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THE FINDING AND PARTIAL PURIFICATION AND CHARACTERIZATION OF THYMONE C

by

Karl Folkers, Henryk Stepien, Teresa Kubiak, Naoki Sakura and Masanori Sakagami

Institute for Biomedical Research, The University of Texas at Austin
Austin, Texas 78712

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SUMMARY

A fraction, isolated from bovine thymus by sequences of ca. ten steps of extractions, gel filtrations, ion exchange chromatography, etc., stimulated the synthesis of both cAMP and cGMP at a level of 100 μg/ml. Thymone A stimulated cAMP, but is chromatographically remote from the fraction. Essentially pure thymone B, which stimulates cGMP, did not stimulate cAMP even at a high level of 100 μg/ml. The presence of thymone B in this fraction is supported by TLC data. This fraction also stimulated the mixed lymphocyte reaction. The cAMP-stimulating entity is designated thymone C until it is characterized and related to or differentiated from products of other investigators.

INTRODUCTION

Investigators, on an international basis, are fractionating thymus extracts, isolating materials, and studying the biological activities of such extracts both in model systems and at the clinical level.

Fraction 5 has been clinically observed (1) to reverse certain immunological parameters which decrease in cancer patients, and in a combined modality, by a randomized protocol, increased both the time and the number of surviving patients with carcinoma of the lung. Varsano et al. (2) and Zaizov et al. (3) clinically investigated a fraction, designated THF (thymic humoral factor), and reported promising results for the treatment of severe disseminated adenovirus and also for therapy of immunosuppressed children who had a lymphoproliferative neoplasia and generalized varicells. Tovo et al. (4) treated 12 immuno-depressed children with malignancies and herpes virus infections with TP-1 (a thymus extract); in 11/12 children, they observed a good and "sometimes dramatic response".

Several peptides have been isolated from thymus extracts and have been chemically characterized, including thymosin α_1 from fraction 5 which has an identified sequence of 28 amino acids (5). Both thymopoietin I and II are peptides having 49 amino acids and differ only in the substitutions in positions 1, 2 and 43. The facteur thymique serique (FTS) is a nonapeptide, sequenced and synthesized (6). The serum factor (SF) was reported (7) to have an MW of <500 and to consist of possibly four amino acids.

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The multiplicity of such factors or hormones is generally understood on the basis that the complexity of the immune system is more likely to be controlled by several rather than by a single hormone.

We have reported (8,9) two peptides from the thymus tissue which have chemical and biological characteristics which differentiate them from previously described thymic peptides. These two peptides are thymone A, which has ca. 68-71 amino acids, and thymone B which has up to 13 individual amino acids and corresponds to a peptide considerably smaller in molecular weight than that of thymone A.

Repetition of our chemical fractionations and bioassays which revealed thymones A and B also revealed the presence of another biologically active entity, designated thymone C, and these results are described herein.

EXPERIMENTAL

Biological Assay Methods

Three biological assays made possible the finding and guided the partial purification and characterization of thymone C. One assay was based upon the incorporation of [^{3}H]-thymidine into DNA by spleen cells from neonatally thymectomized C57BL/6 mice. This assay on incorporation of thymidine guided all the chemical fractionations. The mice were purchased from the Charles River Breeding Labs. Inc., Wilmington, MA. The essential steps of this assay were the same as those summarized for the isolation of thymone A (8). The other two assays were based upon the determinations of cAMP and cGMP.

Determination of cAMP and cGMP

The spleenic lymphocytes, 1.5×10^7, prepared as described (10), were exposed to the fractions to be assayed which had been dissolved in 100 µl of the RPMI-1640 medium at 37°C in a water bath. The biological reaction was stopped by immersion of the incubate into a dry ice-ethanol bath to inactive phosphodiesterase for 1 min. Both assays were performed on fractions that had been lyophilized and resuspended in 0.5 ml of 0.05 M of sodium acetate buffer, pH 6.2, before addition of aliquots to the medium for assay.

The steps of purification and extraction of the cyclic nucleotides were performed according to the method of Yamamoto and Webb (11). After the inactivation of phosphodiesterase, the suspension of cells was placed in a boiling water bath for 4 min. The extraction of the cyclic nucleotides from the samples was performed using 0.2 ml of a Dowex (formate form Dowex AG, 1 - X 8, 200-400 mesh, Sigma Chemical Co., St. Louis, MO)-water slurry (1:1). The Dowex suspension was vortexed 3 times over a 15 min. period and then centrifuged at 2500 rpm for 10 min. The supernatant was discarded. The pellet was washed three times during 10 min. with 0.8 ml of 4 N redistilled formic acid each time. This procedure released both cAMP and cGMP from the Dowex. The formic acid extract was then lyophilized and stored at -20°C until the time of the RIA. The recovery of cAMP and cGMP was estimated by [^{3}H]-radiolabeled cyclic nucleotide to be >85%.

The determinations of the levels of cAMP and cGMP were performed by using the kits from the New England Nuclear, Boston, MA, according to the radioimmunological procedure of Steiner et al. (12). In order to increase the sensitivity of the assay for cGMP, the samples were initially acetylated with a mixture of 5 µl of acetic anhydride and triethylamine (1:2 v/v) according to Harper and Brooker (13).

TABLE I. Incorporation of [^{3}H]-Thymidine into DNA

Substance	Level	cpm±SEM	P
Control	--	8,076	--
*Thymone C and B	25 μg/ml	17,769	<0.01

*Fraction of —

The specificity and cross-reactivity of the cAMP and cGMP antisera with several nucleotides with and without the acetylation step were found to be similar to that reported in the specifications for determination of cAMP and cGMP by the New England Nuclear.

All of the assays were performed in triplicate and the data were calculated as mean values±SEM. The student's t-test was used to determine statistical significance.

Chemical Purification and Partial Characterization

A sequence of steps for purification, which led to the finding of thymone C, were as follows: (a) The thymus glands were cooled with ice immediately after slaughter, and the tissue was shipped with ice, and then stored in the frozen state; (b) The glands were trimmed of extraneous fat and then lyophilized; (c) Methylene chloride was used for defatting; (d) The defatted residue was extracted with methanol; (e) The methanol residue was extracted with acetic acid; (f) The dialysis step which was used for the purification of thymone A (8) was omitted; (g) Stepwise purification was achieved with Sephadex G-50; (h) Fractions, which were identified as containing thymone B by bioassay, were then purified by DEAE-Sephadex A-25; (i) Again, fractions containing thymone B by bioassay were then purified over CM-Sephadex C-25.

The data in Table I show that a fraction from CM-Sephadex C-25 was active at a level of 25 μg in stimulating the incorporation of [^{3}H]-thymidine into DNA.

This same fraction, according to the data in Table II, was found to stimulate the synthesis of both cGMP and cAMP. This fraction, at a level of 100 μg increased ($p<0.001$) the level of cGMP, and was correspondingly effective in stimulating ($p<0.001$) the level of cAMP. The actual level of activity of the fraction was between 10 and 100 μg. This stimulation of the synthesis of cGMP and cAMP by this fraction, at a level of 200 μg, was effective over an incubation period of 1-30 min.

This fraction was subjected to TLC on a silica gel plate (Merck, EtOAc: pyridine:AcOH:H_2O = 5:5:1:3). A spot with an Rf value of ca. 0.48 was evident by the chlorine/o-tolidine reagent. When this TLC was conducted with a reference sample of previously isolated thymone B on the same plate, the Rf values of the entity and of thymone B were the same, 0.48.

Since it is known that thymone B stimulates cGMP but not that of cAMP, the entity which stimulated cAMP is different from thymone B. Since this fraction is chromatographically distinct from fractions containing thymone A, the cAMP stimulating entity is differed from thymone A, and it is designated thymone C.

DISCUSSION

The isolation and characterization of thymones A and B (8,9) was initially based upon the isolation and characterization of very small specimens. In order to obtain larger samples of thymones A and B, purifications were repeated,

TABLE II. Stimulation of cAMP and cGMP

Substance	Level	Incubation	Incorporation			
	µg/ml	min.	cAMP pM/10^7 cells	P	cGMP fM/10^6 cells	P
Control	--	0	4.0		5.00	
*Thymone C and B	200	10	22.95	<0.01	33.88	<0.001
	100	10	11.99	<0.001	20.45	<0.001
	10	10	3.58	n.s.	6.6	n.s.
Control	--	0	4.19		4.88	
*Thymone C and B	200	1	19.3	<0.001	20.4	<0.001
	"	5	13.25	<0.001	15.5	<0.001
	"	10	12.3	<0.001	14.8	<0.001
	"	20	16.5	<0.001	15.7	<0.001
	"	30	16.2	<0.001	12.86	<0.001

*Fraction of —

and changes for improvement are summarized herein. In particular, the dialysis step omitted. Stepwise purifications by Sephadex G-50, DEAE-Sephadex, and CM-Sephadex, were monitored, as before, using the guidance of activity in the assay for stimulation of the incorporation of [^{3}H]-thymidine into DNA; the purification was designed to reisol te thymone B.

After step 10 using CM-Sephadex C-25, the fraction which appeared to contain thymone B was bioassayed; the data in Table I show this fraction was active at 25 µg/ml to stimulate DNA synthesis. This fraction was also bioassayed for the stimulation of both cAMP and cGMP. The data in Table II show that levels of 100 and 200 µg/ml significantly stimulated the synthesis of cAMP as well as the synthesis of cGMP. At a level of 200 µg/ml, the stimulation was effective ($p<0.001$) over incubation periods of 1 through 30 min., and for both cAMP and cGMP.

The presence of thymone B in this fraction from CM-Sephadex was supported by TLC data. By TLC, a spot was evident with an Rf of 0.48 which was the same as the Rf of essentially pure thymone B, as reference, on the same plate.

It was previously reported (9) that essentially pure thymone B did not stimulate the synthesis of cAMP even at the high level of 100 µg/ml. The stimulation of cAMP by this fraction (containing $<100\%$ of thymone B) shows the presence of another biologically active entity in this fraction, which is presently designated thymone C.

When thymones C and B are completely separated, a test on the possible stimulation by thymone C of the incorporation of [^{3}H]-thymidine into DNA can be definitive.

TABLE III. Effect of Thymones B and C on the Mixed Lymphocyte Reaction of Responding Spleen Cells from Neonatally Thymectomized C57BL6 Mice

Substance	Level	Stimulatory Cell	cpm±SEM	P
Control	--	C57BL6	2234±487(a)	
		B6F3	5260±225(b)	
*Thymones C and B	100 μg	C57BL6	1947±421(a)	
		B6F3	7089±502(b)	<0.05

*Fractions of —

(a) syngeneic reaction
(b) allogeneic reaction

The data in Table III show the effect of this fraction on the mixed lymphocyte reaction of responding spleen cells which were obtained from neonatally thymectomized mice (C57BL/6). The assay was conducted like that described by Sunshine et al. (14). Under these specific conditions, the fraction stimulated ($p<0.05$) the mixed lymphocyte reaction. The dose level of the fraction was arbitrary, and may have been too low.

At this time, only the biological activity of thymone C to stimulate the synthesis of cAMP and the activity of the fraction to stimulate the mixed lymphocyte reaction are criteria to allow comparisons with previously described fractions and isolated peptides from thymus tissue. Thymopoietin, in a given system, produced a rapid and transient rise in cGMP levels and slightly decreased cAMP levels (14). In a review (15), it is recorded that the facteur thymique serique (FTS) did not elevate levels of cAMP, and the effect of the human serum thymic factor (SF) to elevate levels of cAMP was ambiguous. Fractions of the thymic humoral factor (THF) have been reported to elevate levels of cAMP, a result which was believed to characterize THF as a hormone.

The thymic control of proliferation of T-cell precursors in bone marrow has been studied by Cohen and Fairchild (16) who proposed that T-cells undergo successive maturation in three tissues. Pre-T-cells mature in the bone marrow. In the thymus, a second maturation takes place. Thirdly, cells from the thymus reach peripheral lymphoid tissues where further maturation to T- and Lyt-types occur. There may be a relationship between one of these three stages of maturation of T-cells and thymones A, B and C.

Other investigators have described fractions of biologically active entities from thymus tissue, lymphocytes, and other tissues. Baker et al. (17) investigated a T-cell growth factor (TCGF). Dabrowski et al. (18) studied a calf thymic hormone (TFX). Lenfant et al. (19) reported on a

spleen derived immunosuppressive peptide (SDIP) from bovine tissue. Soder and Ernstrom (20) isolated from calf thymus a thymocyte specific growth factor, which appeared essentially pure, is acidic, and has a molecular weight of about 10,000 daltons.

The chemical nature of thymone C can be related to or differentiated from these cited and uncited investigations by other investigators when thymone C is chemically and biologically characterized.

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