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## NATURAL AND SYNTHETIC THYMIC PEPTIDES AS THERAPEUTICS FOR IMMUNE DYSFUNCTION

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**Abstract**—Natural thymic peptides have been isolated from calf thymus by mild acid extraction. Pharmaceutical containing natural peptides (Thymalin<sup>®</sup>) was put into practice as immunocorrector. One of the immunomodulatory molecules (L-Glu-L-Trp) has been isolated from Thymalin by reversed-phase high performance liquid chromatography. Pharmaceutical containing this agent (Thymogen<sup>®</sup>) was designed on the base of synthesized dipeptide. A novel immunomodulatory dipeptide was synthesized and termed Vilon<sup>®</sup>. Both natural and synthetic pharmaceuticals activated T-cell differentiation, T-cell recognition of peptide-MHC complexes, induced the changes in intracellular composition of cyclic nucleotides and cytokine [interleukin (IL-2), interferon (IFN)] excretion of blood lymphocytes. Synthetic dipeptides activated neutrophil chemotaxis and phagocytosis. They had no influence on antioxidant response in thymocytes in comparison with natural peptides. Thymalin and Thymogen were used in persons with chronic pathology and immune dysfunction. The results indicate that thymic peptides participate in the regulating mechanisms of inflammatory processes as cytokine antagonists and show the difference between natural and synthetic products. It is important for the drugs designed to prevent immune dysfunction development. © 1998 Published by Elsevier Science Ltd on behalf of the International Society for Immunopharmacology.

**Keywords:** thymus, thymic factors, Thymalin<sup>®</sup>, Thymogen<sup>®</sup>, Vilon<sup>®</sup>, immunoregulation, immunomodulation, immunotherapy

It is well known that thymus involution develops with different extreme conditions, injuries and aging resulting in the reduction of immune reactions and immune dysfunction development. This prepares the ground for using therapeutics containing either natural and synthetic thymic factors as immunocorrectors.

Natural thymic factors (NTFs) have been isolated from calf thymus by mild acid extraction (Morozov & Khavinson, 1991). Pharmaceuticals containing NTFs (Thymalin<sup>®</sup>) are used in clinical practice for prevention and treatment of immunodeficiency.

Pharmaceutical containing one of the immunomodulatory molecules (L-Glu-L-Trp) isolated from Thymalin<sup>®</sup> by reversed-phase high performance liquid chromatography (RP-HPLC) was designed on the base of synthesized dipeptide and termed Thymogen<sup>®</sup>. A novel immunomodulatory thymic dipeptide was synthesized and termed Vilon<sup>®</sup>.

The paper discusses the immunotherapeutic strategies of using pharmaceuticals containing both natural and synthetic thymic peptides.

*Isolation and physicochemical study of Thymalin<sup>®</sup>, Thymogen<sup>®</sup> and Vilon<sup>®</sup>*

For the isolation of NTFs tissue was subjected to dehydration by acetone, homogenization and extraction by aqueous acetic acid (48 h, pH 3.4–4.0) in the presence of ZnCl<sub>2</sub> (0.5–1.0 g l<sup>-1</sup>). The extract was treated by acetone in a 1:5 ratio. The sediment was washed, dried and further dissolved in de-ionized water (25 g l<sup>-1</sup>, pH 6.0–6.5). The solution was subjected to ultrafiltration through an Amicon PM-10 filter and concentrated on a YC-0.5 filter. The fraction was collected, filter-sterilized and dried under vacuum.

Bioactive substances have been isolated from NTFs earlier by ion-exchange chromatography (Morozov *et al.*, 1977). Generally accepted physicochemical approaches were used for their analysis. The study of biological activity of fractions obtained by cation-exchange proved that the major activity was eluted at *ca* 0.7–0.9 M ammonium acetate. The compounds isolated by mild acid hydrolysis were probably peptidic in nature and had molecular mass 1–10 kDa.

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RP-HPLC of NTFs was performed on a Nucleosil 7/C-18 column. The peptides were subjected to gas-phase automatic sequencing, amino acid and mass spectral analysis. Some Trp-containing immunomodulatory molecules were isolated and synthesized. Thymogen<sup>®</sup> was designed on the base of L-Glu-L-Trp by classical chemical synthesis. Vilon<sup>®</sup> was also designed on the base of the Glu-containing dipeptide.

### PHARMACOKINETIC STUDY

The pharmacokinetics of Thymogen<sup>®</sup> was studied in rats after intramuscular (i.m.) administration of [<sup>3</sup>H]L-Glu-L-Trp (sp. act.  $4.8 \times 10^4$  counts per minute (cpm)  $\mu\text{g}^{-1}$ ) in a dose of  $870 \mu\text{g kg}^{-1}$ . For the control [<sup>3</sup>H]L-glutamic acid and [<sup>3</sup>H]L-tryptophan were used. Rat organs and plasma were subjected to basic hydrolysis. Radioactivity of hydrolysates was measured by scintillation counter.

[<sup>3</sup>H]L-Glu-L-Trp was rapidly taken up by the tissues and had a large volume of distribution. After an injection of tested substance liver, adrenals, kidney, lymph nodes and plasma showed the highest concentration of radioactivity. The blood-brain barrier was penetrated with dipeptide. In the liver, adrenals, kidney, lymph nodes, thymus and spleen the total radioactivity was considerably longer than that in other tissues.

The maximal peak plasma concentration of L-Glu-L-Trp occurred after 1–2 h (Fig. 1). Rats secreted *ca* 85% of the dose within 24 h, indicating a rapid elimination.

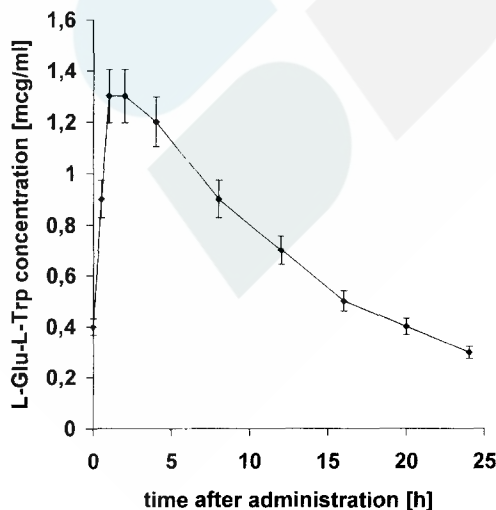


Fig. 1. Plasma concentration time course following i.m. administration of  $870 \mu\text{g kg}^{-1}$  L-Glu-L-Trp in rat.

### PHARMACODYNAMIC STUDY

In a previous study Thymalin<sup>®</sup> and Thymogen<sup>®</sup> restored the immune defense of thymectomized animals and enhanced regenerative processes in the thymus as well as cellular immunity in different extreme conditions, injuries and stress (Morozov & Khavinson, 1978; Grintsevich *et al.*, 1984; Morozov, 1990).

The comparative study of pharmacodynamic properties of Thymalin<sup>®</sup>, Thymogen<sup>®</sup> and Vilon<sup>®</sup> was carried out in adult thymectomized CBA mice which were immunized intravenously by  $1 \times 10^7$  sheep red blood cells (SRBCs) 2 months after thymus removal. The tested pharmaceuticals were injected subcutaneously (s.c.) in a dose of  $0.01 \text{ mg kg}^{-1}$  4 days before immunization. The quantity of plaque-forming cells (PFC) in spleen, the PHA response of spleen lymphocytes as well as the level of cyclic nucleotides (cAMP and cGMP) in splenocytes were determined. The results are shown in Table 1.

After administration of tested substances the stimulation of humoral immune response and the increase of cyclic nucleotide concentration in splenocytes were observed. Thymalin effect was pronounced on PHA response of spleen lymphocytes.

The influence of Thymalin<sup>®</sup>, Thymogen<sup>®</sup> and Vilon<sup>®</sup> on neutrophil leukocytes was studied in adult CBA mice. The tested substances were injected intraperitoneally (i.p.) in the dose of  $0.01 \mu\text{g kg}^{-1}$  during 6 days. Apyretic 0.15 M NaCl was administrated similarly to the control group of animals. 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  $12.5 \text{ mln ml}^{-1}$  concentration with *Staphylococcus aureus* strain. To a 0.1 ml sample of cell suspension 0.05 ml of microbe suspension ( $250 \text{ mln ml}^{-1}$ ) and 0.05 ml of Hanks' solution was added. Phagocytosis by neutrophils was estimated in Giemsa-stained preparations. An average of 1000 cells were examined per slide and the percentage of phagocytic cells was calculated.

Synthetic dipeptides showed a pronounced ability to increase chemotaxis and phagocytosis by neutrophils (Table 2).

The restoration of cellular immunity was discovered in mice with spontaneous tumor development and those induced by carcinogens as a result of NTFs administration Anisimov *et al.* (1989). Chronic treatment of female C3H/Sn mice with Thymalin<sup>®</sup> started at the age of 3.5 months ( $1.0 \text{ mg kg}^{-1}$  s.c. for 5 days once a month up to natural death), prolonged their mean life span by 28%, decreased the rate of tumour

Table 1. Effect of Thymalin<sup>®</sup>, Thymogen<sup>®</sup> and Vilon<sup>®</sup> on spleen cells of thymectomized CBA mice after immunization\*

Animals	No. of animals	Plaque-forming cells per 10 <sup>6</sup> spleen cells	PHA response of spleen cells (cpm × 10 <sup>3</sup> )	cAMP (pmol per 10 <sup>7</sup> cells)	cGMP (pmol per 10 <sup>7</sup> cells)
Normal	15	162.4 ± 28.1	35.6 ± 4.1	8.6 ± 0.5	0.28 ± 0.01
Thymectomized	12	43.9 ± 6.5†	30.2 ± 4.2	7.3 ± 0.4†	0.20 ± 0.01†
Thymectomized + Thymalin <sup>®</sup>	11	186.3 ± 29.1‡	50.3 ± 6.0‡	16.3 ± 1.1‡	0.55 ± 0.05‡
Thymectomized + Thymogen <sup>®</sup>	14	139.6 ± 19.0‡	41.3 ± 5.5	13.5 ± 0.8‡	0.70 ± 0.06‡
Thymectomized + Vilon <sup>®</sup>	10	93.8 ± 13.1‡	39.4 ± 4.9	11.6 ± 0.7‡	0.71 ± 0.08‡

\*Groups of CBA mice were given thymic pharmaceuticals (0.01 mg kg<sup>-1</sup> s.c.) 4 days before immunization with SRBCs and in 2 months post-adult thymectomy.

†*P* < 0.05 in comparison to normal mice.

‡*P* < 0.05 in comparison to thymectomized mice.

Table 2. Thymalin<sup>®</sup>, Thymogen<sup>®</sup> and Vilon<sup>®</sup> activity on leukocyte chemotaxis and phagocytosis in CBA mice\*

Treatment	No. of animals	No. of cells migrated to abdominal cavity (mln ml <sup>-1</sup> )	No. of phagocytic cells (%)
Saline solution	12	40.0 ± 5.0	18.8 ± 0.3
Thymalin <sup>®</sup>	8	45.0 ± 5.5	20.4 ± 0.8
Thymogen <sup>®</sup>	10	60.0 ± 7.0†	26.1 ± 0.6†
Vilon <sup>®</sup>	10	80.0 ± 6.5†	28.7 ± 0.5†

\*Groups of CBA mice were given thymic pharmaceuticals (0.01 mg kg<sup>-1</sup> i.p.) 6 days before i.p. injection of sterile 10% peptone. Cells were extracted from abdominal cavity in 2 h, and then were added by *S. aureus* suspension.

†*P* < 0.05 in comparison to saline treated mice.

incidence by 2.8 times as well as malignant tumour development by 2.6 times. The administration of Thymogen<sup>®</sup> in similar conditions was also followed by a decreased rate of spontaneous tumour development by 1.9 times. The pharmaceutical did not influence the malignant tumour development and life span of animals. The results of the experiments are shown in Table 3.

#### IMMUNOTHERAPEUTIC APPLICATION OF THYMIC FACTORS

Thymalin<sup>®</sup> and Thymogen<sup>®</sup> were used as therapeutics in patients with a variety of immune disorders. In the case of injuries complicated by infection Thymalin<sup>®</sup> treatment improved the inflammation process, prevented infection and destruction of tissues

Table 3. Effect of thymic pharmaceuticals on spontaneous tumour development and life span of C3H/Sn mice\*

Experiment	Treatment	No. of animals	No. of animals with tumours (%)	No. of malignant tumours (%)	Life span of animals (days)
A	Saline solution	21	67	52	487 ± 29
	Thymalin <sup>®</sup>	25	24†	20†	623 ± 25†
B	Saline solution	41	56	27	576 ± 25
	Thymogen <sup>®</sup>	33	30†	21	507 ± 26

\*Groups of C3H/Sn mice were given thymic pharmaceuticals (1.0 mg kg<sup>-1</sup> s.c. for 5 days once a month) from the age of 3.5 months up to their natural death.

†*P* < 0.05 compared with control groups, respectively.

and promoted faster healing of wounds. Thymalin<sup>®</sup> application also improved a number of immune responses as well as clinical state in previously anergic, immunodepressed and elderly patients (Morozov *et al.*, 1978; Khavinson & Morozov, 1981; Khavinson & Morozov, 1991; Morozov & Khavinson, 1996).

The results from clinical study prove that main directions for immunotherapeutic application of thymic pharmaceuticals are chronic inflammation and tissue repair disorders, stress-induced immunodepression, optimization of cancer immunotherapy, chemotherapy and radiotherapy, age-related immune dysfunction. A summary of immunomodulatory effects of thymic factors is presented in Table 4.

### DISCUSSION

The thymus is an essential microenvironment for T-cell development, but our knowledge of the directional recruitment of pro-T cells from bone marrow into the thymus and processes of their differentiation or programmed death remains fragmentary. Numerous data indicate that thymic factors participate in the regulating mechanisms of immune defense and inflammation.

Our results are consistent with the hypothesis on the regulatory signal properties of short immunopeptides generated by metabolic fragmentation of tissue specific thymic factors (Birr *et al.*, 1994). It was reported on *in vitro* bioactivities of synthetic thymic peptides derived from thymosin  $\alpha_1$  (Goldstein *et al.*, 1977). These studies clearly demonstrate the difference between natural and synthetic thymic factors.

According to our concept macromolecule degra-

dition products show qualitatively novel immunomodulatory properties. It is known that some products of protein degradation influence on host defense mechanisms and inflammation. For instance, during the degradation of structure proteins components with chemotactic properties are being formed (Chiang *et al.*, 1978; Senior *et al.*, 1982). However, the significance and mechanisms of action of these products are presently not well understood. Recent studies suggest that protein degradation in the living cell is very selective and has an essential role in the maintenance of cell structure and function (Bergamini, 1992).

One group of immunomodulators has been derived from purine structures and classified as "thymomimetic drugs" (Hadden, 1985). Purine immunomodulators show effects *in vitro* on T-cell proliferative responses and T-cell differentiation. *In vivo* results in mice indicate that purine immunomodulators possess adjuvant effects for both humoral and cellular immune responses (Hadden *et al.*, 1986; Sosa *et al.*, 1994). Thymomimetic drugs are able to reverse the immunosuppression induced by an HIV-derived peptide (Hadden *et al.*, 1991). Thymalin also has an effect on T-cell development, both humoral and cellular immune responses and as a potential immunotherapeutic for early HIV infection (Morozov *et al.*, 1994). It seems likely that natural thymic pharmaceuticals contain either purine and peptide immunomodulators, which cause specific trophic action on T-cells as well as host resistance during the application.

Pharmaceuticals on the basis of synthetic fragments of NTFs and their analogues show distinct immunomodulatory effects. Novel therapeutic opportunities are, therefore, open in connection with the creation of Thymogen<sup>®</sup> and Vilon<sup>®</sup>.

Table 4. Immunomodulatory actions of thymic factors in patients with a variety of immune disorders

#### *Immune state*

Increased in blood lymphocytes and T cells in previously anergic, immunodepressed and elderly patients  
 Increased immune response to thymus-dependent antigens  
 Increased delayed-type hypersensitivity  
 Increased mitogen (PHA, Con A) response by lymphocytes  
 Increased production of MIF, IFN, IL-2, GM-CSF by lymphocytes  
 Decreased production of proinflammatory cytokines  
 Increased chemotactic response by neutrophils  
 Increased phagocytosis by neutrophils and macrophages

#### *Clinical state*

Improved inflammatory response and tissue repair  
 Improved host defense to infection  
 Improved hemopoiesis, blood coagulation and microcirculation  
 Decreased period of treatment

## CONCLUSIONS

The results of experimental and clinical studies reveal that thymic factors regulate immune response as Th1-cell cytokine agonists and inflammatory responses as proinflammatory cytokine antagonists. Natural and synthetic thymic factors have different pharmacodynamic properties. Natural thymic pharmaceuticals contain complex substances, which cause

tissue specific trophic action on thymus, restore T-cell functions as well as host resistance during the application. It may be taken into consideration that synthetic fragments of NTFs and their analogues show distinct immunomodulatory effects. In general, this is very important for the drugs designed to prevent immune dysfunction development. Future studies will likely reveal the significance of protein degradation products in peptide immunoregulation.

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