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The Influence of Bovine Thymus Hypocalcemic Protein, TPl, on the Levels of Cyclic Nucleotides in Murine Thymus Lymphocytes

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The effects of bovine thymus hypocalcemic protein (TP1) on cAMP and cGMP levels in murine thymus cells were studied. TP1 elevated cGMP levels 12.5-fold at a concentration of 0.15 mg/ml after incubation for 5 min but the amount of cAMP in thymocytes was lowered. These results suggest that TP1 stimulates the differentiation of early T-cells to mature T-cell subsets, such as helper cells, but does not stimulate that of stem cells to early T-cells.

Keywords——TP1; thymus hypocalcemic protein; cGMP; cAMP; T-cells; thymic hormone

We previously purified a hypocalcemic protein from bovine thymus[2] and reported its molecular weight, amino acid composition, terminal amino acids, and some other chemical properties.[3] The hypocalcemic protein had a lymphocyte-promoting action[4] and increased the number of antibody-forming cells and rosette forming cells.[5]

It has also been reported that some thymic factors influence the levels of cyclic nucleotides in thymus cells. Thymic factor in human serum (SF) and thymic humoral factor (THF) were shown to elevate cyclic AMP (cAMP) levels[6] but thymosin and thymopoietin raised cGMP levels.[7] It was shown that the change of cAMP was related to the differentiation of stem cells to immature T-cells, while that of cGMP was related to the differentiation of early T-cells to mature T-cell subsets, such as helper, suppressor, and killer cells. We describe here the effects of our hypocalcemic protein on the levels of cellular cyclic nucleotides in thymocytes.

Experimental

Materials——cAMP assay kits and cGMP radioimmunoassay kits, containing tritiated cyclic nucleotides, were purchased from the Radiochemical Centre, Amersham, England.

Samples——Bovine thymus hypocalcemic protein (TP1) was purified according to the methods described previously, as follows.[3,4] The saline extract from the acetone-dried powder prepared from bovine thymus gland was fractionated with ammonium sulfate, and the fraction that precipitated at 15% (w/w) concentration of ammonium sulfate was obtained. After dialyzing the precipitate, the fraction was chromatographed on DEAE-cellulose and the eluted active fraction was gel-chromatographed on Ultrogel AcA 22 (LKB). The

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2) *a*) A. Mizutani, M. Terada, Y. Toda, and K. Yamamoto, *Ann. Rept. Pharm. Nagoya City Univ.*, **17**, 16 (1969); *b*) A. Mizutani, Y. Saito, H. Sato, and M. Saito, *Yakugaku Zasshi*, **90**, 445 (1970); *c*) A. Mizutani, K. Yamamoto, H. Sato, and Y. Saito, *Yakugaku Zasshi*, **91**, 297 (1971).
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fraction eluted at Kav 0.65 (G-2) was further purified by preparative polyacrylamide gel electrophoresis. The fraction TP1, having a relative mobility of 0.68 with respect to BPB on electrophoresis, was obtained. The purity of G-2 was about one-tenth of that of TP1, on the basis of the hypocalcemic activity and the analytical disc gel patterns. In these experiments, G-2 was mainly used as a partially purified TP1. The hypocalcemic activities of G-2 and TP1 were significant; the falls were $11.69+1.47\%$ at a dose of 0.1 mg/kg of rabbit and $8.72\pm2.34\%$ at a dose of 0.01 mg/kg, respectively.

Cell Preparation——C57B1/6 mice (aged 4—5 weeks, weighing 17—20 g) were sacrified by cervical dislocation and the thymus (about 50 mg) was rapidly removed. The thymus was dissected with fine pincetts in 10 volumes of Eagle's MEM medium (Nissui Pharm. Co.) containing glutamine, and the cell suspension was filtered through a stainless steel screen of 200—400 mesh. The solution containing the cells was centrifuged at $250\times g$ for 10 min, then the precipitated cells were resuspended in 0.017 M Tris-HCl buffer containing 0.75% ammonium chloride (pH 7.0) to lyse the red cells.[8] After incubation for 10 min at room temperature and filtration through the above screen, the cells were centrifuged at $250\times g$ for 10 min, then resuspended in Eagle's MEM medium and washed twice by centrifugation. The cell concentration was adjusted to 2.5×10^7 cells/ml. The cells were incubated for 10 min at 37° to allow them to stabilize.

Cyclic Nucleotides Assays——A solution (0.2 ml) containing cells at a concentration 2.5×10^7/ml was mixed with 0.2 ml of Eagle's MEM medium containing TP1 or G-2 and then incubated at 37°. The reaction was stopped by immersion for 1 min in a dry ice-acetone bath followed by 3 min in a boiling water bath according to the reported methods.[7b] Assays for cAMP or cGMP were performed on duplicate samples of the supernatants of a 10 min, $2000\times g$ spin.

cAMP in 0.05 ml aliquots of the supernatants was measured as follows; 0.05 ml of [^{3}H]cAMP (5 μCi/10 ml) and 0.1 ml of the binding protein were added to the supernatants and incubated for 2 hr at 2°. After adding charcoal solution, the mixture was centrifuged at $8000\times g$ and the radioactivity in 0.05 ml of the supernatants was measured with a liquid scintillation counter (Mark II, Nuclear Chicago). The scintillant used consisted of one part of Triton X-100 and two parts of toluene containing PPO (4 g/l) and POPOP (0.1 g/l).

For the measurement of cGMP, 0.1 ml of the supernatants was mixed with 0.05 ml of [^{3}H] cGMP (1.6 μCi/10 ml) and 0.05 ml of antiserum. After incubating the solution at 2° for 1.5 hr, 1 ml of ammonium sulfate solution was added and the mixture was centrifuged at $13000\times g$ for 2 min. The precipitate was dissolved in 1.1 ml of distilled water and 1 ml of the solution was used for scintillation counting. The contents of cyclic nucleotides in unknown samples obtained from cells were calculated from the standard curve, using the mean values of duplicate data for each sample.

Other General Methods——Hypocalcemic assay was carried out according to the method described previously.[3c] Calcium in serum was determined by the atomic absorption method.[9] Protein concentration was determined by the method of Lowry *et al.*[10] Polyacrylamide disc gel electrophoresis was carried out according to Davis.[11]

Results

Figure 1 shows the dose-response relationship between G-2 and cGMP in thymus cells after incubation for 5 min. The contents of cGMP in thymocytes were increased at concentrations of G-2 above 0.125 mg/ml, as shown in Fig. 1. TP1 at a dose of 0.15 mg/ml elevated the value of cGMP obtained 12.5-fold compared with the control. Figure 2 shows the time course of the change of cGMP in cells after administration of G-2 at a concentration of 1 mg/ml. An increase of cGMP was found after incubation for 2 min, as shown in Fig. 2. The increase reached a plateau after incubation for 5 min and did not fall upon incubation for 30 min. It was found that the time course of cGMP levels obtained in control experiments (incubation of cells only) showed no significant change compared with the zero-time level.

Figure 3 shows the time course of cAMP in cells at a concentration of 1 mg/ml of G-2. The content of cAMP was lowered to about 39% of the control value after incubation for 5 min but the level recovered rapidly. The control experiments showed not change of cAMP levels. Figure 4 shows the dose-response relationship of G-2 and cAMP in thymocytes after incubation for 5 min. cAMP in thymocytes was decreased as shown in Fig. 4, and the value (2.3 pmol/5×10^5 cells) obtained on incubation at 0.25—1.0 mg/ml of G-2 was about 50% of the control

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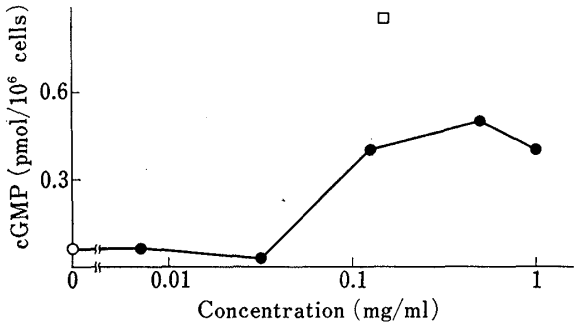

Fig. 1. Dose-response Curve for the Action of Thymus Hypocalcemic Protein on cGMP Levels

●, sample G-2; □, TP1; ○, control.

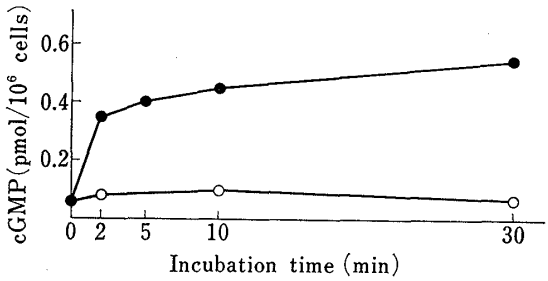

Fig. 2. Time Course of the Effect of G-2 (1 mg/ml) on the Levels of cGMP in Murine Thymocytes

●, sample; ○, control.

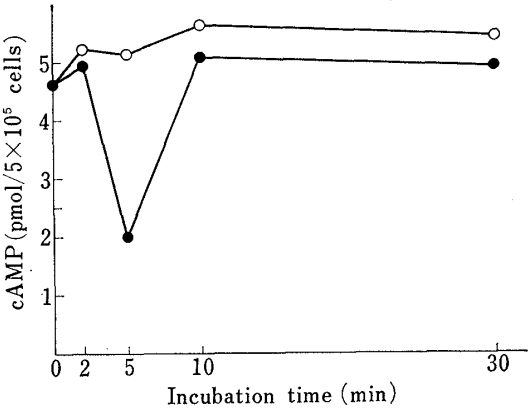

Fig. 3. Time Course of the Effect of G-2 (1 mg/ml) on the Levels of cAMP in Murine Thymocytes

●, sample; ○, control.

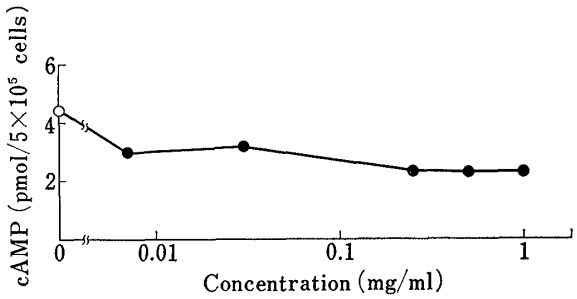

Fig. 4. Dose-response Curve for the Action of G-2 on cAMP Levels

●, sample G-2; ○, control.

(4.4 pmol/5×10⁵ cells). The concentration (0.25 mg/ml) of G-2 affecting cAMP levels was twice that affecting cAMP levels (0.125 mg/ml). A similar lowering effect on cAMP levels was obtained with TP1. TP1 was effective at doses above 0.038 mg/ml, and TP1 at a dose of 150 μg/ml showed longer action than G-2.

It was concluded that TP1 elevated cGMP levels in murine thymus lymphocytes but lowered cAMP levels in the cells.

Discussion

This study suggests that the second messenger for TP1 in activating thymocytes may be cGMP and not cAMP; similar results have also been obtained with thymopoietin and thymosin.

Table I. Comparison of Effects on Cyclic Nucleotides Levels and Other Properties of Thymus Products

	TP1	Thymosin[a)]	Thymopoietin[b)]	THF[c)]	SF[d)]
cAMP	−	+−[e)]	−	++	++
cGMP	++	++	++	ND[f)]	ND
Molecular weight	68000	12000	5560	2900	900
Heat stability	Labile	Stable	Stable	Stable	ND

a) reference 7*b*, *b*) reference 7*a*, *c*) reference 6*b*, *d*) reference 6*a*, *e*) unchanged, *f*) not determined.

TPl had stronger activity than thymosin at a dose of 0.1 mg/ml (thymosin fraction 5 elevated cGMP 2-fold over the control value[7b] but TPl increased it 12.5-fold).

In contrast with the results for TPl, the effects of other thymic factors on the levels of cyclic nucleotides are summarized in Table I, which also shows their molecular weights and heat stabilities. One group (TPl, thymosin, and thymopoietin) of thymic products is of high molecular weight, and these factors elevated the cGMP level. Another group (low molecular weight) includes THF and SF, and these factors elevated the cAMP level. It is probable that the factors in Table I act cooperatively in the complex differentiation of stem cells to mature T-cells. The different effects of thymic factors on cGMP and cAMP levels can be considered as follows.[7b] Factors increasing cAMP levels stimulate the development of stme cells, to earyl T-cells and other factors increasing cGMP levels, such as TPl and thymopoietin, have an important role at the step of development of early T-cells to mature T-cell subsets, such as helper, suppressor, and killer cells, as illustrated in Fig. 5. The results with TPl are consistent with our previous results on the increase of plaque forming cells with TPl,[5] and it appears that TPl might increase the number of helper cells, but not that of B-cells. We also showed that TPl increases the levels of circulating lymphocytes, about 70% of which are T-cells, and rosette-forming cells originated from T-cells.[5]

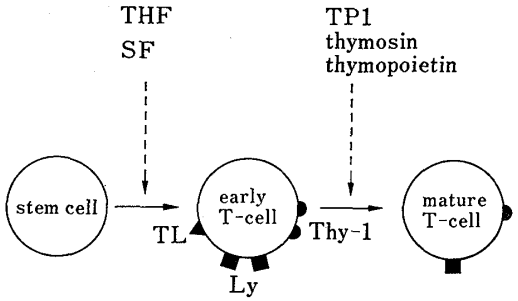

Fig. 5. Possible Points of Stimulation by Thymic Factors

Surface antigens: TL, thymus-leukemia; Thy, Theta; Ly, T lymphocytes.

The results in this work are consistent with a hypothesis of Watson[12] that cGMP levels are elevated by a helper signal from T-cells and that a decrease in the cAMP/cGMP ratio promotes the conversion of B-cells to antibody-forming cells.

The reason why TPl decreased cAMP levels after incubation for 5 min at higher doses than those required for cGMP was not elucidated. Another problem was that the cell suspension used in these experiments might contain many classes of T-cell subsets, so more detailed experiments are required after separation of the T-cell subpopulations.

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