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Antitumor Activity of Host T and Non-T Cells Recovered from Tumor Nodules after Interferon Therapy

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We examined the modification of host T cells of tumor nodules by interferon (IFN) therapy in mouse models. The host cells were recovered from regressing tumor nodules of mice at Day 13 after intradermal tumor inoculation at Day 0 and administration of 5×10^5 U/mouse/day IFN at Day 6 to Day 10. These host cells neutralized *in vivo* Meth A growth in a dose-dependent fashion. *In vitro* treatment of these cells with anti-Thy 1.2 monoclonal antibody and rabbit sera as a source of complement abrogated their tumor-neutralizing activity, but only partially, indicating that both T cells and non-T cells were involved in tumor neutralization. The finding that host cells from regressing tumor nodules of either Meth A or Meth 1, an antigenically distinct fibrosarcoma, neutralized both Meth A and Meth 1 tumors without much selectivity was consistent with possible non-T cell involvement. Most of these characteristics of host cells of regressing nodules of IFN-administered mice were also noted with host cells of progressing nodules of placebo-administered mice and there was no significant difference in neutralizing activity qualitatively or quantitatively between the two sources of host cells. There was no significant difference in host T and B cell numbers and compositions of regressing and progressing nodules either. These essentially negative findings raise the possibility, among others, that the primary target host cells to be modified by IFN were not T cells, although the therapeutic effect of IFN was dependent on the host T cells.

Key words: Tumor nodule — Host cell modification — Interferon-α

Antitumor activity of interferons (IFN)-α, β is reproducibly found in different animal models. However, the mechanisms involved in tumor eradication have not been fully clarified. With regard to the host modifications induced by IFN, antitumor macrophages were proposed to be effector cells responsible for tumor eradication.[1] Based on the failure to identify involvement of any cellular and soluble factors in tumor regression by IFN therapy, another proposal was recently made, that the antitumor effect is mediated by mechanisms that have not been seriously considered heretofore.[2]

On the other hand, we previously reported that the antitumor effect of IFN was achieved in a host T cell-dependent fashion.[3] A further study identified the enhanced production of antitumor T cells in lymphoid organs in tumor-bearing mice treated with IFN.[4] The present study was primarily concerned more directly with the host antitumor effector T cells in tumor nodules after IFN therapy. Paradoxical results were obtained, in that antitumor effector cells consisting of T cell and non-T cell populations were recovered not only from the regressing tumor nodules of IFN-administered mice but also from the progressing tumor nodules of placebo-administered mice, and no qualitative or quantitative difference between the two sources could be found under the present experimental conditions. Further examination of the regressing and progressing nodules did not show any significant difference in T and B cell numbers and compositions between the two sources either. These essentially negative results raise the possibility that the primary target host cells of IFN were not T cells, although the therapeutic effect of IFN was dependent on host T cells.

MATERIALS AND METHODS

Mice and tumors Female BALB/c mice were obtained from Japan Charles River Co., Ltd. (Atsugi, Kanagawa). Meth A fibrosarcoma was supplied by Dr. Y. Hashimoto. Meth 1 fibrosarcoma, supplied in ascitic form by Dr. M. Morimoto, was subcutaneously passaged in our laboratory, and its transplantable fraction was selected.

IFN IFN-α A/D (*Bgl*) was purified from *E. coli* 294 cell lysates by a combination of methods described previously.[5] The purified material was homogenous on polyacrylamide gel electrophoresis and had a specific activity of more than 10^8 U/mg protein when titrated with bovine kidney cells (MDBK). Antiviral activity was titrated against the NIH standard (Gxa 01-901-535). IFN was

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supplied in 0.9% NaCl solution containing 100 μg/ml IFN and 1 mg/ml mouse serum albumin, Fraction 5, prepared from DDY mice. Placebo contained the same ingredients as above except IFN.

Therapy by IFN Groups of mice were inoculated intradermally (id) with 5×10^5 tumor cells/site at both right and left flanks. They were given intravenously 5×10^5 U/mouse/day IFN or placebo during Day 6 to Day 10 after tumor inoculation. Tumor nodules of these mice were surgically resected 3 days after cessation of therapy, when tumor nodules of IFN-administered mice were in the process of regression whereas those of placebo-administered mice continued to progress.

Cell preparation of tumor nodules Single cells were prepared from tumor nodules according to the previous report[6] with slight modifications. The tumor nodules after excision were cut into pieces and minced with scissors, then incubated for 1 h at 37°C in an enzyme mixture consisting of 3200 U/ml collagenase (type IV, Sigma Chemical Co., St. Louis, MO) and 800 U/ml DNase (type IV, Sigma) in Hanks' balanced salt solution with magnetic stirring. The digested mixtures were filtered through 4 sheets of gauze. To separate viable cells from dead cells, the cell suspension was overlaid on Ficoll Paque (Pharmacia Fine Chemicals, Uppsala, Sweden) and centrifuged for 20 min at 700*g*. The top and interface layers were collected. Over 95% cell viability was routinely achieved, and the cells were differentially counted easily for Meth A cells and host cells based on the difference of cell size and shape.

Host cells were separated from the cell mixture by density gradient centrifugation. The cell suspension (2 ml) was layered on a stepwise gradient of 4 ml each of 10% and 15% bovine serum albumin (Miles Laboratories Inc. Naperville, IL). After centrifugation for 20 min at 70*g* at room temperature, the 10% bovine serum albumin layer, in which host cells were accumulated, was collected. The host cell preparation thus prepared contained less than 10% Meth A cells.

***In vivo* tumor neutralization test (Winn test)** The indicated numbers of host cells were mixed *in vitro* with Meth A or Meth 1 cells ($1.5\text{–}2.0 \times 10^5$) and id inoculated into naive mice at the flank. Tumor sizes were measured at the longest (a) and shortest (b) dimensions and expressed as $\sqrt{ab}$.

***In vitro* treatment of host cells** Host cells (10^7/ml) were incubated for 45 min in ice in the absence or presence of anti-Thy 1.2 antibody at 2,000- to 3,000-fold dilution (Olac 1976 LTd., Oxon, England). The mixture was centrifuged at 500*g* and the sediment was suspended in rabbit complement (Low-Tox-M, Cedarlane Laboratories, Ltd., Ontario, Canada), 20-fold diluted unabsorbed solution or 10-fold diluted solution absorbed with BALB/c thymocytes (1.5×10^7/ml) and spleen cells (1.5×10^7/ml) by incubation for 1 h in ice. The cell suspension was incubated at 37°C for 45 min to 1 h. Throughout the experiment, cytotoxicity medium (Cedarlane) was used as the incubation and diluting medium. Under the present experimental conditions T cells of tumor nodules were quantitatively killed and, functionally, tumor neutralizing activity of T cells was completely abrogated in spleen and lymph nodes as reported previously.[4]

Cell composition analysis Host cells were incubated with 1% paraformaldehyde (E. Merck, Darmstadt, W. Germany) in ice for 30 min and fixed. These cells were incubated in ice for 30 min with either anti-Thy 1.2 antibody labeled with fluorescein isothiocyanate (Becton Dickinson Immunocytometry Systems, Mountain View, CA, 280-fold diluted), or goat anti-mouse IgM antibody ($F(ab')_2$ fragment) labeled with rhodamine (Cappel Laboratories, West Chester, PA, 10-fold diluted). The stained cells were observed under a fluorescence microscope (Leitz Dialux 20B, Ernst Leitz Wetzler, Wetzler, W. Germany). More than 200 cells were scored.

Statistics Differences of tumor sizes of mice between two groups were statistically evaluated by means of the t test. Differences in the incidences of tumor-free mice were evaluated by using the Fisher exact test.

RESULTS

Tumor-neutralizing activity of host cells of regressing Meth A nodules Administration of IFN according to the regimen used throughout the present study regressed Meth A nodules and further extensive administration of IFN resulted in complete cure of Meth A-bearing mice as reported previously.[3] In the present study, host cells prepared from regressing tumor nodules of Meth A-bearing and IFN-administered mice were assayed for Meth A-neutralizing activity in naive recipient mice (Fig. 1). The growth of 2×10^5 Meth A cells was completely suppressed by 10^6 host cells, while with smaller numbers of host cells Meth A cells grew. There was thus a host cell dose dependency of Meth A growth inhibition in the present neutralization test.

The involvement of nodule T cells in Meth A neutralization was examined (Fig. 2) since the previous study had shown that the therapeutic effect of IFN depended on the host T cells in this animal model.[3] The results showed that the quantitative *in vitro* killing of T cells by anti-Thy 1.2 antibody and complement abrogated the neutralizing activity of those host cells, but only partially, indicating that both T and non-T cells were involved in the present Meth A neutralization. These results were in sharp contrast with the results on spleen and lymph node cells of Meth A-bearing and IFN-administered mice in

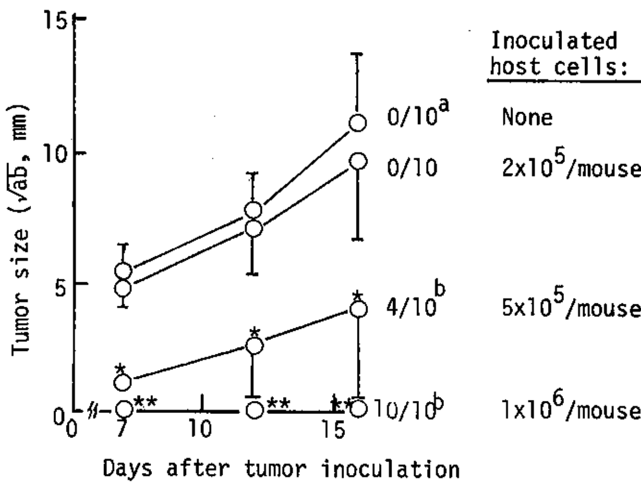

Fig. 1. Dose dependency of tumor-neutralizing activity of nodule host cells in IFN therapy. Meth A-bearing mice were administered with IFN. The indicated numbers of host cells of tumor nodules prepared from these mice were mixed *in vitro* with 2×10^5 Meth A cells and the mixture was id inoculated into naive mice. Tumor sizes were measured at the indicated intervals. Mean values and standard deviations are included. For further details, see "Materials and Methods." Asterisks indicate the statistical significance of differences in tumor size at $P<0.05$ as determined by the *t* test (* vs. "None" group, ** vs. other groups). a. No. of tumor-free mice/total at the termination of experiments (Day 21). b. Statistically significant at $P<0.05$ by the Fisher exact test vs. "None" group.

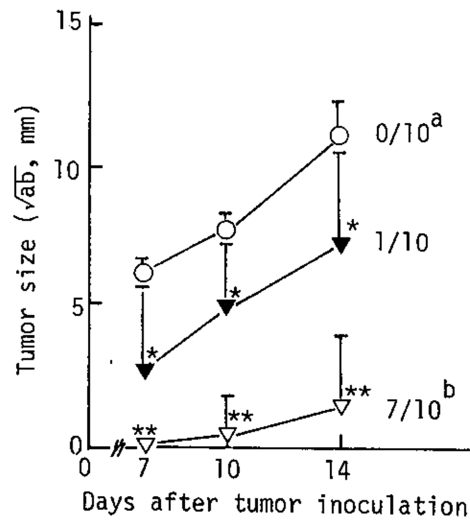

Fig. 2. Tumor-neutralizing activity of T and non-T host cells of regressing nodules. Host cells from tumor nodules of Meth A-bearing mice administered with IFN were treated *in vitro* with either complement alone (▽) or anti-Thy 1.2 antibody and complement (▼). These cells (10^6) were mixed with 2×10^5 Meth A cells and id inoculated into mice. Control mice were id inoculated with 2×10^5 Meth A cells alone (○). Tumor sizes were measured at the indicated intervals. Mean values and standard deviations are included. For further details, see "Materials and Methods." For asterisks, a, and b, see the legend to Fig. 1.

which the same *in vitro* treatment killing T cells completely nullified their Meth A-neutralizing activity.[4]

Tumor-neutralizing activity of regressing and progressing nodules The association of Meth A-neutralizing activity of nodules with the therapeutic effect of IFN was examined. Host cells recovered from regressing Meth A nodules of IFN-administered mice were compared with those cells recovered from progressing Meth A nodules of placebo-administered mice for neutralizing acitivity (Fig. 3). Host cells of both sources suppressed Meth A growth significantly (vs. the control) but there was no significant difference in neutralizing activity between the two sources of host cells. Further study was carried out to look for any difference in their antitumor effector cell populations and tumor selectivity.

Antitumor cell populations of progressing Meth A nodules Host cells of progressing nodules of placebo-administered mice and those of regressing nodules of IFN-administered mice were treated *in vitro* with anti-Thy 1.2 antibody and complement or complement alone and their Meth A-neutralizing activities were compared (Fig. 4). Host cells of both sources treated with complement alone neutralized Meth A and no difference was

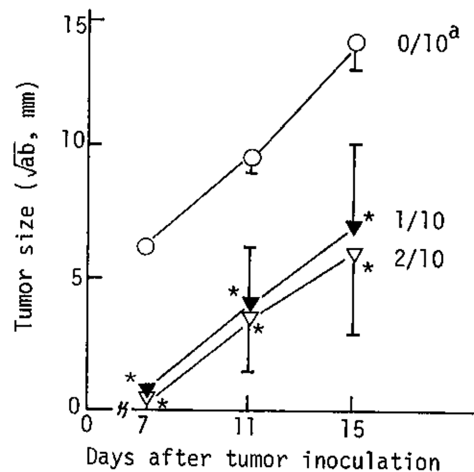

Fig. 3. Tumor-neutralizing activity of host cells of regressing and progressing nodules. Host cells were prepared from regressing Meth A nodules of IFN-administered mice (▼) and from progressing Meth A nodules of placebo-administered mice (▽). These cells (10^6) were mixed with 2×10^5 Meth A cells and id inoculated into mice. Control mice were id inoculated with 2×10^5 Meth A cells alone (○). Tumor sizes were measured at the indicated intervals. Mean values and standard deviations are included. For further details, see "Materials and Methods." For asterisks, a, and b, see the legend to Fig. 1.

found between them. The killing of T cells abrogated their neutralizing activity, but only partially, and again no difference was found in the neutralizing activity left in host cells from progressing and regressing nodules after T cell killing.

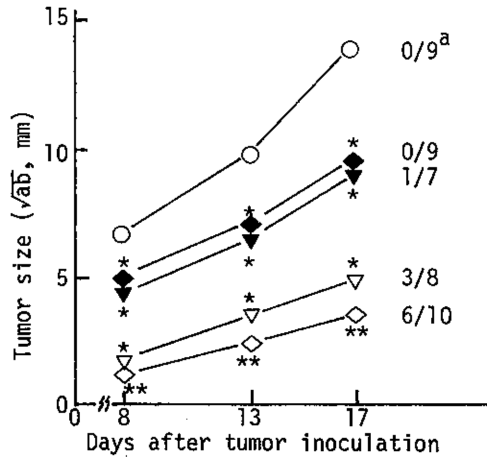

Fig. 4. Tumor-neutralizing activity of T and non-T cells of progressing and regressing nodules. Host cells from progressing Meth A nodules of placebo-administered mice (◇, ◆) and those from regressing Meth A nodules of IFN-administered mice (▽, ▼) were treated *in vitro* with complement alone (◇, ▽) or anti-Thy 1.2 antibody and complement (◆, ▼). These cells (7.5×10^5) were mixed with 2×10^5 Meth A cells and id inoculated into mice. Control mice were id inoculated with 2×10^5 Meth A cells alone (○). Tumor sizes were measured at the indicated intervals. For simplicity, standard deviations are not included. For further details, see "Materials and Methods." For asterisks and a, see the legend to Fig. 1.

Tumor selectivity of host cells of regressing and progressing nodules Tumor nodules of mice bearing Meth A and Meth 1, an antigenically distinct fibrosarcoma, and administered with IFN or placebo were used to examine the difference in tumor selectivity of neutralizing activity of host cells from regressing and progressing nodules. The host cells of regressing Meth A nodules suppressed not only Meth A growth but also Meth 1 growth, showing cross-reactive neutralizing activity (Fig. 5A). This was also the case with the host cells of regressing Meth 1 nodules (Fig. 5B).

The host cells of progressing tumor nodules were examined under the same experimental conditions as above. The host cells from progressing nodules of either Meth A or Meth 1 suppressed both Meth A and Meth 1 tumors (Fig. 6A, B) showing cross-reactive neutralizing activity on both Meth A and Meth 1 progressor nodule cells. These results show that there was no difference between host cells of regressing and progressing nodules in the tumor selectivity of neutralizing activity.

Cell composition of progressing and regressing Meth A nodules The cell compositions of nodules of Meth A-bearing mice treated with placebo and IFN were comparatively analyzed to obtain a clue to the mechanisms of Meth A regression. In terms of cell composition ratio Meth A cells were dominant over host cells in the progressing nodule whereas host cells were dominant over Meth A cells in the regressing nodule (Table I). However, in terms of the absolute numbers per nodule, host cell numbers of the regressing nodule were not significantly different from those of the progressing nodule. A further study was carried out to examine the host cell composition of the progressing and regressing

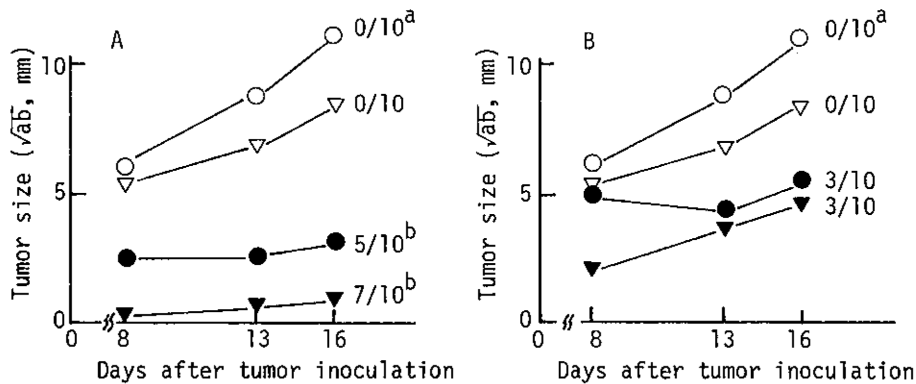

Fig. 5. Tumor selectivity of neutralizing activity of host cells of regressing nodules. Host cells (5×10^5) from regressing tumor nodules of IFN-administered Meth A-bearing (panel A) and Meth 1-bearing (panel B) mice were mixed with 2×10^5 of either Meth A (●) and Meth 1 (▼) cells and id inoculated into mice. Control mice were id inoculated with 2×10^5 Meth A (○) or Meth 1 (▽) cells alone. Tumor sizes were measured at the indicated intervals. For simplicity, standard deviations are not included. For further details, see "Materials and Methods." For a and b, see the legend to Fig. 1.

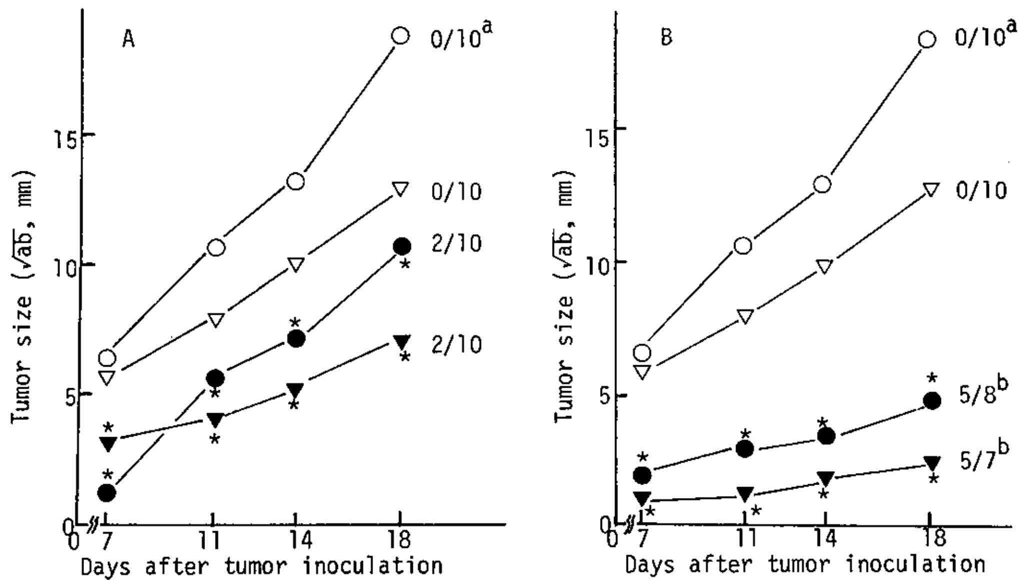

Fig. 6. Tumor selectivity of neutralizing activity of host cells of progressing nodules. Host cells (7.5×10^5) from progressing tumor nodules of placebo-administered Meth A-bearing (panel A) and Meth 1-bearing (panel B) mice were mixed with 1.5×10^5 Meth A (●) or Meth 1 (▼) cells and id inoculated in mice. Control mice were id inoculated with 1.5×10^5 Meth A (○) or Meth 1 (▽) cells alone. Tumor sizes were measured at the indicated intervals. For simplicity, standard deviations are not included. For further details, see "Materials and Methods." For asterisks, a, and b, see the legend to Fig. 1.

Table I. Cell Composition of Meth A Nodules

Meth A-bearing mice treated with:	Weight of nodule (mg)	Total viable cells ($\times10^6$/nodule)	% of total viable cell	
			Meth A cells	Host cells
Placebo	265 ± 28	16.7 ± 5.6	75 ± 3.2 (12.3 ± 3.6)[a]	25 ± 3.2 (4.3 ± 2.0)[a]
IFN	127 ± 15[b]	5.5 ± 1.3[b]	30 ± 4.7[b] (1.7 ± 0.35)[a, b]	69 ± 4.7[b] (3.8 ± 1.0)[a]

Meth A-bearing mice were administered with placebo or IFN from Day 6 to Day 10, and at Day 13 after tumor inoculation, host cells in the tumor nodules were examined. For further details, see "Materials and Methods."

a) $\times10^6$ cells/nodule. Mean±SD of 3 experiments.

b) Statistically significant at $P<0.05$ by the t test vs. the corresponding placebo group.

nodules (Table II). There was no difference in T and B cell populations between the two types of nodules either.

DISCUSSION

The present study was primarily concerned with the antitumor host T cells of tumor nodules responsible for tumor regression in IFN therapy. Host cells from regressing Meth A nodules of mice administered with IFN showed antitumor activity and neutralized Meth A tumor in a dose-dependent fashion. The host cells of the regressing nodules involved in the neutralization were identified as T cells and non-T cells. These host cells neutralized not only Meth A cells but also Meth 1 cells, an antigenically distinct tumor. This was also the case with host cells from regressing Meth 1 nodules; they neutralized not only Meth 1 tumor but also Meth A tumor. These results showed that the tumor-neutralizing activity of host cells was cross-reactive between two antigenically distinct fibrosarcoma cells. On the other

Table II. Host Cell Composition of Meth A Nodules

Meth A-bearing mice treated with:	% of total host cells	
	$Thy1.2^+$ cells	μ^+ cells
Placebo	7.3±0.94[a]	2.3±1.2
IFN	6.3±0.94	3.0±1.4

Meth A-bearing mice were administered with placebo or IFN from Day 6 to Day 10, and at Day 13 after tumor inoculation, host cells of tumor nodules were examined for the markers indicated in the table. For further details, see "Materials and Methods."
a) Mean±SD of 3 experiments.

hand, a study of host cells from progressing nodules showed that these characteristics of the host cells from the regressing nodules were reproduced in the host cells from the progressing nodules of tumor-bearing mice administered with placebo, and there appeared to be no significant qualitative or quantitative difference between host cells from the two sources.

Nevertheless, IFN therapy of Meth A-bearing mice resulted in their complete cure.[3] In fact, tumor cells recovered from the regressing nodules were drastically reduced in number. These apparent paradoxical observations on tumor eradication and antitumor effector activity in tumor nodules were further studied. Examination of host cell composition showed no difference in T and B cell numbers and compositions between progressing and regressing nodules either. These results suggest that IFN did not directly modify T cells as regards either neutralizing activity or cell number.

Thus, the present study did not disclose any clear difference in tumor-neutralizing effector cells between the regressing and progressing nodules. These results, however, do not exclude the involvement of these antitumor effector cells in the eventual tumor eradication but rather point to the existence of other factors which would be modified by IFN therapy and which would contribute to tumor growth suppression along with these antitumor effector cells, either mutually dependently or independently. It may be relevant to note that any single tumor is composed of heterogenous cell populations in many biological senses and, to cure the tumor-bearing host, all of those biologically different cell populations should be properly handled. This would be achieved only by the sum of all the different antitumor systems that the host is endowed with. In fact, we found that IFN produced monocytes in peripheral blood that were capable of suppressing the *in vitro* proliferation of tumor cells without tumor selectivity (unpublished results). Antitumor T cells in collaboration with these monocytes may eradicate the entire population of tumor cells, resulting in complete cure.

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