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Appearance of T-cell Markers in Bone Marrow Rosette-Forming Cells after Incubation with Thymosin, a Thymic Hormone

(cell-mediated immunity/B-cells/azathioprene/thymectomy/anti-lymphocyte serum/anti-theta serum)

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ABSTRACT **After incubation with thymosin, a thymic hormone, normal bone marrow rosette-forming cells acquire T-cell characteristics, including increased sensitivity to azathioprine, anti-lymphocyte serum, and anti-theta serum. This activity of thymosin provides a new sensitive and reproducible bioassay for thymosin, and is well correlated with an *in vivo* graft-versus-host assay. In addition, incubation of spleen cells from adult thymectomized mice with thymosin *in vitro* restores to normal their diminished sensitivity to azathioprine and anti-lymphocte serum.**

Several biological systems have been used to establish the activity of thymosin, a thymic hormone (1, 2) that stimulates lymphocytopoiesis in normal (3), adrenalectomized§, germ-free§, neonatally thymectomized (4), and adult, lethally x-irradiated (5) mice. In addition, thymosin has been demonstrated to function as a hormone in various *in vivo* (4, 6–11) and *in vitro* (10, 11) systems designed to assess immunological competence mediated by lymphoid cells. Of further significance are the observations that thymosin may play a role, both *in vivo* and *in vitro*, in the ontogenesis and maturation of immunologically-competent lymphoid cells (10, 11). An hypothesis has been suggested recently that integrates both of the proposed cellular and hormonal roles of the thymus (10, 12).

We wish to report a new and sensitive bioassay for thymosin based upon the *in vitro* technique of rosette-cell formation (13, 14). The spontaneous rosette-forming cells (sRFC) present in the normal mouse are found in all lymphoid organs. Normally, B-cells (total cell population of the bone marrow) have few if any T-cell markers, whereas all thymus sRFC and 75% of spleen sRFC are T-cells (15). B- and T-sRFC differ in sensitivity to anti-theta serum, azathioprine (Imuran), and anti-lymphocyte serum, (ALS) (15); all three of these agents are considered to be capable of acting upon T-cells (13, 15).

With this information, a new bioassay for thymosin has been developed based upon an evaluation of the sensitivity of bone-marrow sRFC, after *in vitro* incubation with thymosin, to the three agents mentioned above. Preliminary studies describing the activity of a relatively crude thymosin preparation (fraction 3) (3) in this assay have been reported (15, 16). The present communication documents the applicability of this assay to more highly purified preparations of thymosin.

MATERIALS AND METHODS

Thymosin Fractions. The present report includes data obtained in the application of the new *in vitro* assay procedure to several thymosin fractions, each representing either the same fraction from different species or further purification of thymic fractions from a single species (bovine).

The fractions assayed were obtained as follows (all steps, except where indicated, were conducted at 5°C). Fresh calf-thymus, obtained from a local abattoir, was homogenized with 0.15 M NaCl. Centrifugation at low speed removed insoluble cellular debris. The supernatant solution was centrifuged at 105,000 × *g* for 60 min. The clear supernatant solution (fraction 1) was brought to 80°C for 15 min by immersion of a flask, containing the solution, in a water bath with suitable temperature control. The heavy precipitate that forms was removed by a low-speed centrifugation and the clear supernatant was centrifuged at 20,000 × *g* for 60 min. The heat step and centrifugation remove about 80% of nonthymosin protein. The clear supernatant solution (fraction 2) was poured with stirring into 10 volumes of cold (−30°C) acetone. A flocculent precipitate forms that was collected by filtration on a Buchner funnel, washed on the funnel with cold acetone, and dried under reduced pressure (fraction 3). This acetone powder, fraction 3 (3), was obtained from thymic tissue of a 1-week-old infant, 2-month-old calves, and adult hogs.

Calf thymosin (fraction 3) was further purified by $(NH_4)_2SO_4$ fractionation and column chromatography. The more purified thymosin fractions, derived from fraction 3, are the following: Fraction 4 (25–50% $(NH_4)_2SO_4$ cut); fraction 5 (Sephadex G-150 eluant); fraction 6 (ECTEOLA anion-exchange chromatography fraction)§.

Several additional fractions from calf tissues were also tested as controls. These included preparations of fraction 3 made from brain, spleen, liver, and muscle, in a manner identical to that used for the isolation of fraction 3 from calf thymus. An endotoxin from *Salmonella enteritides* was also examined. The data obtained by application of the *in vitro* sRFC technique are compared to those derived from a modified *in vivo* graft-versus-host assay (11).

Abbreviations: RFC, rosette-forming cells; T-cell, thymic-dependent cell; ALS, anti-lymphocyte serum; sRFC, spontaneous rosette-forming cells; B-cells, bone-marrow-derived cells;

§ Manuscript in preparation.

Azathioprine was generously provided by Burroughs-Wellcome as its soluble sodium salt. The anti-theta serum was a gift of Dr. M. C. Raff. The serum was produced by injections of CBA thymus cells into AKR mice (17). The mouse ALS was prepared in rabbits as was described (18). The mice used were either Swiss or CBA strains (Centre d'Elevage des Animaux de Laboratoire, Orleans-La Source, 45 France). Thymectomies were performed by Dr. A. J. S. Davies on CBA mice.

Rosette Assay—Action of Thymosin. The technique used for measuring spontaneous rosette formation has been described (13, 14, 19). 3×10^6 bone-marrow or spleen lymphoid cells that have been incubated in Hank's medium with added bicarbonate (pH 7.2), with thymosin or with control fractions from other calf tissues for 90 min, and with either azathioprine, ALS, or anti-theta serum, were mixed with 12×10^6 sheep erythrocytes. The mixture was centrifuged for 5 min at $200 \times g$ and then resuspended slowly by gentle rotary agitation for 5 min. Aliquots of this mixture were then transferred to a hemocytometer. Duplicate samples of the number of rosettes present in two fields of 6000 cells (each) were compared with similarly incubated control bone marrow or spleen lymphoid cells exposed only to either azathioprine, ALS, or anti-theta serum. The minimal inhibitory concentrations of any one of these three agents is defined as the quantity that reduces by greater than 50% the number of spontaneous rosette-forming cells counted in a given field, as compared to controls.

The protein content of each thymosin fraction was determined by the Lowry method (20). Various concentrations of thymosin (in 0.15 ml) and of azathioprine (usually 0.15–0.20 ml of a solution containing 10 μg/ml of the sodium salt of azathioprine in Hank's solution) were added to 3×10^6 normal bone-marrow cells (in 0.10 ml), and the mixture was incubated for 90 min at 37°C. Previous studies have shown that such concentrations of azathioprine inhibit T-cell sRFC, but do not inhibit B-cell sRFC (21). 12×10^6 erythrocytes (in 0.10 ml) were added, as described above, and rosette formation was assessed. Controls included tubes with thymosin and without azathioprine, as well as with azathioprine and without thymosin. Results are calculated on the basis of minimal inhibitory concentrations of azathioprine and ALS (see Table 1).

The graft-versus-host assay has been described (11), and is a modification of the Simonsen technique (22).

RESULTS

Thymosin fractions

All of the thymosin fractions from calf thymus that were tested increased the sensitivity of bone-marrow cells to azathioprine in a manner similar to that of fraction 3. This increased sensitivity of B-sRFC to azathioprine was now equivalent to that of untreated spleen cells. In contrast, no alteration in the sensitivity of bone-marrow cells to azathioprine was evident after incubation of the cells with preparations from nonthymic tissues. Table 1 gives the minimal active concentration (in μg of protein/0.5 ml) and the number of units/mg of protein for each fraction tested. Table 1 also gives the minimal protein concentration of the preparations, where tested, capable of endowing bone-marrow cells from adult mice with the capacity to induce a graft versus host reaction (11). A relatively good correlation is apparent between the activities obtained by the two assay procedures. Fraction 6 is about 500 times as active as fraction 3 in both assays. Fraction 3 was studied systematically with $\log_2$ dilutions of both thymosin and azathioprine. The data obtained are shown in Fig. 1.

Control preparations with nonthymic fractions may inhibit rosette formation, although in our experience such inhibition was never observed for protein concentrations lower than 100 μg/ml. Such inhibition, when observed, is generally due to toxic products contained in the preparations, as evidenced by decreased cell viability (trypan-blue exclusion test). As already stated, no control preparation used in the present study endowed bone-marrow cells with sensitivity to azathioprine.

TABLE 1. *Activities of thymosin fractions and control preparations in graft-versus-host assay and rosette assay with bone-marrow cells*

Fraction tested	Graft-versus-host assay* (minimal active concentration per 10×10^6 bone-marrow cells, μg)	Rosette assay† (minimal active concentration per 3×10^6 bone-marrow cells, μg)	Units of thymosin activity/mg protein‡
Calf thymosin (fraction 1)	N.T.§	100	10
Calf thymosin (fraction 2)	N.T.	15	66
Calf thymosin (fraction 3)	2–10	10 ± 5	100
Calf thymosin (fraction 4)	0.5–5	12 ± 5	83
Calf thymosin (fraction 5)	0.1–1	1 ± 1	10^3
Calf thymosin (fraction 6)	0.01–0.1	0.01–0.1	10^4–10^5
Hog thymosin (fraction 3)	N.T.	100 ± 40	10
Human thymosin (fraction 3)	N.T.	16 ± 5	62
Calf brain (fraction 3)	Inactive	>50	—
Calf spleen (fraction 3)	100	>100	—
Mouse spleen (fraction 3)	N.T.	>100	—
Salmonella enteritidis endotoxin	Inactive	>100	—

* For details of assay see ref. 11. For a brief description of fractions (unpublished details), see *Methods*.

† For details, see *Methods*. Values in this column are means ± 2 standard deviations.

‡ A thymosin unit is defined as the minimum quantity of thymosin protein in mg that increases the sensitivity of bone-marrow cells to 0.5 μg of azathioprine/0.5 ml of final test solution, resulting in more than a 50% inhibition of rosette formation by the sRFC present in 3×10^6 normal CBA bone-marrow cells.

§ N.T. = Not tested.

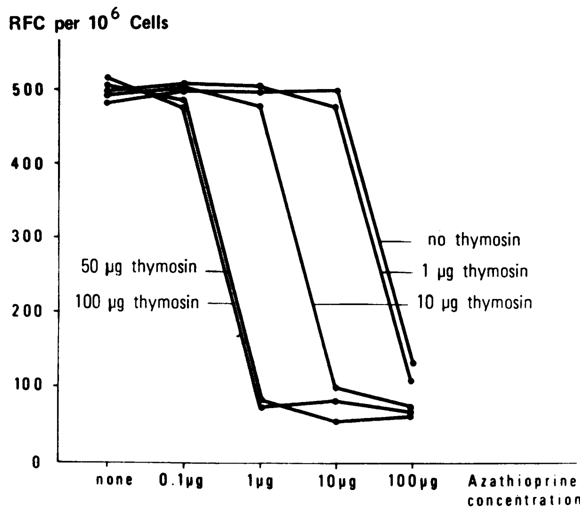

FIG. 1. Appearance of sensitivity to azathioprine in bone-marrow sRFC after 90 min of incubation with thymosin (fraction 3).

Some characteristics of the modification of bone-marrow sRFC by thymosin

Thymosin (fraction 6, 0.1 μg of protein/0.5 ml) was first incubated with bone-marrow cells for 30 min at 37°C. Azathioprine was then added and the cells were incubated for an additional 60 min. Sensitivity to azathioprine was obtained, as described above, whether or not the cells were washed with a thymosin-free medium after the first incubation period with thymosin (before azathioprine was added). However, when the initial incubation with thymosin was performed at 4°C, no thymosin activity could be detected when the cells were washed before azathioprine was added and the assay was then conducted by the standard procedure.

The time required for thymosin to exert its action was studied as follows: Bone-marrow cells were incubated for 15 min at 37°C in order to stabilize cells and medium at this temperature. Thymosin (fraction 6, 0.1 μg of protein/0.5 ml) was then added and incubation was continued for 2, 5, 10, 20, or 30 min.

TABLE 2. *Action of thymosin on sensitivity of normal bone-marrow and spleen cells to ALS and anti-theta serum*

Experimental groups	Rosette inhibition titers* (reciprocals)	
	Bone-marrow cells	Spleen cells
ALS	1000	8000
ALS after calf-thymosin fraction 3†	8000	8000
ALS after calf-spleen fraction 3‡	1000	8000
Anti-theta serum	40	320
Anti-theta serum after calf-thymosin fraction 3†	320	320
Anti-theta serum after calf-spleen fraction 3‡	40	320

* For details see *Methods*.

† 25 μg of protein/3 × 10^6 cells.

‡ 100 μg of protein/3 × 10^6 cells.

At the end of each of these periods, the cell preparation was washed twice and resuspended in thymosin-free medium to which azathioprine (5 μg) was added. Rosette inhibition was observed only when the initial incubation with thymosin was longer than 5 min in duration. Thymosin activity was not apparent after an incubation period of less than 5 min.

Action of thymosin on sensitivity of sRFC to anti-theta serum and ALS

Bone-marrow cells from CBA mice were incubated with thymosin for 30 min at 37°C. The cells were washed once and resuspended in Hank's medium containing different dilutions of ALS or anti-theta serum and complement (fresh guinea pig serum, 1/50 final concentration). After 60 min incubation at 37°C, rosette formation was studied. Negative-control experiments included incubation of bone-marrow cells with (*a*) either ALS or anti-theta serum without thymosin, (*b*) thymosin without ALS or anti-theta serum, (*c*) a spleen fraction with ALS or anti-theta serum, or (*d*) thymosin simultaneously with ALS or anti-theta serum. Two preparations of calf thymosin (fraction 3) were used, at a concentration of 25 μg of protein/0.5 ml.

The data in Table 2 show that in all six experiments, thymosin induced bone-marrow stem cells to acquire a sensitivity to anti-theta serum and ALS that equaled the sensitivity of spleen sRFC to these agents (23). Two spleen fractions

TABLE 3. *Modification by thymosin of the sensitivity to azathioprine and ALS of spleen sRFC from adult thymectomized mice*

Experimental groups	Treatment	Minimal inhibitory concentrations*	
		Azathioprine	ALS (with complement) (titer)
Sham adult thymectomized	—	1.1 μg ± 0.3	1/8000
Adult thymectomized (7 days postoperative)	—	14 μg ± 5	—
Adult thymectomized (7 days postoperative)	Thymosin† (fraction 6)	1.0 μg ± 0.5	—
Adult thymectomized (7 days postoperative)	Spleen‡ (fraction 3)	15 μg ± 5	—
Adult thymectomized (45 days postoperative)	—	6.1 μg ± 2	1/2000
Adult thymectomized (45 days postoperative)	Thymosin‡ (fraction 3)	1.2 μg ± 0.2	1/8000
Adult thymectomized (45 days postoperative)	Spleen‡ fraction 3)	5.8 μg ± 1.2	1/2000

* For 3 × 10^6 spleen cells/0.5 ml. Values in third column are means ± 2 strand deviations.

† 0.1 μg of protein/3.0 × 10^6 spleen cells.

‡ 25 μg of protein/3.0 × 10^6 spleen cells.

from calf and mouse (100 μg of protein/0.5 ml) were not active under these experimental conditions.

Action of thymosin on spleen sRFC from adult thymectomized mice

It has been shown that thymectomy of adult mice decreases drastically the sensitivity to azathioprine and ALS of spleen sRFC obtained 5 days after thymectomy (24). At 21 days after the operation, there is a partial, but significant, recovery of the sensitivity of spleen sRFC (unpublished observations). We report here that decreased sensitivity to azathioprine and ALS of spleen sRFC, obtained from adult mice at 7 days or at 45 days after thymectomy, can be restored by incubation of the cells *in vitro* with thymosin (25 μg of protein/0.5 ml, fraction 3) (Table 3); spleen fraction 3 was inactive.

DISCUSSION

Azathioprine-sensitive spleen sRFC can be considered as thymus-dependent antigen-sensitive cells (T-cells) since (*a*) their presence is necessary for the immune response to sheep-erythrocytes (13, 21), (*b*) their function can be replaced by thymus cells (13, 14), (*c*) they are not found in neonatally thymectomized mice (13, 14), (*d*) they have properties similar to those of cortisol-resistant thymus sRFC (13, 21), and (*e*) they bear the theta antigen (15, 21). Mouse bone-marrow sRFC do not have these properties and may be considered as immunologically immature cells (B-cells). Our data indicate that some of the bone-marrow sRFC can acquire T-cell characteristics (sensitivity to anti-theta serum, azathioprine, and ALS) after incubation with thymosin. 5 min of incubation seems sufficient, but we have not ruled out the possibility that thymosin may adhere to the cells or is taken up by some of the cells which would result in the continuing action of the hormone. In fact, some evidence to the contrary derives from the fact that there was no evidence of thymosin activity when sRFC were incubated for less than 5 min at 37°C or 30 min at 4°C, the cells were washed twice, and the standard rosette assay was then conducted.

The possibility that thymosin contains theta antigen has been excluded by the fact that addition of anti-theta serum (1 ml of anti-theta serum diluted 1:1) to thymosin did not decrease the inhibitory activity of anti-theta serum on sRFC when tested in spleen cells (data not presented). Moreover, thymosin preparations from the thymus of calf, human, or hog were biologically active; it is unlikely that all such preparations contain mouse theta-antigen, whereas the brain preparations (fraction 3) are not active even though mouse brain does contain theta antigen (25). In addition, the presence of theta antigen in thymosin would not explain the action of thymosin on the sensitivity of bone-marrow cells to azathioprine and ALS.

Data have been obtained that suggest that azathioprine (13) and ALS (13) act on sRFC by modifying the cell membrane. Theta antigen is located on membranes. It may therefore be postulated that thymosin modifies a B-lymphocyte surface to endow it with surface characteristics of T-cells. Whether or not the activity of thymosin *in vitro* that we have reported is an *in vivo* model of thymus function remains an unsettled question, but must receive consideration.

Adult thymectomy decreases the sensitivity of spleen sRFC to azathioprine (24, 26) and ALS (24); normal sensitivity of the cells to azathioprine is restored after incubation with thymosin. One interpretation is that thymosin transforms B-precursors of T-cells into T-cells in the thymus. These precursors, normally absent in the spleen, are found in the spleen of the adult thymectomized mouse. It is also possible that a constant supply of thymosin is required in the circulation to maintain the normal sensitivity of T-cells to azathioprine. Thymosin could function in a primary capacity to develop and/or maintain this reactivity to azathioprine and to anti-theta serum.

The question of the specificity of thymosin has been raised recently, since several thymosin preparations (fraction 3) were observed to be pyrogenic in rabbits, suggesting the possible contamination of the fraction with endotoxin (27). Our data show that endotoxin is not involved in the thymosin responses described in this communication, since high concentrations of *Salmonella* endotoxin were not active in the graft-versus-host or RFC assays. Moreover, it is unlikely that thymosin antigenicity could play a significant role in the 90-min *in vitro* RFC assay. The rapidity (<90 min) with which thymosin converts a B-cell *in vitro* into a cell with T-cell characteristics suggests that the maturation of this class of lymphoid cells does not involve a proliferative process, but rather a derepression or activation of a B-cell, which must be previously endowed with the potential capacity to function as a mature T-cell.

The present study indicates that thymosin can function in lieu of the intact thymus with regard to the role of the thymus in the maturation of cells that behave as T-cell sRFC *in vitro.*

NOTE ADDED IN PROOF

It appears from studies in progress that thymosin is acting on a precursor of a T_1-cell, which under appropriate conditions, can give rise to other T-cell populations (24).

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