INCREASE OF CYCLIC GMP INDUCED IN MURINE THYMOCYTES BY THYMOSIN FRACTION 5

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SUMMARY: Using a radioimmunoassay (RIA) for the determination of adenosine 3'5' cyclic monophosphate (cAMP) and an acetylation-RIA procedure to measure guanosine 3'5' cyclic monophosphate (cGMP), we observed that cGMP levels, but not cAMP levels, were significantly elevated in murine thymocytes which had been incubated with preparations containing the thymic hormone, thymosin. Stimulation of intracellular cGMP levels was seen as early as 1 minute after incubation with thymosin fraction 5 and was maximal at approximately 10 minutes. Dose response studies indicated an optimum stimulation of cGMP with a thymosin concentration of 100 µg/ml. A control spleen fraction prepared by an identical procedure as fraction 5 did not affect the levels of either cyclic nucleotide.

The endocrine influence of the thymus on the maturation and function of the immune system has been previously demonstrated in a variety of animal models by use of thymus gland transplantation and by the administration of thymic extracts (1,2). Recently it has been shown that thymosin is also active in increasing T cell numbers and responses in cancer patients (3) and in increasing cell-mediated immunity and resistance to infection in children with thymic dependent primary immunodeficiency diseases (4). Although the effects of thymosin on various cell populations both *in vivo* and *in vitro* have been repeatedly demonstrated, the mechanism by which thymosin activates T cell populations is not known. In the present study we report that one of the first detectable effects of thymosin preparations on thymocytes is to increase intracellular cGMP levels, but not cAMP. This observation suggests that a mechanism of action of thymosin may involve modulation of cGMP levels in murine thymocytes.

MATERIALS AND METHODS

Reagents. Cyclic AMP and cGMP RIA kits were purchased from Schwartz-Mann, Orangeburg, New Jersey. Lipopolysaccharide B (*E. coli* 0111:B4) was obtained from Difco Laboratories, Detroit, Michigan. Thymosin fraction 5 (Lot #BPM 390) and the spleen fraction 5 (Lot #BPM 307) was prepared from calf tissue by Hoffmann-LaRoche, Nutley, New Jersey, as previously reported (5). For convenience thymosin fraction 5 will be referred to as thymosin.

Preparation of phosphodiesterase. Rat brain phosphodiesterase was prepared by homogenizing whole brain from an adult male Sprague Dawley rat (400 g) for 1 minute in an ice bath using 3 volumes of 10 mM Tris buffer containing 1 mM $MgCl_2$ (pH 7.5). The homogenate was spun at 105,000 x g for 60 minutes. The supernatant was frozen in aliquots, and diluted 1:10 before use.

Cell preparation. C57B16/J mice were sacrificed by cervical dislocation and the thymus and/or spleen rapidly removed. Cells were dispersed in HEPES buffered RPMI (Gibco, Grand Island, N.Y.) over a fine mesh screen, aspirated through a 25 GA needle, spun at 250 x g for 10 minutes, and resuspended in tris buffered ammonium chloride (ACT) (6) to lyse the red cells. Following a 10-minute incubation at room temperature and filtration through nylon to remove debris, the cells were centrifuged at 250 x g for 10 minutes. Cells were then resuspended in RPMI-1640, washed twice and incubated at 25 x 10^6 cells/ml for 15 minutes at 37°C in a 5% CO_2, 96% air incubator to allow them to stabilize.

Cyclic nucleotide assays. Cells were incubated with agents to be tested at a final concentration of 12.5 x 10^6 cells/ml. The reaction was stopped by a 1 minute immersion in a dry ice-ethanol bath followed by a 3 minute immersion in a boiling water bath. Assays were performed on the supernatants of a 10-minute 2000 x g spin. In some cases samples were extracted, lyophylized and reconstituted in HRPMI.

Cyclic-AMP was measured directly in the spun supernatant or the extracted lyophylizates using the RIA procedure of Steiner as modified by Schwartz-Mann (7).

Cyclic-GMP was assayed by the procedure of Harper and Brooker (8). To a 0.10 ml aliquot of acetylated cGMP was added .200 ml buffer, .05 ml of I^{125} derivative, and 0.05 ml of antisera. Samples were incubated overnight at 4°C, precipitated by 60% ammonium sulfate, and the pellet counted in a Nuclear Chicago gamma counter. In both assays logit vs. ln concentration plots were used to determine the values for the unknown samples.

RESULTS

A 15-minute *in vitro* incubation with thymosin fraction 5 (100 µg/ml) increased by 1.5 to 3-fold the levels of cGMP in murine thymocytes but had no consistent significant effect on cAMP levels (Table 1). The stimulatory effect of thymosin was similar if the samples and standards were fractionated with a Dowex-1-formate resin according to the procedure of Yamamoto and Webb (9). A control extract of spleen prepared under similar conditions as that

TABLE 1

Cyclic Nucleotide Levels Determined After 15 Minutes Incubation at 37°C with the Agents Indicated

	cAMP pmoles/10^7 cells	cGMP fmoles/10^6 cells
Media Control*	1.70 ± .17**	2.70 ± .63
100 µg thymosin fr. 5	1.38 ± .03 (NS)***	7.55 ± .07 ($p<.001$)
100 µg spleen fr. 5	0.81 ± .03	1.57 ± .21 (NS)

* HRPMI-1640
** Standard deviation for independent triplicate samples
*** Statistical significance based on student's t-test (NS-not significant, $p>.05$)

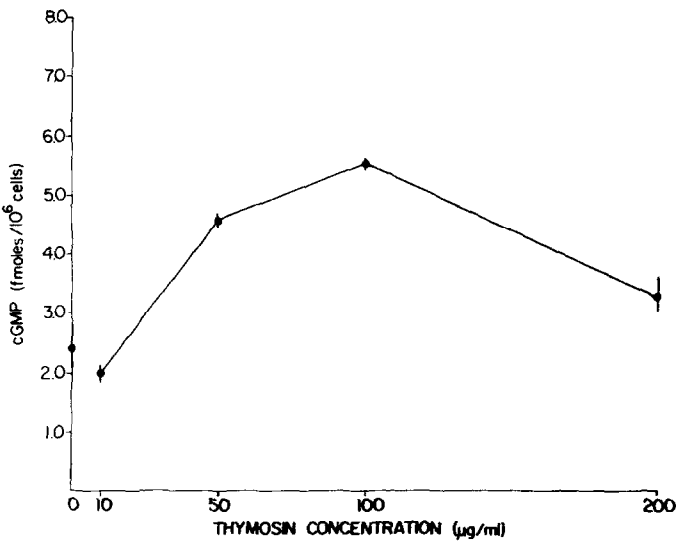

Figure 1. The dose response curve for fraction 5 thymosin has an optimum of 100 µg/ml for murine thymocytes incubated at 37°C for 15 minutes.

of thymosin did not increase cGMP (Table 1). Although in the experiment shown there was a slight decrease in cAMP following incubation with 100 µg/ml of the spleen fraction, this was not seen in other experiments (Table 1). The optimum dose of thymosin was found to be 100 µg/ml (Fig. 1). The increase in cGMP was detectable within 1 minute and maximal as early as 4 minutes (Fig. 2)

Most lots of thymosin contained material which analyzed as cGMP (approximately 8 fmoles/10 µg of thymosin) but not cAMP. This could account for the zero time values of the 0.1 ml aliquots used in Fig. 2. All of the material

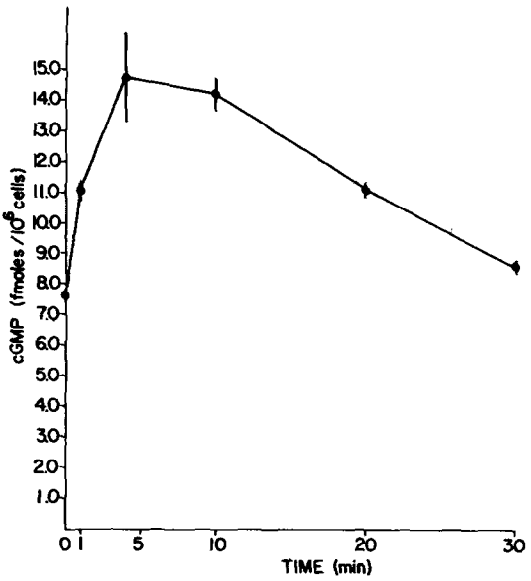

Figure 2. Cyclic GMP levels in murine thymocytes incubated with 100 µg/ml of thymosin fraction 5. The increase is detectable at 1 minute (11.13 ± .28 fmoles/10^6 cells). The value for the cells at zero time is actually the average and standard error for the untreated cells at 0, 10, 20 and 30 minutes. Thus there is no significant shift in the values of cGMP in the non-stimulated cells over the 30 minute incubation period.

measured as cGMP could be destroyed with a crude phosphodiesterase preparation from rat brain. While interference by guanosine nucleotides could account for some of the thymosin cGMP, we feel that the time and dose dependent increases in cGMP reflect changes in the cyclic nucleotide itself. It should be noted that the spleen preparation did not contain a measurable amount of cGMP.

The thymosin that we are using is pyrogen free as determined by the rabbit pyrogen test, and contains less than 1 ng/mg of endotoxin as assayed by the limulus lysate assay (Difco, Detroit, Michigan). We have found that LPS B had no effect on cAMP and cGMP at levels 1000 times that amount.

DISCUSSION

As a result of experiments suggesting that cGMP has an enhancing effect on T cell function and that cAMP generally has an inhibiting effect on lymph-

ocyte responses, a role for cyclic nucleotides in modulating the immune response has been postulated (10,11). Our data is consistent with the hypothesis that the second messenger for thymosin in activating thymocytes may be cGMP, rather than cAMP. Although we have explored several different assays for cAMP including the RIA, kinase binding, and an assay utilizing the prelabeling of intracellular adenine pools, we have been unable to demonstrate a statistically significant stimulating effect of thymosin on cAMP levels in either spleen or thymus cells (Table 1 and unpublished observation). This probably does not eliminate cAMP as an important second messenger for specific T cell responses since cAMP can mimic thymosin in some *in vitro* systems (12, 13). In one system both thymosin and agents which elevate *in vitro* levels of cAMP have been shown to increase the azathioprine sensitive rosette forming cells in adult thymectomized murine spleen cells (12). In another assay, new surface antigens (T1 and thy-1) were induced in bone marrow cells by both thymosin and cAMP stimulating agents (13). Neither of these assays reflects functional changes and these cell surface markers can be induced by agents not derived from the thymus.

In contrast to our findings, Kook and Trainin have shown that THF, another thymic humoral factor, increases cAMP in murine thymocytes (14). Our observations would thus appear to provide additional evidence to support the hypothesis that there is a family of thymic peptides, some of which may activate cGMP systems, others cAMP systems, and still others which may act via a non-cyclic nucleotide route (15). These peptides probably have different biological activities, may effect different subsets of cells, and may or may not work in concert to induce the maturation and function of T cells. The rapid increase in cGMP is expected in light of experiments indicating that thymosin induced changes in membrane markers can occur rapidly (16,17).

A major difficulty in accurately measuring cyclic nucleotide changes induced by thymosin is the extreme heterogeneity of lymphocyte populations and the possibility that only a small population of cells is responding. Cell

separation experiments are currently being used to investigate whether thymosin induces changes in cAMP levels in T cell subsets and to determine which T cells are responding to thymosin by increasing their intracellular cGMP. The probability that we are measuring an increase in a more mature T cell is suggested by the fact that spleen cells respond as well as do those from the thymus (unpublished observation).

It is possible that in the more immature cell populations, cAMP is involved in the initial commitment to differentiate along the thymus cell path while cGMP is more important in the later stages of maturation and final differentiation. Thus, thymosin in its multipotential role could modulate cAMP levels in more immature T cell populations in nude mouse spleen or bone marrow cells while cGMP would be affected in more mature T cell populations such as seen in the thymus and spleen of normal animals. Experiments are in progress to explore this possibility as well as to examine critically the role of thymosin and cyclic nucleotide modulation in assays which reflect functional changes in lymphocytes.

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