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Induction of T-Cell Differentiation *In Vitro* by Thymin, a Purified Polypeptide Hormone of the Thymus

[thymic hormone/cytotoxic inhibition test/bone marrow cells/TL antigen/Thy-1(θ) antigen]

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ABSTRACT **Thymin, a purified polypeptide isolated from bovine thymus, was shown to induce the expression of differentiation antigens characteristic of thymocytes [TL and Thy-1 (θ)] when incubated *in vitro* with mouse bone marrow or spleen cells. This induction occurred in 5–10% of the cells from bone marrow after a 2-hr incubation with subnanogram concentrations of thymin. The induced cells expressed more TL and Thy-1 (θ) antigens than average normal thymocytes.**

The thymus is a compound organ in which the epithelial elements provide an appropriate micro-environment for the differentiation of lymphoid stem cells. These cells arise in hematopoietic tissues, undergo maturation and proliferation within the thymus, and are then distributed to peripheral tissues as immunocompetent "thymus-derived" lymphocytes (T-cells). A humoral secretion of the thymic epithelial cells is believed to induce the stem cells to differentiate and acquire the surface antigens which characterize thymocytes (1). Among these antigens the best studied are the thymus-leukemia antigen (TL) and Thy-1 (θ) (2, 3).

Several laboratories have reported the identification and partial purification from the thymus of substances which affect the development of immune responsiveness (4–6). There have been many inherent difficulties in these studies, especially in the development of appropriate assays (1, 7). Recently, thymic differentiation has been detected more directly by demonstrating the expression of antigens characteristic of thymocytes on previously undifferentiated precursor cells (8, 9).

Thymin is a purified polypeptide hormone isolated from bovine thymus by its effect on neuromuscular transmission (10). It was first detected in experimental autoimmune thymitis (11–13), a laboratory model of the disease myasthenia gravis, in which impaired neuromuscular transmission is regularly associated with thymic abnormalities (14, 15). Thymin was also shown to be produced by the normal thymus (16). These studies of the neuromuscular effects of thymin established that it was, by classical physiological criteria, a hormone of the thymus. However, the major recognized function of the thymus is the differentiation of lymphoid stem cells. We present evidence that purified thymin induces the expression in spleen and bone marrow cells of serologically defined thymic antigens. Since thymin induces this differentiation at subnanogram concentrations, we favor the interpretation that this is the principal physiological role of thymin.

Abbreviations: RCI, relative cytotoxic index; BSA, bovine-serum albumin.

MATERIALS AND METHODS

Animals. Mice of the A/J, C57B1/6J, AKR/J, and C58/J strains were purchased from the Jackson Laboratories (Bar Harbor, Me.). A·TL-mice were bred at New York University from a foundation stock generously given us by Dr. E. A. Boyse. All mice were housed eight to a cage, and maintained on commercial high protein mouse pellets and acidified water.

Antisera. Antibodies to TL antigen were produced by injecting live tumor cells (ASL-1) into A·TL- × C57B1/6J F·1 mice. After five semimonthly injections the animals were bled and the resultant serum pooled, absorbed with A·TL- thymus to remove autoantibodies, and stored in small aliquots at −70°. The serum pool used in these experiments had a cytotoxic titre of 1:2500 when titrated against ASL-1. Anti-Thy 1-2 (θ − C3H) was produced by injecting AKR/J mice with C58/J thymocytes and absorbing the resultant antiserum with AKR/J thymocytes. The serum pool used had a cytotoxic titer of 1:400 when tested against ASL-1.

Thymin. The isolation of thymin has been described in detail (10). Purity was assessed by disc electrophoresis on 7% polyacrylamide gels at pH 8.9 and pH 4.3. Single bands were obtained at both pH with 200 μg of polypeptide applied to each gel (10). In addition, a single major amino-acid sequence was found using an automated protein sequencer, and lysine alone was identified as the C-terminal residue (D. H. Schlesinger, G. Goldstein, and H. D. Niall, manuscript in preparation). Thymin I and Thymin II are closely related polypeptides, each having a molecular weight of 7000; their precise relationship remains to be determined (10). Lot GG83BTI of Thymin I and lot GG81BTII of Thymin II were used in the present experiments. Each was dissolved in phosphate-buffered saline (containing 1 mg/ml of bovine-serum albumin to minimize adsorptive losses), distributed into 2-μg lots, and lyophilized. Each polypeptide was reconstituted in tissue culture medium at the desired concentrations just prior to use. Controls included the polypeptides insulin (Lily) and ACTH (Sigma) and the plant lectin phytohemagglutinin (Burroughs-Wellcome).

Induction of Thymic Antigens. Bone marrow cells were obtained from the femoral marrow. Both spleen and marrow cells were teased into single cell suspensions and washed three times in RPMI 1640 medium supplemented with 10 mM Hepes and 100 U/ml of penicillin and 100 μg/ml of streptomycin. The cells were then suspended in the same medium with 5% adult bovine serum at a concentration of 5.0 ×

10^6 cells per ml; 1 ml of this suspension was incubated with an appropriate dilution of thymin in 16-mm plastic petri dishes (Linbro Ind) in a humidified atmosphere containing 5% CO_2 in air. At the end of the incubation the cells were washed twice and held at 4° at a concentration of 5.0×10^6 cells per ml in culture medium until tested for the presence of TL or Thy-1 (θ).

Target Cells. The ASL-1 lymphoma of A strain mice was used as the target cell in all titrations of the antisera used in these experiments, and in the cytotoxic inhibition test to be described below. This lymphoma is TL positive and Thy 1-2 (θ-C_3H) positive; it was maintained by weekly intraperitoneal passage in A/J mice. Lymphoma cells were labeled with ^{51}Cr as described by Wigzell (17) and used as target cells in the cytotoxic inhibition test.

Cytotoxic Inhibition Test. Antisera to either TL or Thy-1 (θ) were titered to determine the quantity of antibody which was required to kill 50% of the target cells in a standard suspension. The appropriate amount of antiserum was added to each well of a microtiter plate with various dilutions of the cells being tested for their antigen content. The total volume was 0.15 ml. If cells expressed the antigen against which the antiserum was directed, they absorbed the antibody, reducing the amount available to react with the ^{51}Cr-labeled target cells which were added subsequently. The concentration of antibody used was extremely critical to the sensitivity of the test: with excess antibody absorption was not detectable, whereas insufficient antibody failed to produce adequate cytotoxicity. The antisera to TL or Thy-1 (θ) and the cells being tested for their antigen content were incubated together at 4° for 90 min. After this incubation, 50,000 target cells in a volume of 0.025 ml were added, and the incubation was continued in the cold for an additional 30 min to permit the sensitization of the target cells by the remaining unabsorbed antibody. The plates were then centrifuged at 1500 rpm for 10 min and the supernatant fluid removed. The pelleted cells were resuspended in 0.1 ml of RPMI 1640, containing 4% fresh rabbit serum as a source of complement, and incubated for 45 min at 37°. The cells were then repelleted by centrifugation and a 0.075-ml aliquot of the supernatant fluid was counted in an automatic gamma spectrometer to determine the amount of ^{51}Cr released from the target cells. All assays were performed in duplicate and the results were expressed by calculating the number of cells required to reduce the relative cytotoxic index (RCI) to 0.5. The RCI was calculated as follows:

$$\text{RCI} = \frac{\text{CPM released in the presence of absorbing cells} - \text{CPM released without antibody}}{\text{CPM released in the absence of absorbing cells} - \text{CPM released without antibody}}$$

Direct Cytotoxic Test. Bone marrow and spleen cells were obtained by flotation in bovine-serum albumin (BSA) gradients similar to those used by Komuro and Boyse (8). These cells were tested for their susceptibility to anti-TL and anti-Thy-1 (θ) antibodies in a direct cytotoxicity test, using the uptake of trypan blue as a measure of cell death. The test was performed as described by Boyse (18).

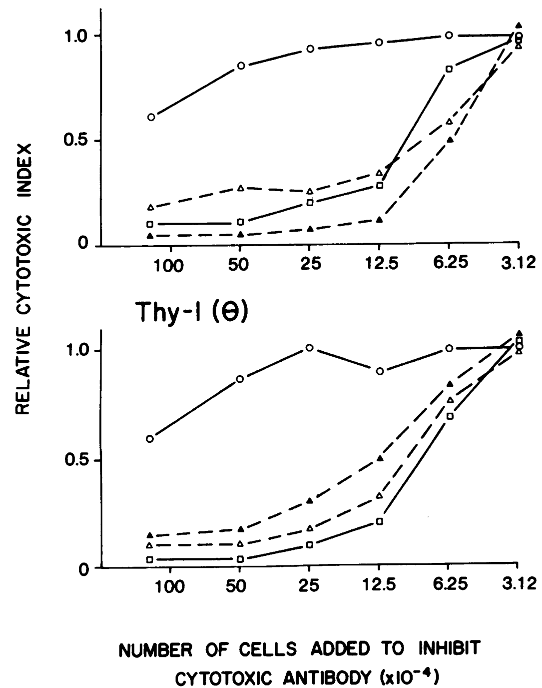

FIG. 1. Absorption of anti-TL and anti-Thy-1 (θ) antibody by bone marrow cells incubated with thymin I or thymin II. Bone marrow cells were incubated with 100 ng/ml of thymin I or thymin II for 18 hr. The control thymocytes from A/J mice were freshly isolated. The absorption of anti-TL and anti-Thy-1 antibody by these thymocytes is indicated by the empty squares (□——□). Absorption of antibody by thymin-treated bone marrow cells is indicated by triangles: Thymin I ▲- - -▲, Thymin II △- - -△. Bone marrow cells which were not treated with thymin absorbed little or no antibody, as indicated by the empty circles (○——○). (The numbers on the abscissa have been multiplied by 10^{-4}.)

RESULTS

Induction of TL and Thy-1 (θ). Fig. 1 shows the results of a cytotoxic inhibition test in which the induction of both TL and Thy-1(θ) antigens on mouse bone marrow cells was demonstrated. In this experiment unfractionated cells were incubated with 100 ng/ml of thymin I or II for 18 hr. At the end of this time these cells absorbed antibodies directed against either TL or Thy-1 (θ). Cells incubated under similar conditions without thymin remained unable to absorb these antibodies, as did cells incubated with 1 μg/ml of insulin, ACTH, or phytohemagglutinin. The relative amount of antigen which these cells expressed was estimated by comparing the minimum number of cells required to produce a reduction of the RCI to 0.5. Bone marrow cell cultures containing thymin expressed 50% more TL and 30% less Thy-1 (θ) than normal thymocyte cultures.

The actual number of cells induced could not be determined in the cytotoxic inhibition assay since this test is a measure of the total antigen present in the culture. The direct cytotoxic test does indicate the actual number of cells which have acquired sufficient antigen to be lysed by antiserum and complement. However, unless sufficient numbers of such cells are present they cannot be detected because of a substantial background usually found in such tests. Thus, we could not detect TL induction in unfractionated cells by direct cyto-

TABLE 1. *Direct cytotoxic detection of TL induction in bone marrow and spleen cells fractionated on discontinuous gradients of bovine-serum albumin*

Source of cells				Dead cells*		
Organ	Mouse strain	Cell† layer	Temperature of incubation	Thymin incubation	BSA control	Cell‡ recovery (%)
Bone marrow	A/J	A	37°	24	3	9
	A/J	B	37°	12	5	34
	A/J	C	37°	4	6	21
	A/J	D	37°	6	7	8
Spleen	A·TL-	A	37°	5	9	9
	A/J	A	4°	7	6	8
	A/J	A	37°	22	4	6
	A/J	B	37°	14	5	22
	A/J	C	37°	6	7	32
	A/J	D	37°	10	8	21

The gradients consisted of layers containing 36%, 29%, 26%, 23%, 21%, and 17.5% BSA in medium 199. The unfractionated cells were suspended in the 29% layer. The gradients were centrifuged for 30 min at 13,000 rpm in an SW 50 rotor of a Spinco ultracentrifuge. The cell populations listed in the table were obtained from the following interfaces: *A*, 17.5–21; *B*, 21–23; *C*, 23–26; *D*, 26–29. The cells were incubated with thymin I (20 ng/ml) for 2 hr. All of the data are the means of triplicate assays.

* % of cells stained with trypan blue after treatment with anti TL antiserum + complement.

† From BSA gradient.

‡ % of total cells (4.0×10^8) applied to the gradient.

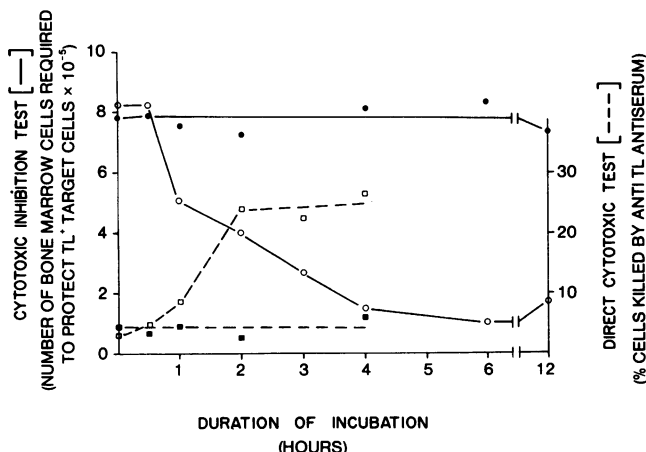

FIG. 2. Kinetics of the appearance of TL antigen on bone marrow cells incubated with thymin. The broken lines indicate the results of the direct cytotoxic test on bone marrow cells obtained from the "A" layer of the BSA gradient. The empty squares (□- - -□) are the results of titrations using bone marrow cells which had been incubated with thymin I (20 ng/ml), while the filled squares (■- - -■) are the results obtained with control bone marrow cells from the same fraction of the gradient. The continuous line indicates the results obtained in the cytotoxic inhibition test using unfractionated bone marrow cells. The left ordinate indicates the number of cells required to reduce the RCI by 0.5. Thymin-treated bone marrow (20 ng/ml) gave the results shown with the empty circles (O) while the control bone marrow gave the values shown with filled circles (●). (The numbers on the left ordinate have been multiplied by 10^{-5}.)

toxic tests, but when bone marrow or spleen cells were fractionated by flotation on discontinuous BSA gradients, significant induction could be demonstrated in the least dense fractions of both tissues (Table 1). Thus, in this experiment, after incubation with 20 ng/ml of thymin I for 2 hr, anti-TL antiserum plus complement caused the death of 18% of the spleen and 21% of the bone marrow cells in the "A" fraction. Values as high as 28% were obtained in other experiments. Bone marrow cells from genetically TL-negative mice did not acquire TL antigen after incubation with thymin, nor did bone marrow cells from TL-positive mice when similarly incubated with thymin at 4° (Table 1).

Time Course of the Induction. Fig. 2 indicates the kinetics of the appearance of TL antigen on bone marrow cells incubated with thymin (20 ng/ml). The results using both the cytotoxic inhibition test and the direct cytotoxicity test are shown. In the former, unfractionated bone marrow was used, while in the latter only cells from the least dense fraction of the BSA density gradients were used. TL antigen was detected in both tests after 1 hr of incubation. In the direct cytotoxicity test the maximum number of cells sensitive to anti-TL antibody and complement was reached after 2 hr. At this time almost one-quarter of the cells in the low density fraction were susceptible to cytolysis by anti-TL antisera. Further incubation did not increase the proportion of susceptible cells. The quantity of TL expressed by these cells, as measured in the cytotoxic inhibition test, continued to increase for 4 hr. This is indicated in Fig. 2 by the decreasing number of cells required to protect the ^{51}Cr-labeled target cells from lysis by anti-TL antibody and complement.

Dose Response. The thymins were extremely potent in inducing the appearance of TL and Thy-1 (θ) antigens. Maximal induction was achieved with 2 ng/ml of thymin I and as little as 20 pg/ml produced a measurable response. A representative dose response for the induction of TL antigen by thymin I is shown in Fig. 3. Thymin I was generally slightly more potent than thymin II.

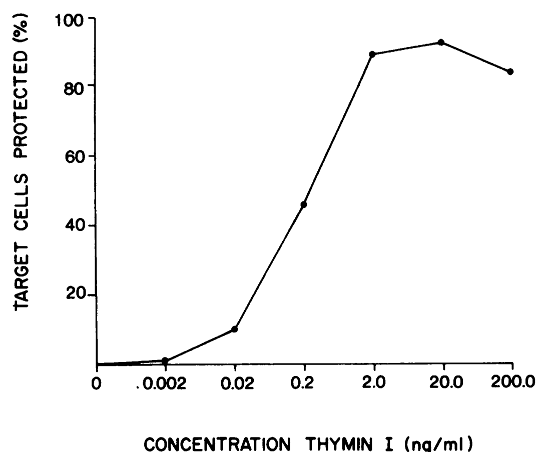

FIG. 3. The relationship between the concentration of thymin and the amount of TL antigen induced. Bone marrow cells were incubated for 4 hr with various concentrations of thymin I. The abscissa represents the percentage of target cells protected from anti-TL mediated cytolysis by 100,000 bone marrow cells incubated with thymin.

DISCUSSION

We have demonstrated that bone marrow cells rapidly acquired characteristic thymic differentiation antigens [TL and Thy-1 (θ)] when incubated in the presence of low concentrations of the purified polypeptides thymin I and thymin II. The concentration at which the thymins acted was within the range usually found with other polypeptide hormones (19), and the lowest detectable concentration (20 pg/ml) represented only several hundred molecules per susceptible cell. No other putative thymic factor has approached this level of activity. The most potent preparation previously reported was that of Bach *et al.*, which was said to be minimally active at a concentration of between 10 and 100 ng per 3×10^6 bone marrow cells (20). Thymin I is active at approximately one-thousandth this concentration.

The quantity of TL and Thy-1 (θ) detected in bone marrow cells treated with optimal amounts of thymin was remarkably high. Although only 5–10% of the cells in the thymin treated marrow expressed sufficient TL to become susceptible to immune cytolysis with anti-TL antiserum, the total amount of TL in the cultures was 50% greater than that found in an equivalent number of thymocytes. This indicated that the bone marrow cells expressing TL actually had 15–20 times more of the antigen than average normal thymocytes. Similarly, these cells had 6–12 times more Thy-1 (θ) than average normal thymocytes. These findings would be consistent with the known events associated with thymocyte maturation. TL disappears from thymocytes while they are still within the thymus (3) and the amounts of Thy-1 (θ) in peripheral "T" lymphocytes is much reduced compared to that on intrathymic thymocytes (2, 21). It appears that the highest concentration of these antigens occurs in the earliest phases of thymocyte differentiation, and that they are progressively diminished on each cell as it matures. The thymin-induced cells, on which we demonstrated such high amounts of TL and Thy-1 (θ), would thus correspond to an early stage in thymocyte maturation.

A surprising finding in earlier studies of induction using partially purified thymus extracts was the rapidity of onset of differentiation, and the short period of time over which maximum numbers of differentiated cells appeared. These findings were reported by Komuro and Boyse (8), using direct cytotoxic assays to measure TL and Thy-1 (θ) expression, and by Bach *et al.* (20), using the development of azathioprine-sensitive, rosette-forming cells. Our results using the direct cytotoxic test to measure TL expression were in general agreement, but our parallel studies using the cytotoxic inhibition test suggested that this suddenness of appearance of differentiation may have been related to the tests used. Reactive cells rather than antigen quantity are measured in these tests. Until a sufficient density of antigen appears on the cell surface, the cells resist cytolysis or rosette formation. Once the critical concentration of antigen is achieved, the tests become positive, and no further changes can be detected. By the indirect cytotoxic test we detected a progressive increase of TL antigen over 4 hr. We believe this reflects more accurately the changes occurring at the cell surface.

Polypeptide hormones in general act by combining with specific receptors in the surface membranes of susceptible cells, this union triggering the responses appropriate to those cells (19). The finding that 5–10% of bone marrow cells were induced to differentiate by extremely low concentrations of pure thymin suggested that these cells were stem cells with appropriate receptors, and thus already committed to eventual thymocyte differentiation. This large population of committed cells would be distinct from the totipotent hematopoietic stem cells, which make up less than 0.1% of the cells in the murine bone marrow (22). Using crude thymus extracts, Komuro and Boyse (8) also demonstrated this large population of mouse hematopoietic cells susceptible to differentiation by a thymus-inducing factor. Furthermore, they demonstrated that these susceptible cells were "pre-thymic," in that they could be identified in athymic nude mice (nu/nu) and in the hematopoietic cells of 14 day embryonic mouse liver obtained before the epithelial thymus had developed (9). The relationship of these cells to the "pre-T cells" identified by El-Arini and Osoba (23) remains to be clarified.

The nature of the inductive process initiated by thymin has yet to be established. The antigens expressed represented products of several unlinked genes (8). The genetic background of the induced cells clearly dictated the nature of the products expressed, since cells from genetically TL negative mice could not be induced to express TL. Metabolic activity seemed to be required, since induction did not occur at 4°. These findings are consistent with the interpretation that thymin combined with specific receptors in the membranes of cells committed to thymocyte differentiation, and that this binding initiated within the cell the complex processes involved in differentiation, including the coordinated expression of several surface antigens.

Our experiments showing that pure bovine thymin induced the differentiation of murine stem cells established that thymin was active across species. This was in keeping with previous experiments using thymus extracts, although the interpretation of these is less clear because of the possibility of nonspecific inducing agents being present in such extracts (24). Activity across species was not unexpected, since it is found for many polypeptide hormones, and bovine thymin had previously been shown to affect neuromuscular transmission in guinea pigs, rats, and mice (10, 25, 26).

We believe that the induction of lymphoid differentiation is the physiological role of thymin. If this be so, it is remarkable that thymin was isolated not by this effect, but by a presumably secondary neuromuscular effect detected in myasthenia gravis. Acetylcholine receptors are widely distributed in the body, being present not only in the nervous system but also in hematopoietic and lymphoid cells (27, 28). Thus, it is possible that the physiological receptors for thymin are related to such acetylcholine receptors, and that the binding site of thymin is acetylcholine-mimetic.

Note Added in Proof. It has been brought to our attention that our term thymin for this polypeptide hormone is being confused with the base thymine (Roth, M., *Nature*, in press). We therefore intend to change our terminology in the future and use the term thymopoietin (Goldstein, G., *Nature*, in press).

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