

## Evaluation of the Immunomodulatory Properties of a Calf Lipid Thymus Extract and Standardization of a Method for Quantification of its Biological Activity

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**Abstract:** The aim of this study was to test the immunobiological properties of a new formulation of the lipid thymus calf extract (Thymsol), prepared for *in vitro* cell culture experiments. At first, we showed that Thymsol enhanced the antigen specific immune response in AO rats *in vivo*, using Keyhole limpet hemocyanin (KLH). At lower concentrations (0.5-2.5  $\mu\text{g mL}^{-1}$ ) the extract increased the proliferation of rat spleen lymphocytes *in vitro*, stimulated with suboptimal concentrations of Concanavalin A (ConA) or anti-T-cell receptor (TCR) monoclonal antibody (mAb), followed by an increase in the production of interleukin-2 (IL-2). Stimulation of IL-2 production was additionally enhanced in the presence of a blocking anti-IL-2 receptor  $\alpha$  mAb. Higher concentrations of Thymsol (20-60  $\mu\text{g mL}^{-1}$ ) showed a dose-dependent inhibition of lymphocyte proliferation. After testing the variability in IL-2 production by anti-TCR mAb-activated splenocytes, using several stimulatory concentrations of different Thymsol samples, and the production of this cytokine by different cultures of splenocytes treated with the same Thymsol sample, we proposed an IL-2-based method for quantification of the immunobiological activity of the extract.

**Key words:** Thymus extract, immune response, proliferation, IL-2 production

### INTRODUCTION

The thymus gland plays a crucial role in the development and maintenance of immunity and tolerance, as it provides the microenvironment for T-cell differentiation, maturation and selection<sup>[1]</sup>. The intrathymic development of T cells results from cell-to-cell interactions between T-cell precursors and microenvironmental cells and the activity of soluble factors. Among them, thymic hormones play the most important role and act not only on thymic T cells but also on peripheral mature T cells<sup>[1,2]</sup>. It is postulated that the involution of the thymus gland and subsequent decline of thymic hormone secretion are involved, to a considerable extent, in the development of the age-associated immune deficiency<sup>[3,4]</sup>. In this regard substitutive therapy with thymic hormones or different thymic extracts was reported to improve or restore age-associated immune defects in both experimental animals and humans<sup>[5-7]</sup>. Later on, the therapeutic application of thymic preparation has been extended to

different fields in clinical medicine such as acute and chronic infectious diseases, chronic allergies, cancer, autoimmune diseases, complication of sepsis, immunosuppression after chemotherapy and radiotherapy and many others<sup>[8,9]</sup>.

Significant therapeutic value of thymus extracts, published up to now in large number of papers, is predominantly related to the improvement of cellular immune functions, especially those of T cells. Thymus extracts have been shown to modulate the development, maturation and activation of T cells<sup>[8-11]</sup>, enhance their responsiveness to mitogens<sup>[12-14]</sup>, stimulate production of interleukin-2 (IL-2)<sup>[12-16]</sup> and T helper 1 (Th1) immune responses<sup>[17]</sup>.

Many commercial preparations of liquid thymus extracts typically contain thymic hormones, but also many other water soluble peptides and components, which contribute additionally to their biological effects. Therefore, for companies and laboratories producing thymus extracts to be used for therapeutic or research procedures, it is very important to establish relevant

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screening tests for quantification of the biological activity of different batches of such preparations.

Several years ago, we started with the research of a new calf thymus extract, predominantly composed of lipids, to be potentially used for clinical application in human and veterinary medicine. A number of results, collected up to now from preclinical studies showed that this calf thymus lipid extract (CTLE) possesses immunomodulatory activity<sup>[18]</sup> (and Vucevic *et al.*, manuscript submitted) and improves wound healing (data not shown). Since there were difficulties in mixing the lipid extract with cell culture medium for evaluation of its immunobiological activity, we prepared a new formulation of CTLE suitable for *in vitro* experiments. The aim of this work was to check the effect of this extract on the immune response *in vivo* and *in vitro* and to standardize a method for quantification of its biological activity.

## MATERIALS AND METHODS

**Animals:** Experiments were performed on Albino Oxford (AO) rats, male, 6 months old, bred at the vivarium of the Institute of Medical Research, Military Medical Academy, Belgrade, under conventional laboratory conditions.

**Preparation of Thymosol:** The calf thymus gland was obtained from a slaughter-house. After removal, the gland was immediately frozen at -20°C and transported to the laboratory. The frozen thymus was milled and then suspended in a polar organic solvent (n-butyl alcohol). Extraction was done by using an azeotropic distillation process. The ballast material was drained and the total extract was concentrated and vacuum dried. The non-lipid component was removed from the dried total extract, using a Folch method<sup>[19]</sup> and rest of the fraction (lipid extract), containing 97.0-97.7% lipids and about 0.3% peptides, was further processed as we previously described<sup>[18]</sup>. The CTLE was solubilized and emulsified in benzyl alcohol, butylated hydroxyanisole, ethyl oleate, benzyl benzoate, Cremophor EL and water for injections. The components of the solvent were obtained from Merck, whereas Cremophor EL was from BASF, Ludwigshagen, Germany. The concentrations of all used excipients were permitted for parenteral administration according to the European Pharmacopea V. After sterilization by filtration, the preparation, named Thymosol, was ampulated at the concentration of 50 µg mL<sup>-1</sup>. The placebo ampules contained the solvent/emulgator components without CTLE.

**Immunization protocol:** Rats were immunized by injection of Keyhole limpet hemocyanin (KLH) (Sigma, Munich, Germany) (2 mg mL<sup>-1</sup>) emulsified in the complete Freund's adjuvant (CFA) (Sigma) in a total volume of 100 µL into right posterior footpads. The experimental group was injected with 2.5 mg 100<sup>-1</sup> µL of Thymosol into the same footpad, one hour before immunization. The same procedure was repeated after 4 days of immunization. Two control groups were used. The placebo group was treated with the same volume of placebo before and after immunization. The pure control group was treated with the same volume of saline. After 10 days draining popliteal lymph nodes were removed and cells were prepared.

**Preparation of lymph node lymphocytes and splenocytes:** Lymph node lymphocytes from immunized animals were aseptically removed, pressed through stainless meshes placed in Petri dishes with addition of phosphate buffered saline (PBS)+5% fetal calf serum (FCS) (ICN, Costa Mesa, CA), filtered through nylon gauze to remove large debris and clumps and then washed twice by centrifugation (580xg) with RPMI medium (Sigma)+5% FCS. Cells were counted and their viability (usually higher than 95%) was determined by trypan blue dye exclusion.

Splenocytes were isolated from intact AO rats using the same procedure as described for lymph node lymphocytes. Red blood cells from splenocyte suspensions were lysed using a hypotonic ammonium chloride buffer, following by two subsequent cell washings (120xg) with RPMI medium+5% FCS. Viability of the cells was between 93-96%.

**Proliferation assays:** Lymph node lymphocytes were resuspended in complete RPMI medium (RPMI medium+10% FCS+2 mM L-glutamine+10 U mL<sup>-1</sup> penicillin+1 mg mL<sup>-1</sup> streptomycin) and adjusted to a concentration of 2.0x10<sup>6</sup> cells mL<sup>-1</sup>. Cells were cultivated for 96 h in 96-well plates (ICN) (200 µL well<sup>-1</sup>) in an incubator with 5% CO<sub>2</sub> at 37° C with different concentrations of KLH. Splenocytes (2.0x10<sup>6</sup> cells mL<sup>-1</sup>, 200 µL well<sup>-1</sup>) were incubated in culture medium alone, different concentrations of Thymosol or corresponding concentrations of placebo for 72 h as described for lymph node cells. Proliferation of splenocytes was induced with a suboptimal concentration (1.25 µg mL<sup>-1</sup>) of ConcanavalinA (ConA) (Sigma) or a suboptimal concentration (0.1 µg mL<sup>-1</sup>) of an anti-T cell receptor (TCR) monoclonal antibody (mAb) (R73) (Serotec, Oxford, UK). During the last 18 h of cultivation, cells were pulsed with 1 µCi well<sup>-1</sup>

[<sup>3</sup>H] thymidine (6,7 Ci mmol<sup>-1</sup>, Amersham, Books, U.K.). After harvesting, the radioactivity was counted using a scintillation counter (Beckman). Results were expressed as mean counts per minute (cpm) of triplicates.

**Production of interleukin-2 (IL-2):** Lymph node lymphocytes from immunized animals were cultivated with KLH (50µL mL<sup>-1</sup>) for 48 h as described for the proliferation assay. Splenocytes, stimulated with R73 mAb with or without a blocking IL-2RαmAb (OX-39) (Serotec, Oxford, UK) at the concentration of 10 µg mL<sup>-1</sup>, were treated with Thymosol or placebo and cultivated for 48 h. Cell-free supernatants were assayed for the presence of IL-2 using a commercial rat IL-2 ELISA kit (R and D), following exactly the instructions of the manufacturer. Results are expressed as pg mL<sup>-1</sup> of triplicates.

**Statistic analysis:** The data are presented as a mean±SD. Student's t-test was used for statistical analysis of the results. The values (p<0.05) or less were considered to be statistically significant. Coefficient of variation (CV) was calculated as follows: CV (%) = standard deviation (SD)/mean value×100.

## RESULTS AND DISCUSSION

**Effect of Thymosol on the antigen-specific immune response *in vivo*:** The first aim of this study was to test whether a new formulation of CTLE (Thymosol), exerts the immunomodulatory activity *in vivo*. For this purpose we immunized rats with KLH, with or without Thymosol, as described in Materials and methods. A control group of rats was treated similarly with the same volume of placebo.

As shown in Fig 1A, the treatment of animals with Thymosol resulted in significantly higher cellularity of regional lymph nodes, compared with both control groups. Lymph node cells, isolated from immunized animals, showed a dose-dependent proliferation *in vitro* in the presence of KLH (Fig 1B). Although cellular proliferation in cultures from the placebo group was a bit higher compared to the pure control group (rats immunized with KLH, only), the proliferative response of lymphocytes from the experimental group was statistically significantly higher compared to both control groups and these results correlated with higher production of IL-2 (Fig 1C).

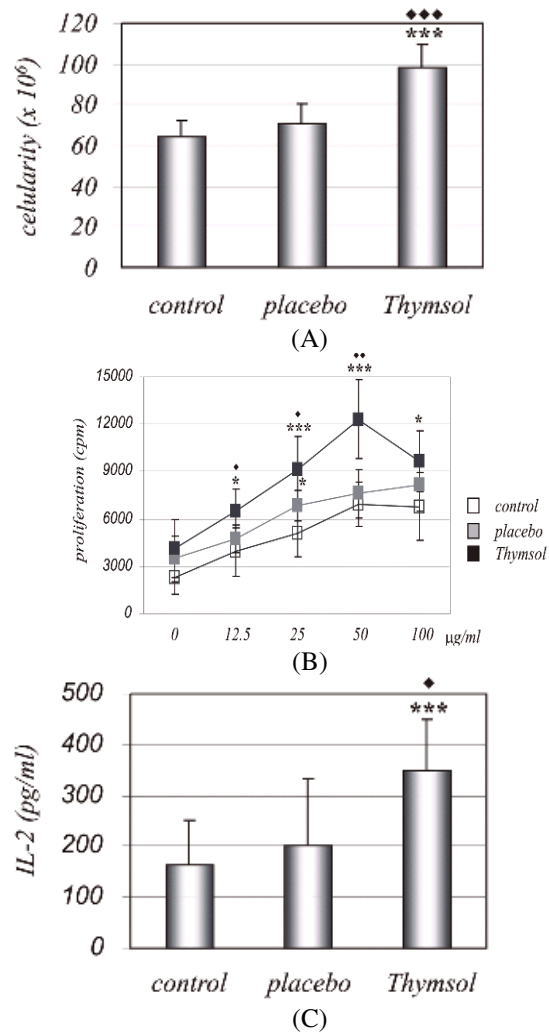


Fig. 1: Effect of Thymosol on cellularity of lymph nodes (A), proliferation (B) and production of IL-2 (C) by lymph node lymphocytes isolated from rats immunized with KLH. Rats (n = 6/group) were immunized by KLH as described in Materials and methods. After 10 days draining popliteal lymph nodes were removed and cells were prepared. Proliferation was measured by a [<sup>3</sup>H]-thymidine method, after stimulation of lymph node lymphocytes with different concentrations of KLH. The levels of IL-2 in culture supernatants (24h after cultivation) were determined by ELISA, as described in Material and methods. \* = p<0.05, \*\*\* = p<0.005 compared to corresponding controls, • = p<0.05, •• = p<0.01, ••• = p<0.005 compared to corresponding values of placebo

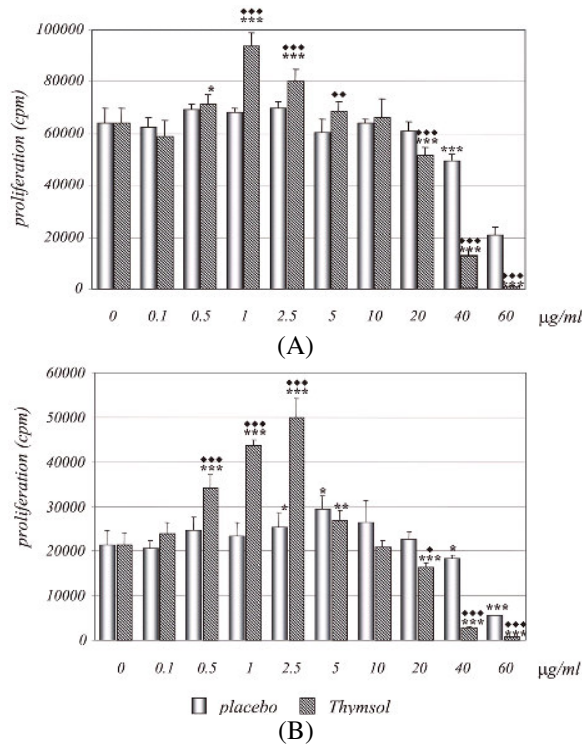


Fig. 2: Effect of different concentrations of Thymysol or placebo on the proliferation of rat splenocytes in cultures stimulated with ConA (A) or R73 mAb (B) Splenocytes were stimulated with ConA (1.25 µg mL<sup>-1</sup>) or R73 (0.1 µg mL<sup>-1</sup>) for 3 days. The cultures were treated with different concentrations of Thymysol or placebo. Cellular proliferation was measured by a [<sup>3</sup>H]-thymidine method as described in Materials and methods. Values are given as mean cpm±SD of triplicates of one representative experiment (of 4 with similar results). = p<0.05; \*\* = p<0.01; \*\*\* = p<0.005 compared to corresponding cultures without Thymysol or placebo, • = p<0.05; •• = p<0.01, ••• = p<0.005 compared to corresponding placebo cultures

**Effect of different concentrations of Thymysol on the proliferation of rat splenocytes *in vitro*:** Based on the previous results we examined the effect of different concentrations of Thymysol and placebo on the proliferation of rat splenocytes *in vitro*. For stimulation of the cells, two T-cell mitogens were used: a suboptimal concentration of ConA (1.25 µg mL<sup>-1</sup>) and a suboptimal concentration of R73 (anti-TCR) mAb. Fig 2A shows that Thymysol at concentrations between 0.5 and 5.0 µg mL<sup>-1</sup> augmented the proliferation of ConA-stimulated splenocytes, whereas the concentrations

between 20-60 µg mL<sup>-1</sup> showed a dose-dependent inhibition of cellular proliferation. The proliferation of splenocytes triggered by R73 mAb was lower compared to ConA, but the stimulatory effect of Thymysol was significantly higher. Similarly, higher doses of Thymysol were inhibitory in this assay (Fig 2B).

The stimulatory activity of Thymysol, compared to placebo, was confirmed in four independent experiments. However, the maximum stimulation varied between the experiments and the results can be summarized as follows. The lowest stimulation: 1.2 fold increase at 2.5 µg mL<sup>-1</sup> of Thymysol (ConA), 1.7 fold increase at 1.0 µg mL<sup>-1</sup> of Thymysol (R73 mAb), the highest stimulation: 2.1 fold increase at 1.0 µg mL<sup>-1</sup> of the extract (ConA), 3.4 fold increase at 2.5 µg mL<sup>-1</sup> of the extract (R73 mAb).

Stimulatory and inhibitory activity of placebo was also evident at certain lower or higher concentrations, respectively, in both assays, but its effect was less pronounced compared to the corresponding concentrations of Thymysol (Fig 2A and Fig 2B).

**Production of IL-2 *in vitro* by R73-stimulated splenocytes:**

Previous results clearly showed the stimulatory effect of Thymysol *in vitro*. In order to check how the phenomenon correlates with the production of IL-2, we added several concentrations of the extract ranging between 0.1-3.0 µg mL<sup>-1</sup> to R73-stimulated splenocytes. Parallel cultures were treated with an IL-2Rα blocking mAb (OX-39).

At first, we prepared three different samples of the same batch of Thymysol in order to study the variability of the results, obtained with the same preparation of splenocytes. Results given in Table 1 show that R73-stimulated spleen lymphocytes produced a significantly higher level of IL-2 in Thymysol-treated cultures, compared to both control - untreated cultures and corresponding placebo-treated cultures. As expected, the production of IL-2 was significantly higher in the presence of OX-39 mAb. Comparable to the results from the proliferation assay, a slight stimulation of IL-2-production (compared to the control-untreated cultures) was observed with certain concentrations of placebo.

The variability in the cytokine production between different samples of Thymysol was 12.0%, as judged by the coefficient of variation (CV). The maximum stimulation (451 pg mL<sup>-1</sup> of IL-2) was obtained with sample 1 (2.0 µg mL<sup>-1</sup> of Thymysol), normalized to the level of IL-2 in the corresponding culture of placebo. The minimum stimulation (366 pg mL<sup>-1</sup> of IL-2) was

Table 1: Effect of different concentrations of three Thymosol samples and corresponding placebo samples on the production of IL-2 by the same population of rat splenocytes stimulated with anti-TCR antibody with or without a blocking anti-IL-2R $\alpha$  antibody (OX-39)

	- OX-39	+ OX-39	- OX-39	+ OX-39	- OX-39	+ OX-39
Control	149.3 $\pm$ 24.5	479.3 $\pm$ 58.6	149.3 $\pm$ 24.5	479.3 $\pm$ 58.6	0	0
Sample 1 of Thymosol/Placebo	Thymosol		Placebo		Thymosol-placebo differences	
Concent (µg mL <sup>-1</sup> )						
0.1	159.0 $\pm$ 36.1	402.8 $\pm$ 30.2	217.4 $\pm$ 40.2	488.4 $\pm$ 39.9	58.4	-85.6•
0.5	221.3 $\pm$ 16.2	504.4 $\pm$ 30.2	201.0 $\pm$ 21.3	422.2 $\pm$ 43.0	20.3	82.2
1.0	251.2 $\pm$ 20.1***•	620.0 $\pm$ 41.2**•	197.7 $\pm$ 8.2*	501.3 $\pm$ 28.0*	53.5	118.7
1.5	296.1 $\pm$ 23.2***•••	754.4 $\pm$ 37.2***•••	182.2 $\pm$ 20.6	403.6 $\pm$ 30.2	113.9	350.8
2.0	367.1 $\pm$ 20.8***•••	864.4 $\pm$ 50.1***•••	210.2 $\pm$ 13.1*	413.4 $\pm$ 50.0	156.9	451.0
2.5	208.5 $\pm$ 12.8*	534.1 $\pm$ 30.6•	218.0 $\pm$ 29.6*	452.4 $\pm$ 20.0	-9.5	81.7
3.0	196.3 $\pm$ 20.8	528.6 $\pm$ 48.1•	175.5 $\pm$ 13.2	377.6 $\pm$ 40.8*	20.8	151.0
Sample 2 of Thymosol/Placebo	Thymosol		Placebo		Thymosol-placebo differences	
Concent (µg mL <sup>-1</sup> )						
	- OX-39	+ OX-39	- OX-39	+ OX-39	- OX-39	+ OX-39
0.1	148.1 $\pm$ 20.6	481.2 $\pm$ 31.7	138.6 $\pm$ 17.3	455.2 $\pm$ 60.0	95.0	26.0
0.5	205.5 $\pm$ 22.8*	601.3 $\pm$ 34.4**•	180.3 $\pm$ 22.8	475.3 $\pm$ 50.1	25.2	126.0
1.0	214.4 $\pm$ 13.9*•	721.3 $\pm$ 63.6***•••	160.4 $\pm$ 18.8	355.3 $\pm$ 40.8*	54.0	366.0
1.5	231.2 $\pm$ 30.6**••	641.2 $\pm$ 50.1***•••	139.1 $\pm$ 10.8	293.0 $\pm$ 25.5**	92.1	348.2
2.0	230.6 $\pm$ 18.7**	540.6 $\pm$ 30.9***•••	188.2 $\pm$ 20.6	254.2 $\pm$ 18.7***	-23.6	286.4
2.5	181.2 $\pm$ 22.9	508.4 $\pm$ 70.1•	171.3 $\pm$ 11.6	384.1 $\pm$ 30.1	9.9	124.3
3.0	100.3 $\pm$ 27.7	468.3 $\pm$ 29.4**•	123.4 $\pm$ 14.2	359.6 $\pm$ 28.1*	-23.1	108.7
Sample 3 of Thymosol/Placebo	Thymosol		Placebo		Thymosol-placebo differences	
Concent. (µg mL <sup>-1</sup> )						
	- OX-39	+ OX-39	- OX-39	+ OX-39	- OX-39	+ OX-39
0.1	121.3 $\pm$ 10.2	462.0 $\pm$ 38.6	136.4 $\pm$ 14.6	402.0 $\pm$ 21.4	-15.1	60.0
0.5	135.6 $\pm$ 25.1	688.3 $\pm$ 52.3***•	127.3 $\pm$ 18.8	543.1 $\pm$ 39.6	8.3	145.2
1.0	241.3 $\pm$ 61.4	692.1 $\pm$ 70.3**•	150.2 $\pm$ 26.1	473.5 $\pm$ 51.4	91.1	218.6
1.5	269.2 $\pm$ 30.6***•••	864.0 $\pm$ 101.3***•••	149.8 $\pm$ 15.2	492.0 $\pm$ 22.6	119.3	372.0
2.0	265.1 $\pm$ 20.1***•••	694.3 $\pm$ 38.8***•••	183.1 $\pm$ 9.2	386.2 $\pm$ 40.6	82.0	308.1
2.5	201.3 $\pm$ 36.1	511.3 $\pm$ 60.8	131.4 $\pm$ 26.9	416.3 $\pm$ 31.4	69.9	95.0
3.0	141.3 $\pm$ 10.1	351.8 $\pm$ 30.7*	125.2 $\pm$ 22.4	292.6 $\pm$ 22.3	16.1	59.2

Splenocytes from one AO rat were cultivated for 48 h with three samples of Thymosol or placebo as described in Materials and methods. The levels of IL-2 in culture supernatants were detected by ELISA. Values are given as mean  $\pm$  SD (pg/ml) of triplicates. \* = p<0.05, \*\* = p<0.01, \*\*\* = p<0.005 compared to corresponding cultures without Thymosol or placebo, • = p<0.05, •• = p<0.01, ••• = p<0.005 compared to corresponding placebo cultures

calculated in the culture of sample 2 (1.0 µg mL<sup>-1</sup> of Thymosol).

After these experiments, we compared the variability in the IL-2 production by three different samples of splenocytes (prepared from three separate spleens of AO rats), using the same sample of Thymosol and a control sample of placebo was tested in each experiment. Results presented in Table 2 show that the variability between different samples of target cells was higher (CV = 20.1%). In this study maximal stimulation of IL-2 production was obtained with the sample 1 of splenocytes (548.0 pg mL<sup>-1</sup>, 1.5 µg mL<sup>-1</sup> of Thymosol) whereas minimum stimulation of IL-2 production was measured in the culture of sample 2 of splenocytes (365.4 pg mL<sup>-1</sup>, 1.0 µg mL<sup>-1</sup>, of Thymosol).

In our previous work<sup>[18]</sup> and (Vucevic *et al.*, manuscript submitted) we presented pharmacological and immunological characteristics of a CTLE. The result relevant for this paper showed that except the

lipids, as a dominant component, the extract contains about 0.3% bioactive peptides. CTLE extract stimulated the immune response in young and aged mice and rats and also improve wound healing. Although CTLE showed the immunomodulatory activity *in vitro* it was difficult to assess its effect properly due to difficulties in mixing the lipid component with water solution such as culture medium. This was the reason why we prepared a new formulation of the lipid extract, named Thymosol, using Cremophor EL as a lipid emulgator. Preliminary experiments demonstrated that this emulsion preparation was much better soluble in culture medium than the former CTLE. Therefore the main aim of this study was to check whether Thymosol retains its immunobiological effect *in vivo* and whether *in vitro* tests could be used for screening and standardization of this activity.

Based on our previous results (Vucevic *et al.*, manuscript submitted) which showed that a single dose

Table 2: Effect of different concentrations of Thymosol and corresponding placebo on the production of IL-2 by three different populations of rat splenocytes stimulated with anti-TCR antibody with or without a blocking anti-IL-2R $\alpha$  antibody (OX-39)

Sample 1 of splenocytes							
Concent. ( $\mu\text{g mL}^{-1}$ )	Thymosol		Placebo		Thymosol-placebo differences		
	- OX-39+ OX-39	- OX-39	+ OX-39	- OX-39	+ OX-39		
0	201.0 $\pm$ 16.4	493.2 $\pm$ 26.1	201.0 $\pm$ 16.4	493.2 $\pm$ 26.1	0	0	
0.1	206.2 $\pm$ 18.3	481.6 $\pm$ 24.2	188.2 $\pm$ 20.1	424.3 $\pm$ 36.2	18.0	57.3	
0.5	321.4 $\pm$ 28.1***●	724.3 $\pm$ 46.1***●●●	243.0 $\pm$ 25.1	489.6 $\pm$ 35.1	78.4	234.7	
1.0	336.2 $\pm$ 28.8***●●●	717.3 $\pm$ 51.1***●●●	218.6 $\pm$ 18.2	508.4 $\pm$ 33.6	117.6	208.9	
1.5	492.4 $\pm$ 33.1***●●●	1065.2 $\pm$ 48.2***●●●	233.9 $\pm$ 30.1	517.2 $\pm$ 40.0	258.5	548.0	
2.0	488.2 $\pm$ 32.8***●●●	803.1 $\pm$ 55.2***●	246.2 $\pm$ 37.7	601.2 $\pm$ 59.3*	242.0	201.9	
2.5	402.6 $\pm$ 30.2***●●●	950.2 $\pm$ 59.9***●●●	250.2 $\pm$ 18.7*	561.3 $\pm$ 42.2	152.4	388.9	
3.0	301.0 $\pm$ 50.2*	591.3 $\pm$ 60.8*	235.0 $\pm$ 30.6	488.1 $\pm$ 20.0	66.0	103.2	
Sample 2 of splenocytes							
Concent. ( $\mu\text{g mL}^{-1}$ )	Thymosol		Placebo		Thymosol-placebo differences		
	- OX-39+ OX-39	- OX-39	- OX-39	+ OX-39			
0	168.3 $\pm$ 10.2	399.4 $\pm$ 25.1	168.3 $\pm$ 10.2	399.4 $\pm$ 25.1	0	0	
0.1	175.1 $\pm$ 12.3	406.3 $\pm$ 22.0	170.1 $\pm$ 11.3	388.2 $\pm$ 20.1	5.0	18.1	
0.5	198.3 $\pm$ 26.8	430.0 $\pm$ 31.1	182.2 $\pm$ 11.0	408.2 $\pm$ 25.2	16.1	21.8	
1.0	291.3 $\pm$ 20.8***	803.4 $\pm$ 36.6***●●●	231.1 $\pm$ 30.2**	438.0 $\pm$ 30.6	60.2	365.4	
1.5	267.0 $\pm$ 31.4**	724.3 $\pm$ 40.1***●●●	217.2 $\pm$ 18.0*	456.2 $\pm$ 26.0	49.8	268.1	
2.0	181.4 $\pm$ 9.8	504.2 $\pm$ 30.1**●	201.3 $\pm$ 18.2	433.2 $\pm$ 12.8	-19.9	71.0	
2.5	198.3 $\pm$ 10.2*	528.4 $\pm$ 16.2***●●●	179.3 $\pm$ 12.0	359.6 $\pm$ 22.6	19.0	168.8	
3.0	150.2 $\pm$ 8.8	388.3 $\pm$ 18.9	164.0 $\pm$ 12.2	401.2 $\pm$ 12.8	-13.8	-12.9	
Sample 3 of splenocytes							
Concent. ( $\mu\text{g mL}^{-1}$ )	Thymosol		Placebo		Thymosol-placebo differences		
	- OX-39+ OX-39	- OX-39	- OX-39	+ OX-39			
0	180.2 $\pm$ 20.8	478.4 $\pm$ 36.2	180.2 $\pm$ 20.8	478.4 $\pm$ 36.2	0	0	
0.1	159.0 $\pm$ 36.1●	402.8 $\pm$ 30.2●	217.4 $\pm$ 40.2	488.4 $\pm$ 39.9	58.4	-85.6	
0.5	221.3 $\pm$ 16.2	504.4 $\pm$ 30.2●●	201.0 $\pm$ 21.3	422.2 $\pm$ 43.0	20.3	82.2	
1.0	251.2 $\pm$ 20.1*●	620.0 $\pm$ 41.2**●	197.7 $\pm$ 8.2	501.3 $\pm$ 28.0	53.5	118.7	
1.5	296.1 $\pm$ 23.2***●●●	754.4 $\pm$ 37.2***●●●	182.2 $\pm$ 20.6	403.6 $\pm$ 30.2*	113.9	350.8	
2.0	367.1 $\pm$ 20.8***●●●	864.4 $\pm$ 50.1***●●●	210.2 $\pm$ 13.1	413.4 $\pm$ 50.0	156.9	451.0	
2.5	208.5 $\pm$ 12.8	534.1 $\pm$ 30.6●	218.0 $\pm$ 29.6	452.4 $\pm$ 20.0	-9.5	81.7	
3.0	196.3 $\pm$ 20.8	528.6 $\pm$ 48.1●	175.5 $\pm$ 13.2	377.6 $\pm$ 40.8*	20.8	151.0	

Splenocytes from three AO rats were cultivated separately with the same sample of Thymosol or placebo, for the same sample of Thymosol or placebo 48 h as described in Materials and methods. The levels of IL-2 in culture supernatants were detected by ELISA. Values are given as mean $\pm$ SD (pg mL $^{-1}$ ) of triplicates. \* = p<0.05, \*\* = p<0.01, \*\*\* = p<0.005 compared to corresponding cultures without Thymosol or placebo, ● = p<0.05, ●● = p<0.01, ●●● = p<0.005 compared to corresponding placebo cultures

(5 mg/rat) of CTLE enhanced an allogeneic immune response *in vivo*, in this work we used the same dose of Thymosol in a model of KLH antigen-specific immune response, but the dose was divided into two parts ( 2.5 mg at the time of immunization and the second half of dose four days later).The results showed that Thymosol stimulated the immune response, as documented by higher cellularity of regional lymph nodes, higher proliferative response of lymph node lymphocytes following their restimulation with KLH *in vitro* and subsequent increased production of IL-2. These results are generally in agreement with already published results with other different preparations of thymus extracts or thymic peptides isolated from these extracts<sup>[8,10]</sup>. However, the extent of the stimulatory activity varied depending on investigated species, type

of thymic preparation, its dose and the type of immunization protocol. Since the content of peptides in Thymosol is low, our working hypothesis, to be tested in next experiments, is that lipid components, especially gangliosides, act synergistically with peptides in stimulating the immune response and that lipids predominantly stimulate antigen-presenting cells (APC). It has been shown that thymic peptides are also responsible for stimulation of the components of the innate immune system. Tzeheval *et al.*,<sup>[20]</sup> demonstrated that thymosin  $\alpha$ 1 and  $\beta$ 4 potentiate the antigen-presenting functions of macrophages. Macrophages pretreated with thymosin  $\alpha$ 1 significantly enhanced the production of proinflammatory cytokines and this finding correlated with upregulated expression of Toll-like receptors and nucleotide-binding

oligomerization domain (Nod)2 protein<sup>[21]</sup>. Thymosin  $\alpha 1$  also induces the functional maturation and IL-12 production by dendritic cells through the p38 mitogen activated protein kinase/NF- $\kappa$ B pathway<sup>[22]</sup>.

The main result from this study was showed that Thymosol exerted bimodal influence on splenocyte proliferation: stimulation of lower concentrations and inhibition at higher concentrations. Both phenomena were relatively stable in four independent experiments, conducted within two months, but the increase in the proliferative activity and the maximal stimulatory dose varied between experiments. Better stimulatory activity was seen when an anti-TCR mAb was used for cell stimulation, compared to ConA. Under both experimental conditions the response of proliferating T cells within total splenocytes depends on APC and Thymosol was ineffective on purified T cells (data not shown). A slight stimulation of lymphocyte proliferation was observed with some dilutions of placebo and our preliminary results suggest that this effect was caused by Cremophor EL.

The proliferation (clonal expansion) of T cells *in vitro* and *in vivo* is a consequence of produced IL-2 which binds to IL-2R expressed on T cells upon activation<sup>[23]</sup>. Therefore it was logic to study whether in our *in vitro* system an increase in T-cell proliferation was accompanied by increased IL-2 production. We confirmed this relationship and showed that, generally, the level of IL-2 in culture supernatants correlated with the extent of cellular proliferation. However, the differences in the levels of IL-2 between Thymosol-treated cultures and corresponding controls were more pronounced when a locking anti-IL-2R mAb was added.

Up to now, a large number of publications demonstrated that an increase in IL-2 production is one of the main biological effects of thymus extracts or synthetic thymic peptides, but our work is the first report showing the same activity of a lipid thymus extract. Lin and Low<sup>[24]</sup> used three different thymic extracts (one bovine and two porcine thymus extracts) and showed that all three preparations at the concentrations ranging between 12.5 and 50  $\mu$ g mL<sup>-1</sup> increased lymphocyte proliferation as well as production of IL-2, interferon- $\gamma$  (IFN- $\gamma$ ) and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) *in vitro* by human cord blood lymphocytes. The differences in stimulatory potential between the extracts were evident, suggesting that the different immunostimulating effects may reflect not only the species differences but also the difference in

preparation procedures. This finding also suggests that different components in these thymic

Extracts may be responsible for different biological activity. Zatz and Goldstein<sup>[25]</sup> found that thymosin fraction 5, containing one or more biologically active components, caused a two-to threefold enhancement of the proliferative response and production of IL-2 when murine thymocytes were cultured with alloantigen stimulator cells. In addition, the production of colony stimulatory factor (CSF) was increased up to seven fold. Similarly, thymic humoral factor (THF) promoted IL-2 production in intact and thymus-deprived mice both *in vitro* and *in vivo*<sup>[26]</sup>.

THF-gamma 2, a synthetic octapeptide, increased production of IL-2 by lymphocytes from human cord blood<sup>[12]</sup>. Goso *et al.*,<sup>[27]</sup> also reported that a few nanograms of THF-gamma 2, injected in ageing mice *in vivo* raised the frequency of mitogen-responsive T cells in the thymus and spleen and restore IL-2 production by mitogen-stimulated spleen cells. The effect was 400-fold efficient in stimulation of the cell activity than thymosin  $\alpha 1$ .

Cordero *et al.*,<sup>[11]</sup> demonstrated that prothymosin  $\alpha 1$  enhanced IL-2R expression by normal human T cells. This enhancement was dose dependent and paralleled by increased proliferative response to PHA. Prothymosin  $\alpha 1$  increased the anti-CD3 activated killer cytotoxicity of human peripheral blood mononuclear cells (PBMNC) from healthy donors, the expression of adhesion molecules and IL-2R, and production of IL-2 and IL-1 $\beta$ . In addition this biological response modifier induced autologous tumor-reactive CD8<sup>+</sup> and CD56<sup>+</sup> lymphocytes with cytotoxic activity, suggesting its significance as an alternative protocol aimed to improve the clinical results in cellular adoptive immunotherapy of cancer<sup>[28]</sup>. In line of these findings are the results of Beppler<sup>[29]</sup> who showed that thymosin  $\alpha 1$ , derived from prothymosin  $\alpha 1$ , restores normal T-cell functions by increasing IL-2 production and expression of IL-2R in normal mitogen-stimulated T cells both in human and mice.

It seems that thymic preparations modify the Th1/Th2 balance such they stimulate Th1 and inhibit Th2 immune response. This hypothesis was confirmed by Cillari *et al.*,<sup>[30]</sup> who showed that thymopentin (TP) increased the production of Th1 cytokines (IL-2, TNF- $\alpha$  and IFN- $\gamma$ ) and down-regulated the production of Th2 cytokines such as IL-3, IL-4 and IL-5. TP was also efficient *in vivo* in mice with experimental gut-derived sepsis. The significantly reduced mortality of the animals was followed by reduced production of IL-4

and increased production of IL-2<sup>[17]</sup>. Similarly, thymosin  $\alpha 1$  treatment induced a significant increase in production of IL-2 and a decrease in production of IL-4 and IL-10 by T cells from patients with chronic hepatitis C<sup>[31]</sup>.

Our results showed that higher concentrations of Thymosol inhibited proliferation of spleen T cells in a dose dependent manner. A part of the observed effect was probably a consequence of the components of solvent. Although the mechanisms at the immunosuppressive effect of this extract were not investigated, we believe that lipid components are responsible for the most of the suppressive activity. However, there were some publications about the suppressive activity of peptide fractions. The inhibitory effect of thymosin fraction 5 on IL-2 production by PBMNC was found in some patients with active rheumatoid arthritis<sup>[32]</sup>. In addition, the study from the same laboratory also confirmed that thymosin fraction 5 inhibited the proliferation of human PBMNC as well as IL-2 production in the presence of suboptimal concentrations of PHA<sup>[33]</sup>. Similarly, Grinblat *et al.*,<sup>[34]</sup> showed that THF inhibited T-cell proliferation if added after ConA stimulation.

An important question resulting from this work and similar studies is related to the choice of the assay for evaluation of the immunobiological activity of thymic preparations. According to our results, we believe that lymphocyte proliferation assay, based on [<sup>3</sup>H]-thymidine incorporation and IL-2 production assay could be standardized for these purposes. The limitation factor in using the proliferation assay is considerable inter-assay variability and needs for radioactive materials. Our attempts to replace the [<sup>3</sup>H]-thymidine method with an alternative assay, such as MTT test, was unsuccessful due to lower sensitivity of MTT. Based on our previous experience with thymic extracts and other biological modifiers<sup>[35]</sup>, including the results presented in this work, we concluded that the level of produced IL-2 could be the most reliable parameter for testing the immunobiological activity of thymus extracts. Our results also suggest that the extent of stimulation in IL-2 production is more pronounced if IL-2R $\alpha$  are blocked, and because of that utilization of the secreted IL-2 is prevented. Using such an approach with splenocyte cultures stimulated with an anti-TCR mAb, we showed that variability between three samples of the same batch of Thymosol tested on the same population of target cells was about 12%. This CV is very acceptable for biological tests. As expected the variability in the production of IL-2 between different samples of target cells, treated with the same sample of the extract was higher (about 20%). Higher variability in the assay could be explained by different

immunological status of the individual animals. The difference in the *in vitro* lymphocyte response to mitogens and other stimuli strongly depends on the activation properties of accessory cells (DC and macrophages) present in the total population of splenocytes<sup>[36]</sup>. This variability is especially seen in older rats (data not shown) which were used in this study. However, as mentioned before, older animals are more convenient for screening the immunobiological activity of thymus preparations than young animals<sup>[4,7]</sup>. The use of some permanent T-cell lines will be an alternative for screening assay instead of total lymphocytes. Unfortunately, Thymosol is not efficient in triggering T-cell activation and IL-2 production without accessory cells.

In conclusion, according to the obtained results we propose a method for quantification of the immunobiological activity of Thymosol. The method is based on the stimulation of IL-2 production by rat splenocytes in culture stimulated with an anti-TCR mAb in the presence of a blocking anti-IL-2R mAb. We suggest that several stimulatory concentrations of the extract (at least three different samples) should be tested on the same population of target cells (total splenocytes). The highest mean concentration of produced IL-2 subtracted from the highest mean concentration of IL-2 in culture with placebo could be the quantitative indicator of its immunobiological effect. We also suggest that one pg mL<sup>-1</sup> of such calculated value of IL-2 corresponds to one unit (U) of the biological activity of Thymosol. It remains to be tested in future studies whether this parameter is stable and reliable marker for assessing the biological activity, not only different batches of Thymosol, but also similar thymus extracts.

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