

CARCINOGENESIS AND AGING.

IV. EFFECT OF LOW-MOLECULAR-WEIGHT FACTORS OF THYMUS, PINEAL GLAND AND ANTERIOR HYPOTHALAMUS ON IMMUNITY, TUMOR INCIDENCE AND LIFE SPAN OF C3H/Sn MICE

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SUMMARY

The low-molecular-weight polypeptide factors were obtained from bovine thymus (TF), pineal gland (PF) and anterior hypothalamus (AHF). Both TF and PF administration enhanced the rejection of skin allograft and stimulated the immunological response to sheep erythrocytes in adult CBA mice. Treatment of CBA mice with AHF increased the graft survival and inhibited antibody formation to sheep erythrocytes. Chronic TF or PF administration decreased spontaneous tumor development and prolonged the life span of female C3H/Sn mice. Administration of AHF failed to influence the life span and the tumor incidence of female C3H/Sn mice. The role of immunity and hormonometabolic shifts in mechanisms of both aging and the age-associated increase in cancer incidence are discussed.

INTRODUCTION

The life span and spontaneous tumor incidence are especially important among integral biological characteristics of species. Both parameters are determined by a number of events developing during ontogenesis at various levels of organization (subcellular, cell, tissue and organism) and which are in many cases closely interrelated. The data on the identity of both mortality curves and cancer mortality curves for human from 10 to 80 years stress the profound interrelation between gerontogenesis and oncogenesis [1]. However, up to now, there is no uniform opinion on the nature of this interrelation, and on the reasons of age-associated increase of cancer incidence.

From the oncological point of view the mechanisms of this process may be explained as follows. Firstly, the sensitivity of the organism to carcinogens does not change during aging, and therefore the age-associated increase in tumor incidence should be considered as a result of carcinogen dose summation and/or a result of increased exposure

time [2, 3]. Secondly, the age-associated increase of tumor incidence should be considered as a result of the age-associated hormonometabolic and immunological shifts which increase the sensitivity to carcinogen [4, 5].

From the gerontological standpoint the age-associated increase of cancer incidence may be explained by the theories of "errors" or as a consequence of the realization of the genetic programme of aging and the formation of age-associated pathology.

Analysis of data on chemical carcinogenesis does not lead to a uniform conclusion on the increase of the organism sensitivity to the carcinogen action [6]. This sensitivity is determined by age dynamics of some factors: metabolizing enzyme activity, capacity to form mutagenic adducts, the value of their binding with DNA, accuracy in DNA repair, proliferative activity of target tissues [6-8]. There is a great difference in the age-associated sensitivity of the organism to the action of radiation, virus, and hormonal carcinogenic agents [9, 10].

We suppose that one of the approaches to the study of mechanisms determining the close interrelation between aging and carcinogenesis may consist of the evaluation of the effect of some drugs that prolong the life span on spontaneous tumor incidence. According to the concept of the leading role of the thymus in age-associated lesions in immunity, Makinodan [11] has supposed that the improvement of immunological functions by thymocyte transplantation or administration of thymic hormones may prevent the development of some age-associated pathological processes and lead to prolongation of the life span. This paper deals with the effect of immunostimulators—polypeptide thymic factor and pineal factor—and immunoinhibitor—polypeptide factor of the anterior hypothalamus—[12-17] on immunity, life span and tumor incidence in female C3H/Sn mice.

MATERIAL AND METHODS

Animals

Two-month-old virgin female C3H/Sn mice, male CBA, CC57Br and B6D2F₁ mice, and outbred female rats were purchased from the Rappolovo breeding nursery of the Academy of Medical Sciences of the U.S.S.R. The animals were kept 7-8 per plastic cage under lighting conditions of 14 hours light : 10 hours dark, and with standard lab chow and tap water *ad libitum*.

Preparation of thymic, pineal and anterior hypothalamic factors

The physiologically active substances were prepared from bovine thymus, pineal gland and anterior hypothalamus as described earlier [12-15]. The native tissues at -4 °C were kept in acetone for 48 hours. After the acetone was poured off the tissues were homogenized and extraction was performed in 3% acetic acid at a ratio of 1:6 (v/v) in the presence of ZnCl₂ for 72 hours. After the final extraction, and centrifugation for 20 min at 3000 rpm, acetone was added to the supernatant (at -4 °C; 1:8). After precipitate formation the acetone was poured off. The precipitate was treated with

acetone and ether in the filter until a white powder was formed. The powder was dissolved, sterilized and lyophilized.

When studying the prepared substances by ion-exchange chromatography on the carboxyl cationite Biocarb (U.S.S.R), gel-filtration on Sephadex G-25 (Pharmacia, Sweden) and electrophoresis on cellulose layers (Filtrak, G.D.R.), it was found [15] that the above substances are complexes of polypeptides. The characteristics of polypeptides prepared by ion-exchange chromatography are presented in Table I. The total preparations dissolved in normal saline were used in all experiments.

Reaction of allogenic skin transplant rejection

The method of Billingham and Medawar [17] was used with slight modification. Skin samples (1.5 × 1.5 cm) from the donor's back (3-month-old male CC57Br mice) were placed in media 199 (Institute of Poliomyelitis and Viral Encephalitis, Moscow, U.S.S.R.) and were cleaned of subcutaneous fat. Recipients (3-month-old male CBA mice) under hexenal anaesthesia were administered procain subcutaneously (0.5 ml of a 0.25% solution) into the area of transplantation. Then the skin was separated without the subcutaneous fat from the back. The donor skin graft was fixed on the recipient's back with gum.

The mice were injected subcutaneously with the test substances at a dose of 0.1 mg per mouse for three days before grafting and for four days afterwards. The control animals were injected with normal saline.

Antibody titer to sheep erythrocytes

The preparations being tested were administered to 3-month-old CBA mice subcutaneously daily for four days, the dose being 0.1 mg per mouse. The control animals

TABLE I
PHYSICOCHEMICAL CHARACTERISTICS OF COMPONENTS EXTRACTED FROM BOVINE THYMUS, PINEAL GLAND AND ANTERIOR HYPOTHALAMUS

<i>Preparation</i>	<i>Components</i>	<i>Content in total preparation (%)</i>	<i>Molecular weight</i>	<i>Isoelectric point (pH units)</i>
Thymic factor (TF)	Fraction 1	80	660	2.5
	Fraction 2	15	5000	4.0
	Fraction 3	5	5000	9.5
Pineal factor (PF)	Fraction 1	74	250	3.0
	Fraction 2	16	11000	5.0
	Fraction 3	10	1200	10.5
Anterior hypothalamus factor (AHF)	Fraction 1	70	900	2.7
	Fraction 2	18	8800	5.5
	Fraction 3	12	3200	10.5

were treated with normal saline. On the fifth day a suspension of 1×10^7 sheep erythrocytes was administered intravenously into the tail vein of mice. The antibody levels were determined 3, 5, 7 and 10 days later by hemagglutination reaction: 0.25 ml of a 3% suspension of sheep erythrocytes was added to 0.25 ml of serum in sequential dilutions. The titer of hemagglutination was evaluated in 24 hours.

Study of the effect of thymic (TF), pineal (PF) and anterior hypothalamus (AHF) factors on life span and tumor incidence

Eighty-six female C3H/Sn mice were divided into four groups. From the age of 3.5 months the animals were treated subcutaneously with 0.2 ml of normal saline (control group) or with 0.5 mg of TF, PF or AHF dissolved in 0.2 ml of normal saline for five consecutive days once a month up to their natural death.

Pathological examination

All the dead animals were autopsied. Any tumors discovered were fixed in 10% neutral formalin and were covered with paraffin. Slices 5–7 μm thick were stained with haematoxylin and eosin. All the tumors were classified according to Turusov's classification [18].

Transplanted tumors

Leukemia L1210 cells (10^6) were inoculated intraperitoneally in 0.2 ml of normal saline into 16 B6D2F₁ mice 3 months' old. Walker-256 carcinoma and Pliss lymphosarcoma were grafted subcutaneously to rats by a routine method. TF in the dose of 2 mg per animal per day was administered daily from the first day after L1210 inoculation and from the third day after transplantation of solid tumors. Leukemia-inoculated mice were observed up to their death. The tumor-bearing rats were sacrificed on the 12th day after tumor transplantation, and the tumors were weighed.

Statistics

For statistics Student's *t*-test, chi-square test, the Wilcoxon–Mann–Witney *U*-test and *P*-value method were used [19, 20]. The life-table method was used for estimating the cumulative incidence of tumors [21].

RESULTS

Effect of TF, PF and AHF on cell-mediated and humoral immunity in young adult CBA mice

Table II shows that TF and PF administration enhances the rejection of skin grafts (31.8% and 16.5%, respectively), and that AHF administration increases the time of graft survival by 45.9%. Study of the kinetics of antibody levels in immunized mice revealed the peak response on the fifth day after erythrocyte injection in all tested groups. Table II shows that up to the fifth day administration of TF and PF stimulated the immunolog-

TABLE II

EFFECT OF TF, PF AND AHF ON THE REACTIONS OF CELL-MEDIATED AND HUMORAL IMMUNITY IN ADULT CBA MICE

Treatment	Time of rejection of skin allograft (days)		Titre of serum hemagglutinins (\log_2)	
	No. of mice	Mean \pm S.E.M.	No. of mice	Mean \pm S.E.M.
Saline	10	8.5 \pm 0.2	12	8.7 \pm 0.19
TF	10	5.8 \pm 0.4*	12	9.5 \pm 0.28*
PF	9	7.1 \pm 0.2*	12	9.2 \pm 0.37*
AHF	8	12.4 \pm 0.3*	12	8.2 \pm 0.19*

*The difference compared with saline is significant, $p < 0.05$.

ical response to sheep erythrocytes, and treatment with AHF inhibited antibody formation in immunized mice.

Effect of chronic administration of TF, PF and AHF on spontaneous tumor development in female C3H/Sn mice

The tumor incidence in TF- or PF-treated female C3H/Sn mice decreases by 2.8 and 2.1 times, respectively, in comparison to saline-treated control animals (Table III). The effect of AHF administration was not significant ($p > 0.05$) in chi-square and P -value tests. Calculation of the cumulative tumor incidence by the life-table method also revealed a non-significant difference between AHF- and saline-treated mice (Table III, Fig. 1a).

The effect of TF and PF on the development of mammary adenocarcinomas was very pronounced. The mammary tumor incidence decreased by 2.6 and 2.9 times, respectively; the multiplicity of mammary tumors also decreased (Table III). The percentage of mammary adenocarcinomas in AHF-treated mice was diminished in comparison to saline-treated control mice ($p < 0.01$ in chi-square test). However, calculation of the expected incidence of these tumors by the P -value method showed a non-significant level of differences ($p > 0.05$).

It should be stressed that no leukemias were found in TF-treated mice compared to 14.3% of cases in control mice ($p < 0.03$ in P -value test).

Effect of chronic TF, PF and AHF administration on life span of female C3H/Sn mice

Chronic treatment of female C3H/Sn mice with TF or PF significantly (by 28% and 31%, respectively, $p < 0.01$) prolonged their mean life span (Table IV). At the same time the maximum life span increased by 2.5 and 3.5 months, respectively. On the other hand, the average life span of AHF-treated mice was 1 month shorter, and the maximum life span 2.5 months shorter than in the control group. As Fig. 2a shows, the survival curves of control and AHF-treated mice are similar. At the same time, the survival curves of mice chronically treated with TF or PF were significantly shifted to the right. The calculation of mortality rate (R) for mice of separate experimental groups showed that the aging

TABLE III
TUMOR INCIDENCE AND LOCALIZATION IN TF-, PF- AND AHF-TREATED FEMALE C3H/Sn MICE

Treatment	No. of mice	No. of tumor-bearing mice		No. of tumors	Localization and type of tumors								
		Absolute (%)	Relative (%)		Cumulative (%)	Total	Per mouse	Mammary adenocarcinoma	Leukemia	Hepatoma	Ovarian tumor	Others	
Saline	21	14	66.7	83.8	20	0.95	11	52.4	15	3	1	1	—
TF	25	6*	24.0	38.0	8	0.32	5*	20.0	5	0*	—	1	2 ^a
PF	22	7*	31.8	50.9	8	0.36	4*	18.2	4	3	1	—	—
AHF	18	7	38.9	71.6	7	0.39	2	11.1	2	4	—	—	1 ^b

*The difference compared with saline is significant $p < 0.01$.

^aFibroma of abdomen wall; adenomyoma of uterus.

^bEndometrial polyp.

TABLE IV
THE LIFE SPAN OF TF-, PF- AND AHF-TREATED FEMALE C3H/Sn MICE

Treatment	Total No. of mice	Life span (days)			Mice without tumors		Tumor-bearing mice	
		Mean \pm S.E.M.	Median	Maximum	No. of mice	Mean (\pm S.E.M.) survival time (days)	No. of mice	Mean (\pm S.E.M.) survival time (days)
Saline	21	487 \pm 29.4	511	776	7	462 \pm 59.2	14	499 \pm 36.8
TF	25	623 \pm 24.6*	602	863	19	649 \pm 29.3*	6	542 \pm 55.1
PF	22	640 \pm 33.1*	674	885	15	650 \pm 38.5*	7	617 \pm 48.7*
AHF	18	455 \pm 33.3	470	706	11	472 \pm 34.2	7	429 \pm 71.7

*The difference compared with saline is significant, $p < 0.05$.

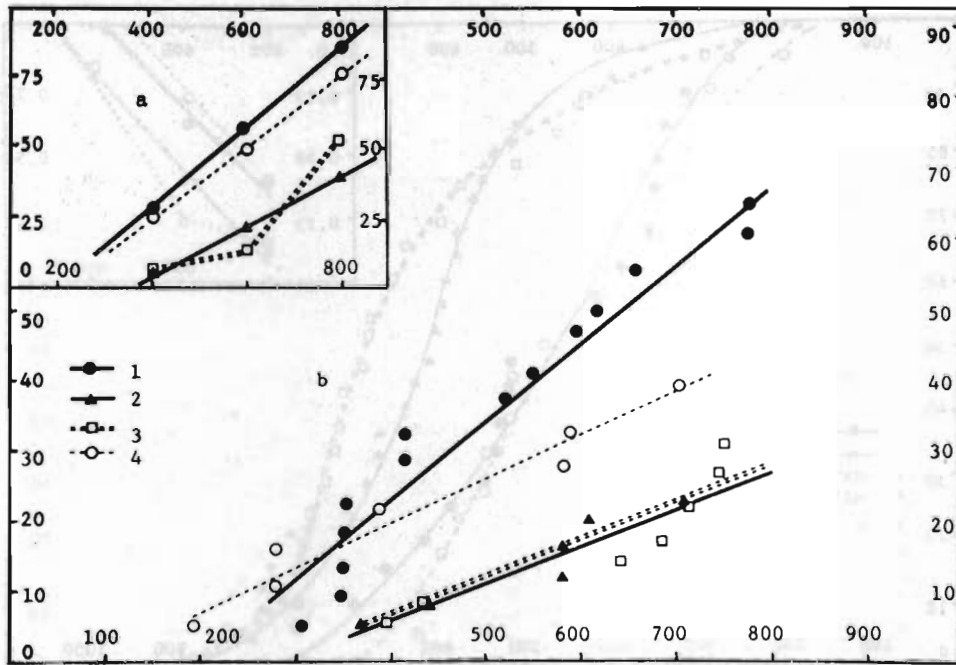


Fig. 1. Total tumor yield in female C3H/Sn mice treated with TF, PF and AHF. (a) Cumulative tumor incidence; (b) relative tumor incidence. Ordinate = number of tumor-bearing mice (%); abscissa = age (days). 1 = saline; 2 = TF; 3 = PF; 4 = AHF.

rate* of saline-, AHF- and TF-treated mice was similar. However, in TF-treated mice the aging onset was delayed (Fig. 2b). The aging rate of PF-treated mice was significantly decreased up to the 600th day of their life, but then increased (Fig. 2b).

Effect of TF administration on the development of transplantable tumors

The mean survival time of B6D2F₁ mice inoculated with leukemia L1210 was 7.5 ± 0.4 days. The treatment of L1210-bearing mice with TF (2 mg per day per mouse) prolonged their survival time to 9.1 ± 0.6 days (by 21.3%). TF administration failed to influence the growth of subcutaneously grafted Walker-256 carcinoma and Pliss lymphosarcoma (Table V).

DISCUSSION

According to the immunological theory of aging, the age-related dysfunction of immunity determines the decrease with age of resistance to infection and is the predispos-

*Aging rate = $\ln \frac{dR}{dt}$ as calculated by Gompertz equation.

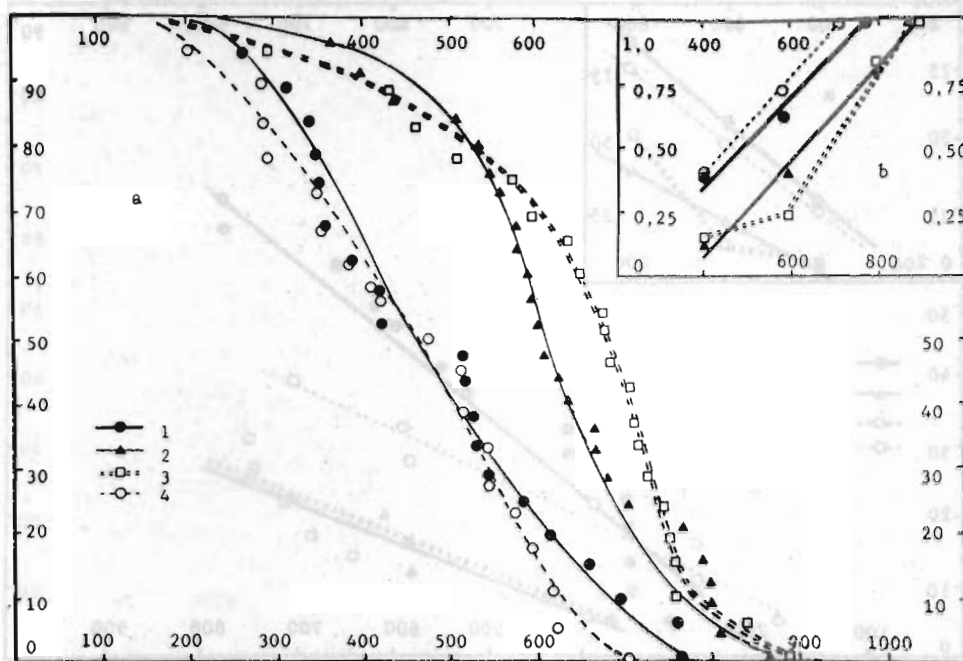


Fig. 2. The life span in female C3H/Sn mice treated with TF, PF and AHF. (a) Survival curves: ordinate = number of mice (%); abscissa = age (days). (b) Mortality curves: ordinate = mortality rate (R); abscissa = age (days). 1 = saline; 2 = TF; 3 = PF; 4 = AHF.

TABLE V
EFFECT OF TF ON GROWTH OF WALKER-256 CARCINOMA AND PLISS LYMPHOSARCOMA IN RATS

Tumor strain	Treatment	No. of rats	Tumor weight (g) (mean \pm S.E.M.)
Walker-256 carcinoma	Saline	12	23.5 \pm 2.1
	TF	12	22.2 \pm 1.9
Pliss lymphosarcoma	Saline	15	19.3 \pm 2.6
	TF	15	21.1 \pm 2.3

ing factor in the development of autoimmune diseases and cancer [4, 22–25]. The main role in these processes belongs to the shift in T-lymphocytes and their precursor functions, including the decrease in their capacity to proliferate [22, 23, 26]. Also shown has been the age-associated decrease in synthesis and secretion of specific thymic hormones [27–29], which, as supposed, play a key role in the age-related deterioration of immunity [22, 23, 26].

There are some available data that the transplantation of young donor lymphocytes or thymocytes to old recipients could improve some characteristics of immunity [11, 30].

Thymus grafting or T-cell administration to short-living NZB mice prolonged their mean life span [31, 32]. A single injection of lymph node cells to dwarf Snell-Bagg mice increased their survival threefold, while bone marrow or thymic cells were not effective [33]. The geroprotective influence of thymic tissue grafted or injected into AKR mice was not constant [31, 34].

The separation of some thymic polypeptide factors (hormones) that stimulate immune functions [12, 15, 27, 35–38] opens up possibilities for studying immunological aging mechanisms and retardation of aging. There are some data on the capacity of various thymic factors which improve humoral and cell-mediated immunity in old animals [39, 40]. In our experiments, administration of one of the above thymic factors to C3H/Sn mice prolonged their survival time. Similar effects of PF were also shown. AHF administration failed to change the mean life span of mice, but it decreased their maximum life span. The mean life span of tumor-bearing mice was increased only by PF administration (Table IV). At the same time, the number of mice that failed to develop any tumors and the mean life span of TF- and PF-treated mice increased by TF or PF treatment in comparison to control animals (by 40.5% and 40.7%, respectively). AHF administration did not influence these parameters. Thus, it is suggested that the prolongation of life span of C3H/Sn mice treated with TF or PF is due to an antitumor effect of the substances used. However, the specific geroprotector effect of TF and PF is also significant.

In our experiments the incidence of mammary adenocarcinomas was significantly decreased in both TF- and PF-treated C3H/Sn mice. In recent studies we found an inhibiting effect of these preparations on 7,12-dimethylbenz(a)anthracene (DMBA)-induced development of mammary adenocarcinomas in female rats [41, 42] (Table VI). The similar effect of thymosin was also shown [43]. The incidence of mammary tumors in (1 × C3H)F₁ female mice treated with BCG vaccine as immunostimulator for 11 months was 30%, and in control mice 50% [44].

In our experiments the inhibition of spontaneous leukemogenesis in C3H/Sn mice and the prolongation of survival time of mice inoculated with L1210 both treated with TF were shown. Thymosin administration inhibits the development of transplanted monocyte Dunning leukemia in rats [45]. In this study the control rats died 10–17 days

TABLE VI
EFFECT OF TF AND PF ON 7,12-DIMETHYLBENZ(a)ANTHRACENE (DMBA)-INDUCED MAMMARY CARCINOGENESIS IN FEMALE RATS

Treatment	No. of rats	No. of mammary adenocarcinoma-bearing rats		No. of mammary adenocarcinomas	
		Total	%	Total	Per rat
DMBA + saline	32	22	68.8	39	1.77
DMBA + TF	22	4*	18.2	4	1.00
DMBA + saline	37	30	81.1	43	1.43
DMBA + PF	35	9*	25.7	10	1.11

*The difference compared with control is significant, $p < 0.05$.

after leukemia inoculation, and 10–80% of thymosin-treated animals survived for up to one year. The effect of purified thymic extract on X-ray-induced leukemia in mice was dependent on the dose of the preparation [46]. Recently, Barker *et al.* [47] revealed the inhibition effect of thymosin on production of endogenous oncornavirus by thymocytes of young mice. It could be supposed that TF inhibits the development of leukemia in C3H/Sn mice in a similar manner. BCG administration decreased the leukemia incidence and increased the life span in a strain of mice with a high incidence of spontaneous leukemias [48]. However, other authors [49] failed to find the BCG effect on leukemia incidence in the same mice.

In our experiments TF administration did not influence development of Pliss lymphosarcoma and Walker-256 carcinoma in adult rats. As Zisblatt *et al.* [50] have shown, thymosin administration to neonate mice increased their resistance to Moloney sarcoma growth. The thymic extract containing thymosin, thymopoietin 1 and 2, and thymic humoral factor, has no effect on the growth of DS sarcoma in adult rats [51]. The causes of the occurring contradictions are still obscure.

The results of our experiments with PF are in accordance with data on the inhibitory effect of pineal tissue transplantation or of melatonin-free pineal extract administration on growth of chemical carcinogen-induced tumors and some transplanted tumors [41, 52]. It could be suggested that the antitumor effect of PF and TF is due to their capacity to delay age-related decrease of immunity [53]. It is well known that tumor incidence, as a rule, increases in immunodepressant-treated patients or laboratory animals as well as in an immunodepressive state [54, 55]. On the other hand, immunostimulators, such as levamisol, restore the decreased immune characteristics in old animals [56] and show an antitumor effect in some experimental models [57]. At the same time, some data contradict the idea of an exclusive role for age-related changes in immunity in the mechanism of spontaneous tumor development [11, 58–60]. It is possible that the age-associated increase in tumor incidence is determined by complex deterioration of neuroendocrine and immune systems. The data presented by Fabris *et al.* [33] and Dilman [5] are in accordance with this suggestion.

It is very interesting that calorie restriction which effectively prolongs the life span and decreases tumor incidence in animals [61, 62], delays the aging of immune system [63]. At the same time, it is suggested that the geroprotective effect of calorie restriction is caused by retardation of age-related changes in pineal function [64]. In our experiments PF administration stimulated some characteristics of humoral and cell-mediated immunity ([16, 53]; present paper), significantly increased the life span of female rats [21] and mice (present paper) and decreased tumor development ([21, 41]; present paper). It could be suggested that the normalizing influence of PF on some hormonal-metabolic shifts which develop during aging and promote tumor development (see ref. 52) play the main role in the mechanism of its antitumor and geroprotector effects. The age-associated elevation of the hypothalamic threshold of sensitivity to hormonal feedback control [65], on the one hand, and exchange of lipids, insulin and glucocorticoid serum levels, increase in fatty acid oxidation (forming the syndrome of metabolic immunodepression) [5], on the other hand, are supposed to play the key role in the devel-

opment of the above-mentioned shifts. It must be stressed that administration of anti-diabetic biguanides, which normalize some of these changes and abolish metabolic immunodepression [5], also prolonged the life span and decreased tumor development in rats and mice [66, 67].

CONCLUSIONS

The data presented demonstrate that administration of the immunostimulator TF to C3H/Sn mice decreased the development of spontaneous tumors and increased the life span of animals. Chronic administration of PF, which normalizes some age-associated hormonal-metabolic shifts and delays aging of immune system, decreased the tumor incidence, increased tumor latency and acted as a geroprotector in female C3H/Sn mice. The results of our own experiments, as well as the available data on similar effects of calorie restriction and antidiabetic biguanides, could be considered to support both the immunological theory of aging and an immunological mechanism for the age-associated increase of cancer incidence. However, the leading role of the hormonal-metabolic pattern in the modulation of age-associated changes of immune function and the development of immune-mediated pathologic processes must be stressed.

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