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Enhancement of the Immune System of Chemotherapy-treated Cancer Patients by Simultaneous Treatment with Thymic Extract, TP-1

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Summary. *Thirty patients with incurable gastrointestinal cancer were treated in a randomized clinical trial, either with combination chemotherapy (5-fluorouracil, vincristine, and methyl-CCNU) or with the same chemotherapy and the thymic extract TP-1. T-lymphocyte counts slowly and steadily,* increased *in the TP-1-treated patients over a period of 4–5 months, in contrast to* decreasing *counts in the patients treated by chemotherapy only. The changes in both groups were significantly different from pretreatment counts ($P < 0.01$). Skin tests for primary sensitization with DNCB were converted to positive or improved after TP-1 treatment ($P < 0.05$ against control patients). In conclusion, TP-1 had a progressively accumulating effect on T-cell lymphopoiesis and activity (skin tests), which was strong enough to overcome chemotherapy-induced immunosuppression without any appreciable side effects.*

Introduction

Thymic hormones have been under consideration in the last few years as potential immunotherapeutic agents against cancer [11]. Thymic hormones regulate the activity of T lymphocytes and improve their effector functions [2]. These functions can contribute to the host defense against his cancer [7]. However, in the cancer patient, immune functions become impaired both by the growing tumor [14] and by the treatment [13]. In vitro studies on peripheral blood lymphocytes of cancer patients indicate that thymic hormonal preparations can apparently correct measurable defects in T-lymphocyte characteristics [15, 25]. Moreover, thymic hormonal treatment of tumor-bearing mice was shown to have an impact on the survival of the animals [5, 16]. These considerations support the growing trend of testing thymic hormones in cancer treatment.

Several thymic hormonal preparations exist today [2, 8–10, 12, 17, 18]. Although some of these preparations, when tested comparatively, were shown to have certain similarities in their immunological effects, they nevertheless differ from each other in several important aspects of their activity [29]. It is, therefore, widely accepted today that the thymus may secrete more than one active factor [2]. As we do not know what preparation will be most appropriate for cancer treatment, it will be of advantage to test more than one of the available preparations. So far, only thymosin has been studied systematically in randomized clinical trials, with conflicting results: shortened disease-free interval in melanoma patients treated with thymosin, BCG, and DTIC [21], as opposed to prolonged survival of chemotherapy-treated patients with small-cell lung cancer [6].

The thymic extract selected by us for this study, TP-1, was isolated and partially purified by Bergesi and Falchetti [3]. The extraction and purification procedures (see Materials and Methods) of TP-1 are different, in some important details, from those used for other preparations [8, 12, 17, 18], and it may conceivably contain other active thymic factor(s). TP-1 was shown to be effective in replacement therapy of congenital cell-mediated immune deficiencies [1]. It was also shown to induce the appearance of mouse T-cell differentiation antigen (Thy-1), to increase the responsiveness of mouse spleen cells to PHA and ConA but not to lipopolysaccharide, and to stimulate the capacity of allogeneic mouse marrow cells to induce a graft-versus-host response in irradiated mice [9]. In order to avoid confusion with other, previously described thymic factors,TP-1 was recently renominated as "Thynostimuli".

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In the present study, patients with incurable gastrointestinal cancer were treated in a randomized trial with combination chemotherapy [20] and the thymic extract TP-1. The goals at this stage of the study were to evaluate the safety and immunological efficiency of this type of continuous immunomodulation. It will be shown that TP-1 had a profound effect on T-cell lymphopoiesis and function strong enough to overcome chemotherapy-induced immunosuppression without any appreciable side effects.

Materials and Methods

Thymus Extract

The thymic extract was prepared by Bergesi and Falchetti [3] from Instituto Farmacologico Serono by the following procedure. Calf thymuses were minced and extracted with ammonium acetate. The extract was heated to 70° C, filtered, and precipitated with ammonium sulfate. The precipitate was dissolved in water and subjected to ultrafiltration on an Amicon PM-10 membrane. The filtrate was desalted on Sephadex G-25 and gel-filtered on Sephadex G-50. The fractions that on polyacrylamide gel electrophoresis showed two characteristic bands with RF 0.22 and 0.42 were combined and called TP-1. The extract was lyophilized and its activity expressed as units of T-cell rosette formation per milligram of protein. It did not contain endotoxin. In toxicological studies, the extract did not cause any toxic or other side effects in doses up to 100 mg/kg when administered to mice for 21 days or to rats for 31 days or when administered to rats or dogs for 180 days in doses up to 50 mg/kg [9; R. Falchetti, personal communication]. The extract did not alter neuromuscular transmission either in vitro or in vivo [9]. This preparation was kindly provided by Instituto Farmacologico Serono, Rome, Italy.

Patients

Thirty patients with histologically proven adenocarcinoma of the stomach, pancreas, or colon-rectum entered the study in the first year. All of them were under 70 years of age, with local extensive and/or metastatic disease, but not bedridden (Karnofsky index ≥ 60) and without previous chemotherapy or radiotherapy. Of the 30 patients, 21 completed at least two cycles of treatment. The nature of the treatment was explained to the patients and informed consent was obtained.

Study Design and Evaluation of Clinical Effect

Patients were randomly allocated into three treatment groups (Table 1). Randomization was done separately for each of six subgroups according to the site of the primary tumor (stomach, pancreas, colon-rectum) and to the presence of measurable disease. Treatment was allocated by a person who did not see the patient and did not know his clinical condition. All patients received combination chemotherapy with three drugs, as specified in Table 1. Arm I was the 'control', i.e., chemotherapy alone, whereas patients in arms II and III were treated, in addition, with IM injections of TP-1 in two dose levels. Complete evaluation of the extent of disease (physical examination, liver function tests and liver scan, chest X-rays, bone scan, and other studies indicated in individual cases) was made before treatment and before every other cycle of the treatment (i.e., approximately every 3 months). The evaluation of clinical effect was based on measurements of tumor masses or hepatomegaly. In addition, survival data and indirect criteria were used, including change in performance status, weight, liver function tests, and ascites. Patients that showed progressive disease after two cycles of treatment or no change in measurable disease after three cycles of treatment, or who failed to follow the protocol were taken off the study.

Evaluation of Immunological Competence

This evaluation was based on skin tests and several in vitro assays. The skin tests included primary sensitization with dinitrochlorobenzen (DNCB), performed as suggested by Catalona et al. [4]. Both primary sensitization (with 2 mg DNCB in 0.1 ml acetone) and challenge (50 μg DNCB) were done before treatment. The challenge was repeated every second cycle. In addition, each patient was tested with five recall antigens: (1) PPD, intermediate strength (50 tuberculin units/ml, Ministry of Health, Israel); (2) tetanus toxoid (10 L_f/ml, Rafa Labs., Israel); (3) Candida and (4)

Table 1. Study design: chemoimmunotherapy for incurable gastrointestinal cancer[a]

Stratifiction:	1) Site: Stomach, colon, pancreas 2) Measurable and nonmeasurable disease	}	6 stratification categories
Randomization:	3 treatment groups I Chemotherapy alone II Chemotherapy + TP-1, low dose (0.1 mg protein/kg, IM) III Chemotherapy + TP-1, high dose (0.5 mg protein/kg, IM)		
Chemotherapy:	1) 5-FU 10 mg/kg/day	days 1–5	IV
	2) VCR 1 mg/m^2	day 1	IV
	3) MeCCNU 100 mg/m^2	day 2	PO
	To be repeated every 6 weeks (= treatment cycles)		
Immunotherapy:	TP-1 1) 10, 7, and 3 days before the 1st cycle 2) day 5 (last day of chemotherapy), then 3) 2 injections/week for 2 weeks 4) 1 injection/week for 3 weeks		

[a] Abbreviations: 5-FU, 5-fluorouracil; VCR, vincristine; MeCCNU, 3-*cis*-(2-chlorethyl)-1-notrosourea; TP-1, thymic product-1 of Serono

Trychophyton (0.01% suspension each. Israel Institute for Biological Research); (5) streptokinase-streptodornase (400/100 μ/ml, Lederle). The antigens (0.1 ml of each) were injected intradermally. These tests were also performed before treatment and before every second cycle. More frequent repetition of skin tests may sensitize and improve response [22, 28]. Results were read as suggested by Sokal [28], with induration graded from 0 to 4. Because of the limitations of the measurement procedure, only changes from negative to positive (or vice versa) or changes of at least two grades in a previously positive reaction were considered significant changes.

For the in vitro immunological studies, mononuclear cells were separated on Ficoll-Hypaque from patients' venous blood collected with heparin (10 μ/ml). These cell preparations contained 92%–97% mononuclear cells; 20%–25% of them were latex-eating cells. They will be referred to, conventionally, as peripheral blood lymphocyte (PBL) samples. Of the cells, 95%–100% were viable as tested by Eosin Y exclusion.

These PBL preparations were tested for the E rosettes with sheep red blood cells (SRBC), both total and active rosette formation (TR and AR, respectively), as described [26]. Briefly, for total rosettes, 50 μl PBL suspension (10^7/ml) was mixed with 100 μl 1% SRBC suspension and 50 μl inactivated fetal calf serum, centrifuged at 200 *g* for 5 min, incubated for 60 min at room temperature and read immediately. For active rosettes, 50 μl PBL suspension was incubated first with 50 μl fetal calf serum (60 min, room temperature) and then with 100 μl SRBC suspension (4×10^7/ml), after which the mixture was centrifuged and read immediately.

PBL were also tested in stimulation assays: mixed lymphocyte tumor culture (MLTC) with Raji lymphoma cells, as well as lectin stimulation with concanavalin A (Con A) and phytohemagglutinin (PHA), as described [27]. Briefly, 100 μl PBL suspension (10^6/ml) was mixed with 100 μl stimulator cell suspension (2×10^5/ml mitomycin-treated Raji cells) or various concentrations of PHA and Con A in the wells of round-bottom, microwell plates in Dulbecco's modified Eagle's medium with 10% inactivated pooled human AB serum. The cultures were incubated for 3 days for lectins and 4–5 days for MLTC, in a 5% CO_2 incubator; ^{3}H-thymidine (1 μCi) was added 18 h before termination of the assay.

The in vitro tests were repeated twice before treatment and then after the initial three TP-1 injections, and also before each chemotherapy treatment cycle. A complete blood count with differential was obtained on each of these occasions, as well as weekly throughout the study period.

Statistical Analysis

Statistical tests included analysis of covariance [30] to compare overall significance of differences among groups during the treatment cycles and further analysis, i.e., comparison between groups by linear contrasts. Other results were analyzed according to Student's *t*-test or Fisher's exact probability test for 2×2 tables.

Results

Clinical Data on Patients

Data about the patients that entered the protocol are summarized in Table 2. The distribution of the patients among the three treatment arms was good. Twenty-one of the patients completed at least two cycles of treatment and could be evaluated for the effect of the treatment on the immune system. (Skin tests were repeated every other cycle.) This number was, however, too small for meaningful clinical evaluation.

The side effects of the chemotherapy were gastrointestinal and hematological, as expected, and were well tolerated. No toxic or allergic, local or systemic, side effects attributable to TP-1 were noted.

Skin Tests

Table 3 describes the results of the skin tests in 20 patients in whom these tests were performed before treatment and after two cycles of it. The response to primary sensitization with dinitrochlorobenzen was initially poor (grade 1–2; 8 cases) or absent (12 cases). After treatment only one of the control

Table 2. Destribution of protocol patients in the three treatment arms

	I	II	III	Total
No. of patients entered	9	11	10	30
No. of patients died	4	8	6	18
Sex: Males	4	8	5	17
Age: Mean ± SEM[a]	61.7 ± 1.9	56.2 ± 3.0	63.2 ± 2.2	
Primary tumor: Stomach	3	4	4	11
Pancreas	1	2	1	4
Colon	5	5	5	15
Metastasis	6	5	5	16
Measurable disease[b]	7	7	5	19
Indirect criteria[c]	6	5	5	16

[a] No significant age difference
[b] Primary or metastasis
[c] Ascites, abnormal liver function tests etc.

Table 3. Skin tests of protocol patients

	Chemotherapy only (group I)				Chemotherapy + TP-1 (groups II and III)				P^f
	No. improved[c]/ No. tested	%[d]	Mean response[e]		No. improved[c]/ No. tested	%[d]	Mean response[e]		
			Before Rx	After Rx			Before Rx	After Rx	
DNCB test[a]	1/7	14.3	0.7	1.3	11/13	87.5	0.7	2.9	< 0.05
Recall antigens[b]	2/7	28.6	1.0	1.3	9/13	69.2	0.8	1.4	NS

[a] Evaluation of the intensity of DNCB reaction was done by grading (0–4) (see ref. 28) the response to challenge dose (or primary sensitization, if positive), before and after two cycles of treatment. No reaction (grade 0, 12 cases), or a weak one (grades 1–2, 8 cases) was observed before treatment (mean grade 0.7 in both groups)

[b] Response to recall antigens was evaluated by a change in the intensity of the reaction to one or more of the five antigens used (PPD, tetanus toxoid, Candida, Trychophyton and Streptokinase-streptodomase).

[c] No. of patients with improved skin test reactivity after two cycles of treatment. Improvement is either conversion from negative to positive reaction or upgrading of a positive reaction by at least two grades

[d] % of patients with improved skin tests

[e] Mean grade of response (grade 0 = no response; grade 4 = maximal response)

[f] P of difference between group I and II–III for number of patients improved. Fisher's exact probability test

patients converted, and the rest showed no change. In contrast, 11 of 13 of the TP-1-treated patients either converted or had significantly stronger reactions (more than one degree change in reaction intensity). A similar trend was noticed with the five recall antigens tested, although the results were not statistically significant.

Formation of T-cell Rosettes

In patients treated with chemotherapy only (group I) there was some trend toward decline in the percentage of rosettes (both TR and AR), which was, however, statistically insignificant. On the other hand, there was a highly significant increase in the percentage of both TR and AR in TP-1-treated patients (groups II and III). This effect was progressively accumulating (Table 4).

Lymphocyte Counts

Total number of lymphocytes, total T lymphocytes and active T lymphocytes per cubic millimeter were calculated from the WBC and the percentage of the respective cell types in the differential counts and the two E-rosette assays, all of which were performed on the same blood sample. For the present analysis we took two values: (1) the nadir counts (usually 2–4 weeks after chemotherapy), which represent the maximum depression of the bone marrow and immune system by the chemotherapy. (2) The recovery counts: the counts before the chemotherapy of the next cycle. These values were compared with the baseline before treatment. The baseline represents the mean of two consecutive counts taken before treatment. These counts were in good agreement with each other. In addition no difference in baseline counts was found among the treatment groups (Table 5). This validates the comparison of treatment effects.

The nadir counts were depressed by about 50% or more against the baseline. No effect of TP-1 treatment on either leukocyte or lymphocyte counts was demonstrated.

In contrast, the end-cycle (recovery) counts were significantly increased in TP-1-treated patients (Table 5). This increase was most pronounced for 'active' T lymphocytes and progressively less so for the total T cells, lymphocyte count, and white blood count. It is further emphasized by the significant decrease in counts noticed in the control, chemotherapy-treated, patients. These striking differences are clearly demonstrated when the counts are expressed as mean ± SEM of the posttreatment/pretreatment ratio (Fig. 1). The TP-1 effect was progressively accumulating and become more pronounced as the treatment continued. Indeed, no difference was found in PMN counts between TP-1-treated and -untreated patients (Table 5).

Lymphocyte Stimulation Assays

Lymphocytes from the protocol patients were stimulated by either Raji lymphoma cells or the plant lectin PHA and Con A. The changes in these assays

Table 4. Change in total and active E-rosettes inprotocol patients during treatment[a]

	% Rosettes					
	I ($n = 6$)		II ($n = 8$)		III ($n = 7$)	
	TR[c]	AR[c]	TR[c]	AR[c]	TR[c]	AR[c]
Before Rx	53.4 ± 3.8	23.5 ± 3.9	50.7 ± 3.0	24.0 ± 2.8	49.6 ± 4.0	25.4 ± 3.5
After TP × 3[b]	–	–	56.7 ± 2.3	28.3 ± 3.3	51.1 ± 2.2	27.1 ± 4.1
After 1st cycle[b]	52.1 ± 4.4	21.0 ± 1.5	58.4 ± 2.0	38.1 ± 3.9	58.6 ± 5.0	32.6 ± 4.3
2nd cycle	48.2 ± 1.7	21.5 ± 1.3	60.0 ± 3.1	36.8 ± 4.3	55.8 ± 5.2	37.5 ± 5.7

[a] Statistical analysis
Student's *t*-test for paired data:
Group I: No significant change in TR or AR after treatment
Group II and III: Elevation of percent TR or AR after initial three injections of TP-1 of borderline significance ($P = 0.06$ and 0.1, respectively). This change becomes highly significant after two cycles of treatment ($P < 0.005$ for TR and $P = 0.005$ for AR)

[b] Total and active rosettes were determined for each patient before treatment, after the first three injections of TP-1, which precede chemotherapy, and at the end of each treatment cycle of combined therapy (see Table 1 for details of treatment)

[c] Abbreviations: TR, total rosettes; AR, active rosettes

Table 5. White blood counts at the end of each treatment cycle (recovery counts)

	Treatment group[a]	Cell counts per mm^3					P[c]
		B[b]	C1	C2	C3		
WBC	I	9250 ± 1040	8600 ± 1005	7300 ± 998	5620 ± 606		< 0.02 ↓
	II	9830 ± 2115	6766 ± 488	8760 ± 1736	7900 ± 1238		NS
	III	8150 ± 722	6757 ± 801	7585 ± 724	6550 ± 386		NS
						II + III	NS
PMN	I	6927 ± 927	6396 ± 1187	5566 ± 619	4543 ± 257		NS
	II	7594 ± 1964	5069 ± 523	6797 ± 1646	4626 ± 683		NS
	III	6127 ± 603	4489 ± 492	5769 ± 617	4544 ± 301		NS
						II + III	NS
Lymphocytes	I	1518 ± 281	1745 ± 351	1044 ± 177	755 ± 200		< 0.01 ↓
	II	1505 ± 184	1437 ± 310	1624 ± 146	2831 ± 564		< 0.05 ↑
	III	1172 ± 129	1404 ± 462	1705 ± 325	1920 ± 276		NS
						II + III	< 0.01 ↑
T lymphocytes (TR)	I	911 ± 201	1089 ± 201	465 ± 101	379 ± 93		< 0.01 ↓
	II	769 ± 110	719 ± 149	996 ± 94	1815 ± 344		0.02 ↑
	III	531 ± 105	887 ± 465	968 ± 283	1276 ± 197		< 0.05 ↑
						II + III	< 0.01 ↑
T lymphocytes (AR)	I	356 ± 10	347 ± 78	190 ± 49	148 ± 40		< 0.01 ↓
	II	319 ± 46	492 ± 129	683 ± 109	1048 ± 229		< 0.01 ↑
	III	245 ± 84	459 ± 187	431 ± 81	632 ± 19		NS
						II + III	< 0.01 ↑

[a] I, $n = 6$; II, $n = 7$; III, $n = 5$

[b] Abbreviation: B, baseline counts (counts before treatment); C, treatment cycle; WBC, white blood counts; PMN, polymorphonuclear leucocytes; TR, total rosettes; AR, active rosettes

[c] For the statistical analysis the data were tested, after logarithmic transformation, by analysis of covariance. For this analysis we had one grouping factor, one trial factor and one covariate (baseline counts) that is constant over the trial. This test showed highly significant ($P < 0.001$) interaction between groups and treatment cycles for all the lymphocyte counts, and less significant interaction ($P = 0.028$) for WBC. Therefore, each group was tested separately for treatment effect by using linear contrast of the form $\bar{x}_3 - \bar{x}_1$ and the resulting P values of this test are shown here

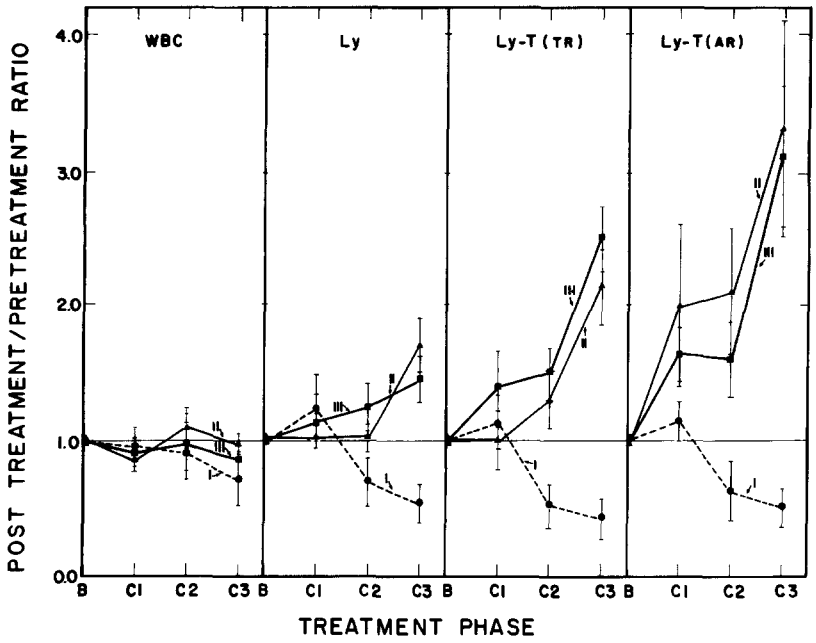

Fig. 1. Posttreatment/pretreatment ratios in cell counts in treatment arms I, II, and III in different treatment phases

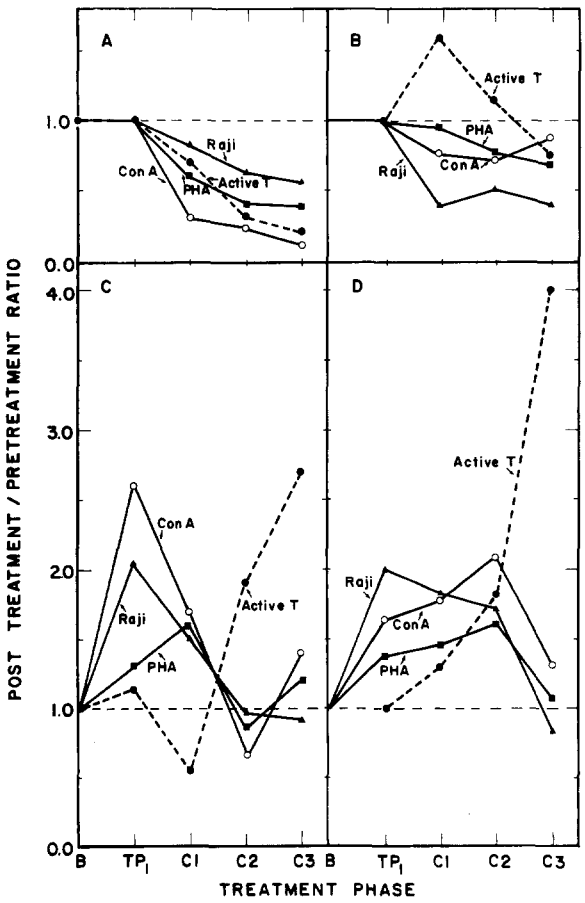

Fig. 2. Posttreatment/pretreatment ratios in active T lymphocyte counts and in response in 3 stimulation assays in the different treatment phases. Four individual cases: A and B (treatment arm I) = control; c (arm II) and D (arm III) = TP-1 treated

during the follow-up are less clearly related to TP-1 treatment than are the changes in lymphocyte counts and skin tests. Therefore, a larger number of patients is needed to test the possible correlation between treatment, clinical condition, and changes in these assays. Thus, while 11 of 12 TP-1-treated patients increased their T-lymphocyte counts, and 5 of 6 control patients decreased them, with the stimulation assays, only 8 TP-1-treated patients had better responses after treatment, and only 4 of them exhibited this improvement in all the three stimulation assays. These four patients had the best clinical course[1]. It has to be mentioned, however, that increase in response in these assays, when it occurred after TP-1 treatment, was only a transient one, in contrast to the progressive increase in lymphocyte counts (Fig. 2). In the control group, three patients had lower responses, one remained unchanged, and two had higher responses after treatment, but only to one of the lectins.

1 The four patients had local extensive adenocarcinoma of the stomach. At the end of two cycles of treatment all the four had improved Karnofsky index, three had gained weight, and one had objective response of his local tumor (> 50% reduction). Two of these patients are still alive, 18 and 15 months after randomization, one died after 13 months, because of an unrelated reason and one after 14 months because of his cancer

Discussion

The results presented here indicate a profound effect of the tested thymic extract on the immune system of the treated patients. This was demonstrated in two of the three types of assay used in this study: T-lymphocyte counts and skin tests. The lymphocyte stimulation assays did not give a clear-cut picture.

The parameter most strongly linked with TP-1 treatment is the *increase* in T-lymphocyte count in 11 of the 12 TP-1-treated patients, as compared with the *decrease* in the counts in five of six control patients. These changes do not seem to be related to disease state, but rather to the treatment per se: chemotherapy causes a progressive decline in the lymphocyte (and leukocyte) count and the addition of TP-1 causes a remarkable increase in the lymphocyte population. The TP-1 effect was maximal on the number of active T lymphocytes, with progressively diminishing effect on the number of total T lymphocytes, total lymphocytes, and white blood count (Fig. 1), as if the main responding population is represented by the active T lymphocytes and enumeration of the total populations progressively dilute the demonstrable effect.

Lymphocytopoietic effect of thymic extracts was recognized long ago [23]. Metcalf observed that the injection of saline extract of thymus into baby mice was followed by transitory lymphocytosis [19]. Here we have demonstrated, in a randomized clinical trial, that such an effect can be progressive and can overcome the combined natural and chemotherapy-induced immunosuppression of cancer patients. Moreover, the effect on cell number is accompanied by improvement in cell function, as judged from the results of the skin tests.

The results with the rosette assays (both TR and AR) (Table 4) and the calculated lymphocyte counts (Table 5) at the end of the TP-1 treatment period tended to be higher than those in our normal adult controls [26]. These differences have not yet reached statistical significance. However, it is quite possible that with chronic treatment, the accumulating effect does not stop at the restoration of the immune response to normal activity, as suggested by other studies [24, 25]. Instead, a higher-than-normal effect may be reached, analogous to the effect of chronic administration (or pathological hypersecretion) of other hormones, such as steroids, somatotropin, etc. Therefore it will be of interest to determine T-lymphocyte number and function after longer periods of treatment with the thymic extract.

Skin test reactivity to primary sensitization with DNCB was significantly improved after TP-1 treatment, and this was accompanied by a similar trend for improvement in reactivity to recall antigens (Table 3). These results are less clear-cut than those with the lymphocyte counts, because skin tests may convert or improve without treatment. This was shown to happen with DNCB [22] and recall antigens [28]. Possible causes for such spontaneous conversions are immune hyporeactivity with prolonged latency, repeated local sensitization, or improvement in the patient's condition, which may accelerate the appearance of the reaction. The control group without TP-1 accounts for such conversions, but nevertheless, we are left with a significantly higher rate of conversion in the TP-1-treated group.

As the treatment group was rather small and the patients had far advanced disease we did not expect conclusive *clinical* results. Lack of toxic or allergic side effects of TP-1 and its potent effect on T-cell number and function were, however, clearly demonstrated. TP-1 in combination with chemotherapy also prolonged the life of tumor-bearing mice and increased their cure rate against those achieved with chemotherapy alone [16]. These results, taken together, call for further clinical trials in which an efficient mode of cytoreductive treatment will be combined with a thymic hormonal preparation such as TP-1 as an immunomodulating agent.

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