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Modulation of natural killer activity by thymosin alpha 1 and interferon

Cartesio Favalli[1], Teresa Jezzi[1], Antonio Mastino[1], Cristina Rinaldi-Garaci[2], Carlo Riccardi[3], and Enrico Garaci[1]

[1] Department of Experimental Medicine, Faculty of Medicine, II University of Rome, Rome, Italy
[2] Institute of General Pathology, University of Rome, Rome, Italy
[3] Institute of Pharmacology, University of Perugia, Perugia, Italy

Summary. A single injection of αβ-interferon (αβ-IFN) (30000 units/mouse), a major biological modifier of natural killer (NK) cytolytic activity, strongly stimulated NK activity in normal mice, as expected, while the same treatment did not statistically alter the NK response in cyclophosphamide (CY)-suppressed animals.

We investigated the possibility of thymosin α_1 cooperating with αβ-IFN in boosting NK activity in CY-suppressed animals.

The results show that treatment with thymosin α_1 (200 μg/kg) for 4 days, followed by a single injection of αβ-IFN 24 h before testing, strongly restored NK activity in CY-suppressed mice. Thymosin α_1 was, moreover, able to accelerate the recovery rate of NK activity in bone marrow reconstituted murine chimeras.

Taken together the data support the concept that the synergic effect between thymosin α_1 and αβ-IFN could be the result of effects on differentiation of the NK lineage at different levels.

Introduction

Interferon (IFN) represents a group of glycoproteins that play an important role in the regulation of the natural killer (NK) system [10, 11, 3]. Most work has focused on the in vitro enhancing effects of IFN on NK activity [22, 23, 16]. In vivo administration of IFN has also been reported to boost NK activity both in human [12, 14, 5] and murine [4, 7] experimental models. However, treatment of cancer patients with IFN has not always led to an increased NK response [18, 2].

Recent studies have emphasized that thymic hormones could regulate NK activity in normal and immunosuppressed animals [15, 1]. Since thymosin α_1, a well-known synthetic polypeptide of thymic origin [8, 17], restored NK activity in immunosuppressed mice [24, 19], we hypothesized that thymosin α_1 could cooperate with IFN in boosting NK activity in immunosuppressed animals.

To investigate this possibility we carried out the present study to examine: (a) the effects on NK activity of thymosin α_1, given in association with α β-IFN to cyclophosphamide (CY)-suppressed mice and (b) the effects of thymosin α_1 on the recovery rate of NK activity in bone marrow reconstituted murine chimeras.

Offprint requests to: C. Favalli, Department of Experimental Medicine, Faculty of Medicine, II University of Rome, via Orazio Raimondo I-00173 Rome, Italy

Materials and Methods

Mice

Female C57Bl/6 mice 6 to 7 weeks old, purchased from Charles River Italia, Calco, Milano, Italy were used.

Drugs and treatments

Thymosin α 1. Thymosin α_1, generously provided as a lyophilized preparation by Hoffman-La Roche (Nutley, N.J., USA), was dissolved in sterile 1.4% $NaHCO_3$ at a concentration of 300 μg/ml and stored at −20 °C. Mice were injected with 200 μg/kg of thymosin α_1 in 0.2 ml of phosphate buffered saline (PBS) i. p.

Interferon. IFN (α and β mixture), kindly supplied by Dr. F. Belardelli (Istituto Superiore di Sanità, Roma, Italy), was prepared from suspension cultures of mouse sarcoma 243 cells inoculated with Newcastle disease virus. It was administered as a single injection i. p. 24 h before the NK assay at a dose of 30000 units in 0.1 ml of PBS.

Cyclophosphamide. CY 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 4 days before the NK assay.

In vitro cytotoxicity assay

Preparation of effector cells. Spleen cells were obtained from normal, treated or bone marrow reconstituted mice, by gentle teasing of individual spleens in RPMI 1640 (Flow Laboratories, Irvine, Ayrshire, UK). The resultant cell suspension was filtered through a Nytex mesh, washed once with RPMI 1640 and the pellet was then resuspended in the assay culture medium (RPMI 1640 supplemented with 10% fetal bovine serum (FBS) (Flow Lab.), 200 mM L-glutamine (Flow Lab.), 25 mM Hepes (Flow Lab.) and 50 μg/ml gentamicin (GRS, Shering Co. Kenilworth, N.J., USA)

Target cells. YAC-1, a Moloney virus-induced mouse T cell lymphoma of A/SN origin was used as the target cell in the chromium release assay. A cell suspension of 5×10^6 YAC-1 cells in 0.9 ml of culture medium was labelled with 100 μCi of sodium chromate (^{51}Cr) (New England Nuclear, Boston, Mass., USA) for 60 min at 37 °C in a CO_2 incubator. After labelling, the cells were washed three times

in RPMI 1640 and resuspended in the complete culture medium at 1×10^5 cells/ml.

NK assay. The NK activity of effector cells was measured by a 4-h ^{51}Cr release assay using labelled YAC-1 target cells as described by Herberman et al. [9].

Briefly, effector cells were adjusted to varying concentrations and added to 1×10^4 ^{51}Cr-labelled YAC-1 cells in U shaped 96-well microtiter plates (Flow Lab.) 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 50 g for 10 min, 0.1 ml of supernatant was collected and the radioactivity measured using a Beckman Biogamma Counting System (Beckman Instruments, Irvine, Ky., USA). All assays were performed in quadruplicate and three effector-target cell ratios were employed. The baseline ^{51}Cr release was that of labelled YAC-1 cells incubated alone in 0.2 ml of culture medium, and in no case did it exceed 10% of the total counts incorporated by target cells. Experimental results were expressed as percentage cytotoxicity measured by specific ^{51}Cr release and were calculated as follow:

$$\frac{\text{test cpm} - \text{baseline cpm}}{\text{total cpm incorporated} - \text{baseline cpm}}$$

Bone marrow chimeras

Mice were lethally irradiated by exposition to γ-rays (850–950 rads) using a ^{60}Co irradiator (Hot Spot, MKIV, Harwell, UK). After 4–5 h 1×10^7 syngeneic bone marrow cells were inoculated i. v. in 0.5 ml of RPMI 1640.

Statistical analysis

Data obtained from animals of different experimental groups were analysed for differences by the Student's t-test.

Results

Effect of αβ-IFN and thymosin α_1 on NK activity in normal mice

In order to evaluate the effects of αβ-IFN and thymosin α_1 on the cytotoxic activity of NK cells in normal mice, the animals were randomized, divided into four experimental groups and treated respectively with (a) control diluent at the same time as the treated animals, (b) a single i. p. injection of αβ-IFN 24 h before testing, (c) four daily i. p. injections of thymosin α_1 starting 4 days before testing, (d) the same treatment as group (c) followed by the treatment of group (b) 3 h after the last pre-treatment injection Dosages and treatment schedules were selected on the basis of the best results in preliminary trials.

The experiment was performed twice. Because of highly concordant data in corresponding experimental groups, they are expressed as cumulative results.

As shown in Table 1 the animals inoculated with αβ-IFN showed a predictable and significant increase in NK activity in accordance with other reports [4, 7], while treatment with thymosin α_1 had a slight but not significant effect. No difference was observed between mice treated with αβ-IFN alone and mice treated with thymosin α_1 associated with αβ-IFN.

Table 1. Effect of thymosin α_1 and IFN on NK activity in normal mice

Group[a]	Mean percentage cytotoxicity ± SEM against YAC-1		
	E:T ratio		
	100:1	50:1	25:1
Control	33.8 ± 1.7[b]	25.7 ± 2	18.6 ± 1.1
Normal + IFN	40.5 ± 2.9[c]	36.7 ± 1.6[c]	31.0 ± 3.1[c]
Normal + α_1	36.9 ± 2.7	31.9 ± 2.3	24.1 ± 2.7
Normal + α_1 + IFN	39.6 ± 2.4	32.1 ± 1.9	29.8 ± 2.6

[a] C57Bl/6 mice were randomized, divided into four groups and treated respectively with control diluent, αβ-IFN 30,000 units (day 3), thymosin α_1 200 μg/kg (days 0, 1, 2, 3), thymosin α_1 200 μg/kg (days 0, 1, 2, 3) plus αβ-IFN 30,000 units (day 3). On day 4 mice were sacrificed and spleen cell NK activity was tested.

[b] cumulative results of two independent experiments (total $n=10$)

[c] $P < 0.001$ by Student's t-test against control

As shown in Table 1 the animals inoculated with αβ-IFN showed a predictable and significant increase in NK activity in accordance with other reports [4, 7], while treatment with thymosin α_1 had a slight but not significant effect. No difference was observed between mice treated with αβ-IFN alone and mice treated with thymosin α_1 associated with αβ-IFN.

Effects of αβ-IFN and thymosin α_1 on NK activity in CY-suppressed mice

In a parallel study we examined the possible synergistic effect of αβ-IFN with thymosin α_1 on NK activity in mice pre-treated with CY.

The mice were injected with CY 4 days before the assay. On the same day they were randomized, divided into four experimental groups and treated as previously described for normal mice. Again the experiment was repeated twice and concordant data were obtained.

Cumulative results presented in Table 2 show that αβ-IFN was not able to increase the CY-induced reduction in NK activity. Thymosin α_1 only slighthly increased the NK activity. Conversely, when αβ-IFN was given in association with thymosin α_1, a marked increase in NK activity was noted.

Effects of thymosin α_1 on regeneration of NK cell activity of bone marrow chimeras

To evaluate the influence of thymosin α_1 on the in vivo growth and differentiation of less differentiated non-cytolytic and IFN-insensitive progenitor NK cells in immuno-

Table 2. Effect of thymosin α_1 and IFN on NK activity in CY-suppressed mice

Group[a]	Mean percentage cytotoxicity ± SEM against YAC-1		
	E:T ratio		
	100:1	50:1	25:1
Control	33.8 ± 1.7[b]	25.7 ± 2.0	18.6 ± 1.1
CY	10.6 ± 2.6	6.6 ± 1.5	3.2 ± 1.4
CY + IFN	12.0 ± 2.7	8.0 ± 2.3	4.6 ± 2.1
CY + α_1	15.4 ± 2.1	10.0 ± 3	5.4 ± 1.3
CY + α_1 + IFN	26.4 ± 3.8[c]	17.3 ± 2[c]	9.5 ± 2.1[c]

[a] Each animal received 200 mg/kg CY i.p. on day 0. Starting on the same day mice were randomized into four groups and treated as outlined in Table 1
The control group was represented by untreated animals

[b] Cumulative results of two independent experiments (total $n=10$)

[c] $P < 0.001$ by Student's t-test against CY-treated animals

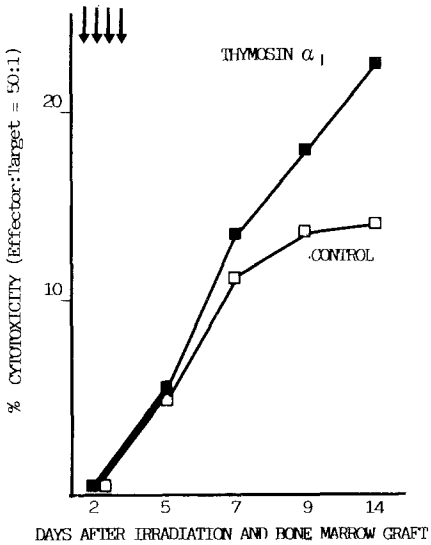

Fig. 1. Bone marrow chimeras. Mice were irradiated by exposure to y-rays (850–900 rads) using a 60 Co irradiator (Hot spot, MKIV, Harwell, UK). After 4–5 h 10^7 syngenic bone marrow cells were inoculated i. v. (in 0.5 ml/mouse). Animals were then randomized in two groups: one was treated with four injections of thymosin α_1 (200 μg/kg) for 4 days consecutively, starting on the transplantation day, the second group was treated with control diluent. Each experimental group consisted of ten animals. ($p < 0.001$ by Student's t-test against control at day 7, 9, 14)

suppressed animals, we studied a different model, in which bone marrow chimeras were employed.

Bone marrow reconstituted mice were randomized in two groups: the first one was treated with four injections of thymosin α_1 for 4 days consecutively starting on the transplantation day; the second group was treated with control diluent.

As show in Fig. 1, thymosin α_1 induced no significant early variation in splenic NK activity in bone marrow reconstituted animals compared to the control group. However, 48 h later, a significantly higher reconstitution in NK activity was evident under thymosin α_1 treatment.

Our data showed that after lethal irradiation and bone marrow reconstitution, the depressed NK activity recovered to normal control levels within 12–24 days. When NK activity was tested at different times after irradiation and bone marrow graft in mice treated with thymosin α_1 (day 7–14), there was a significant increase in NK reconstitution.

Discussion

The present results suggest that αβ-IFN, which is one of the major stimulators of NK activity in normal mice, seems to lose its effects when it is given to CY-suppressed animals (Table 2). This evidence was also witnessed by Maluish et al. [18] indicating that prolonged IFN treatment of advanced cancer patients fails to increase the low NK activity. Interestingly, a similar hyporesponsiveness state to IFN-induced NK activity boosting, was recently evidenced by Saito et al. [21] in a different model of NK suppression. Furthermore, thymosin α_1 induced a slight increase in NK activity in normal and CY-suppressed animals (Tables 1 and 2). On the other hand we found that the association of αβ-IFN and thymosin α_1 resulted in a marked increase in NK activity in CY-suppressed mice. In this context, our results suggest that these two biological response modifiers could have a synergistic effect on the regulation of NK activity.

One possible explanation for these data could be the possible partial or total lack of NK cells targets for IFN stimulation, in CY-treated animals. Thymosin α_1 might then induce maturation of less differentiated non-cytolytic and IFN-insensitive progenitor cells which, in turn, could result in the increase in NK activity in CY-suppressed mice.

In fact, results obtained in bone marrow chimera experiments suggest that thymosin α_1 can influence the in vivo process of bone marrow progenitor cell differentiation to cytolytic effectors. The hypothesis is supported by the data of Umeda et al. [24], demonstrating that thymosin α_1 could increase NK activity in tumor-bearing mice and implicating the NK progenitor cell.

It seems likely that the stimulator activity of thymosin α_1 could be associated with the stimulation of maturation of progenitor NK cells to a low reactive NK cell population (pre-NK). These pre-NK cells could then be susceptible to the IFN boosting activity that results in maturation to cytolytic NK cells [20].

Our results might somehow correlate with those obtained by Hurme et al. [13], who demonstrated that the first splenic NK cells derived from bone marrow progenitors, express the thy-1 antigen.

In conclusion, thymosin α_1 and IFN cause synergistic effects which could be the result of the effect on differentiation of the NK cell lineage at different levels. However, on the basis of our results, we cannot exclude the hypothesis that thymosin α_1 could exert its activity indirectly, by influencing the maturation of helper T cells, which in turn could regulate NK activity, as recently proposed [6]. Further studies are in progress in our laboratories to define the mechnism involved more clearly.

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