.2 ± 2.1	4.78 ± 0.77	12.55 ± 1.30
33	22.8 ± 2.4	3.33 ± 0.39	11.13 ± 1.02
Tumor-bearing animals under modified treatment			
8	1.0 ± 0.1	0.10 ± 0.01	0.48 ± 0.03
13	21.5 ± 1.8	1.72 ± 0.13	17.87 ± 1.71
23	14.8 ± 1.3	4.04 ± 0.32	7.41 ± 0.61
33	20.6 ± 1.5	2.62 ± 0.18	13.45 ± 1.12
Tumor-bearing animals without treatment			
3	19.3 ± 0.5	2.14 ± 0.20	12.51 ± 0.95
5	25.0 ± 2.3	6.35 ± 0.45	12.03 ± 1.17
7	23.6 ± 2.0	5.69 ± 0.43	12.11 ± 1.20
Normal values			
	21.8 ± 2.0	3.97 ± 0.34	14.62 ± 1.25

Discussion

This report stems from the observation that the described modified polychemotherapy improves the survival of Yoshida sarcoma-bearing experimental rats. By the experiments presented we tried to substantiate the rationale for this observation. Notably, survival curves for YAS-bearing animals under the two types of treatment never reached plateau within the time span of experiments.

Table 3

Spleen. Methodology is described under 'Material and Methods'. 'Er', 'Gr' and 'Ly' stand for erythroid, granuloid and lymphoid elements. Concerning 'Days', see text to Table 1

Days	Weight (mg)	Cellularity $\times 10^6$	Total number $\times 10^6$		
			Er	Gr	Ly
Tumor-free animals under conventional treatment					
5	894 ± 31	295 ± 20	7.3 ± 0.7	6.6 ± 0.3	237 ± 14
10	1115 ± 40	424 ± 15	20 ± 1.0	27 ± 1.3	285 ± 17
20	706 ± 18	451 ± 35	26 ± 1.7	18 ± 0.8	300 ± 26
30	939 ± 79	488 ± 17	19 ± 0.9	34 ± 3.0	315 ± 11
Tumor-free animals under modified treatment					
5	313 ± 23	165 ± 15	0.8 ± 0.01	12 ± 0.8	150 ± 13
10	695 ± 39	868 ± 52	169 ± 9.7	84 ± 2.9	599 ± 30
20	535 ± 36	369 ± 26	2.6 ± 0.1	38 ± 1.5	325 ± 18
30	470 ± 13	379 ± 28	11 ± 0.9	58 ± 1.8	308 ± 21
Tumor-bearing animals under conventional treatment					
8	500 ± 40	116 ± 10	0	8 ± 0.7	106 ± 9
13	2300 ± 98	956 ± 74	57 ± 4.3	156 ± 11	731 ± 34
23	895 ± 61	350 ± 27	4 ± 0.3	64 ± 4.2	280 ± 12
33	1300 ± 72	530 ± 41	15 ± 1	95 ± 8	417 ± 22
Tumor-bearing animals under modified treatment					
8	661 ± 38	110 ± 10	0	11 ± 0.9	98 ± 6
13	3007 ± 96	1662 ± 66	80 ± 6.4	249 ± 18	1324 ± 76
23	1109 ± 58	352 ± 17	7 ± 0.5	51 ± 3.6	290 ± 13
33	1192 ± 64	674 ± 31	3 ± 0.2	95 ± 6.2	569 ± 23
Tumor-bearing animals without treatment					
3	1165 ± 61	440 ± 18	0	92 ± 7.1	342 ± 11
5	1202 ± 68	610 ± 21	0	72 ± 5.2	536 ± 36
7	3200 ± 89	871 ± 34	0	132 ± 8.7	726 ± 48
Normal values					
	430 ± 5	375 ± 17	9.7 ± 0.6	33 ± 2.0	330 ± 22

We did not pursue this matter since all animals with this experimental tumor, under these types of treatments, invariably develop metastases and die.

Experiments were performed using excessive tumor burden. This approach selected since, in our opinion, it provides more rapid data acquisition than analogous experiments performed with an order of magnitude lower tumor burden. Survival curves show that untreated animals died within 8 days following the tumor inoculation. The modified treatment was more than twice as efficient as the conventional protocol in preserving the life of inoculated animals 33 days after the inoculation. There was a time-dependent increase in volume of ascites. The cellularity did not parallel this increase, due probably to limited oxygen and nutrient supply. Both types of treatment delayed the appearance of ascites and lowered the cellularity, and the modified regimen was more efficient. Metastatic growth in the spleen was significantly reduced by the conventional treatment and suppressed by the modified protocol within the time of observation. The basis for the benefit of the modified protocol in comparison with the conventional treatment in reducing the population of rapidly growing Yoshida cells remains unclear at present.

The analysis of the peripheral blood cell count revealed two facts. First, the expected cytotoxic effect was followed by recovery, confirming that we were dealing with animals with normal physiology. Notably, erythropenia was avoided in case of tumor-free animals under modified treatment. Second, YAS-bearing animals under modified treatment displayed an exceptional peak in the leukocyte count. This strongly suggests the existence of factors of hematopoietic recovery related to the delayed application of CY. As CY is known to be either immunosuppressor or immunostimulator, depending on the therapy regimen, and antineoplastic agents often have overlapping immuno- and myelomodulating properties (12), this finding is not surprising.

In the bone marrow we observed the expected intial aplasia as consequence of the cytotoxic therapy. It was more pronounced in tumor-bearing animals. This might be due to the induction of a suppressor activity in the bone marrow associated with some tumors (17). On the other hand, the bone marrow analysis did not reveal such suppression in tumor-bearing animals not receiving cytostatic treatment. In tumor-free animals under modified treatment, there was after the aplasia phase significant increase of the bone marrow cellularity. This finding agrees with reports demonstrating hemopotentiating effects of CY therapy after proper dosing and timing (18, 19). These phenomena have complex mechanisms which probably are partly connected with preferential killing of suppressor T cells and induction of humoral stimulating activity (HSA). These potentiating properties of the modified protocol were not observed in tumor-bearing animals, probably because devastating initially induced aplasia could not be compensated for. In untreated tumor-bearing animals, a small but significant increase of the bone marrow cellularity was probably due to bleeding associated with rapid tumor growth.

The spleen analysis demonstrated dramatic increase of the organ weight in all cases except tumor-free animals under modified treatment. This suggests that the modified treatment exerts less stress on the organism than the conventional treatment. On the other hand, in tumor-bearing animals much higher increase of the spleen weight was induced by the modified than by the conventional treatment. This phenomenon suggests higher ability to recruit hemocompensatory activities of the spleen after modified treatment. In the absense of therapy, the tumor alone induced a 7–8-fold increase of the spleen weight. This clearly shows how serious trauma was the tumor burden exerted in terms of compensatory activity of the spleen. The conventional protocol, but not the modified protocol lowered this increase. The spleen cellularity did not parallel the variations of the weight, which underlines the benefit of the modified protocol. As expected, an initial cellular

aplasia of the spleen was observed. In untreated tumor-bearing animals a transient increase of the spleen cellularity was found, comparable to that of tumor-bearing animals under conventional treatment, but remarkably lower than in tumor-bearing animals under the modified protocol. This was the most striking feature of the described modality of therapy sequencing and probably the dominant parameter responsible for improved survival of animals inoculated with Yoshida sarcoma.

We hypothesize that beneficial properties of the modified treatment are mostly based on two facts. 1) On the time scale of experiments, properly timed CY-induced HSA peak partly counterbalances unavoidable cytotoxicity. 2) The preferential killing of suppressor T cells is more effective in case of the modified regimen due to triggering of resting hematopoietic cells into proliferation by the first application of cytotoxic drugs.

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