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Immunomodulatory Effects of Alpha Interferon and Thymostimulin in Patients with Neoplasias

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In this report, we have evaluated the immunological effects following administration of alpha interferon (IFN-α) in combination with thymostimulin (TP-1), as well as of IFN-α and TP-1 alone in patients with neoplasias who underwent surgery and were subsequently treated with conventional chemotherapy. Data suggest that the combination of IFN-α and TP-1 is the most effective in the up-regulation of some immune parameters such as the $CD4^+$-$CD8^+$ cell-dependent antibacterial activity. Since this immune function plays an important role in the host protection against different targets such as invading microorganisms and/or neoplastic cells, the administration of TP-1–IFN-α is advisable for patients with neoplasias under chemotherapy.

In patients with neoplasias, certain immune alterations have been reported such as defective lymphocyte proliferation in cell cultures (18), decreased interleukin 2 (IL-2) production (6), or impaired in vitro release of tumor necrosis factor alpha (25). Since some immunological functions are specifically devoted to the destruction of tumor targets, i.e., cytotoxic activities exerted by T lymphocytes, macrophages, natural killer (NK) cells, and antibodies (23), many immunotherapeutic attempts have been made to induce an immune reaction resulting in tumor destruction (16, 24).

Antineoplastic activity of alpha/beta interferon (IFN-α/β) (10) has been reported in the course of certain diseases such as hairy cell leukemia (10), epidemic Kaposi's sarcoma (10), malignant melanoma (13), and breast cancer (4, 17). Concerning the mode of action of IFN-α on lymphocytes, it is well known that IFN-α induces the expression of major histocompatibility class I molecules in primary breast carcinoma cultures. This suggests that a possible indirect immunomodulatory effect of IFN-α could be mediated by the potentiation of cancer cell antigenicity (19). Additionally, the use of immunomodulators in combination with other cytotoxic drugs such as 5-fluorouracil (5-FU) or folinic acid has had a profound effect upon metastatic disease (7, 12, 15). Clinical studies have been performed combining IFN-α and 5-FU to potentiate the effect of 5-FU by increasing levels of active 5-FU metabolites in serum. Moreover, IFN-α can reverse resistance to 5-FU by inhibiting the overexpression of thymidylate synthetase (11). Thymic hormones have been repeatedly demonstrated to be immunomodulating agents through their effects on the immune system (9, 20). They are able to modulate bone marrow stem cell proliferation and differentiation (1, 2) and to induce the release of IFN-γ (22) and IL-2 and the expression of the membrane receptor for IL-2 (20). In particular, thymostimulin (TP-1) is able to increase cytotoxic activity of NK cells (20, 21).

On the basis of these properties, in a group of cancer patients we have investigated putative alterations of monocyte (MO), polymorphonuclear leukocyte (PMN), and $CD4^+$ and $CD8^+$ cell functional activities. At the same time, the efficacy of IFN-α–TP-1 combination therapy and of IFN-α and TP-1 alone on the correction of depressed immune functions has been evaluated.

In our study, 31 patients with breast carcinoma (18 females) or colorectal carcinoma (male/female ratio, 8/5) (age range, 27 to 52 years) were selected and randomized into four treatment groups for immunotherapy. In group 1, six patients with breast carcinoma were treated with only conventional chemotherapy. This group served as the control. In group 2, 12 patients with breast carcinoma were administered TP-1 (Serono, Rome, Italy) at a dose of 1 mg/kg/day during the first week and at the same dose three times weekly during the second, third, and fourth weeks. In group 3, five patients with colorectal carcinoma were treated with IFN-α (Serono) at the dose of 3×10^6 IU/day for 3 weeks. In group 4, eight patients with breast carcinoma were administered a combination of IFN-α–TP-1 following the same schedules as groups 2 and 3. All patients underwent surgery, and 2 weeks after the operation they received conventional chemotherapy (5-FU at 375 mg/m^2 for 1 to 5 days; folinic acid at 200 mg/m^2 for 1 to 5 days) for six cycles every 21 days (for a total of 180 days). For groups 2, 3, and 4, immunotherapy was carried out throughout the chemotherapeutic cycles. All patients were monitored before receiving chemotherapy-immunotherapy (time zero [T_0]) and at the end of the therapy (ET). Ten subjects (male/female ratio, 5/5) (age range, 25 to 51 years) were enrolled in this study to serve as normal controls.

Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) density gradient centrifugation (5), washed twice with Hanks' balanced salt solution (HBSS), and resuspended in RPMI 1640 (Flow Laboratories, Milan, Italy) supplemented with penicillin (100 IU/ml), streptomycin (100 μg/ml), glutamine (2 nM), and heat-inactivated fetal calf serum (5%) (complete medium).

To isolate PMN, cell pellets, after Ficoll-Hypaque density gradient centrifugation, were washed twice with HBSS and mixed with 6% dextran (molecular weight, 40,000) in HBSS. After sedimentation for 1 h at 37°C, PMN-rich supernatants

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TABLE 1. Percentage of ABA in cancer patients under chemotherapy administered with immunomodulators

Group	Sample time	Mean (SE) at effector/target ratio:			
		1:3	1:1.5	1:0.7	1:0.3
Healthy controls		51.5 (4.3)	43.5 (4.9)	39.0 (4.7)	26.9 (37.1)
Group 1, control patients	T_0	38.7 (6.8)	30.1 (5.5)	23.6 (5.9)	14.3 (6.0)
	ET	24.8 (8.8)	18.4 (9.1)	10.7 (4.9)	24.8 (8.9)
	P value	0.181	0.128	0.042[a]	0.216
Group 2, TP-1 patients	T_0	37.0 (5.5)	24.0 (5.1)	16.2 (4.3)	5.5 (2.8)
	ET	35.9 (6.3)	24.9 (5.3)	13.6 (4.5)	35.9 (6.3)
	P value	0.928	0.937	0.741	0.003[a]
Group 3, IFN-α patients	T_0	44.0 (7.0)	27.7 (5.9)	16.2 (7.6)	11.9 (5.3)
	ET	33.1 (9.6)	24.0 (8.6)	19.1 (8.0)	33.1 (9.6)
	P value	0.292	0.675	0.801	0.106
Group 4, TP-1–IFN-α patients	T_0	22.4 (4.7)	16.3 (4.8)	10.2 (4.5)	3.2 (1.3)
	ET	50.5 (4.4)	39.9 (4.1)	26.6 (4.7)	50.5 (4.4)
	P value	0.003[a]	0.004[a]	0.064[a]	<0.005[a]

[a] Significant differences for $\alpha < 0.10$.

were collected, centrifuged at 400 × *g* for 10 min, and subjected to osmotic lysis by a cold mixture of 10 ml of phosphate-buffered saline (PBS) plus 20 ml of distilled water for 1 min. Then, cells were washed again with HBSS by centrifugation (400 × *g* for 10 min) and resuspended in complete medium. Their morphology was examined by Wright's stain (about 95% PMN).

The above cell preparations, resuspended in complete medium, were used for the following assay. To measure the capacity of PMN and MO to engulf and digest antigens, *Candida albicans* was used as a target, according to a widely employed methodology (14). Briefly, 10×10^6 PBMC or PMN were incubated with 30×10^6 *C. albicans* cells for 30 min at 37°C. Thereafter, phagocytosis was microscopically evaluated by counting the number of phagocytes which engulfed *C. albicans*. Killing was calculated by lysing phagocytes and counting CFU developed depending on the number of live organisms in the cells.

For antibacterial activity (ABA) assay (3), 10^4 *Salmonella typhi* bacteria (target cells) contained in a final volume of 0.1 ml were put into 5-ml conical tubes together with RPMI and centrifuged at 1,300 × *g* for 10 min at 4°C. The lymphomononuclear cell suspension (0.1 ml) (effector cells) was then added to bacteria at different effector-to-target ratios (1:3, 1:1.5, 1:0.7, 1:0.3), and tubes were again centrifuged at 500 × *g* for 5 min at 4°C. The experimental and control tubes, which contained bacteria and RPMI but not cells, were then incubated at 37°C for 2 h. At the end of the incubation period, appropriately diluted aliquots of cells were placed on petri dishes containing agar tryptose. After overnight incubation, CFU were counted. The percentage of ABA was calculated as follows: 100 − [100 × (number of CFU in experimental tubes)/(number of CFU in control tubes without lymphocytes)]. Surface phenotypes of PBMC were analyzed by monoclonal antibodies to lymphocyte surface antigens. Absolute numbers of $CD3^+$, $CD4^+$, $CD8^+$, $CD14^+$, and $CD20^+$ cells were determined by using fluorescein isothiocyanate-conjugated anti-Leu4, anti-Leu3, anti-Leu2, anti-LeuM3, and anti-Leu16 monoclonal antibodies, respectively (Becton Dickinson, Milan, Italy). PBMC suspensions (10^6) were incubated with 10 μl of fluorescein-conjugated monoclonal antibodies for 30 min at 4°C. Then, cells were washed twice in cold PBS, resuspended in the same medium, and examined with a microscope equipped for epi-illumination fluorescence and phase contrast. The percentage of positive cells was calculated by scoring at least 300 cells per slide.

Changes in mean values from T_0 to ET were tested for

TABLE 2. Percentage of phagocytosis and killing activities of PMN and MO in cancer patients under chemotherapy administered with immunomodulators

Group	Sample time	Mean (SE) for:			
		Phagocytosis		Killing	
		PMN	MO	PMN	MO
Healthy controls		78 (1.6)	76.6 (2.9)	96.4 (2.0)	92.3 (1.6)
Group 1, control patients	T_0	45.5 (6.1)	35.9 (4.4)	66.8 (10.0)	69.8 (11.7)
	ET	41.8 (3.8)	46.4 (6.2)	77.15 (5.4)	78.3 (6.3)
	P value	0.573	0.062[a]	0.464	0.605
Group 2, TP-1 patients	T_0	46.3 (4.3)	40.4 (3.1)	67.4 (7.5)	66.5 (7.7)
	ET	54.6 (2.7)	39.8 (2.4)	81.3 (4.5)	81.8 (2.9)
	P value	0.108	0.849	0.205	0.078[a]
Group 3, IFN-α patients	T_0	45.1 (5.7)	34.1 (3.3)	64.7 (8.8)	63.4 (11.2)
	ET	49.7 (3.2)	39.0 (3.4)	79.3 (7.4)	72.2 (8.7)
	P value	0.388	0.372	0.339	0.669
Group 4, TP-1–IFN-α patients	T_0	50.7 (4.1)	41.8 (3.8)	82.8 (2.9)	89.4 (2.6)
	ET	51.9 (2.5)	46.2 (4.2)	87.0 (6.4)	88.4 (2.4)
	P value	0.827	0.482	0.561	0.77

[a] Significant differences for $\alpha < 0.10$.

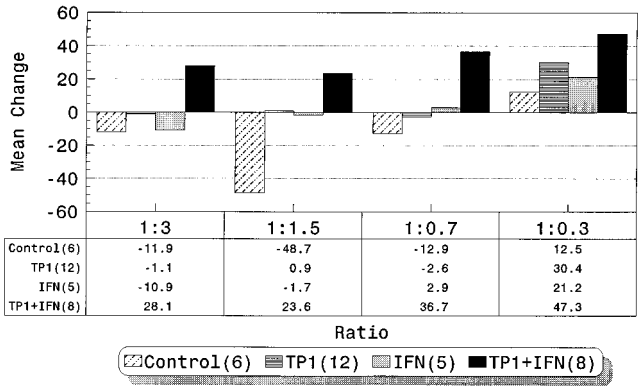

FIG. 1. Percentage of ABA in cancer patients under chemotherapy receiving different immunomodulating regimens. Shown is the mean change from T_0 to ET. ABA was determined by incubating various effector (PBMC)/target (*S. typhi*) ratios overnight at 37°C. Actual salmonella killing was evaluated on the basis of CFU developed in tryptose soy agar plates. The number of samples is shown in parentheses.

significance by using the paired *t* test. The level of significance in this study was set to 0.10.

In our study, it is evident that all patients enrolled exhibited multiple deficits in terms of PMN and MO phagocytosis and killing and ABA. This last activity is sustained by $CD4^+$ and $CD8^+$ cells, and in particular, in areas where salmonellae are endemic (e.g., the Bari area, southern Italy) this function is extremely enhanced (3). All the above results are expressed in Tables 1 and 2.

With particular reference to the immunomodulators used, our data show that the combination TP-1–IFN-α was able to significantly increase ABA without modifying PMN and MO-impaired functions. These results are illustrated in Tables 1 and 2 and in Fig. 1.

On the other hand, absolute numbers of $CD3^+$, $CD4^+$, $CD8^+$, and $CD20^+$ cells fluctuated within normal ranges, regardless of the immunomodulating regimen used (data not shown). In the case of the combined treatment TP-1–IFN-α, an increase of $CD14^+$ cells (predominantly MO) was evident at ET, as also confirmed by differential leukocyte counts (data not shown).

Conversely, single administration of TP-1 and IFN-α, respectively, did not lead to a substantial modification of the immunodepression observed at T_0, in terms of both phagocyte and T-cell-dependent functions.

The explanation of these results may rely on the capacity of TP-1 and IFN-α to synergistically act on the same target. In fact, TP-1 acts on T cells via production of IL-2 and IFN-γ (22), while IFN-α is a potent enhancer of cytotoxic T cells (19). On the other hand, there is a general agreement on the incapability of both IL-2 and IFN-α to correct PMN and MO functional deficits, since these cell types are scarcely responsive to the above cytokines (8).

Finally, in clinical terms, patients administered the combination TP-1–IFN-α could complete chemotherapeutic cycles without interruptions and underwent fewer infectious events in comparison with individuals receiving a different therapeutic regimen (data not shown).

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