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Research article

Immunomodulatory synthetic dipeptide L-Glu-L-Trp slows down aging and inhibits spontaneous carcinogenesis in rats

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Abstract

Immunomodulatory molecule L-Glu-L-Trp was isolated from natural calf thymic peptide complex Thymalin by reverse-phase high performance liquid chromatography. On the basis of the synthesized dipeptide a pharmaceutical was designed containg this compound, which later receives the brand name Thymogen®. The agent activated T-cell differentiation, T-cell recognition of peptide-MHC complexes, induced changes in intracellular composition of cyclic nucleotides, and activated neutrophilic chemotaxis and phagocytosis. The effect of dipeptide on survival, life span and spontaneous tumor development was studied in female rats. Seventy-six, five-month-old outbred female rats were randomly subdivided into two groups and were subcutaneously injected with 0.2 ml of normal saline (controls, 32 rats) or with 5 μg/rat of the dipeptide L-Glu-L-Trp, dissolved in 0.2 ml of saline (44 rats), 5 times per week for 12 months. Animals were monitored up to their natural death and all the tumors discovered were studied microscopically. Mean life span of rats in both groups was similar but that of 10% maximum survived control rats constituted 949 $\pm$ 16.1 days, whereas in the dipeptide-treated rats this value was 1048 $\pm$ 21.1 days ($P < 0.001$). Six out of 44 rats treated with the drug survived over the maximum life span of control rats (965 days). The aging rate indicated as α in the Gompertz equation, was 0.0071 days^{-1} in controls and 0.0041 days^{-1} in rats exposed to L-Glu-L-Trp. Total tumor incidence was 1.5 times lower ($P < 0.01$), malignant tumor incidence 1.7 times lower ($P < 0.01$), and hematopoietic malignancies (leukemias and lymphomas) 3.4 times lower ($P < 0.02$) in rats exposed to the dipeptide in comparison with controls. Thus, treatment with L-Glu-L-Trp delayed aging rate and decreased spontaneous tumor incidence in rats.

Introduction

The problem of preventing premature aging and prolonging life span is one of the most important and difficult medico-biological questions that has ever existed. The search for effective anti-aging therapies is seriously aggravated by: (1) existence of several controversial theories of aging; (2) incompleteness of information on possible side effects of existing geroprotective agents (for example, some geroprotectors, such as antioxidants and chelating agents, posess carcinogenic properties, whereas a number of other agentsor factors inhibit growth and reproductive function (Anisimov 1987, 1989). According to the immunological theory of aging, age-related immune dysfunction results in a reduced resistance to infection and increased risk of autoimmune diseases and cancer (Walford 1974; Kay and Makinodan 1986). Therefore, experimental attempts have been performed to stop or, at least, slow down the age-associated decrease of immunity of old-aged laboratory animas using lymphocyte or thymocyte transplantation from young donors (Kay and Makinodan 1986; Zatz and Goldstein 1985; Ghanta et al. 1991).

Natural calf thymic peptide preparation Thymalin® was shown to possess effective immunostimulating capacity to delay age-related immune dysfunction in rodents and in humans, to slow down aging and to inhibit spontaneous or chemically or ionizing radiation induced carcinogenesis in rodents (Morozov et al. 1977, 1994; Morozov and Khavinson 1978, 1996, 1997; Anisimov et al. 1982, 1989). The immunomodulatory molecule L-Glu-L-Trp was isolated from Thymalin® by reverse-phase high performance liquid chromatography with the use of a Nucleosil 7/C-18 column. As a result, on the basis of the dipeptide, synthesized by a classical chemical synthesis, a pharmaceutical was designed, later termed Thymogen® (Morozov and Khavinson 1991, 1996a). This work presents the effect of the synthetic dipeptide L-Glu-L-Trp on survival, life span and spontaneous tumor development in female rats.

Materials and methods

Pharmacokinetics assays

The pharmacokinetics of L-Glu-L-Trp was studied in rats after intramuscular injection of [^{3}H]L-Glu-L-Trp (sp.act. 4.8×10^4 counts per minute (cpm) μg^{-1}) dissolved without any carrier in normal saline in a dose of 870 μg/kg. For control, [^{3}H]L-glutaminic acid and [^{3}H]L-tryptophan were used. The radioactivity was estimated different organs and plasma of rats 30 min, 1, 2, 4, 8, 24 and 48 h after injection. Six rats were sacrificied for each time-point. Rat organs and plasma were subjected to basic hydrolysis. The radioactivity of hydrolysates was evaluated with the aid of a scintillation counter. Elimination of the radioactivity was estimated in the urine of rats kept 24 hours in individual metabolic cages. Six rats were in each group.

Pharmacodynamics assays

The pharmacodynamic properties of L-Glu-L-Trp were studied in adult thymectomized CBA mice which were immunized intravenously with 1×10^7 sheep red blood cells two months after thymus removal. The dipeptide was injected subcutaneously in a dose 0.01 μg/kg four days before immunization. The quantity of plaque-forming cells in the spleen, the PHA response of spleen lymphocytes, as well as the level of cyclic nucleotides (cAMP and cGMP) in splenocytes were determined.

Neutrophyl leukocyte assay

The effect of the dipeptide on neutrophil leukocytes was studied in adult CBA mice. L-Glu-L-Trp was injected intraperitoneally in a single daily dose of 0.01 μg/kg for six days. Control mice were injected with apyretic 0.15 M normal saline. The final stage included i.p.injection of sterile 10% peptone.The cells containing 95–98% of neutrophils were extracted from abdominal cavity in 2 h, and then were incubated in 12.5 mln/ml concentration with a *Staphylococcus aureus* strain. To a 0.1 ml sample of cell suspension there were added 0.05 ml of microbe suspension (250 mln/ml) and 0.05 ml of Hanks' solution. Phagocytosis by neutrophils was estimated in Giemsa-stained preparations. There were estimated 1000 cells per slide, on average, and the share of phagocytic cells was calculated.

Survival and pathology studies

L-Glu-L-Trp effect on survival and spontaneous tumor incidence was studied in female LIO rats (Anisimov et al. 1989a) 76 animals, 3 months old when obtained from the Rappolovo Animal Farm of the Russian Academy of Medical Sciences, were divided at random into two groups. Animals were kept six per polypropylene cage at 22–23 °C under a 14 h light/10 h dark light cycle and were fed with standard lab feed and water *ad libitum*.

Forty-four rats (group 1) were injected with L-Glu-L-Trp dissolved in normal saline subcutaneously in a single daily dose 5 μg/animal for 5 consecutive days weekly, during 12 months, starting at the age of 5 months. At the age 16–17 months the switching-off of the estrous function have take place in females of the strain used (Anisimov et al. 1989a). Control animals (group 2) numbering 32 rats were treated with normal saline in accord with the same schedule as animals of group 1. All rats were under observation until their natural death and were weighed monthly. Fallen animals and those sacrificed in an agonal state were autopsied, the neoplasia detected being detected as incidental or fatal (Gart et al. 1986) and fixed in 10% of neutral formalin. Subsequent to routine histological processing, the tissues were embedded in paraffin. Sections, 5–7 μm thick were stained with hematoxylin and eosin and examined microscopically, the tumors being classified as recommended by IARC (Turusov and Mohr 1990). Statistical analysis of the data on neoplasia in different groups was performed simultaneously with respect to

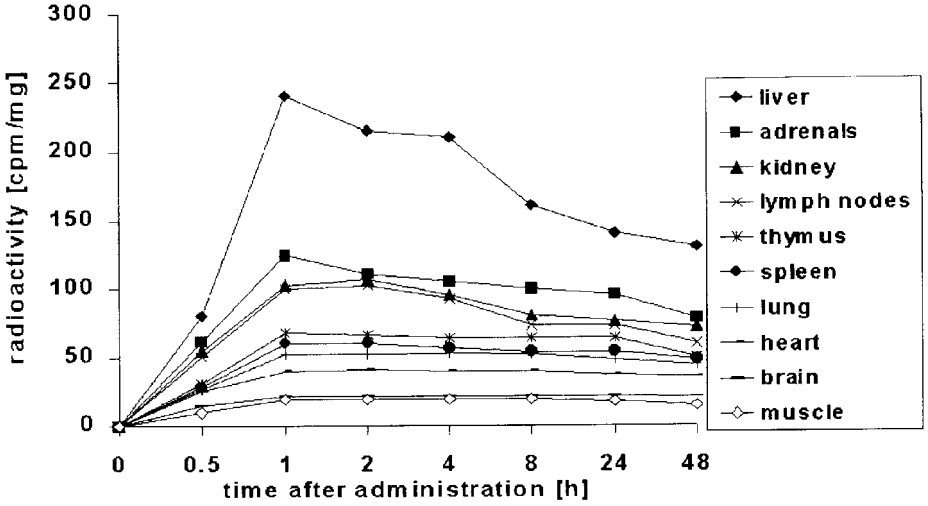

Figure 1. Distribution of [^{3}H] L-Glu-L-Trp in rat tissues. Six rats were sacrificed in each time point.

Table 1. The effect of dipeptide L-Glu-L-Trp on spleen cells of thymectomized CBA mice after immunization.[a]

Parameters	Intact mice	Thymectomized mice	
	Saline	Saline	L-Glu-L-Trp
Number of animals	15	12	14
Plaque-forming cells per 10^6 spleen cells	162 ± 28.1	44 ± 6.5[b]	140 ± 19.0[c]
PHA response of spleen cells (cpm $\times 10^3$)	35.6 ± 4.1	30.2 ± 4.2	41.3 ± 5.5
cAMP (pmol per 10^7 cells)	8.6 ± 0.5	7.3 ± 0.4[b]	13.5 ± 0.8[c]
cGMP (pmol per 10^7 cells)	0.28 ± 0.01	0.20 ± 0.01[b]	0.70 ± 0.06[c]

[a]Groups of CBA mice were given saline or the dipeptide (0.01 μg/kg s.c.) 4 days before immunization with SRBCs and in 2 months post-adult thymectomy.
[b]$P < 0.05$ in comparison with intact mice.
[c]$P < 0.05$ in comparison with thymectomized mice.

tumor incidence and the time of detection, using the life-table method of combining contingency tables by neoplasm detection time jointly for fatal and incidental tumors, the procedure involving a correction for intercurrent mortality. Moreover, Fisher's exact method and Student's *t*-test were used. Computations were performed using the (cartest) program running on an IBM PC/AT (Gart et al. 1986).

Results and discussion

The pharmacokinetics of the dipeptide was studied in rats after injection of [^{3}H]L-Glu-L-Trp. [^{3}H]L-Glu-L-Trp was rapidly taken up by the tissues and had a large scale of distribution (Figure 1). After administration of the test dipeptide, liver, adrenals, kidneys, lymph nodes and plasma showed the highest concentration of radioactivity. The blood-brain barrier was penetrated with dipeptide. In the liver, adrenals, kidney and lymph nodes, thymus and spleen the radioactivity remained longer than in other tissues. The

Table 2. The effect of dipeptide L-Glu-L-Trp on leukocyte chemotaxis and phagocytosis in CBA mice.[a]

Parameters	Saline	L-Glu-L-Trp
Number of animals	12	10
Number of cells migrated to abdominal cavity (mln/ml)	40.0 ± 5.0	60.0 ± 7.0[b]
Number of pagocytic cells (%)	18.8 ± 0.3	26.1 ± 0.6[b]

[a]Groups of CBA mice were given saline or the dipeptide (0.01 μg/kg i.p.) 6 days before i.p. injection of sterile 10% peptone. Cells were extracted from abdominal cavity in 2 h, and then *S. aureus suspension* was added.
[b]$P < 0.05$. in comparison with saline treated mice.

Table 3. Survival of rats exposed to saline or L-Glu-L-Trp.

Group	Number of rats surviving to the age (in days)										
	0–200	201–400	401–500	501–600	601–700	701–800	801–900	901–1000	1001-1100	1101–1200	>1201
Saline	32	32	32	32	27	24	16	4	0	0	0
L-Glu-L-Trp	44	44	41	40	37	33	22	14	4	1	0

maximal peak plasma concentration of [^{3}H]L-Glu-L-Trp occurred 1–2 h after administration. As it has been shown by estimation of the radioactivity of urine, rats discharged 85% of the dose administered within 24 h, demonstrating rapid elimination.

The study of the pharmacodynamic properties of L-Glu-L-Trp was conducted in adult thymectomized CBA mice which were immunized intravenously with sheep red blood cells 2 months after thymus removal. The results are shown in Table 1. L-Glu-L-Trp treatment caused stimulation of humoral immune response and the increase of cyclic nucleotide concentration in splenocytes.

The effect of the dipeptide on neutrophil leukocytes was studied in adult CBA mice. L-Glu-L-Trp showed a pronounced ability to increase chemotaxis and phagocytosis by neutrophils (Table 2).

L-Glu-L-Trp effect on survival and spontaneous tumor incidence was studied in female rats. The mean life span of rats was identical in both groups of rats, whereas the maximal life span of group 1 animals was longer by 4.6 months than that of control rats (group 2). Six out of 44 rats of group 1 out-lived the group 2 animal that showed the longest lifetime (Table 3). The calculation of the rate of aging, defined as α in the Gompertz equation ($R = R_0 e^{\alpha t}$ where R is mortality rate, $R_0 = R$ at the time $t = 0$, e is natural logarithm base and α is the constant) has shown that L-Glu-L-Trp slowed down the aging rate of rats. Thus, the value of α for the control group was estimated as 0.007 082 days^{-1}, whereas for group 1 as 0.004 123 days^{-1} (Table 4). The treatment with the dipeptide did not resulted in a decrease of the body weight, as compared to intact controls (data are not shown). Thus, L-Glu-L-Trp-induced slow down of the aging rate was not due to any decrease in food consumption and by retardation in body weight gain.

The occurrence of tumors, in general, and malignant neoplasia, in particular, in rats of group 1 was 1.5 and 1.7 times lower, respectively, in comparison with controls (Table 5; Figure 2).

Table 4. The effect of dipeptide L-Glu-L-Trp on survival of female rats.

Parameters	Saline	L-Glu-L-Trp
Number of rats	32	44
Mean life span, days	773 ± 18.4	786 ± 26.2
Mean life span of 10% maximum long-living rats, days	949 ± 16.1	1048 ± 21.1*
Maximum life span, days	965	1104
Aging rate (α), days^{-1}	0.007082	0.004123

Statistically significant, $^*P < 0.001$.

Table 5. The incidence and site of tumours in female rats treated with saline or dipeptide L-Glu-L-Trp.

Parameters	Saline	L-Glu-L-Trp
Effective number of rats[a]	31	40
Number of tumor-bearing rats:		
total tumors	12 (38,7%)	10 (25.0%)
malignant tumors	5 (16.1%)	3 (7.5%)*
Total number of tumors	14	12
Tumor site and type		
Pituitary adenoma	2	—
Mammary gland: fibroadenoma	7 (6)[b]	9(8)[b]
Hematopoietic system: leukemia	3	1
Malignant lymphoma	2	1
Stomach: adenocarcinoma	—	1

[a]Number of rats survival the time of 1st tumour detection.
[b]One rat had two tumors.
$^*P < 0.01$ (Peto's test).

Complex peptide preparation Thymalin®, as well as other thymic peptide immunomodulators, obviously inhibited spontaneous carcinogenesis. Furthermore, thymalin and some other immunomodulators were in some cases capable to prolong the life span of animals (Ghanta et al. 1991; Anisimov et al. 1982; Morozov and Khavinson 1996, 1997). The effect of thymic peptides was shown to materialize predominantly on the level of T-cell precursors and to involve specific peptides and lymphocyte membrane binding, a buildup of intracellular cAMP and cgMP contents of thymo-

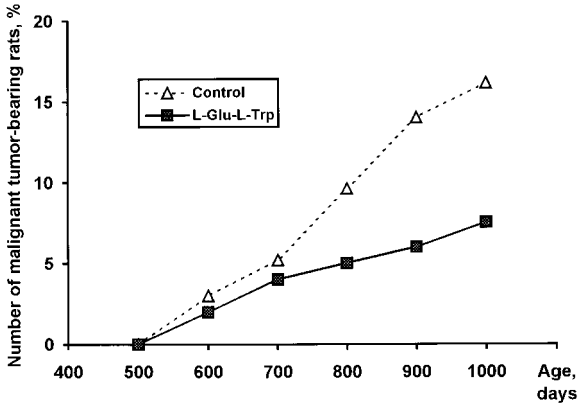

Figure 2. Effect of the exposure to L-Glu-L-Trp on malignant tumor yield in female rats.

cytes and a higher activity of cAMP-dependent protein kinases in the thymic and splenic lymphocytes at early stages of activation (Morozov et al. 1977; Morozov and Khavinson 1996, 1997).

L-Glu-L-Trp has been primarily isolated from the polypeptide complex preparation Thymalin® and synthesized. It restores thymus-dependent lymphocyte population in irradiated animals (Morozov et al. 1977; Morozov and Khavinson 1996). The use of these immunomodulators for treatment of cancer and HIV-positive patients lowers the incidence of complications, prevents the decline of immune parameters induced by infection and ionizing radiation and restores immunity (Morozov et al. 1994; Morozov and Khavinson 1996, 1997). Thus, the ability of a synthetic dipeptide L-Glu-L-Trp to inhibit spontaneous carcinogenesis and to prolong life span has been established for the first time in our experiments.

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