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Combination treatment using thymosin α1 and interferon after cyclophosphamide is able to cure Lewis lung carcinoma in mice

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Summary. A combination treatment with thymosin α1 (200 μg/kg) for 4 days, followed by a single injection of murine interferon α/β (3×10^4 international units/mouse), starting 2 days after cyclophosphamide treatment (200 mg/kg, single injection) demonstrated a dramatic and rapid disappearance of tumor burden in mice bearing Lewis lung carcinoma (3LL) tumor. The effectiveness of this new chemoimmunotherapy protocol was evident even on the long-term survival in a high percentage of animals, and was statistically significant when compared to treatment with the single agents in conjunction with chemotherapy or to chemotherapy itself. The same combination immunotherapy treatment strongly stimulated natural killer activity and cytotoxicity against autologus 3LL tumor cells in 3LL-tumor-bearing mice treated with cyclophosphamide, whereas treatments with each agent singly did not alter or only slightly modified the cytotoxic activity towards Yac-1 or 3LL target cells. Selective depletion with antibodies showed that killer cells stimulated by combination chemoimmunotherapy treatment bear phenotypic characteristics of asialo-GM1-positive cells. A histological study has shown a high number of infiltrating lymphoid cells in the tumors obtained from mice treated with combination chemoimmunotherapy.

Introduction

In our previous studies we demonstrated that thymosin α1, a synthetic polypeptide of thymic origin [10], given in combination with interferon (IFN) α/β to cyclophosphamide-treated or tumor-bearing immunosuppressed mice strongly stimulated natural killer (NK) activity, while none of the single agents, when administered at the same doses and at the same time, statistically altered the NK response [5, 6]. Since it has been substantiated that NK activity appears to play an important role in host defence against tumors [13, 14, 25, 26], the possibility of stimulating this parameter of the immune response could have therapeutic potential. As a consequence, in a recent study we examined the possible correlation between the restored NK response and the effect on tumor growth. In fact we have demonstrated that a combination therapy with thymosin α1 and IFN was able to inhibit the growth rate and to prolong the survival time of some experimental tumors [9]. The results, however, although encouraging, did not highlight modifications in the long-term survival of tumor-bearing animals treated with the combined immunotherapy protocol, insofar as all the animals from the various experimental groups nonetheless died.

It would appear to be rational, therefore, to associate our protocol of combined immunotherapy with thymosin α1 and IFN with a chemotherapy treatment. In fact, chemotherapy could potentiate the immunotherapeutic action, which is well known to be more effective on small masses, by reducing the neoplastic bulk, or by different mechanisms that are not fully understood [19].

It is for this reason that in this work we have evaluated the effects of thymosin α1 and IFN, singly or in a combination of the two, directly on the tumorous growth of Lewis lung carcinoma (3LL) in tumor-bearing mice treated with chemotherapy. The results have shown a potent therapeutic effect of combination immunotherapy in association with cyclophosphamide (CY). Furthermore, in order to explain, at least in part, the efficacy of this new schedule of treatment, we have studied the cytotoxic activity of spleen cells and the lymphoid cell infiltration at tumor level in 3LL-tumor-bearing mice of different treatment groups. Combination immunotherapy associated with CY was able to stimulate the cytotoxic activity significantly against both an NK-sensitive cell line and autologus 3LL tumor cells, and histological analysis demonstrated a dramatic increase of tumor-infiltrating lymphocytes in the same group, when compared to the other treatments.

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Materials and methods

Animals. Male C57BL/6NcrlBR mice, 4 weeks old, purchased from Charles River Italia (Calco, Como, Italy) were used.

Tumor. Lewis lung carcinoma (obtained originally from Dr. Zupi, Istituto Regina Elena, Rome, Italy) was maintained in vivo by serial subcutaneous transplantations in C57Bl/6NcrlBR male mice. In these experiments the tumors, freshly excised from tumor-bearing animals, were minced in Hanks' balanced salt solution (HBSS) (Flow Laboratories, Irvine, Ayrshire, UK) and strained through a fine stainless-steel mesh. Cells were then collected by centrifugation, washed twice, counted and re-suspended to the desired concentration. Viability, as determined by trypan blue exclusion, ranged between 10% and 20%. Mice of different experiments were inoculated with 2×10^5 viable 3LL cells in 0.1 ml HBSS subcutaneously in the right flank. Tumors were measured daily bidimensionally using calipers, and the survival time was controlled.

Drugs. Thymosin α1, kindly provided as a lyophilized synthetic preparation by Alpha 1 Biomedicals Inc (Washington, D. C.), was dissolved in sterile 1.4% $NaHCO_3$ at a concentration of 1 mg/ml and stored at –20° C. Mice were injected with 200 μg/kg thymosin α1 in 0.2 ml phosphate-buffered saline (PBS) i. p. Interferon (a mixture of α and β), was purchased from Lee Biomolecular Research Lab. Inc. (San Diego, Calif.); 3×10^4 international units (IU) in 0.1 ml PBS was administered i. p. cyclophosphamide, purchased from Sigma Chemical Co. (St. Louis, Mo.), was dissolved in 0.85% NaCl sterile solution immediately before use and injected i. p. in a volume of 0.2 ml. Each CY-treated animal received a single dose of 200 mg/kg.

Treatment schedules. The animals were randomized 8 days after tumor inoculation (t. i.) and divided into different experimental groups, which received or did not receive CY (200 mg/kg); they were then treated with: (a) control diluent, (b) thymosin α1 i. p. 200 μg/kg for 4 days starting on day 10 after t. i., (c) a single injection i. p. of mouse IFNα/β 3×10^4 IU/mouse 13 days after t. i., (d) the same treatment as group (b) followed by treatment as group (c) 3 h after the last injection. Dosages and treatment schedules were selected on the basis of our previous studies and of the best results in preliminary trials.

Preparation of effector cells. Spleen cells were obtained from normal or tumor-bearing mice by gentle teasing of spleens in RPMI-1640 (Flow Laboratories). The resultant cell suspension was filtered through a Nytex mesh, washed once with RPMI-1640 and the pellet was then re-suspended in the assay culture medium, which consisted of RPMI-1640 supplemented with 10% fetal bovine serum (Flow Laboratories), 200 mM L-glutamine (Flow Laboratories), 26 mM HEPES (Flow Laboratories) and 50 μg/ml gentamycin (GRS, Shering Co., Kenilworth, NJ).

Target cells. YAC-1, a Moloney-virus-induced mouse T cell lymphoma of A/SN origin, and 3LL tumor cells were used as target cells in the chromium-release assay. 3LL cells were obtained from donor tumor-bearing mice and utilized after 2 days of culture in RPMI-1640 medium, supplemented with 10% fetal calf serum, 2 mM glutamine, 4% non-essential amino acids, streptomycin (100 μg/ml), and penicillin (100 μg/ml). Before testing cytotoxic activity, non-adherent cells were removed from the culture and adherent 3LL cells were harvested after short exposure (less than 1 min) to 0.02% EDTA solution. Suspensions of 5×10^6 target cells in 0.9 ml culture medium were labelled with 100 μCi sodium [^{51}Cr]chromate (New England Nuclear, Boston, Mass.) for 60 min at 37° C in a CO_2 incubator. After labelling, the cells were washed three times in RPMI-1640 medium and re-suspended in the complete culture medium at 1×10^5 cells/ml.

Cytotoxicity assay. The cytotoxicity of effector cells collected from mice was measured by a 4-h ^{51}Cr-release assay as described by Herberman et al. [15].

Briefly effector cells were adjusted to varying concentrations and added to 1×10^4 ^{51}Cr-labelled target cells in U-shaped 96-well microtiter plates (Flow Laboratories) in a total volume of 0.2 ml. The plates were then incubated for 4 h at 37° C in a CO_2 incubator. After the incubation period, the plates were centrifuged at 350 *g* for 10 min. Successive 0.1-ml aliquots of the supernatant were collected and the radioactivity was measured using a Beckman Biogamma counting system (Beckman Instruments, Irvine, Ky.). All assays were performed in quadruplicate and three effector/target cell ratios were employed.

The baseline ^{51}Cr release was that of labelled cells incubated alone in 0.2 ml culture medium, and in no case did it exceed 10% of the total radioactivity incorporated by target cells. Experimental results were expressed as lytic units (LU), calculated from regression-line analysis of the specific cytotoxicity obtained at three different (100 : 1, 50 : 1, 25 : 1) effector/target cell ratios. The number of cells required to produce *n* % specific cytotoxicity is recorded as LU_n. Specific cytotoxicity was calculated as follows:

$$\text{Specific cytotoxicity} = \frac{\text{test } ^{51}\text{Cr release (cpm)} - \text{baseline release (cpm)}}{\text{total } ^{51}\text{Cr incorporated} - \text{baseline release}}$$

Cell depletion. The phenotype of spleen cells obtained from normal and tumor-bearing mice, treated or not with CY and/or single or combination therapies with thymosin α1 and IFN, was examined by treating fresh spleen cells with appropriate predetermined dilutions of the antibodies described below, plus low-toxic rabbit complement (Cedarlane Laboratories Ltd., Ontario, Canada; final dilution 1 : 8), before testing the cytotoxicity. Cells obtained from mice of each experimental group on day 14 after tumor inoculation were pooled, adjusted to 10^7/ml and incubated for 30 min at 4° C. After being washed twice, the cells were incubated for 1 h with complement. The cells were then washed twice again and the cytotoxicity against YAC-1 target cells was determined as described above. Cell numbers were not readjusted after antibody and complement treatment to prevent selective enrichment of surviving cells. Experimental groups are described in "treatment schedules". A group of normal, untreated mice was also examined. The following antibodies were used: monoclonal antibodies rat anti-(mouse Thy1.2) (clone 30H12) and anti-Lyt2 (clone 53-6.7) (Becton Dickinson, Mountain view, Calif.), monoclonal antibody rat anti-(mouse macrophage) (clone M1/70.15) (Sera-lab Ltd., Crawley Down, W. Sussex, England) and rabbit antiserum anti-(asialo-GM1) (Wako Chemicals GmbH, Neuss, FRG).

Histology. 3LL tumors from mice belonging to different experimental groups, were harvested on day 14 after t. i. (i. e. 24 h after the last inoculation). Mice were treated as described in the treatment schedules. Tumors were fixed in Bouin's fixative for 4 h and maintained in 70% ethanol for 24 h. Paraffin sections (5 μm) were prepared and stained with hematoxylin and eosin.

Statistical analysis. Data obtained from animals of different experimental groups were analyzed for differences by Student's *t*-test (parametric) or the Fisher exact test (non-parametric).

Results

Effect of thymosin α1 and IFN on the 3LL tumor growth in CY-treated mice

As shown in Fig. 1 combined administration with CY and thymosin α1 plus IFN induced a tumor regression evidently more effective than results detected in the groups treated with CY alone or with CY and single agents. Combination immunotherapy alone without CY causes only a slight, but significant, effect on tumor growth. If we consider the results of the same experiment in terms of the percentage of tumor-free mice, we clearly observe the disappearance of tumor burden in a high number (7/10) of mice after combination chemoimmunotherapy.

The chemotherapy treatment in conjunction with the combined immunotherapy protocol has proved itself to be

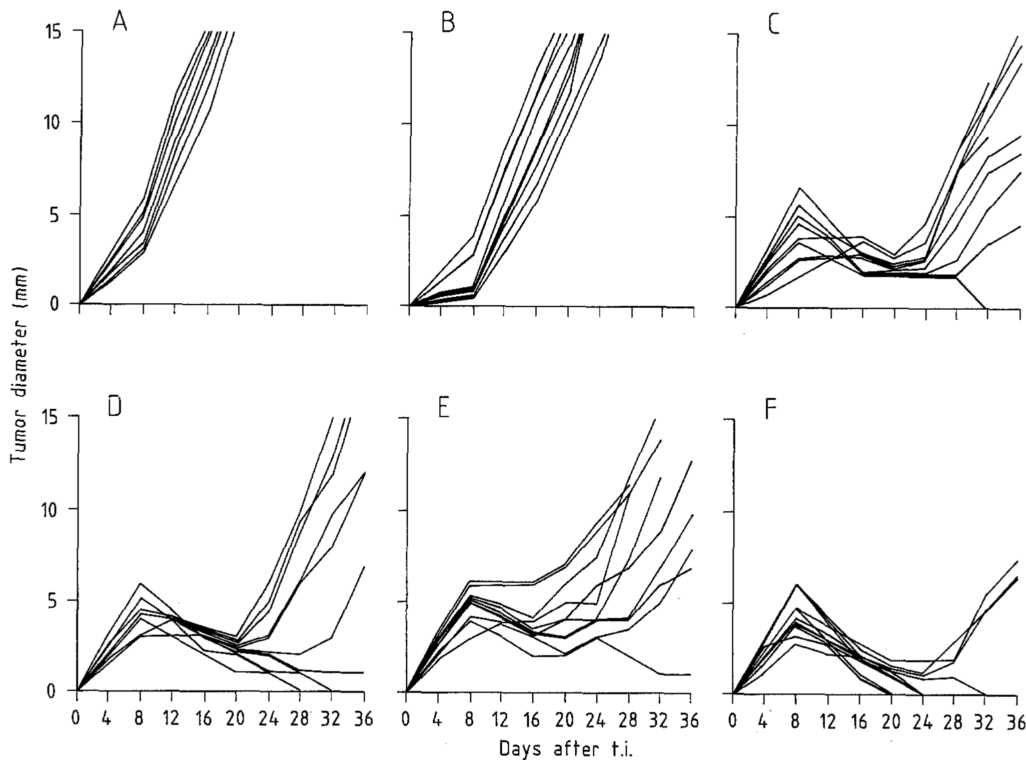

Fig. 1. A–F Growth curves of Lewis lung carcinoma (3LL) tumors in chemoimmunotherapy-treated mice. C57BL/6NCrlBR mice were injected s.c. with 3LL tumor cells on day 0 (tumor inoculation: *t. i.*). The curves illustrate tumor growth of the individual tumor in untreated mice (**A**), mice treated with thymosin α1 on days 1–4 plus interferon (IFN) on day 4 (**B**), cyclophosphamide (CY) on day 8 (**C**), CY on day 8 and thymosin α1 on days 10–13 (**D**), CY on day 8 and IFN on day 13 (**E**), CY on day 8 and thymosin α1 on days 10–13 plus IFN on day 13 (**F**)

Table 1. Therapeutic effects on Lewis lung carcinoma (3LL) experimental tumor of thymosin α1 (TA1) and murine interferon α/β (IFN) in combination with cyclophosphamide (CY)

Group[d]	Treatment			Died/treated		
	CY[a]	TA1[b]	IFN[c]	Expt. 1	Expt. 2	Total (%)
1	No	No	No	10/10	10/10	20/20 (100%)
2	No	Yes	No	10/10	N.D.	10/10 (100%)
3	No	No	Yes	10/10	N.D.	10/10 (100%)
4	No	Yes	Yes	10/10	N.D.	10/10 (100%)
5	Yes	No	No	9/10	9/10	18/20 (90%)
6	Yes	Yes	No	6/10	7/10	13/20 (65%)
7	Yes	No	Yes	9/10	9/10	18/20 (90%)
8	Yes	Yes	Yes	3/10	2/10	5/20 (25%)[e]

[a] CY (200 mg/kg) was administered i. p. on day 8
[b] Thymosin α1 (200 μg/kg) was administered i. p. on days 10–13
[c] IFN (3×10^4 IU/mouse) was administered i. p. on day 13
[d] C57BL/6NCrlBR 3LL-bearing mice, were randomized, divided into different experimental groups (ten animals each) and treated as described above. Results are expressed as absolute numbers and percentages of dead mice detected for each experimental group 60 days after tumor inoculation.
[e] Statistically significant difference of group 8 from group 6 ($P < 0.02$) and other groups ($P < 0.001$) by Fisher exact test

very effective even on the long-term survival of the animals treated, as is demonstrated in Table 1. Here we note that repeated experiments have demonstrated how such a treatment is able to cause long-term survival in a high percentage of animals. This survival rate of the animals, demonstrated by observations made even after 60 days with autopsies, corresponds to the total regression of the tumor in the animals. No effect was observed on long-term survival in mice treated with each immunotherapy protocol separately, while slight but not significant effects were observed in mice treated with chemotherapy alone or chemotherapy plus single immunotherapy.

Effect of thymosin α1 and IFN on cytotoxicity against YAC-1 cells in 3LL-inoculated, CY-treated, tumor-bearing mice and phenotypical characterization of cytolytic cells

The results shown in Table 2 demonstrate that the combined treatment with thymosin α1 and IFN evidently stimulate the cytolitic response against YAC-1, NK-sensitive target cells, in 3LL-inoculated, CY-treated, tumor-bearing mice. In fact, 14 days after t.i., the NK activity in 3LL-tumor-bearing mice appears to be evidently depressed when compared to the activity in control, non-tumor-bearing animals, as previously observed [18]. Chemotherapy treatment on day 8 after t.i. does not evidently alter this NK-depressed response, when tested 14 days after t.i. Treatments with each agent, thymosin α1 or IFN, singly do

Table 2. Natural killer activity and phenotypic characterization by selective depletion with antibodies plus complement of cytotoxic cells from 3LL-tumor-bearing mice treated or not with cyclophosphamide and stimulated in vivo by thymosin α1 (TA1) and murine interferon α/β[a]

Antibody treatment[b]	Cytotoxicity ($LU_{20}/10^6$ effector cells); mean [(mean + SD) – (mean – SD)]					
	Normal c.d.	3LL c.d.	3LL CY	3LL CY+TA1	3LL CY+IFN	3LL CY+TA1+IFN
None	3.79 (4.11–3.50)	1.75 (1.85–1.66)[*3]	1.44 (1.63–1.29)[*3]	1.61 (1.71–1.52)[*3]	1.90 (2.02–1.79)[*3*4]	3.18 (3.57–2.83)[*5]
Complement	3.76 (4.19–3.38)	N.D.	1.22 (1.40–1.06)[*3]	N.D.	N.D.	2.98 (3.23–2.75)[*1*5]
Anti-Thy1.2	3.42 (3.77–3.10)	N.D.	1.19 (1.37–1.03)[*3]	N.D.	N.D.	2.75 (2.96–2.55)[*1*5]
Anti-Lyt2	2.91 (3.19–2.65)[*6]	1.55 (1.78–1.34)[*3]	1.12 (1.23–1.01)[*2*6]	1.60 (1.68–1.52)[*3#]	1.92 (2.04–1.81)[*2*5]	2.88 (3.11–2.67)[*5]
Anti-aGm1	0.10 (0.23–0.04)[*7]	0.12 (0.26–0.06)[*7]	0.05 (0.09–0.03)[*7]	0.09 (0.16–0.06)[*7]	0.07 (0.11–0.04)[*7]	0.01 (0.03–0.04)[*7]
Anti-Mθ	3.61 (4.07–3.20)	1.67 (1.77–1.58)[*3]	1.23 (1.39–1.09)[*3]	1.45 (1.52–1.38)[*3]	1.80 (2.00–1.61)[*3*4]	2.42 (2.64–2.22)[*1*5*6]

[a] Spleen cells obtained from mice of each experimental group on day 14 after tumor inoculation were pooled, adjusted to 10^7/ml and treated or not with the following antibodies plus complement: rat anti-(mouse Thy1.2 clone 3OH12) and anti-(Lyt2 clone 53-6.7), rat anti-[mouse macrophage (Mθ) clone M1/70.15) and rabbit antiserum anti-(asialo-Gm1). The cytotoxicity against YAC-1 target cells was determined. Results are expressed as geometric means of $LU_{20}/10^6$ effector cells, plus and minus standard deviations, calculated from quadruplicate values of cytotoxicity obtained at three different effector/target cell ratios. A group of normal untreated mice (Normal) was also examined. Statistical analysis by Student's one-sided *t*-test. *1 $P < 0.01$, *2 $P < 0.001$ and *3 $P < 0.0001$ against corresponding antibody treatment of normal untreated mice; *4 $P < 0.01$ and *5 $P < 0.0001$ against corresponding antibody treatment of CY treated 3LL-tumor-bearing mice; *6 $P < 0.01$ and *7 $P < 0.0001$ against no antibody treatment within the same experimental group

[b] C57BL/6NCrlBR 3LL-bearing mice were randomized, divided into five groups and treated respectively with control diluent (c.d.), CY 200 mg/kg on day 8 after tumor inoculation (CY), CY 200 mg/kg on day 8 plus TA1 200 μg/kg on days 10–13 (CY+TA1), CY 200 mg/kg on day 8 plus IFN 30000 IU on day 13 (CY+IFN), CY 200 mg/kg on day 8 plus TA1 200 μg/kg on days 10–13 and IFN 3×10 IU on day 13 (CY+TA1+IFN)

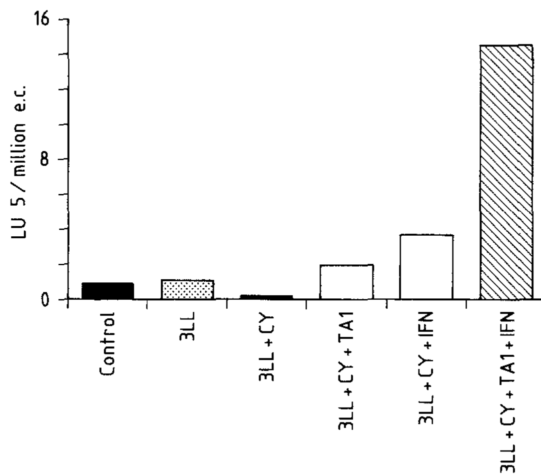

Fig. 2. Cytotoxic activity against autologus 3LL tumor cells of spleen cells from 3LL-tumor-bearing mice. C57BL/6NCrlBR 3-LL-bearing mice were randomized, divided into five groups and treated respectively with control diluent (*3LL*), CY 200 mg/kg on day 8 after tumor inoculation (*3LL+CY*), CY 200 mg/kg on day 8 plus thymosin α1 200 μg/kg on days 10–13 (*3LL+CY+TA1*), CY 200 mg/kg on day 8 plus IFN 30000 IU on day 13 (*3LL+CY+IFN*), CY 200 mg/kg on day 8 plus thymosin α1 200 μg/kg on days 10–13 and IFN 3×10^4 IU on day 13 (*3LL+CY+TA1+IFN*). Spleen cells obtained from mice of each experimental group on day 14 after tumor inoculation were pooled, adjusted to 10^7/ml and the cytotoxicity against 3LL target cells was determined. Results are expressed as LU_5/million effector cells. A group of normal untreated mice (*CONTROL*) was also examined

not or only slightly modify the cytotoxic activity towards the target cells YAC-1 in CY-treated 3LL-tumor-bearing mice, as was already apparent in our previous studies on mice suppressed either with chemotherapy only or with a tumor only. Whereas combined treatment exerted a powerful stimulating action on NK activity in CY-treated 3LL-tumor-bearing mice. Moreover, in order to characterize the cells involved in this cytolytic activity, we have tested the NK activity after selective depletion of the major murine spleen cell subsets. Results also illustrated in Table 2 show that the cytotoxic activity we observed would appear to be due to cells phenotypically characterized principally as asialo-GM1-positive cells. In fact, only the pre-treatment with anti-asialo-GM1 antibodies and complement almost completely eliminates the cytotoxicity of splenic cells. By contrast, this activity is not reduced or is slightly reduced by anti-Thy1.2, anti-Lyt2 or anti-macrophage antibodies.

Effect of thymosin α1 and IFN on cytotoxicity against 3LL autologus tumor cells, in 3LL-inoculated, CY-treated, tumor-bearing mice

The results shown in Fig. 2 demonstrate that the combined treatment with thymosin α1 and IFN is clearly able to stimulate the cytolytic response in spleen cells collected from 3LL-inoculated, CY-treated, tumor-bearing mice against autologus 3LL cells also. Treatments with each agent, thymosin α1 or IFN, singly evidently do not enhance the cytotoxic activity towards the target 3LL cells in CY-treated 3LL-tumor-bearing mice. Results obtained in the control group, constituted by normal untreated mice, indicate, as expected, that 3LL tumor cells are not sensitive to NK cell cytolytic activity.

Histological study

The results of the histological study on the effect of combined chemoimmunotherapy with CY followed by thymosin α1 and IFN at tumor level, are shown in Fig. 3. As illustrated in Fig. 3A, tumors collected from control untreated mice show the characteristic feature of neoplastic tissue: 3LL tumor cells appear in close contact with neighboring cells, few cells have two or more nuclei and the

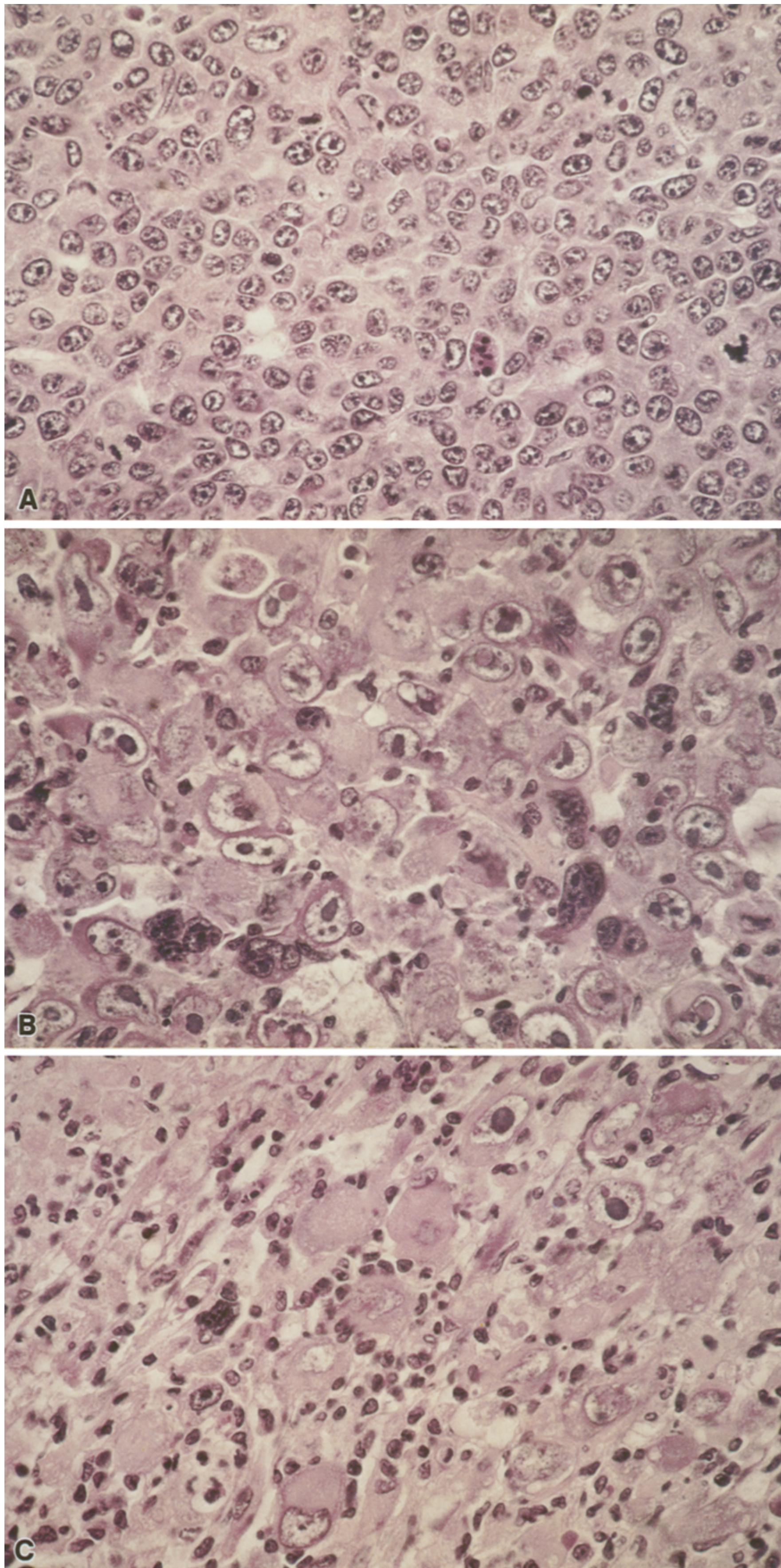

Fig. 3. A. Histological analysis of 3LL tumors growing subcutaneously in untreated C57BL/6NCrlBR mice 14 days after tumor inoculation (t. i.); **B** modifications caused by cyclophosphamide treatment (single injection on day 8 after t. i.) when observed on day 14 after t. i.; **C** effect of combined chemoimmunotherapy treatment (CY on day 8 after t. i. plus thymosin α1 on days 10–13 and IFN on day 13 after t. i.). A tumor cell completly surrounded by lymphoid cells is shown. All images were obtained at the same amplification and in the same conditions. (Original magnification ×125)

intensity of lymphoid infiltration within the tumor tissue is very low. Modifications caused by CY treatment 6 days after CY inoculation show evident areas of necrosis, with many dead tumor cells (Fig. 3B). The damage in surviving tumor cells is indicated by the presence of many multinucleated cells and by an evident increase in the mean cellular volume. A certain infiltration of lymphoid cells is easily detectable. Tumor tissue of mice treated with combined chemoimmunotherapy shows a distinct histological pattern (Fig. 3C). Surviving tumor cells are difficult to detect, whereas lymphoid cell infiltration is clearly evident. Some tumor cells are completely surrounded by lymphoid cells, forming a characteristic rosette feature. Similar effects were not observed in mice treated singly with thymosin α1 or IFN after CY, in which only a certain increase in lymphoid cell infiltration with respect to mice treated with CY alone was observed (data not shown).

Discussion

The effectiveness of a combined treatment protocol with CY followed by thymosin α1 plus IFN in the control of neoplastic growth in the experimental model of 3LL murine tumor, is clearly shown. In fact, we submit the evidence that combined chemoimmunotherapy treatment is able to induce tumor regression, without the appearance of any toxic or side effect, even if it is started when the tumor burden is developed and easily detectable. Experimental therapies effective in tumor control often consist of protocols in which the treatment is started before tumor appearance.

Our results allow us to explain, at least in part, the mechanism by which the combined treatment with thymosin α1 and IFN exerts this powerful anti-tumorous action in animals treated with CY. In fact, the results obtained on the stimulation of cytotoxic activity of the splenic cells by a combined use of thymosin α1 and IFN after CY against 3LL tumor cells as target cells would seem to indicate that such stimulation might play an important role in the anti-tumorous mechanism of combined chemoimmunotherapy. We have also observed a stimulation of typical NK cells in the spleen. This result is in accordance with those, previously obtained by us, demonstrating a high synergistic effect between thymosin α1 and IFN α/β in stimulating cytotoxicity in CY-depressed or tumor-bearing mice [5, 6]. Perhaps NK cell stimulation could not be directly related to the anti-tumorous action of our combined chemoimmunotherapy schedule in this NK-resistant tumor experimental model, but it indicates the possibility of also successfully utilizing this treatment schedule in NK-sensitive primary tumors or metastases. Moreover the histological study strongly supports the hypothesis that combined chemoimmunotherapy with CY followed by thymosin α1 and IFN could induce a high infiltration of activated effector cells directly within the tumor tissue.

As a consequence, one possible explanation for the phenomenon might derive from the fact that chemotherapy reduces, to a certain extent, the quantity of tumor cells which then could be eliminated by the effectors that are stimulated by the immunotherapeutic treatment. It has been recently demonstrated that large granular lymphocytes are able to respond to different stimuli with a prompt migration [22]. The lymphoid cell infiltration after CY treatment could be thus related to the release of chemotatic or stimulating molecules at tumor level. Interestingly, a positive correlation between the number of $CD4^+$ lymphocytes (well known to be involved in interleukin-2 production) in the tumors and better prognosis in breast cancer has been reported [1]. Activated effector cells with different phenotypes and different functions could be thus responsible for the phenomenon we observed.

However, other factors might well come into play. CY treatment could be effective in rendering the tumorous cells more vulnerable to the immune response effectors. This hypothesis could explain the highly synergistic effect of the combination of CY plus thymosin α1 and IFN in curing 3LL tumor. In fact CY or the combination of thymosin α1 plus IFN alone, even if administered in an early phase, is able to inhibit tumor growth but not to cure it (data not shown). On the other hand, in our experimental model it seems unlikely that immunomodulating effect of CY could in itself play an important role in inducing tumor regression. In effect, the stimulation mechanism on the part of CY on the immune response, connected to the inhibition of suppressor cells, is obtained at lower dosages than the ones we used [28]. Even the described rebound phenomenon of the cytolytic activity that follows the treatment with CY [2] does not seem to be connected to the regression of the primary tumorous mass, considering that the rebound takes place at a moment that chronologically follows the effects we observed here.

Together with the documented stimulation of NK activity by a combination of thymosin α1 and IFN [5, 6, 23], we must consider that thymosin α1 alone is able to induce an evident maturation of T helper cells in vivo, and to stimulate interleukin-2 (IL-2) production and IL-2 receptor expression in vitro [8, 24, 29]. It has recently been demonstrated that killer cells activated by IL-2 with different methods tend to localize preferably in the tumorous area after chemotherapy treatment of animals and humans, and that a chemoimmunotherapeutic model consisting of CY and IL-2 administered in vivo exerts an evident antitumor action [3, 7, 12, 16, 20, 21]. Tumoricidal cells could be thus locally activated by IL-2 producing cells stimulated by thymosin α1. Moreover these mechanisms might explain, at least in part, the evident partial effectiveness of the treatment with CY and thymosin α1 alone. In fact some evidence for a certain antitumoral effect of thymosin and other thymic hormones has been reported in humans as well as in mice [4, 11, 17, 27]. Also in our experimental model, thymosin α1 alone, following CY, was able to exert a certain antitumoral effect; but this effect was much less evident when compared to that obtained in combination with IFN.

In short, it is feasible that several mechanisms and several stimulated cellular effectors, may contribute to the powerful immune anti-tumorous action that is clearly apparent. Further studies are in progress in our laboratories to characterize fully the anti-tumorous cells stimulated by this new combination chemoimmunotherapy and to extend our

studies to other experimental tumor models, in order to transfer it to humans.

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