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EFFECT OF LOW-MOLECULAR-WEIGHT FACTORS OF THYMUS AND PINEAL GLAND ON LIFE SPAN AND SPONTANEOUS TUMOUR DEVELOPMENT IN FEMALE MICE OF DIFFERENT AGE

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SUMMARY

Female SHR mice, aged 3.5 or 12 months, were exposed monthly to 5-day long courses of subcutaneous injections of 0.1 mg thymus-derived or pineal gland-derived polypeptide factors (TF and PF, respectively) or 0.9% sodium chloride solution (control). PF treatment increased life span of both young and middle-aged mice by 20% and 17%, respectively, and TF increased the life span only in young mice. Both factors when administered to young mice caused a decrease in both overall tumour incidence and incidence of mammary adenocarcinomas (TF, 1.8-fold decrease; PF, 2.6-fold decrease). TF administration to mature mice did not produce any antitumour effect, whereas PF possessed certain anti-tumour activity, but the response was far less pronounced than in young animals. The results obtained give additional evidence of the geroprotective and anti-tumour effect of thymus and pineal gland-derived peptide factors. The mechanisms of action of TF and PF and perspectives of clinical use of these agents as geroprotectors are discussed.

Key words: Thymus; Pineal; Peptides; Mouse

INTRODUCTION

The problem of human life prolongation is one of the most important and difficult medico-biological questions ever existed. The search of effective geroprotective means is seriously aggravated by: (i) existence of several controversial theories of ageing; (ii) incompleteness of information on possible side effects of existing geroprotective agents (for example some geroprotectors such as antioxi-

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dants and chelating agents possess carcinogenic properties whereas a number of other agents or factors inhibit growth and reproductive function) [1—5].

According to the immunological theory of ageing, age-related immune disfunction results in a reduction of resitance to infection, and an increase of the risk to autoimmune diseases and cancer [6—9]. Therefore, experimental attempts to stop or at least slow-down the age-associated decrease in immunoreactivity of aged laboratory animals using lymphocyte or thymocyte transplantation from young donors [9] are justified. It is noteworthy that low molecular weight peptide factors obtained from the thymus and bone marrow may also be of value in this respect [9—11].

In recent times a number of reports have appeared in the literature on a regulatory role of the pineal gland in ageing and anti-tumour resistance. Thus, neuroendocrine and immune system-modulating function of the pineal gland was shown to be altered [12—14] in ageing and cancer development [15—18] and pineal gland hormone administration suppressed tumour growth and increased life span in animals [18—23].

We have previously shown in mice and rats that long-term administration of low molecular weight peptides (cytomedines) extracted from thymus and pineal gland (the treatment was commenced when animals were 2—3.5 months old) increased the life span and reduced the incidence of spontaneous tumours [18,19,24,25]. However, the problem when best to initiate the treatment schedule needs to be resolved; i.e. in early post-pubertal period, middle age or onset of ageing.

In the present report the results of a comparative study on the effect of administration of thymus- and pineal gland-derived factors on life span and spontaneous tumour incidence in young and middle-aged mice are described.

MATERIALS AND METHODS

Animals

Two-month-old virgin females of Swiss-derived outbred SHR mice were obtained from "Rappolovo" Animal Farm of the Academy of Medical Sciences of the USSR. The animals were kept 7—8 per plastic cage at 21—23 °C under a 14 h light/10 h dark light cycle and were fed with standard laboratory chow and water *ad libitum*.

Low molecular weight peptide factors of thymus and pineal gland

The physiologically active substances were prepared from bovine thymus and pineal gland, as described earlier with an additional purification of the substances by ion-exchange chromatography with subsequent preparative isoelectrofocusing [11,19,26,27]. The detailed descriptions of the thymus-derived (TF) and pineal gland-derived (PF) factors were presented elsewhere [19,27]. The commercial drug forms of these factors (TF-thymalin, PF-epithlamin) are permitted by the Pharmaceutical Committee of the USSR Ministry of Health for medical use and are in use for treatment of patients in clinics.

The study of TF and PF influence on the life span and spontaneous tumour incidence

Mice (101) aged 3.5 months and 104 mice aged 12 months were randomly divided into 6 groups (3 groups of each age marked respectively as "young" and "middle-aged"). From the above mentioned ages until natural death the animals received monthly a 5-day course of injections of 0.1 ml 0.9% sodium chloride solution (control) or the same volume of solution containing 0.1 mg TF or PF per mouse, respectively.

Pathological examination

All animals were kept until death or killed when moribund. Complete necropsies were performed and skin, brain, thymus, liver, lung, kidney, spleen, gonad and other tissues showing macroscopic lesions were fixed in 10% buffered formalin. Paraffin sections 5—7 μm thick were stained with haematoxylin and eosin and examined histologically. Contexts of observation were registered for all tumours [28]. All the tumours were classified according to IARC recommendations [29].

Statistics

The results of the experiments were treated statistically using "life-table" method, adjusted for intercurrent mortality, for fatal and incidental tumours in combination [28]. Student's *t*-test was also used. Statistical treatment of the results was carried out using Canon AS-100 mini-computer (Canon Inc., Japan).

RESULTS

TF and PF influence on the life span of female SHR mice

The comparison of life duration data in two control groups observed from the age of 3.5 and 12 months (see Table I) and comparison of the survival curves in these two groups (Fig. 1) shows homogeneity among groups. The difference in maximal life span between "young" and "middle-aged" mice is a result of chance as it is seen from Fig. 1. The calculation of the ageing rate for young and middle-aged control groups has shown approximately similar values. The ageing rates were calculated as values of α in Gompertz equation

$$R = R_0 \times e^{\alpha t}$$

where R = mortality, R_0 = mortality at t (time) = 0, e = base of the natural logarithms, α constant.

Long-term treatment of young and middle-aged female mice with PF resulted in 20% and 17% increase of mean life span in respective age groups (taking into account only time from the commencement of treatment). At the same time, the maximal life span of middle-aged TF- and PF-treated mice was shorter than that in the young ones (Table I). Figure 2 shows that treatment with PF markedly shifted

TABLE I

LIFE SPAN OF TF- AND PF-TREATED YOUNG AND MIDDLE-AGED SHR MICE

Age of mice (months)	*Group*	*No. of mice*	*Life span*			*Tumour-free mice*		*Tumour-bearing mice*	
			Mean (M ± m) (Days)	*Med.*	*Max.*	*No.*	*Mean life span (M ± m) (Days)*	*No.*	*Mean life span (M ± m) (Days)*
3.5	1,CONTROL	31	564 ± 22.3	558	843	14	553 ± 38.2	17	574 ± 26.8
3.5	2,TF	38	605 ± 19.1	560	871	21	609 ± 25.7	17	598 ± 29.5
3.5	3,PF	32	627 ± 20.9	634	827	20	643 ± 25.8	12	599 ± 35.7
12	4,CONTROL	41	576 ± 18.3	575	795	18	557 ± 29.5	23	591 ± 23.2
12	5,TF	30	563 ± 16.6	573	750	18	585 ± 25.1	12	531 ± 14.5
12	6,PF	33	612 ± 13.6	594	768	18	626 ± 19.7	15	595 ± 18.2

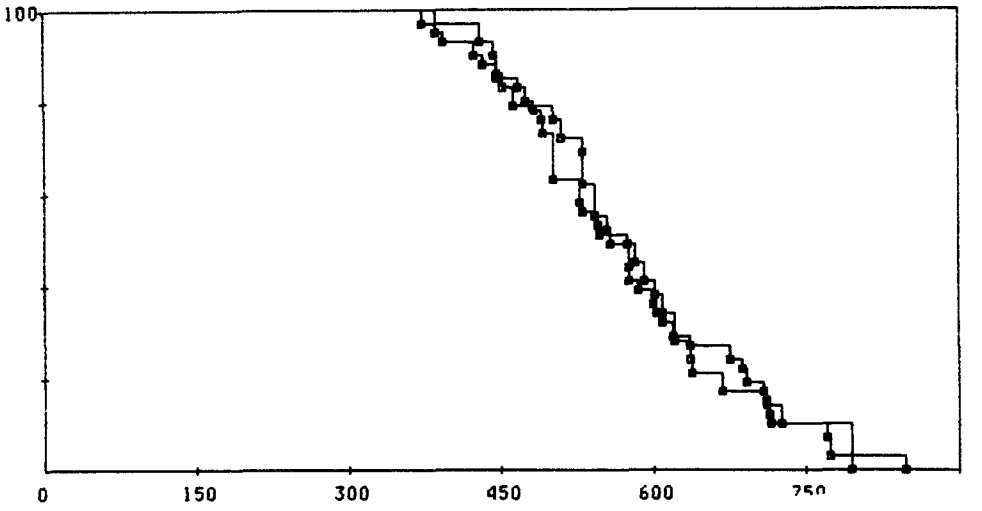

Fig. 1. Kaplan-Meier estimation of animal survival in young and middle-aged age control groups (only animals that survived more than 12 months were included into analysis). OX — age (days); OY — % survived; open squares — young animals; filled squares — middle-aged animals.

survival curves of both age groups to the right comparing to corresponding controls. TF effect was much weaker and was detected only in young mice. The ageing rate calculation in young and middle-aged groups treated with TF or PF revealed the slow-down of ageing in young animals compared with middle-aged ones: α(TF young) = 0.0065 day^{-1} *vs.* α(TF middle-aged) = 0.0071 day^{-1}, α(PF young) = 0.0052 day^{-1} *vs.* α(PF middle-aged) = 0.0077 day^{-1}.

It is noteworthy that TF and PF administration increased life span of tumour-free young mice by 12% and 20% ($P < 0.05$) respectively, i.e. exerted true geroprotective effect. In middle-aged animals this increase was 15% and 36%, respectively ($P < 0.05$) (see Table I).

TF and PF influence on spontaneous tumour incidence in SHR female mice

TF and PF administration to young mice caused significant reduction in spontaneous tumour incidence, first of all because of decrease of mammary tumour incidence — 1.8-fold and 2.6-fold, respectively, $P < 0.025$ (Tables II and III).

As it is shown in Fig. 3a, TF and PF administration shifted curves of tumour-free survival to the right in young mice, whereas in middle-aged group only PF treatment exerted similar, but feebly marked effect (see Fig. 3b).

It is clear from Fig. 4a that in mice treated with TF and PF from the age of 3.5 months, mammary tumour development was significantly inhibited. This result is also supported by the fact of decrease of number of mammary adenocarcinomas per tumour-bearing animal: 1.46 — control group; 1.22 — in TF-treated mice; 1.20 —

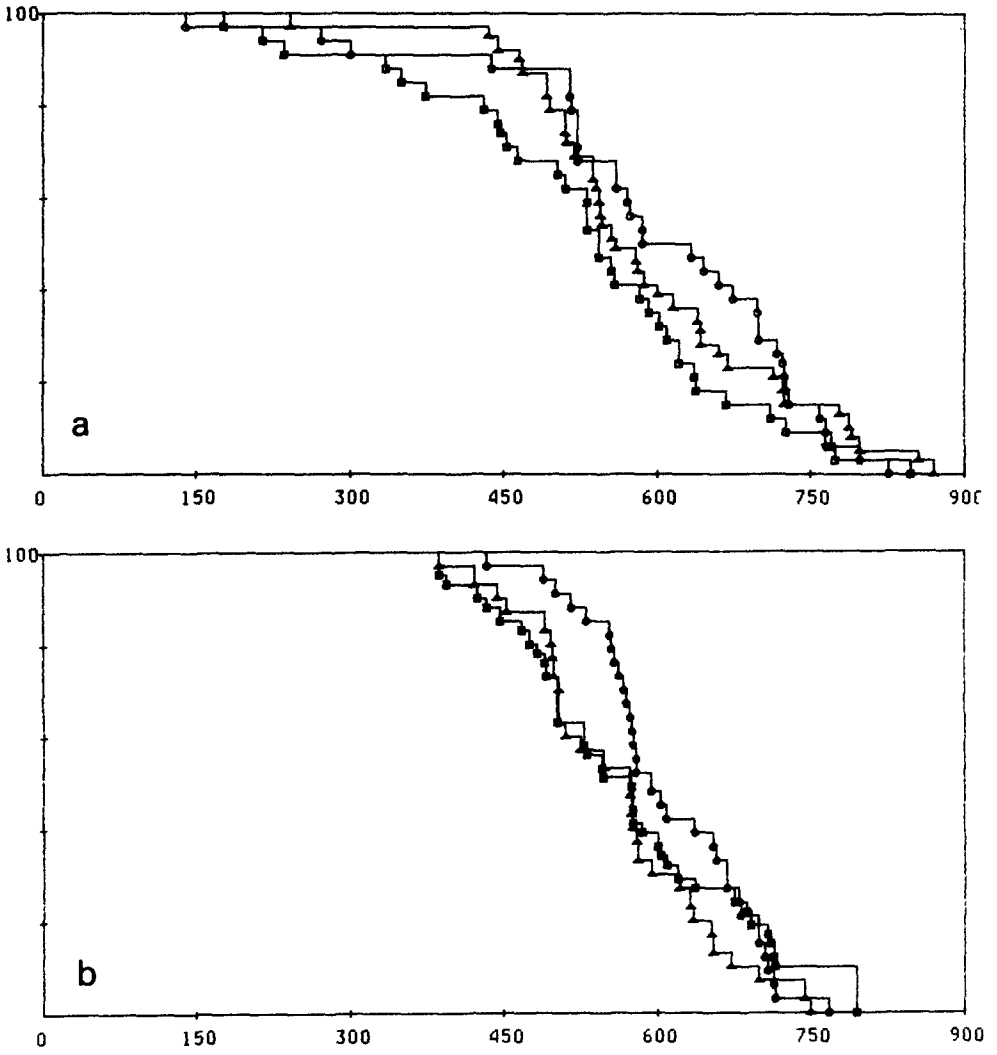

Fig. 2. Survival estimation in young (a) and middle-aged (b) animals of all experimental groups (Kaplan-Meier method). OX — age (days); OY — % survived; open squares — young animals, control; open triangles — young animals, TF; open circles — young animals, PF; filled squares — middle-aged animals, control; filled triangles — middle-aged animals, TF; filled circles — middle-aged animals, PF.

in PF-treated mice. In middle-aged group this effect was absent, at the same time the response to PF administration was reduced comparing to young mice (Fig. 4b).

TF and PF treatments did not significantly influence any other than mammary gland tumour development. It must be mentioned, however, that a tendency towards decrease of incidence of leukaemias and other tumours in middle-aged animals, treated with TF and PF (Table II) was observed.

TABLE II

TUMOUR TYPES AND LOCALIZATIONS IN THE EXPERIMENTAL GROUPS

Age of mice (months)	*Group*	*No. of mice*	*No. of mice with tumours*			*No. of tumours*		*Tumour type and localization*					
			Abs.	*Rel. (%)*	*Cum. (%)*	*Total*	*Per mouse*	*Mammary AdCa*			*Leuk-aemia*	*Lung AdCa*	*Other*
								No. mice	*%*	*No. tumours*			
3.5	1,CONTR.	31	17	54.6	83.8	27	1.59	13	41.9	19	4	1	3(a)
3.5	2,TF	38	17	44.7	58.2	26	1.53	9	23.7	11	3	4	8(b)
3.5	3,PF	32	12	37.5	55.2	14	1.17	5	15.6	6	3	2	3(c)
12	4,CONTR.	41	23	56.1	87.9	32	1.39	10	24.4	11	10	2	9(d)
12	5,TF	30	12	40.0	66.4	13	1.08	7	23.3	7	5	1	0
12	6,PF	33	15	45.5	71.0	18	1.20	6	18.2	7	5	2	4(e)

(a) Two uterine polyps; (b) 2 uterine polyps and 1 Ca *in situ*, 4 skin papillomas, 1 mammary fibroadenoma; (c) 1 kidney tumour of mesenchymal origin, 1 uterine polyp, 1 skin papilloma; (d) 4 uterine AdCa, 1 skin cancer, 1 lung adenoma, 1 haemangioma, 2 haemangiosarcomas; (e) 2 skin cancers, 1 skin papilloma, 1 haemangioma.

TABLE III

LOG-RANK TEST COMPARISON OF TUMOUR RATES IN THE EXPERIMENTAL GROUPS

Comparison	*Expected no. (observed/expected ratio) in group*						*Log-rank chi-sq.*	*df*	*P-value*
	1	*2*	*3*	*4*	*5*	*6*			
(a) Groups 1,2,3, 4,5,6	11.61 (1.46)	20.84 (0.82)	18.99 (0.63)	17.46 (1.32)	10.60 (1.13)	16.49 (0.91)	10.00	5	0.075
(b) Groups 1,2,3,	10.51 (1.62)	18.73 (0.91)	16.76 (0.72)	—	—	—	5.70	2	0.058
(c) Groups 4,5,6	—	—	—	20.33 (1.13)	11.80 (1.02)	17.87 (0.72)	0.90	2	0.637
(d) Groups 1,2	12.43 (1.37)	21.57 (0.79)	—	—	—	—	3.87	1	0.049
(e) Groups 1,3	11.52 (1.48)	—	17.48 (0.69)	—	—	—	4.53	1	0.033
(f) Groups 1,4	16.02 (1.06)	—	—	23.98 (0.96)	—	—	0.11	1	0.745
(g) Groups 4,5	—	—	—	21.86 (1.05)	13.14 (0.88)	—	0.17	1	0.678
(h) Groups 4,6	—	—	—	20.33 (1.13)	—	17.67 (0.85)	0.84	1	0.358
Observed nos.	17	17	12	23	12	15			

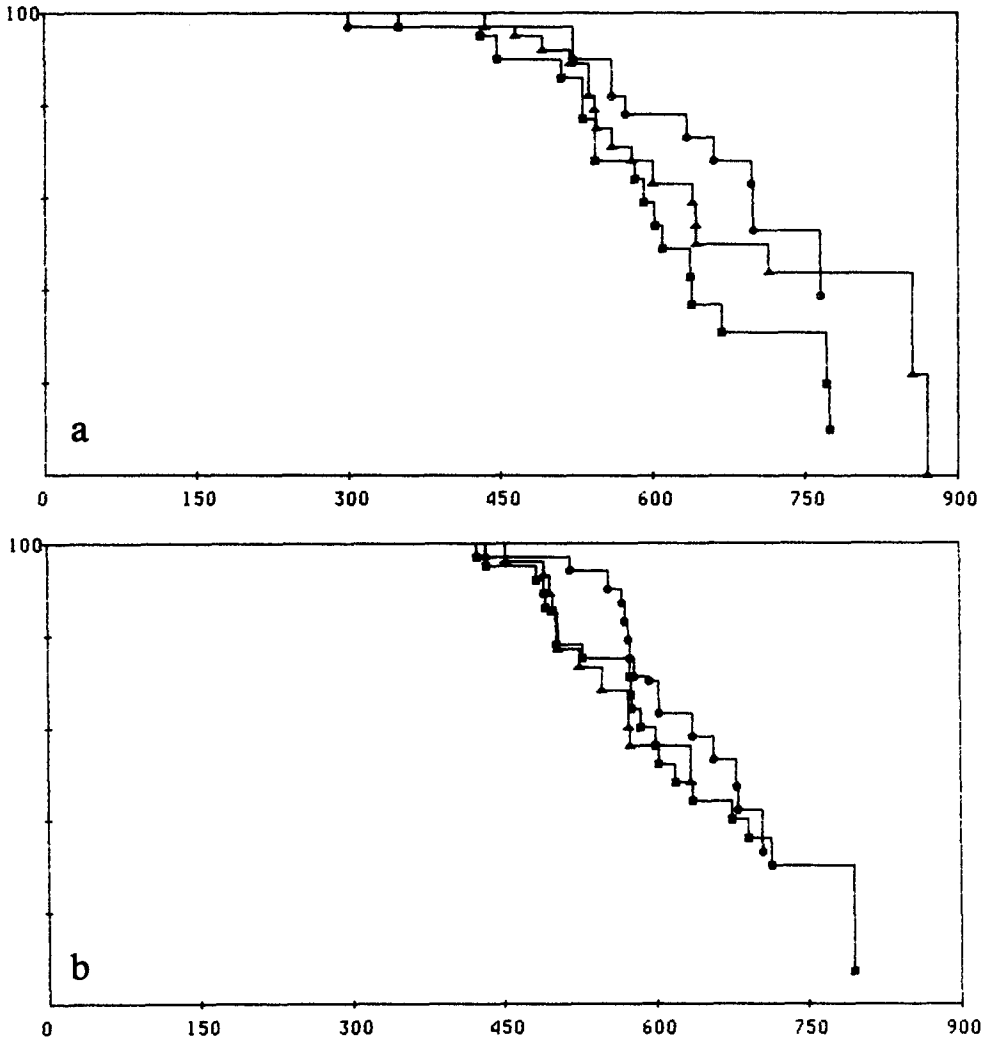

Fig. 3. Time-to-tumour estimation in young (a) and middle-aged (b) animals of all experimental groups (Kaplan-Meier method). OX — age (days); OY — % survived without any tumour. Symbols for experimental groups are as in Fig. 2.

DISCUSSION

The present experiments have shown that geroprotective effect of TF and PF in SHR mice was less pronounced than in previous experiments in C3H/Sn mice [19]. This may result from both strain peculiarities of the animals used and preparation dosage differences. In the experiments with C3H/Sn mice, TF and PF were administered using the same schedule, but a daily dose was 0.5 mg per mouse, providing as a

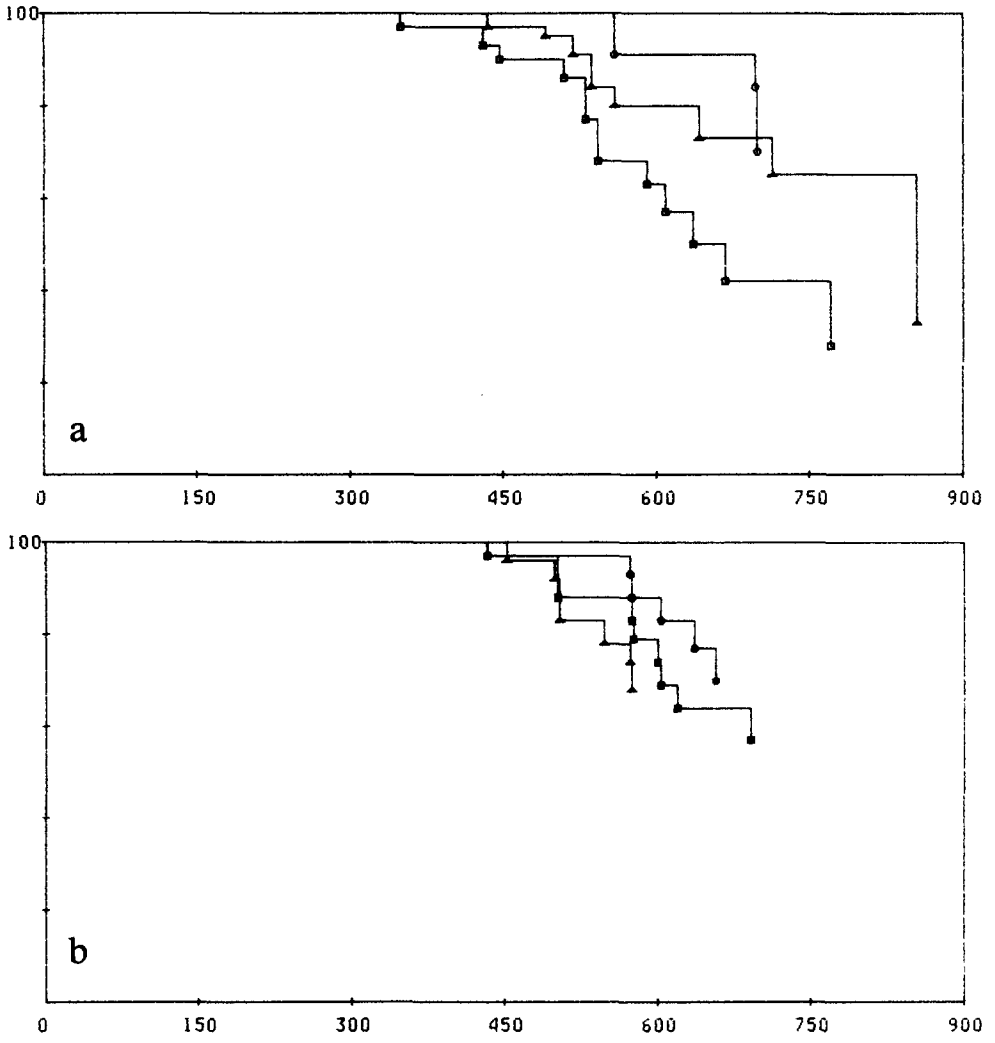

Fig. 4. Time-to-tumour estimation for mammary adenocarcinomas in young (a) and middle-aged (b) animals of all experimental groups (Kaplan-Meier method). OX — age (days); OY — % survived without mammary adenocarcinomas. Symbols for experimental groups are as in Figs. 2 and 3.

result life span prolongation by 28% and 31%, respectively [19], whereas in the present experiment in SHR mice a daily dose was 0.1 mg per animal. In rats we have previously shown geroprotective and antitumour effect of PF to be dose dependent [24]. It is interesting that PF possessed geroprotective and anti-tumour effect when administered both in young and in middle age, while TF was effective only in young mice. It is also noteworthy that the mean life span in young and middle-aged treated groups was about the same but the maximal life span was reduced in middle-aged mice. The reasons of this phenomenon are obscure now. One suggestion is the dif-

ference in the slope of survival curves (ageing rate) in TF- and PF-treated young and middle-aged mice. Similar regularities of the same relationship between ageing rate, mean and maximal life span were summarized elsewhere [5]. At the same time there is a high correlation between ageing rate and tumour incidence in animals untreated or treated with geroprotectors [2,5]. These findings seem to serve a good explanation for more pronounced anti-tumour effect of TF and PF in young mice compared with the middle-aged group.

These observations allow us to propose that TF administration may be recommended first of all at a relatively young age, whereas PF is effective in a wider age range. This is in accordance with the data that the thymus-derived agent thymosin restores immunological response in young mice more than in old ones [30], and repeated thymus grafting from newborn mice increases life span only in the case of commencement of this procedure in young age [31]. As was previously described [32], dietary calorie restriction results in an increase in life span and decrease in spontaneous tumour frequency after caloric restriction not only in young (2—3 months), but also in mature (12—17 months) age. However, in mice of older age groups this effect was less evident than in young ones. Despite the fact that the geroprotective effects of TF and PF on the one hand, and caloric restriction on the other hand, are different (the latter increases maximal life span, whereas peptide preparations did not increase maximal but only mean life span), some mechanisms of geroprotective action of all these influences could be similar.

The mechanism of geroprotective action of caloric restriction appears to be complex and dependent on slow-down of ageing of immune system [32] and neuroendocrine system, in particular on slowing of age-related decrease of functional activity of pineal gland [33], exerting multifactorial modulating influence upon both systems mentioned [5, 12—14, 20]. Previously it was shown that TF and PF application delays age-associated immunity alterations in mice [19,32]. This phenomenon may be of importance in the mechanisms of geroprotective and anti-tumour action of these cytomedines. On the other hand, it is noteworthy, that if thymus-derived factor stimulates specifically cell-mediated immunologic reactions [11,19,27,34], then PF, alongside with immunomodulating effect provides normalizing influence on a series of naturally occurring age-related hormone-metabolic shifts facilitating development of neoplasia [5,19,24,35,36]. Besides, it has been shown that long-term administration of PF delays age-related loss of estral function in female rats, restores fertility in old female rats with persistent estrus, and decreases the threshold of sensitivity of the hypothalamo-pituitary system to estrogens by feedback inhibition [5,24]. It is quite probable that this property of PF causes multiple effects at the level of different homeostatic systems of the organism and, eventually, defines its geroprotective and anti-tumour effects, because increase in the threshold of sensitivity of hypothalamus to inhibition seems to play a leading role in the elevation mechanism of ageing and formation of age-related pathology including cancer [35,36].

We have also studied the influence of TF and PF on the development of tumours induced by different chemical carcinogens (7,12-dimethylbenz[*a*]anthracene and nitrosomethylurea) and whole-body X-ray irradiation. In all these cases carcinogenesis-inhibiting action of the preparations tested was observed [5,18,37—40].

Thus, there is a growing body of evidence [5,18,19,24,34, present work] indicating on the capacity of low molecular weight peptide factors extracted from thymus and pineal gland to prolong animals' life span, simultaneously inhibiting spontaneous tumour development. These observations provide an evidence supporting a concept on the possibilities of practical application of these agents in clinical situations for the prolongation of the period of active life and primary cancer prevention not only in young, but also in mature age. At the same time the problem of further isolation, and characterization of individual active peptides and investigation of their effects on life span and tumour development is an actual task of our current experiments.

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