ENRICHMENT OF IN VITRO AND IN VIVO IMMUNOLOGIC ACTIVITY OF PURIFIED FRACTIONS OF CALF THYMIC HORMONE*  

BY YESHAYAHU YAKIR, ABRAHAM I. KOOK, AND NATHAN TRAININ$  
(From the Department of Cell Biology, The Weizmann Institute of Science, Rehovot, Israel)  

A partially purified preparation of calf thymus extract (THF) has been shown by us to participate in the processes which lead to maturation and acquisition of immunocompetence of mouse thymus-derived lymphoid cells (1, 2). The specific capacity of THF to restore the immunocompetence of lymphoid cells from neonatally thymectomized (NTx) animals or to induce maturation of thymus-derived lymphoid cell populations has been demonstrated by a variety of bioassays. For example, THF was shown to improve the competence of T lymphocytes to participate in mixed lymphocyte (3) and graft-versus-host (GvH) (4) reactions, to increase their reactivity to T lectins (5) and to augment their capacity to kill tumor cells (6). The activity of THF was also confirmed in vivo, since single or repeated injection of this material increased the T-helper capacity of cells in the antibody response against sheep erythrocytes (7), restored the GvH reactivity of spleen cells from NTx mice (4), and raised the anti-tumor reactivity of lymphocytes obtained from tumor-bearing mice against syngeneic tumors. Moreover, it was demonstrated recently that the administration of THF to humans suffering either from primary or from secondary immunodeficiencies resulted in the restoration of cellular immunocompetence, while progressive clinical improvement of these patients was observed (8-11). Immune maturation of thymus-derived lymphoid cells, after in vitro (12, 13) or in vivo exposure to THF occurs via an obligatory rise in cellular cAMP levels, thus establishing the hormonal nature of THF in agreement with the criteria postulated by Sutherland (14).  

The above results justified further studies performed in our laboratory to purify and characterize the active component of THF responsible for the effects described. THF was isolated by a procedure which consisted in a stepwise gel
INCREASED REACTIVITY OF THYMIC HORMONE

filtration through Sephadex columns. When tested in terms of the acquisition of competence by spleen cells from NTx mice in an in vitro assay of GvH reactivity it appeared that the pure material of THF is a polypeptide of 3,000 mol wt which migrates as a single band in isoelectric focusing and sodium dodecyl sulfate polyacrylamide gel electrophoresis (15). Amino acid analysis of acid hydrolysates revealed that purified THF contains approximately 30 residues. At this point of our investigations it was necessary to test whether this active component of THF is responsible for all the biological activities obtained with the crude material (2). Such a screening of activities with the pure THF material was also felt necessary, since Hooper et al. reported that thymosin α1, a chemically defined peptide isolated from thymosin fraction 5 (16), was more active in certain respects than the cruder fraction, while a loss of activity was manifest in other tests (17). This suggested that in addition to thymosin α1, fraction 5 contained other components necessary to elicit full immunologic reactivity.

In the present experiments THF was administered either in vitro or in vivo and its activity was tested by a second in vitro assay of immune competence (mixed lymphocyte culture) and by an in vivo GvH assay in addition to measurements of cAMP levels in the cells examined. The results presented here suggest that one single peptide with the chemical characteristics described previously is responsible for endowment of reactivity to T cells which participate in cell-mediated immunity. Moreover, this final active peptide obtained by stepwise purification was found to be $2 \times 10^4$-fold more active than the dialyze of crude thymus extract which served as a starting material.

Materials and Methods

**Mice.** Newborn C57BL/6 and 6- to 8-wk old C57BL/6 and (C3H/eb x C57BL/6)F1 hybrids of both sexes were used in the present experiments. Thymectomy was performed within 24 h after birth. Animals found to contain a thymic remnant were discarded from the experiments. (C3H/eb x C57BL/6)F1 newborn mice served as hosts for the in vivo GvH assay.

**Preparation of Cell Suspensions.** Spleens or thymuses from 6- to 8-wk old mice were removed aseptically and gently dispersed through a stainless steel mesh and the cells were suspended in Eagle's medium (EM), supplemented with penicillin 100 U/ml, and streptomycin 100 μg/ml. Cells were washed twice in EM and counted in Turk solution with a hemocytometer. Exclusion of 0.05% trypan blue solution was used as a measure of viability.

**Preparation of Organ Extracts.** THF was prepared from calf thymus. The standard preparation of crude dialyzates consisted in homogenization, ultracentrifugation, and exhaustive dialysis of the material. The dialyze obtained was stored at $-20\degree$C (4, 15). No cAMP or DNA could be detected in the thymic preparations (15). In contrast to proteolytic digestion treatment of the thymus extract with RNase did not abolish its activity (15). Thus the concentration of active material used in the present experiments is expressed as protein per milliliter. The more purified fractions of THF were prepared as described (15). Spleen extracts were prepared in a way identical to that of thymus extract (4). In addition to spleen extracts the following substances were tested as controls: glucagon (Sigma Chemical Co., St. Louis, Mo.), bovine serum albumin (BSA) (Sigma Chemical Co.), phosphate-buffered saline 0.05 M (PBS).

**Biological Assays for Testing the Activity of THF and other Control Materials.** The various fractions obtained during the process of purification of THF as well as control materials were tested in the following in vitro and in vivo assays.

**cAMP Determination**

**In vitro treatment.** $10^7$ Thymus cells per milliliter PBS were exposed to the various
fractions of THF for 5 min at 37°C in water bath. The cells were washed three times with large volumes of PBS and intracellular cAMP content was determined.

**In vivo treatment.** The various purified THF fractions or each of the control materials were injected i.m into 4 wk old C57BL/6 NTx mice daily for 14 days. At the 15th-day spleens were removed and their cellular cAMP content was determined.

**Assay.** Intracellular cAMP was measured by a slight modification of Gilman’s procedure (18). The samples were placed in an 80°C water bath for 3 min and then immediately transferred to an ice water bath and neutralized to pH 7.5 by 0.25 M Tris/20 mM EDTA buffer pH 12.3. Cellular levels of cAMP were determined with the cAMP kit provided by the Radiochemical Centre, Amersham, England (Code TRK, 432).

**Mixed Lymphocyte Culture (MLC) Assay**

**In vitro treatment.** 10⁷ Spleen cells from intact and NTx 6 wk old C57BL/6 mice were preincubated with the various purified THF fractions for 1 h, and washed twice with large volumes of EM before the antigenic stimulation in the MLC assay.

**In vivo treatment.** Spleen cells from 6 wk old NTx C57BL/6 mice injected i.m. during 14 days with the various purified THF fractions or with the control materials were assayed for their ability to react in the MLC assay.

**Assay.** The experiments were carried out in Falcon 2058 12 x 75-mm tubes with caps (BioQuest, BBL & Falcon Products, Cockeysville, Md.). Cells were suspended in RPMI-1640 medium (Grand Island Biological Co., Grand Island, N. Y.), buffered with sodium bicarbonate, and supplemented with penicillin 100 U/ml, streptomycin 100 µg/ml, and 5% heat-inactivated fetal bovine serum (Reheis Chemical Co., Chicago, Ill.). Stimulating spleen cells from intact C57BL/6 or (C3H/eb × C57BL/6)F₁ mice were brought to a concentration of 1 × 10⁷ cells/ml and incubated with 25 µg/ml mitomycin-C (Sigma Chemical Co.) at 37°C for 30 min. The cells were then washed three times in a large volume of EM and brought to a concentration of 2 × 10⁶ cells/ml RPMI-1640 medium. 0.5 ml Containing 10⁶ cells was added to the Falcon tubes. Responding spleen cells either from intact or from NTx mice were also brought to a concentration of 2 × 10⁶/ml and 0.5 ml containing 10⁶ cells was added to the tubes. The tubes were capped, mixed lightly, and incubated at 37°C in a humidified incubator in an atmosphere of 95% air and 5% CO₂. 72 h after the initial time of incubation 2 µCi of tritiated thymidine (Amersham, Buckinghamshire, England) sp act of 5 Ci/mmol was added in 50 µl of RPMI medium. 18 h later cells were removed, washed with saline, and precipitated with 5% trichloroacetic acid on a Whatman glass fiber filter GF/A size 1.5 × 19.5 cm (W. and R. Balston, Ltd., England). Filters were put in scintillation vials with simultaneous addition of 10 ml scintillation fluid and the cpm were monitored. Triplicate cultures were used for each group in each experiment. The proliferation of spleen cells from parental C57BL/6 mice stimulated by mitomycin-C-treated spleen cells from (C3H/eb × C57BL/6)F₁ mice was compared to the degree of proliferation exhibited by the parental spleen cells in the presence of syngeneic spleen cells treated with mitomycin-C. As previously indicated (19) the data are expressed as an increase in net cpm.

**In vivo GvH Assay.** Spleen cells from 6 wk old NTx C57BL/6 mice injected i.m. daily with each of the various THF fractions or each of the control materials were brought to a concentration of 3 × 10⁸ in 0.05 ml PBS and injected intraperitoneally into 1 day old (C3H/eb × C57BL/6)F₁ mice. Recipient litters included mice injected with each type of donor cell as well as uninjected controls. The recipients were sacrificed 10 days after challenge and the ratio of mg spleen:10 g body weight was measured according to the method of Simonsen et al. (20). Spleen index was obtained by dividing the spleen:body weights by the corresponding value of the injected control animals, since spleen weight of uninjected controls is equivalent to mice injected with syngeneic cells (4).

**Statistical Analysis.** The Student’s t test was used to analyze the statistical significance of the results. Results are expressed as mean ± standard error.

**Results**

We have recently reported the procedure of isolation of THF as outlined in Fig. 1. The partially purified dialyze of the thymus extract (4, 15) was further
fractionated by gel filtration through a Sephadex G-10 column and the activity checked by the in vitro GvH assay was recovered in the void volume (Fig. 1 A). The void volume active fraction (hereafter fraction A) was further fractionated by gel filtration on a Sephadex G-25 superfine column and activity (again measured by GvH in vitro assay) was consistently found in one peak only (Fig. 1 B). The active fraction eluted from the Sephadex G-25 column (hereafter fraction B) was then fractionated by anion exchange chromatography on DEAE Sephadex A-25 column. The biological activity measured as before was concentrated in one peak, eluted at 0.15 M NaCl (Fig. 1 C). This material is represented by one active peptide (hereafter fraction C) with an isoelectric point (pI) of 5.6 (15). The in vitro GvH assay which was used throughout the purification procedure described is characterized by an all or none type of response. It was therefore of cardinal importance to use other biological assays to estimate the enrichment of biological activities obtained with the various purified THF fractions.

Enrichment of Biological Reactivity of THF Fractions by Their Progressive Purification as Measured by the MLC Assay. Spleen cells from NTx mice exhibit a reduced capacity to respond in the MLC assay (21, 22) and this could
be restored by pretreatment with the partially purified dialyzate of THF (3). We studied here whether the various THF fractions isolated by further stepwise purification could also restore the impaired MLC response. In parallel, the effect of the various THF fractions was also checked on spleen cells from intact mice. Spleen cells from NTx mice were first preincubated with the various THF fractions (fractions A, B, and C) in different protein concentrations and then submitted to the MLC test. As can be seen (Fig. 2 a–d) cell reactivity was raised to a level similar to that of normal spleen cells. Moreover, fractions A, B, and C significantly increased the response of spleen cells from intact mice above that of untreated controls. We also observed that a progressively lower protein concentration was required for the induction of maximal MLC augmentation where more purified fractions were studied. Thus, as can be seen in Fig. 2, 0.005 μg protein of fraction C was equivalent to 0.1 μg of fraction B, to 20 μg of fraction A, or to 80 μg of dialyzate of crude thymus extract. A parallel can also

Fig. 2. In vitro effect of different concentrations of the various active THF fractions on the activity of spleen cells from both intact and NTx C57BL/6 mice in the MLC assay. a. Dialyzate of crude thymus extract. b. Active peak eluted from Sephadex G-10 column (fraction A). c. Active peak eluted from Sephadex G-25 superfine column (fraction B). d. Active peak eluted from DEAE Sephadex A-25 column (fraction C). Spleen cells from intact mice (○—○) and NTx mice (O—O). Bars represent mean of triplicate samples ± SE. Data represent one out of five such experiments.
The in Vitro Effect of the Active Fractions Obtained during the Process of Stepwise Isolation of THF on the Intracellular cAMP Levels in Thymocytes from C57BL/6 Mice

| Fractions                                      | Optimal THF dose | pmol of cAMP ± SE*|10^7| thymocytes |
|------------------------------------------------|------------------|-------------------|-----------|
| Dialyzate of crude thymus extract              | 80               | 4.2 ± 0.75        |
| Void volume fraction of Sephadex G10 column (fraction A) | 20               | 4.5 ± 0.80        |
| Active peak of Sephadex G-25 column (fraction B) | 0.1              | 4.1 ± 0.80        |
| Active peak of DEAE Sephadex A-25 column (fraction C) | 0.005            | 3.9 ± 0.70        |

* Data represent one out of five such experiments. Each experiment was done in triplicate. SE = Standard error.

be seen between the increasing concentrations of the respective fractions tested and the MLC response which reaches a plateau after a certain point. It should be stressed that each of the peaks obtained during the stepwise purification of THF fractions (A, B, and C) which were found inactive in the in vitro GvH test, were also tested in the MLC assay. Each of these inactive peaks was also devoid of activity in this assay.

Increase of Intracellular cAMP Levels in Thymocytes by Progressively Lower Concentrations of THF Fractions A, B, and C. Intracellular levels of cAMP were measured in thymus cells rather than in spleen cells since they represent a more homogeneous lymphoid cell population. Furthermore, thymus cells are known to exhibit low levels of cellular cAMP (Kook, A. I., Yakir, Y. and Trainin, N., unpublished results) thus permitting us to study the in vitro effect of the various purified fractions of THF. Exposure of thymus cells for 5 min to the various THF fractions was followed by an increase in the cAMP level of these cells (Table I). Moreover the activity of fractions A, B, and C, respectively, was equal to increasingly reduced doses. Thus, 0.005 µg protein of fraction C was equivalent to 0.1 µg of fraction B, to 20 µg of fraction A, or to 80 µg of dialyzate of crude thymus extract. A striking finding was the similarity between the pattern of the MLC response and the cellular cAMP levels observed with progressively purified THF fractions. Peaks of fraction A, B, and C, inactive in the in vitro GvH and MLC assays were also devoid of activity in the intracellular cAMP assay.

The in Vivo Effect of THF Fractions on the Restoration of Immunocompetence of Neonatally Thymectomized Mice. At this point in the investigation it seemed to us essential to determine whether the increased immunological reactivity obtained with the various purified THF fractions in vitro could also be observed in vivo. For this purpose 4 wk old C57BL/6 mice thymectomized at birth were injected with 0.1 ml of each of the THF fractions containing different amounts of protein diluted in PBS. On the 15th-day after 14 daily i.m. injection, cell suspensions were prepared from the spleen of these animals and the
Effect of repeated injections of active THF fractions on the capacity of spleen cells from NTx C57BL/6 mice to elicit an in vivo GvH response upon inoculation into newborn (C3H/eb × C57BL/6)F1 mice. a. Spleen cells from normal 2 mo old (C57BL/6 × C3H/eb)F1, newborn mice. b. Spleen cells from NTx 2 mo old C57BL/6 mice inoculated into syngenic C57BL/6 newborn mice. c. Spleen cells from NTx 2 mo old C57BL/6 mice injected 14 times with 40 μg protein/mouse of dialyze of crude thymus extract inoculated into syngenic C57BL/6 newborn mice. d. Spleen cells from NTx 2 mo old parental C57BL/6 mice inoculated into (C57BL/6 × C3H/eb)F1, newborn mice. e. Spleen cells from NTx 2 mo old parental C57BL/6 mice injected 14 times with 40 μg protein/mouse of dialyze of crude thymus extract inoculated into (C57BL/6 × C3H/eb)F1, newborn mice. f. Spleen cells from NTx 2 mo old parental C57BL/6 mice injected 14 times with 10 μg protein/mouse of fraction A inoculated into (C57BL/6 × C3H/eb)F1, newborn mice. g. Spleen cells from NTx 2 mo old parental C57BL/6 mice injected 14 times with 0.1 μg protein/mouse of fraction B inoculated into (C57BL/6 × C3H/eb)F1, newborn mice. h. Spleen cells from NTx 2 mo old parental C57BL/6 mice injected 14 times with 0.002 μg protein/mouse of fraction C inoculated into (C57BL/6 × C3H/eb)F1, newborn mice. i. Spleen cells from 2 mo old parental intact C57BL/6 mice inoculated into (C57BL/6 × C3H/eb)F1, newborn mice. Bars represent mean of triplicate samples ± SE. Data represent one out of five such experiments.

Immunocompetence of the cells was simultaneously evaluated by the in vivo GvH assay and by the mixed lymphocyte reaction. In addition, changes in intracellular cAMP was measured in these cells.

In Vivo GvH. Dissociated spleen cells (3 × 10⁶/0.05 ml of PBS) from NTx C57BL/6 mice treated with the THF fractions A, B, or C, or control spleen cells from both NTx and intact C57BL/6 mice were injected into 1- to 2-day-old (C3H/eb × C57BL/6)F1 hybrids and the index of splenomegaly determined 10 days later according to the assay of Simonsen et al. (20). Splenomegaly induced by cells from NTx-treated mice was significantly higher than that induced by cells of control thymectomized mice and reached a level similar to that obtained when normal spleen cells were injected. Moreover, the restoration of immunocompetence observed here by the injection of the various THF fractions was similar to that obtained above by the in vitro incubation of spleen cells with the same fractions. As can be seen in Fig. 3, treatment with daily injection of 0.002 μg of fraction C restored the capacity of C57BL/6 NTx mice to induce in (C3H/eb × C57BL/6)F1, newborn recipients an index of splenomegaly similar to that obtained by treatment of 0.1 μg of fraction B, 10 μg of fraction A, or 40 μg of the dialyze of thymus extract.

Mixed Lymphocyte Reaction. Spleen cells obtained from NTx mice injected with THF fractions A, B, or C and from un.injected NTx or intact C57BL/6 mice
were tested for their ability to respond in the MLC assay. It is worthwhile to emphasize that the cells tested here were portions of the cell populations used for the in vivo GvH test described above and for the experiments on cAMP determination described below.

As seen in Fig. 4, spleen cells from NTx mice exhibited a low level of reactivity. Injection of the various THF fractions into NTx mice augmented the ability of their spleen cells to respond in the MLC assay. Moreover, this response was raised to a level similar to that obtained with spleen cells from intact mice. Again the biological reactivity of the various purified THF fractions injected increased progressively with THF purification.

Measurement of Intracellular Levels of cAMP. As it is known from previous experiments (13, 14) and also seen above, THF stimulates adenyl cyclase activity and increases intracellular cAMP levels in lymphoid cells after a very short incubation. It is of interest to determine whether in those spleen cells obtained from NTx mice treated with the various THF fractions and thus having gained higher levels of immunocompetence, the intracellular levels of cAMP would also be augmented. As seen in Table II the basal cellular cAMP levels of spleen cells from NTx mice are lower than those of spleen cells from intact mice. Injection of the various THF fractions into NTx mice raised cAMP levels of spleen cells to a level even higher than that of intact mice. Using the cAMP assay, although there was a slighter increase with fractions B and C, we could estimate the degree of purification of the purified THF fractions, since the pattern was similar to that observed by the immunological GvH and MLC tests.

Specificity of the in Vivo Effect of THF. Along with these studies of purification it was essential to establish the specificity of activity observed by
TABLE II
The in Vivo Effect of Various Active THF Fractions on the Intracellular cAMP Levels in Spleen Cells from NTx C57BL/6 Mice

| Fractions                                      | Spleen cells | Optimal THF dose | pmol of cAMP ± SE/10⁷ spleen cells |
|-----------------------------------------------|--------------|------------------|-----------------------------------|
| Dialyzate of crude thymus extract             | NTx          | -                | 2.9 ± 0.45                        |
| Void volume fraction of Sephadex G-10 column  | NTx          | 40               | 6.5 ± 0.80                        |
| (fraction A)                                  |              | 10               | 7.1 ± 0.90                        |
| Active peak of Sephadex G-25 column (fraction B) | NTx          | 0.1              | 5.9 ± 0.55                        |
| Active peak of DEAE Sephadex A-25 column (fraction C) | NTx          | 0.002            | 5.8 ± 0.60                        |
| Normal                                        |              | -                | 5.2 ± 0.45                        |

* Data represent one out of five such experiments. Each experiment was done in triplicate. SE = Standard error.

Fig. 5. Specificity of the in vivo effect of the dialyzate of crude thymus extract and of THF fraction C on spleen cells from NTx C57BL/6 mice measured by in vivo GVH and in vitro MLC assays. a. Spleen cells from NTx C57BL/6 mice. b. Spleen cells from NTx C57BL/6 mice injected 14 times with 40 µg BSA/mouse. c. Spleen cells from NTx C57BL/6 mice injected 14 times with 40 µg protein of spleen extract/mouse. d. Spleen cells from NTx C57BL/6 mice injected 14 times with 10 ng glucagon/mouse. e. Spleen cells from NTx C57BL/6 mice injected 14 times with 40 µg protein/mouse of dialyzate of crude thymus extract. f. Spleen cells from NTx C57BL/6 mice injected 14 times with 10 ng glucagon/mouse of fraction C of THF. g. Spleen cells from NTx C57BL/6 mice injected 14 times with 10 ng protein/mouse of peak II (inactive) obtained from DEAE Sephadex A-25 column. The in vivo GVH and MLC assays were performed as described in Fig. 3 and 4. Bars represent mean of triplicate samples ± SE. Data represent one out of five such experiments.

The stepwise process employed. Therefore, in each of the experiments described above, control materials were tested in amounts equivalent to those of the dialyzate of crude thymus extract and to fraction C. As representative of these control experiments, Fig. 5 shows the results when these materials were injected into NTx mice and the spleen cells of these animals tested in vivo in the GVH model and in vitro in the MLC assay. Control materials were: (a) spleen extract 40 µg protein daily; (b) BSA 40 µg protein daily, both (a) and (b) as controls for dialyzate crude thymus extract; (c) glucagon, (mol wt 3,460) as a peptide control of a similar molecular size to fraction C of THF; and (d) peak II obtained from DEAE Sephadex A-25 column found to be inactive in the in vitro
tests. No activity whatsoever was shown by any of the controls tested. Therefore these experiments lead us to conclude that our purification procedure was adequate to isolate the active component of THF.

Discussion

We have recently described the process of isolation of the active principle of thymus extract (THF) consisting of homogenization, ultracentrifugation, dialysis, and stepwise filtration of the crude material through Sephadex columns of different sizes (15). The bioassay used to monitor these procedures consisted of measurement of the capacity of the various purified fractions obtained to restore the immunological impairment of spleen cells from NTx mice to induce an in vitro GvH response. Since this bioassay is an all or none type of response it was necessary to introduce more adequate tests to quantitate the level of immunocompetence acquired by the responding cells. It was also necessary to test whether the purified material was active upon injection in vivo. To serve these purposes the in vitro mixed lymphocyte reaction and the in vivo GvH assay were used after in vitro or in vivo treatment of the cells with the various fractions obtained during the purification of THF. In addition intracellular cAMP levels were measured in the target cell populations, since they correlate with the level of immune competence of these cells.3

The results described here suggest that the procedures used permitted the isolation of active fractions (A, B, or C) progressively more active than the original dialyzate tested. Thus results with the three present assays are parallel and confirm the previous results. Moreover, progressively lower protein concentrations of fraction A, B, or C manifested equivalent activity as measured in vitro after preincubation of target cells from both intact and NTx mice. Thus, the DEAE Sephadex A-25 fraction of THF (fraction C) was estimated to be $1 \times 10^4 - 2 \times 10^4$-fold more active than the crude dialyzate of thymus extract. The fact that equal concentrations of the various fractions tested produced similar effects on the immunological reactivity as well as in the rise of cellular cAMP of the lymphoid cells studied, gives further strength to our hypothesis that intracellular cAMP participates in the kinetics involved in lymphoid cell maturation. Moreover, the fact that restoration of immunocompetence is observed after in vitro or in vivo treatment of lymphoid cell populations before their antigenic stimulation, points to the physiological role of THF in the differentiation of thymus-derived cells. During the performance of these experiments particular attention was given to verifying the specificity of the effects obtained with the various active THF fractions. Spleen extract was prepared in a way identical to that of the dialyzate of crude thymus extract. BSA was tested as an antigenic protein material, since thymus extracts were from bovine source; glucagon was tested as a polypeptide hormone of mol wt (3,460) similar to that of fraction C, the active principle of THF. Finally, peak II of DEAE Sephadex A-25 anion exchange chromatography was used as a protein control of the same calf thymus origin. The total lack of activity of all these materials gives further strength to the conclusion that the final active product of thymus extract has been isolated by the procedures described here.

The biochemical nature and biological activity of other thymic and serum
factors deserve further comment. Comparison of the amino acid sequence of thymopoietin (23) with the amino acid composition of THF reveals no homology. Moreover, thymopoietin is known to induce its effect on pre T cells (prothymocytes) to express Thy 1,2 antigen, but could not increase the immune competence of such lymphocytes (24) while THF confers immunocompetence on target cells which have already undergone some thymic processing. Thymosin α, also differs from THF greatly in amino acid composition and cannot elicit full immunological responsiveness on immature T lymphocytes (17). The material isolated by Bach et al. (25) from pig serum, designated thymic factor TF, resembles some of the biological properties of THF obtained from calf thymus, but until we determine the amino acid sequence of THF we cannot conclude whether or not TF represents a portion of THF. Variation in molecular weights or in some of the biological activities studied with these substances may be the result of different preparatory procedures which may have altered the conformation of the molecules, abolishing their activity in some biological assays.

Summary

Thymus humoral factor (THF), a thymus hormone which participates in the processes leading to acquisition of immunocompetence of lymphoid cells has been isolated in our laboratory by a stepwise gel filtration through various Sephadex columns. THF so isolated appears to be a polypeptide of 3,000 mol wt which contains approximately 30 amino acid residues.

Here we have tested the biological activity of THF fractions of successive degrees of purity upon lymphoid cells from both intact and neonatally thymectomized mice.

The lymphoid cell populations were treated with the various THF fractions by in vitro incubation for a short time and by repeated injection in vivo. The treated cells evidenced increased ability to react in the graft-versus-host assay in vivo and in mixed lymphocyte cultures in vitro concomitantly with the rise of intracellular cAMP. On the other hand no activity whatsoever was shown by any of the control materials tested. These bioassays permitted isolation of fractions progressively more active than the original crude dialyze of thymus extract tested. Thus the active peptide component of THF eluted from DEAE Sephadex A-25 column was estimated to be $2 \times 10^4$-fold more active than the crude dialyze of thymus extract which served as a starting material.

We wish to thank Mrs. Y. Chacham, Mr. A. Levy, and Mr. I. Serusi for their excellent technical assistance.

Received for publication 22 February 1978.

References

1. Trainin, N. 1974. Thymic hormones and the immune response. Physiol. Rev. 54:272.
2. Trainin, N., M. Small, D. Zipori, T. Umiel, A. I. Kook, and V. Rotter. 1975. In Biological Activity of Thymic Hormones. D. W. van Bekkum and A. M. Kruisbeek, editors. Kooyker Scientific Publications, Rotterdam, The Netherlands. 117:144.
3. Umiel, T., and N. Trainin. 1975. Increased reactivity of responding cells in the mixed lymphocyte reaction by a thymic humoral factor. Eur. J. Immunol. 5:85.
INCREASED REACTIVITY OF THYMIC HORMONE

4. Trainin, N., and M. Small. 1970. Studies on some physicochemical properties of a thymus humoral factor conferring immunocompetence on lymphoid cells. J. Exp. Med. 732:885.

5. Rotter, V., and N. Trainin. 1974. Increased mitogenic reactivity of normal spleen cells to T lectins induced by Thymus Humoral Factor (THF). Cell. Immunol. 16:413.

6. Carnaud, C., D. Ilfeld, I. Brook, and N. Trainin. 1973. Increased reactivity of mouse spleen cells sensitized in vitro against syngeneic tumor cells in the presence of thymic humoral factor. J. Exp. Med. 138:1521.

7. Rotter, V., A. Globerson, I. Nakamura, and N. Trainin. 1973. Studies on characterization of the lymphoid target cell for activity of thymus humoral factor. J. Exp. Med. 138:130.

8. Varsano, I., Y. Danon, L. Livni, B. Shohat, Y. Yakir, A. Shneyour, and N. Trainin. 1976. Reconstitution of T-cell function in patients with subacute sclerosing panencephalitis treated with thymus humoral factor. Isr. J. Med. Sci. 12:1168.

9. Kook, A. I., and N. Trainin. 1975. Isolation and partial chemical characterization of THF, a thymus hormone involved in immune maturation of lymphoid cells. Cell. Immunol. 19:151.

10. Hooper, J. A., C. M. McDaniel, G. B. Thurman, G. H. Cohen, R. S. Schulof, and A. L. Goldstein. 1975. Purification and properties of bovine thymosin. Ann. N. Y. Acad. Sci. 249:125.

11. Goldstein, A. L., T. L. K. Low, M. McAdoo, J. McClure, G. B. Thurman, J. Rossio, C. Y. Lai, D. Chang, S. S. Wang, C. Harvey, A. H. Ramel, and J. Meienhofer. 1977. Thymosin α1: isolation and sequence analysis of an immunologically active thymic polypeptide. Proc. Natl. Acad. Sci. U. S. A. 74:725.
22. Takiguchi, T., W. H. Adler, and R. T. Smith. 1971. Cellular recognition in vitro by mouse lymphocytes. *J. Exp. Med.* 133:63.
23. Schlesinger, D. H., G. Goldstein, M. P. Scheid, and E. A. Boyse. 1975. Chemical synthesis of a peptide fragment of thymopoietin II that induces selective T-cell differentiation. *Cell.* 5:367.
24. Goldstein, G. 1974. Isolation of bovine thymosin: a polypeptide hormone of the thymus. *Nature (Lond.)* 247:411.
25. Bach, J. F., M. Dardenne, J. M. Pleau, and M. A. Bach. 1975. Isolation, biochemical characteristics and biological activities of a circulating thymic hormone in the mouse and in the human. *Ann. N. Y. Acad. Sci.* 247:186.
26. Pleau, J. M., M. Dardenne, Y. Blouquit, and J. F. Bach. 1977. Structural study of circulating thymic factor: a peptide isolated from pig serum. *J. Biol. Chem.* 252:8046.