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Immunomodulating Activity of Thymosin Fraction 5 and Thymosin α_1 in Immunosuppressed Mice

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Summary. *We found that both thymosin from calf thymus and its constituent peptide α_1 prepared by chemical synthesis restore cell-mediated immunity following its suppression in mice by injection of 5-FU. Conditions suitable for assessing the thymosin activity by means of footpad reaction were established in such immunosuppressed mice. In this new animal model, thymosin α_1-peptide showed activity at a low dose of 5–50 μg/kg, which was 100–1,000 times less than that required for thymosin F-5 preparations.*

Further studies utilizing the adoptive transfer technique showed that α_1-peptide corrects the 5-FU-induced suppression of mature T cells, transferring the DTH response as well as that of macrophage function responsible for the expression of footpad reaction. Furthermore, regeneration of lymph node and bone marrow cells as well as CFU-c (progenitor cells of macrophages and granulocytes) was enhanced by thymosin α_1 in the 5-FU-treated mice. All these results indicate that thymosin α_1 accelerates the replenishment and maturation of haematopoietic cells, including not only T cells but also macrophages, when they have been severely damaged by the 5-FU treatment.

Indroduction

The thymus is an endocrine gland indispensable for the development and subsequent maturation of T cells. A number of studies concerning the humoral functions of thymus led to the discovery of various humoral factors such as thymosin [6], serum thymic factor [2], thymic humoral factor [14], and thymopoietin [7].

The activities of these factors have been evaluated in various types of in vivo, ex vivo, and in vitro immunological assay systems as reviewed by Bach [1]. However, since most of these assays are rather complicated and are influenced by a number of factors, the results are often conflicting and both the identity and the mechanism of action of various factors that might possibily be ascribed to the thymus remain uncertain.

Of these factors, thymosin Fraction 5 (first named by A. L. Goldstein), a partially purified preparation from calf thymus, has been studied most extensively in clinical trials. It was proven to be quite effective in patients with some immunodeficiency diseases [17]. Fraction 5 in combination with regular chemotherapy was further shown to prolong the survival of patients with oat cell carcinoma of the lung [4].

With the aim of establishing a simple in vivo model for the evaluation of thymosin and related compounds, we have examined their effects in mice immunosuppressed by the administration of 5-fluorouracil (5-FU). By utilizing the delayed-type hypersensitivity response, we have shown in the present work that this model can be used to assess the immunomodulating activity of thymosin F-5 and α_1-peptide. Further studies on their mode of action indicated that thymosin corrects the damage caused not only to T cells but also to macrophages and their progenitor cells (CFU-c) by 5-FU administration.

Materials and Methods

Animals. Female BDF_1 mice (C57BL/6 × DBA/2) were purchased from Shizuoka Agricultural Cooperative Association for Laboratory Animals (Hamamatsu, Japan).

Reagents. 5-Fluorouracil (5-FU, Roche) was dissolved in physiological saline (10 mg/ml). Chicken red blood cells (CRBC) obtained from Nippon Biotest (Tokyo) were suspended at 2×10^9 cells/ml in physiological saline and 10^8 cells were injected in a volume of 50 μl per mouse.

Thymosin Preparations. Thymosin F-5 was prepared from calf thymus gland following the procedure of Hooper et al. [8], both at Hoffmann-La Roche, Nutley, NJ, USA and in our laboratory. In our case, however, F-5 was processed by ultrafiltration with a PM-30 and then with a PM-10 Amicon membrane in place of a DC-2 hollow fiber. All preparations were tested for endotoxin (LPS) by *Limulus* lysate assay (Teikokuzōki Pharmaceuticals, Tokyo, Japan) and were found to contain less than 10 ng *E. coli* LPS equivalent per mg protein. Chemically synthesized thymosin α_1 and fractions equivalent to F-5 prepared from the extracts of calf spleen and liver were gifts of Hoffmann-La Roche, Nutley, NJ, USA.

Delayed-Type Hypersensitivity (DTH). For active immunization mice were sensitized by injection of 10^8 CRBC in the left footpad and 4 days later challenged with 10^8 CRBC in the right footpad. The footpad swelling was measured with a dial thickness gauge 24 h after the challenge. The DTH response was expressed by the difference in the thickness of footpad between the two hind feet. Seven mice were used for each group.

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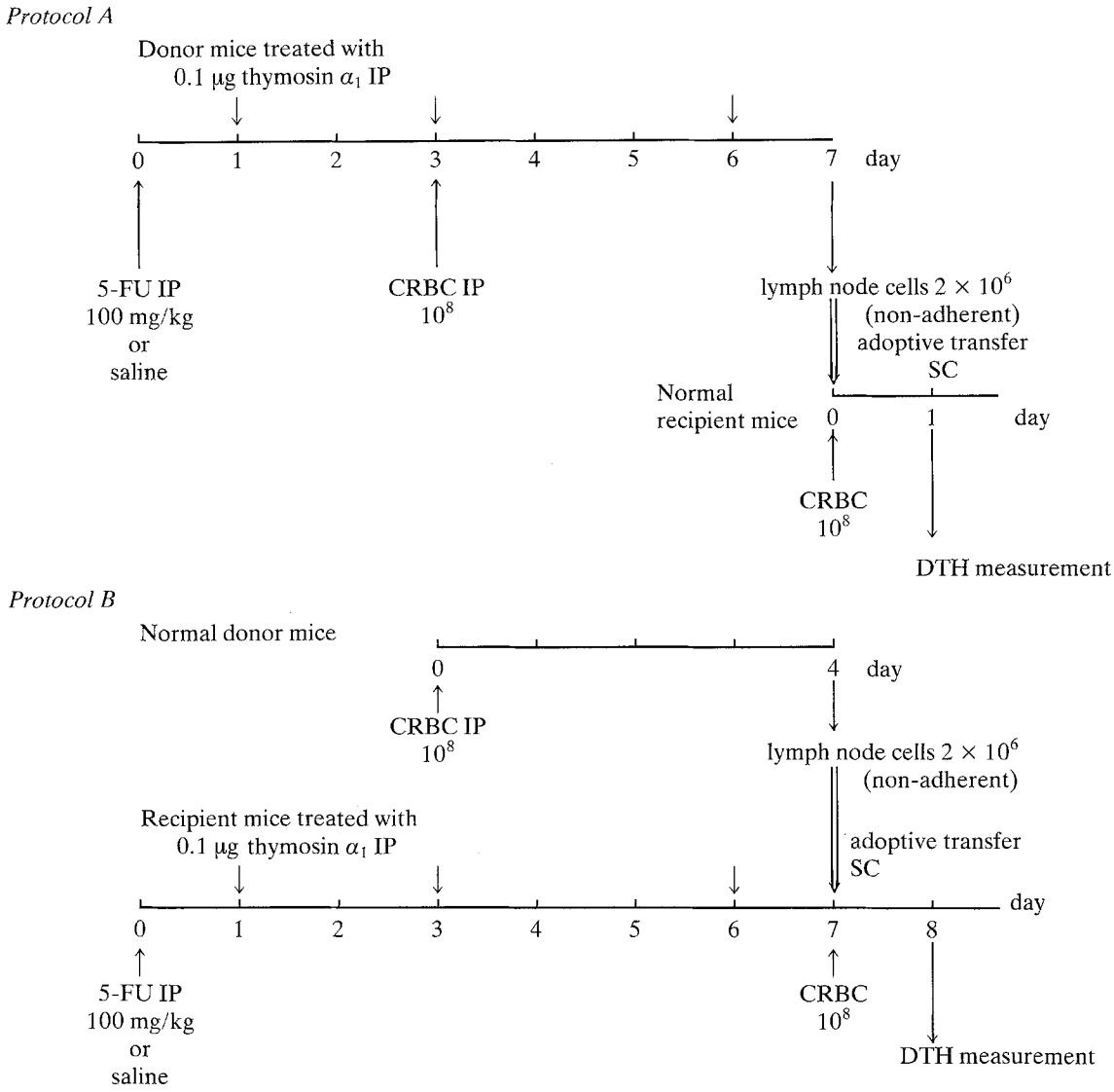

Fig. 1. Experimental protocols for the adoptive transfer of the lymph node cells prepared from the sensitized mice

Adoptive Cell Transfer. Cervical, axillary, mesenteric, and inguinal lymph node cells were collected from each group of donor mice ($n = 5$ or 8, depending on the protocols indicated in Fig. 1) 4 days after sensitization with CRBC (10^8, IP). The pooled cell suspension (10 ml) was laid on the surface of a plastic culture bottle (Corning 25115) and incubated for 30 min at 37° C in a 5% CO_2 incubator to eliminate adherent cells. The same procedure was repeated once again on the surface of the other side of the bottle. Then, 2×10^6 viable cells in the supernatant were injected with 10^8 CRBC into the right hind footpad of the recipient mice ($n = 7$). The DTH measurement was done 24 h after the transfer.

CFU-c Assay. The assay was carried out in the soft agar system described by Bradley and Metcalf [3]. Briefly, bone marrow cells pooled from five mice in a group were suspended in a lukewarm agar medium. The medium used was Eagles MEM (Grand Island Biological Co.) containing a final concentration of 0.3% agar (Difco), 7.5×10^{-3}% DEAE dextran (Sigma St. Louis, USA), 20% horse serum (Pel Freeze), and 10% of conditioned medium from L929 cells. The conditioned medium was used as the source of colony-stimulating factor. A 1-ml portion (10^5 cells) was placed into a 35 mm Falcon petri dish in quadruplicate and, after gelling, incubated at 37° C in a humidified atmosphere of 5% CO_2 in air. Colonies (more than 50 cells) were counted 7 days later under a dissecting microscope.

Statistics: Average and standard error (SE) were calculated using routine statistical methods. Statistical significance was evaluated using Student's t-test.

Results

Evaluation of the Thymosin Activity by the DTH Response in Immunosuppressed Mice

Based on our previous results on the immunosuppressive effects of 5-FU in mice [11], factors possibly affecting the system were evaluated by a number of preliminary experi-

Table 1. Effect of mouse age on restorative activity

Treatment	DTH response (0.1 mm) mean ± SE (%)[a]			
	Age of mice			
	6 weeks old	8 weeks old	10 weeks old	12 weeks old
Saline (control)	13.0 ± 1.7 (100)	13.2 ± 0.6 (100)	10.3 ± 0.9* (100)	9.0 ± 1.0** (100)
5-FU alone	13.8 ± 0.9 (106)	10.7 ± 0.9 (81)	5.5 ± 1.5 (53)	3.8 ± 1.2 (42)
5-FU plus thymosin F-4 (E-19)	11.8 ± 1.0 (91)	11.5 ± 0.6 (87)	7.6 ± 1.8 (74)	8.3 ± 1.0* (89)
5-FU plus thymosin F-5 (E-20)	13.8 ± 1.0 (106)	12.8 ± 0.9 (97)	10.2 ± 1.1* (99)	9.3 ± 1.5* (103)
5-FU plus thymosin F-5 (c-100496)	13.7 ± 1.0 (105)	11.7 ± 0.5 (89)	7.6 ± 1.8 (74)	7.0 ± 1.6* (78)

[a] P value: different from the 5-FU-treated group
* $P < 0.05$; ** $P < 0.01$

Table 2. Dose responses of thymosin F-5 and thymosin α_1

Mice treated with	Dose (μg/kg)	DTH response[a]	
		Mean ± SE (0.1 mm)	%
Expt A			
Saline (control)	–	15.6 ± 0.9**	100
5-FU alone	–	8.0 ± 1.0	51
5-FU plus thymosin F-5	2,500	11.7 ± 1.7	75
	5,000	12.1 ± 1.0*	77
	10,000	8.4 ± 1.3	53
5-FU plus thymosin α_1	50	12.6 ± 0.8**	80
	500	11.2 ± 0.7*	71
Expt B			
Saline (control)	–	11.6 ± 0.6***	100
5-FU alone	–	3.6 ± 0.5	31
5-FU plus thymosin α_1	0.05	6.3 ± 1.8	54
	0.5	8.0 ± 1.2**	69
	5	10.1 ± 1.3***	87
	50	7.0 ± 1.4*	60
Expt C			
Saline (control)	–	15.6 ± 0.8	100
Thymosin F-5 alone	5,000	16.8 ± 0.8	107
	10,000	16.1 ± 0.8	103

[a] P value: statistical difference from the corresponding group treated with 5-FU alone
* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

ments to find conditions suitable for demonstrating the immunomodulating activity of thymosin. These factors included the dose of 5-FU needed to cause a significant immunosuppression, the rate of natural recovery in mice of various ages, and the dose and schedule of thymosin administration. The footpad reaction with chicken erythrocytes (CRBC) was used as an indicator of the delayed-type hypersensitivity (DTH) response.

Results of a typical experiment with mice of various ages are shown in Table 1. In this experiment, a group of mice ($n = 7$) were treated with 5-FU (100 mg/kg, IP) at day 0 and subsequently with various preparations of thymosin from calf thymus (5 mg/kg, IP) at days 1, 4, and 7. Other groups of mice were treated with either saline (control) or 5-FU alone. All mice were sensitized with CRBC (10^8 cells, SC) at day 4 and challenged with the same dose of antigen at day 8 for DTH measurement at day 9.

The comparison of the data among groups of mice at various ages which had received only the treatment with 5-FU clearly indicated that the rate of natural recovery from the 5-FU-induced immunosuppression is more rapid in younger than in older mice. In fact, in mice 6 weeks old recovery was almost complete (as seen by the footpad reaction), so that no significant effect of thymosin could be expected in these circumstances. On the other hand, the activity of thymosin to accelerate the natural recovery was clearly seen with the mice 12 weeks old by comparing groups without and with the thymosin treatment. Therefore, further assessment of the thymosin activity was carried out with mice 12 weeks old utilizing the above dosing schedule.

When normal mice without 5-FU administration were treated with thymosin according to the above schedule, thymosin did not show any effect on their DTH response (Table 2, expt C).

The dose-response relationship of this new model was examined with several thymosin preparations from calf thymus and α_1-peptide prepared by chemical synthesis. The results with a representative preparation of thymosin F-5 and α_1-peptide are shown in Table 2, expts. A, B. A clear dose-response with a maximum was seen in both cases. The potency of α_1-peptide was about 100–1,000 times of that of F-5 preparations.

The specificity of the system was examined by substituting thymosin with preparations corresponding to F-5 derived from calf spleen and liver. No activity could be found with either preparation with the dose range of 2.5–10 mg/kg. Bacterial lipopolysaccharide (LPS), which possibly contaminates these preparations from calf tissues, also did not show any activity at a dose of 0.5 μg/kg, the maximum level of possible contamination.

Effect of Thymosin on Adoptive Transfer of the DTH Response in Immunosuppressed Mice

It is known that sensitized T cells can adoptively transfer the DTH response into non-immunized recipients and that macrophages play an important role in the expression of the delayed footpad reaction. In an attempt to clarify the mechanism action of thymosin was examined separately for its effect on the induction phase and on the expression phase (footpad swelling) of the DTH response by the adoptive transfer technique.

A) Effect of Thymosin α_1 on the Induction Phase. Protocol A, shown in Fig. 1, was used. Briefly portions of non-adherent

Table 3. Effect of thymosin α_1 on lymph node cells of donor immune mice adoptively transferred into normal recipients[a]

Group	Donor mice treated with	DTH response[b] Mean ± SE (0.1 mm)
Expt 1		
1	Saline (control)	3.7 ± 0.4***
2	5-FU alone	2.2 ± 0.3
3	5-FU plus thymosin α_1	4.1 ± 0.3***
4	Donor control	(8.0 ± 1.0)
Expt 2		
1	Saline (control)	3.8 ± 0.4*
2	5-FU alone	2.7 ± 0.3
3	5-FU plus thymosin α_1	3.7 ± 0.3**
4	Thymosin α_1 alone	3.8 ± 0.2**
5	Donor control	(7.1 ± 0.7)

[a] The study was performed according to protocol A in Fig. 1
[b] P value: statistical difference from group 2 (5-FU alone)
* $P < 0.1$; ** $P < 0.05$; *** $P < 0.01$

Table 4. Effect of thymosin α_1 on recipient non-immune mice in which lymph node cells from sensitized normal donor mice were adoptively transferred [a]

Group	Donor mice treated with	DTH response[b] Mean ± SE (0.1 mm)
Expt 1		
11	Saline (control)	3.7 ± 0.4*
12	5-FU alone	3.0 ± 0.2
13	5-FU plus thymosin α_1	3.9 ± 0.2**
14	Donor control	(8.0 ± 1.0)
Expt 2		
11	Saline (control)	3.8 ± 0.4**
12	5-FU alone	2.9 ± 0.3
13	5-FU plus thymosin α_1	3.9 ± 0.3**
14	Thymosin α_1 alone	3.9 ± 0.3**
15	Donor control	(7.1 ± 0.7)

[a] The study was performed according to protocol B in Fig. 1
[b] Statistical difference from group 12 (5-FU alone)
P value: * $P < 0.1$; ** $P < 0.05$

lymph node cells pooled from groups of sensitized donor mice which had received saline (control), 5-FU alone, or 5-FU and thymosin α_1 ($n = 5$ each) were transferred with the antigen to groups of normal recipient mice ($n = 7$ each) for their footpad reaction. The results are shown in Table 3. The response observed following the transfer of cells from the donor mice which had received both 5-FU and thymosin α_1 was significantly higher than that caused by the transfer of cells from the donor mice which had received only 5-FU, and was almost equal to that observed following the transfer of cells from the control donor mice. These results indicate that the T cells damaged by 5-FU can be restored to normal by an additional treatment with thymosin α_1. It was also seen in these experiments that the yield of lymph node cells was somewhat lower in the 5-FU-treated donor mice (75% of yield from mice) but close to the normal level in the mice treated with 5-FU and then with thymosin α_1.

B) Effect of Thymosin α_1 on the Expression Phase. Protocol B shown in Fig. 1 was used. Briefly, non-adherent lymph node cells from a group of sensitized normal mice ($n = 8$) were transferred with the antigen to groups of recipient mice which had previously received saline (control), 5-FU alone , or 5-FU and thymosin α_1 ($n = 7$ each) for assessment of their footpad reactions.

The results are shown in Table 4. Again, the response in the recipient mice which had received both 5-FU and thymosin α_1 was significantly higher that that in the recipient mice which had received 5-FU alone, and was almost equal to that in the control group.

These results suggest that the macrophages damaged by 5-FU can be restored to normal by an additional treatment with thymosin α_1.

Additional results on the effect of thymosin α_1 on donor or recipient mice which had not received 5-FU are also shown in Table 3 (expt. 2, group 4) and Table 4 (expt. 2, group 14). No effect was seen in either case.

Effect of 5-FU and Thymosin on the Progenitor Cells of Macrophages (CFU-c)

Since it was suggested in the previous section that thymosin α_1 probably affects the macrophage function, thymosin α_1 was examined for its effect on the development of macrophages.

Table 5. Restoration by thymosin of CFU-c suppressed in the 5-FU-treated mice

Mice treated with	Dose of thymosin (μg/kg)	BM cells		CFU-c, mean ± SE[a]			
		Per leg × 10^5	(%)	Per 10^5 cells	(%)	Per leg	(%)
Expt A							
Saline (control)	–	54.0	(100)	171.5 ± 2.3***	(100)	9,260 ± 724***	(100)
5-FU alone	–	14.6	(28)	19.8 ± 1.0	(12)	300 ± 15	(3)
5-FU plus thymosin F-5	5,000	27.0	(50)	60.8 ± 3.9***	(36)	1,640 ± 105***	(18)
Expt B							
Saline (control)	–	81.1	(100)	114.0 ± 4.7***	(100)	9,245 ± 381***	(100)
5-FU alone	–	29.5	(36)	23.5 ± 4.1	(21)	693 ± 121	(8)
5-FU plus thymosin α_1	50.0	55.3	(66)	64.8 ± 4.4***	(57)	3,454 ± 235***	(37)
	5.0	29.6	(36)	54.3 ± 4.8***	(56)	1,903 ± 142***	(21)
	0.5	31.3	(39)	16.0 ± 1.6	(14)	501 ± 50	(5)

[a] P value: statistical difference from the corresponding 5-FU-treated group. *** $P < 0.001$

CFU-c (colony forming units in culture) which differentiate from multipotential haemopoietic stem cells are known to be progenitor cells of macrophages and granulocytes.

Groups of mice injected with 100 mg 5-FU/kg ($n = 5$) were subsequently treated IP with either saline (control), thymosin F-5 or thymosin α_1, 4 h and 1 and 2 days after the 5-FU injection. The mice were sacrificed for the CFU-c test 3 days after the 5-FU injection. As shown in Table 5, both the number of bone marrow cells and the proportion of CFU-c in bone marrow were markedly reduced by 5-FU. The subsequent treatment with thymosin α_1 as well as thymosin F-5 restored both the number of bone marrow cells and the proportion of CFU-c to a very significant degree. These results suggest that the damage caused to the bone marrow and the CFU-c can be corrected quite significantly, though only partially.

Discussion

The present work shows that thymosins from calf thymus and α_1-peptide obtained by chemical synthesis restore cell-mediated immunity when this has been suppressed by injection of 5-FU in mice. Conditions suitable for assessing the thymosin activity were established in such immunosuppressed mice by means of delayed footpad reactions against nucleated chicken erythrocytes (CRBC). The activity of thymosin could be shown in older mice (12 weeks old) by acceleration of the natural recovery of their DTH response suppressed by injection of 5-FU. Such accelerating activity could be seen neither with similar preparations from calf spleen and liver nor with *E. coli* LPS, indicating the specificity of this animal model.

A clear dose-response relationship was seen with both calf thymus thymosins and synthetic α_1-peptide. They seem to have optimal doses (bell-shape response) as seen in the case of other immunomodulators. However, since thymosin showed no enhancing effect on the DTH response in normal mice without prior 5-FU injection, thymosin seems to act as an immunomodulator rather than an immunostimulator. Thus, this animal model seems to be useful in evaluating the thymosin activity in vivo. Synthetic thymosin α_1-peptide showed an activity at low doses of 5–50 μg/kg, which were 100–1,000 times lower than the doses required for thymosin preparations from calf thymus.

Primus et al. have reported that thymosin could not restore the mitogen response of spleen cells and tumour allograft rejection in adult thymectomized mice [13]. Martinez et al. also reported that thymopoietin, ubiquitin, and FTS (synthetic serum thymic factor) were ineffective in both systems [10]. Although a number of factors may be considered possibly to affect these models, complete removal of the thymus may also deprive the organism of some thymus-derived factors other than thymosin which are required for such restoration. Furthermore, the conditions used in these models may not have been optimal for showing the activity of these preparations.

Our present studies utilizing the adoptive transfer technique have indicated that 5-FU causes severe damage to the cell populations responsible, both in the induction phase and in the expression phase of the delayed footpad reaction, and that thymosin α_1 restores the cells affected by this damage. This means that thymosin α_1 restores the function (or number) not only of the mature T cells transferring the delayed footpad reaction to the recipient mice, but also of the macrophages responsible in the expression phase of the delayed footpad reaction. Okuda et al. have reported a similar restoration by *C. parvum* of the depressed macrophage function in the expression phase of the delayed footpad reaction in EL-4 tumour-bearing mice [12].

Furthermore, the present studies showed that progenitor cells of macrophages and granulocytes, CFU-c, are also sensitive to 5-FU and that the subsequent treatment with thymosin caused a significant increase of the CFU-c depressed by 5-FU. This probably contributes to the restoration of the depressed macrophage function, as seen in the DTH response. Therefore, the action of thymosin in our animal model seems to be due to replenishment and maturation of haematopoietic cells necessary for the DTH response, which had been suppressed by the 5-FU administration. The finding that thymosin stimulates regeneration of the depressed CFU-c is quite interesting, since it seems to provide an additional function to thymosin which has been known as a stimulator for differentiation/maturation of T cells. However, it remains to be resolved whether thymosin acts directly on the myeloid progenitor cells or stimulates them through its effect on T cells.

The activity of thymosin to stimulate regeneration of both lymphoid and myeloid cells would predict its broad clinical application in patients in an immunodeficient state. It may be particularly useful in cancer patients with certain malignant haematological disorders or receiving intensive chemotherapy or radiotherapy. The restoration of some of the defects in their host defence systems may prevent microbial infections and tumour metastasis. There have been some reports that thymosin is active in regeneration of T cells or lymphoid tissue during radiation therapy in men and mice [5, 9]. Ishitsuka et al. observed that thymosin is effective in preventing not only opportunistic infections but also metastasis of some tumours in immunosuppressed mice [15, 16].

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