

## HORMONE-LIKE ACTIVITY OF A THYMUS HUMORAL FACTOR ON THE INDUCTION OF IMMUNE COMPETENCE IN LYMPHOID CELLS\*

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Participation of a thymus humoral factor (THF)<sup>1</sup> in the induction of immune reactivity has been demonstrated in the last few years. Thus, partial recovery of cell-mediated and humoral immune reactions were observed in thymectomized mice implanted with thymus tissue contained in cell-impermeable chambers (1-4). It was subsequently demonstrated that administration of extracts from thymus tissue prepared from different species to neonatally thymectomized (NTx) mice partially restores their ability to produce an immune response to sheep red blood cells (5, 6), as well as to reject skin and tumor allografts (7). Furthermore, spleen cells from NTx mice gained the capacity to elicit an in vitro graft-vs.-host (GvH) response when incubated in THF preparations (8). The above observations prompted us to investigate the biochemical mechanisms by which THF confers immunological reactivity upon noncompetent lymphoid cell populations in the hope that this will provide insight into the pathways leading to maturation of lymphoid cells. Since recent work in our laboratory suggested that THF selectively activates thymus-derived (T) cell population (9-11, footnote 2), it was of interest to follow specific metabolic changes occurring in this cell population.

The in vitro GvH test (12) was used as an assay to evaluate the induction by THF of immunological maturation in spleen cells from NTx mice. Previous results obtained using this system suggested that lymphoid cells gain competence following brief exposure to THF (13), thus indicating possible membranal changes. Therefore, activation of membranal enzymes in lymphoid cells exposed to THF were also studied.

A preliminary report of this investigation has been presented elsewhere (14).

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<sup>1</sup> Abbreviations used in this paper: D-B cAMP, dibutyryl cAMP; E.M., Eagle's medium; GvH, graft-vs.-host; NTx, neonatally thymectomized; O.C.M., organ culture medium; PGE<sub>2</sub>, prostaglandin E<sub>2</sub>; Poly(A:U), polyadenylic-uridylic acid; SE, spleen extract; THF, thymus humoral factor.

<sup>2</sup> 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 a thymic humoral factor. *J. Exp. Med.* **138**:1521.

### Materials and Methods

*Mice.*—C57BL/6 and (C3H/eb × C57BL/6)F<sub>1</sub> hybrids were used in most of the experiments. Thymectomy was performed within 24 h after birth by a modification of Miller's technique (15). Animals found to contain a thymic remnant were discarded from the experiments. In some experiments, adult thymectomized (Balb × C57BL/6)F<sub>1</sub> hybrid female mice were used.

*Preparation of Cell Suspensions.*—Spleens were removed aseptically from 6- to 8-wk old intact or neonatally thymectomized C57BL/6 or from intact (C3H/eb × C57BL/6)F<sub>1</sub> mice. Thymuses were obtained from 6- to 8-wk old C57BL/6 animals. Both sexes were equally used as donors. The organs removed were dispersed by pressure through a stainless steel mesh into organ culture medium (O.C.M.) (Eagle's basal medium, supplemented with 10% Difco horse serum [Difco Laboratories, Detroit, Mich.] and 5% chick embryo extract), or Eagle's basal medium (E.M.). The cells were further dissociated by means of a syringe with 27 gauge needle. Erythrocytes were removed from spleen cell suspensions by washing the cells in 0.83% solution of NH<sub>4</sub>Cl pH 7. Aliquots of cells were stained with Turk's solution and nucleated cells were counted in a hemacytometer. Exclusion of 0.05% trypan blue solution was used as a measure of viability. To obtain bone marrow-derived and thymus-derived spleen cells (Balb × C57BL/6)F<sub>1</sub> hybrid female mice 2-mo old were thymectomized, irradiated (750 R and injected within 1 h intravenous with 30 × 10<sup>6</sup> syngeneic bone marrow cells or 100 × 10<sup>6</sup> syngeneic thymus cells. 7 days later their spleens were removed and these spleen cell populations were considered as B and T cells, respectively (16).

*Preparation of Extracts.*—Partially purified calf thymus and spleen extracts were used in this investigation. The extracts were prepared as previously described (13), and stored at -10°C at a concentration of 1 mg protein/ml of 0.005 M Na Phosphate buffer pH 7.4. Chemical analysis revealed the presence of small amounts of RNA but no DNA in the thymus preparation. In contrast with proteolytic digestion, treatment of the thymus extract with RNase did not abolish its activity (Kook and Trainin, unpublished observations). Thus the concentration of active material used in the present experiment is expressed as protein per milliliter. In experiments where the activity of THF was compared to that of controls, either equal amounts of spleen extract protein or equal volumes of phosphate buffer were added to control samples.

*Materials Tested.*—In addition to THF and spleen extracts (SE), the following substances were tested for their capacity to activate spleen cell populations: polyadenylic-uridylic acid (Poly[A:U]), double strand, physiological salt (P-L Biochemicals Inc., Milwaukee, Wis.); theophylline, (theophylline)<sub>2</sub> · ethylenediamine (Sigma Chemical Co., St. Louis, Mo.); dibutyryl cAMP (D-B cAMP), N<sup>6</sup>,O<sup>2'</sup>-dibutyryl adenosine 3',5'-cyclic monophosphoric acid, monosodium salt, (Sigma); prostaglandin E<sub>2</sub>(PGE<sub>2</sub>) and flufenamic acid [*N*-( $\alpha,\alpha,\alpha$ -trifluoro-*m*-tolyl) anthranilic acid] were kindly donated by Dr. U. Zor, Department of Biodynamics, Weizmann Institute of Science, Rehovot, Israel.

*Test of Immunocompetence In Vitro.*—Immunocompetence of spleen cells was evaluated by the ability of cell suspensions from NTx C57BL/6 mice compared to cell suspensions from (C3H/eb × C57BL/6)F<sub>1</sub> mice to induce enlargement of C3H/eb × C57BL/6)F<sub>1</sub> newborn spleen explants, according to the in vitro method developed by Auerbach and Globerson (12). As described previously (8), cultures were considered reactive when the index of splenomegaly obtained was 1.2 or more after the 4th day of the test.

*Induction of Competence in Spleen Cells.*—Exposure of spleen cells to the different materials tested was carried out either by addition of the materials to the medium for the 4-day duration course of the GvH assay, or by incubation of spleen cell suspensions with the tested materials for 30 min before assay of immunocompetence. When preincubated, cell suspensions consisting of 20-25 × 10<sup>6</sup> cells per ml of E.M. were used unless specified otherwise. The cells were then washed, resuspended in O.C.M., and counted before assay for immunocompetence. In both

cases the test materials were added at the specified concentrations. As control to the test materials, equal volumes of 0.005 M Na-phosphate buffer pH 7.4 were used.

*Measurement of Adenyl Cyclase Activity and Intracellular Levels of cAMP.*—Adenyl cyclase activity was measured by the procedure of Krishna et al. (17), after incubation of spleen cells in E.M. for 1 h with thymus extract (20  $\mu$ g protein per ml), spleen extract (20  $\mu$ g protein per ml), or equal volumes of 0.005 M Na-phosphate buffer pH 7.4. The assay is based on the conversion of exogenous [ $^3$ H]ATP by membranous adenyl cyclase to [ $^3$ H]cAMP. The labeled cAMP is then isolated by chromatography on Dowex 50-H $^+$  columns followed by precipitation of all nucleotides and inorganic phosphate by ZnSO $_4$ -Ba(OH) $_2$ . This treatment leaves cAMP in solution (17). The purity of the cAMP fractions obtained by this method was verified by the chromatographic procedures established by Krishna et al. (17). [ $^3$ H]ATP sp act 19 Ci/mmol was obtained from Schwarz/Mann Div., Orangeburg, N.Y. Dowex 50-H $^+$  was obtained from Bio-Rad Laboratories, Richmond, Calif.

Changes in intracellular levels of cAMP in thymus cells were measured at different time intervals after exposure to THF stimulation, or at one time point after incubation with increasing concentrations of THF. Intracellular cAMP levels were measured by a slight modification of Gilman's procedure (18), using the cAMP assay kit provided by The Radio-chemical Centre, Amersham, England (code TRK.432). After incubation, the cells were spun in the cold, boiled in the assay buffer, and homogenized in a homogenizer with a motor-driven Teflon pestle using a total of 30 strokes. Greater and consistent recoveries of cAMP have been demonstrated by the use of such procedure (19) than by precipitating the cells with TCA as suggested by Gilman (18).

#### RESULTS

In parallel to our findings on THF activity (5, 8, 13), it has been demonstrated that complexes of homoribopolymers of Poly (A:U) can increase the immunological competence of lymphoid cells (20–24), and restore the immune reactivity of spleen cells obtained from NTX mice (25). Previous experiments performed in our laboratory (26) have shown that at relatively high concentrations Poly (A:U) and polyadenylic acid did not restore the competence of lymphoid cells obtained from NTx mice to induce a GvH response *in vitro*. Therefore, we decided to extend these experiments by testing a range of relatively lower concentrations of Poly (A:U) (22). As shown in Table I, Poly (A:U) added to the culture medium at concentration of 0.001  $\mu$ g per ml re-

TABLE I  
*Effect of Various Concentrations of Poly(A:U) on the Induction of In Vitro GvH Response by Spleen Cells from NTx C57BL/6 Mice*

Substance tested	Concn. ( $\mu$ g/ml medium)	Incidence of reactive cultures			Culture response
					%
—	—	0/5	0/5	0/5	0
THF	20*	5/5	4/5	4/5	87
Poly(A:U)	0.00016	0/5	0/5		0
Poly(A:U)	0.001	4/5	3/4	4/5	79
Poly(A:U)	1	0/5	0/5		0

\*  $\mu$ g protein per culture.

stores the competence of spleen cells taken from NTx mice to produce a GvH response which is similar to the response conferred when 20  $\mu\text{g}$  protein per ml THF is added to the medium (8, 13, 27). Relatively lower or higher concentrations of Poly (A:U) such as 0.00016  $\mu\text{g}$  per ml or 1  $\mu\text{g}$  per ml of incubation medium, respectively, were not effective.

Since it has been shown that poly(A:U) increases adenylyl cyclase activity in mouse spleen cells (28), it was of interest to explore whether THF would also modify adenylyl cyclase activity. Thus, the activity of this enzyme in lymphocytes was measured after their incubation with THF. As shown in Fig. 1, 1-h

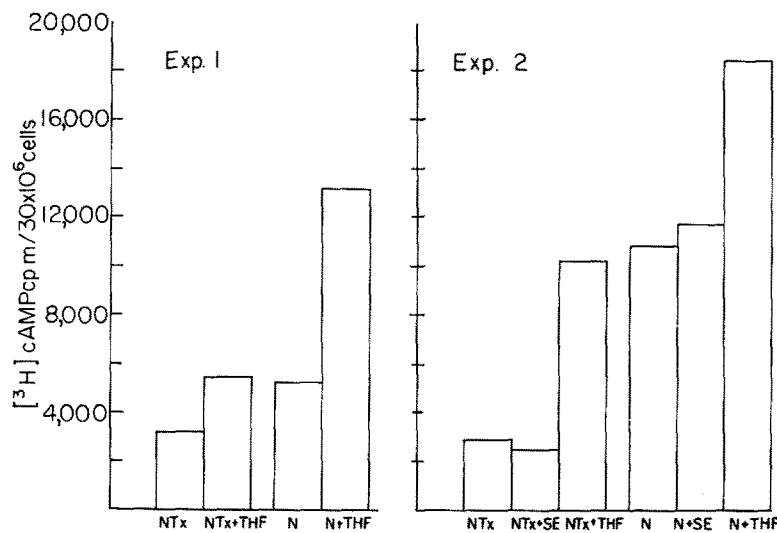


FIG. 1. The effect of THF on adenylyl cyclase activity in spleen cells from NTx and normal (N) C57BL/6 mice. SE was used as control. Cells were incubated in the presence of 20  $\mu\text{g}$  protein per ml of THF or SE for 1 h at 37°C in E.M.

incubation of spleen cells from NTx or normal C57BL/6 mice in the presence of THF (20  $\mu\text{g}$  protein per ml of E.M.) significantly increased adenylyl cyclase activity in those cells, compared to controls. It can be seen that calf spleen extract prepared by a procedure identical to that used for preparation of THF (8, 13) did not modify the activity of adenylyl cyclase of spleen cells from either NTx or normal mice compared to that of controls (Fig. 1, exp. 2). It can also be observed from the results presented in Fig. 1 that THF restores the activity of adenylyl cyclase in spleen cells of NTx mice to that of spleen cells obtained from normal mice.

Since spleen cells from either normal or NTx mice contain bone marrow-derived, as well as thymus-derived lymphocytes (9, 29), it was of interest to determine whether or not the effect exerted by THF on the activity of adenylyl cyclase in spleen cells is due to activation of the enzyme in both cell types. For

that purpose  $30 \times 10^6$  bone marrow cells or  $100 \times 10^6$  thymus cells from (Balb  $\times$  C57BL/6) $F_1$  donors were injected i.v. into adult thymectomized and irradiated (750 R) syngeneic hosts. 7 days later the hosts spleen cells were obtained and were considered B and T cells, respectively. (16). These cell populations were incubated simultaneously for 1 h in the presence of THF under the same conditions described in the experiments presented in Fig. 1. The level of adenylyl cyclase activity in the cells was determined, and the results are shown in Fig. 2. In both experiments shown here, it can be noted that THF increased the level of adenylyl cyclase activity in thymus-derived spleen cells only. No increase in

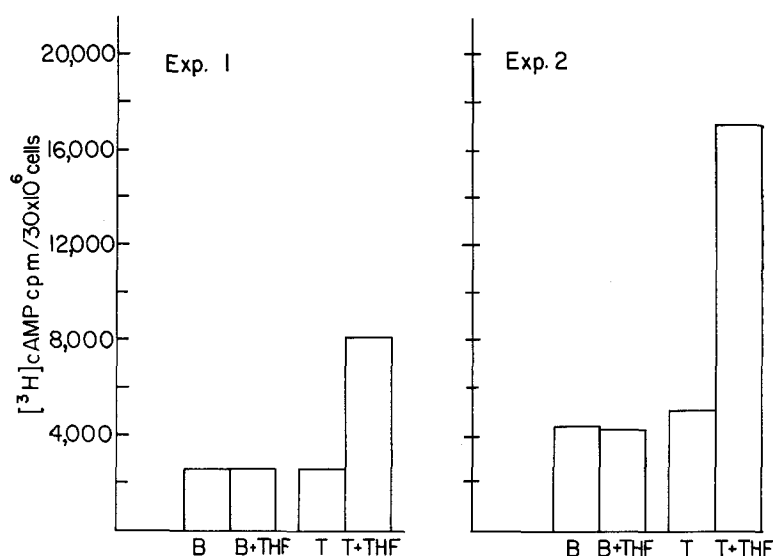


FIG. 2. The effect of THF on adenylyl cyclase activity in spleen cells from adult (Balb  $\times$  C57BL/6) $F_1$  mice thymectomized, irradiated (750 R) and reconstituted with syngeneic bone marrow (B) or thymocytes (T). Cells were incubated in the presence of 20  $\mu$ g protein per ml of THF for 1 h at 37°C in E.M.

the enzymatic activity was observed in bone marrow-derived spleen cells. Thus, it appears that the cell responding to THF stimulation is a "T" cell.

The measurement of adenylyl cyclase activity reported in the above experiments is based on the conversion of exogenous [<sup>3</sup>H]ATP to [<sup>3</sup>H]cAMP (17). It was of interest to investigate, in addition, the changes in endogenous cAMP in lymphocytes induced by THF, and to follow the time course of such a response. The above results indicated that the cells responding to THF are thymus-derived cells. Since thymus cells, with respect to T and B cells, represent a homogeneous lymphoid cell population compared to spleen cells, thymus cells from C57BL/6 mice were chosen for that purpose.  $25 \times 10^6$  cells were incubated with 50  $\mu$ g of THF protein per ml of incubation medium. Control sus-

pensions of thymocytes were incubated with equal volumes of phosphate buffer. Changes in the intracellular levels of cAMP were measured at specified time intervals. The results are shown in Fig. 3. It can be seen that cellular cAMP level increases rapidly following incubation with THF. A threefold increase over control levels was observed within 2 min, the earliest observation made. Thereafter, intracellular cAMP level gradually declined, but was still higher than control level at 60 min. Fig. 4 demonstrates the changes in the intracellular level of cAMP in thymocytes after 5 min incubation with increasing concentra-

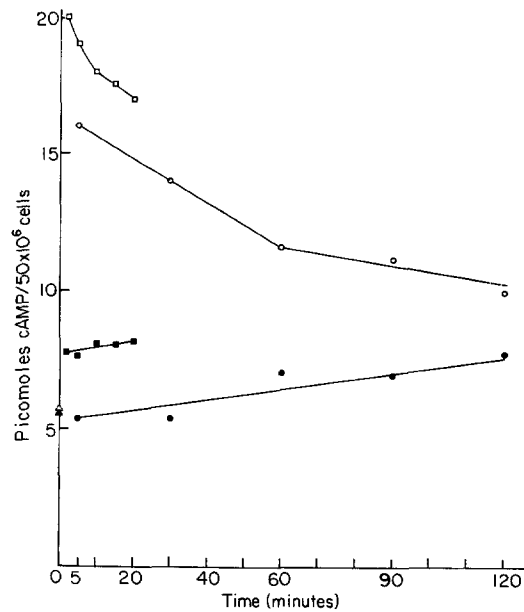


FIG. 3. The effect of THF on intracellular levels of cAMP in thymocytes of C57BL/6 mice.  $50 \times 10^6$  cells were incubated in 2 ml of E.M. at  $37^\circ\text{C}$ . Incubation started by addition of  $50 \mu\text{g}$  protein per ml of THF. (○—○), (□—□), THF present; (●—●), (■—■) THF absent; (△), (▲) normal levels of cAMP.

tions of THF. It can be seen that cAMP levels increase with higher concentrations of THF up to  $200 \mu\text{g}$  protein per ml of THF. THF concentration of  $300 \mu\text{g}$  protein per ml does not seem to induce any further increase in cellular cAMP levels. However, only two determinations of cAMP levels were made at this THF concentration and the variation obtained does not permit a definite conclusion. Nevertheless, it can be observed that cAMP levels increase in a linear fashion with increasing concentrations of THF up to about  $100 \mu\text{g}$  protein per ml. Thereafter the curve begins to flatten, indicating that a maximum effect has been reached by THF under the present experimental conditions.

To eliminate the possibility that the effects on the cellular levels of cAMP ob-

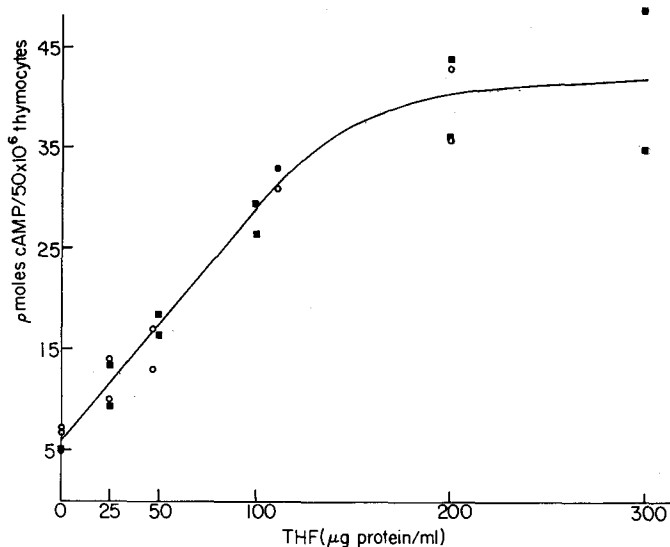


FIG. 4. The effect of increasing THF concentrations on intracellular levels of cAMP in thymocytes of C57BL/6 mice.  $50 \times 10^6$  cells were incubated in 2 ml of E.M. at  $37^\circ\text{C}$  for 5 min. Incubation started by the addition of THF. The figure represents results of two experiments each done in duplicates.

served with increasing concentration of THF are due to contaminating amounts of cAMP in the preparations, several THF preparations including those used in the above experiments were assayed for their cAMP contents. No cAMP could be demonstrated even in volumes twice as concentrated as those used in the above experiments.

The observations that THF stimulates adenylyl cyclase activity and induces a rapid rise in intracellular cAMP levels in lymphocytes bring forward the possibility that these cellular changes are closely linked to the induction of competence by THF. This implies that THF exerts its effect on lymphoid cell populations by a mechanism similar to that demonstrated for other hormones on their respective target tissues (30). This prompted us to investigate whether substances like D-B cAMP and theophylline which are known to modify intracellular cAMP levels would also influence the immunological behavior of non-competent lymphoid cell populations. The effect of various concentrations of D-B cAMP on the capacity of spleen cells from NTx mice to induce an *in vitro* GvH response was assayed. The results are shown in Table II. It can be seen that D-B cAMP at concentration of  $0.001 \mu\text{g}$  per ml incubation medium can be a substitute for THF in the *in vitro* GvH assay. Lower or higher concentrations of D-B cAMP were inactive, thus resembling the effects obtained with various concentrations of Poly(A:U) (see Table I). In the next experiment, when theophylline, an inhibitor of cyclic nucleotide phosphodiesterase known to

increase cellular concentrations of cAMP (30), was tested, it was found that it also enables spleen cells from NTx mice to respond in the in vitro GvH assay (Table III). In light of the above results, it appears that induction of competence by THF in spleen cells obtained from NTx mice is due to an increase in intracellular levels of cAMP.

The GvH tests described above were performed vis-a-vis allogeneic antigens present in the assay system. However, we have previously shown that THF does

TABLE II  
*Effect of Various Concentrations of D-B cAMP on the Induction of In Vitro GvH Response by Spleen Cells of NTx C57BL/6 Mice*

Substance tested	Concn ( $\mu\text{g/ml}$ medium)	Incidence of reactive cultures			Culture response
					%
—	—	0/5	1/8	0/5	6
THF	20*	4/5	6/8		77
D-B cAMP	0.0005	0/5	0/8		0
D-B cAMP	0.001	3/5	6/8	3/5	67
D-B cAMP	0.005	1/5	1/8		15

\*  $\mu\text{g}$  of protein per 1 ml culture medium added at the start of cultures.

TABLE III  
*The Effect of Theophylline on the Induction of In Vitro GvH Response by Spleen Cells of NTx C57BL/6 Mice*

Substance tested	Concn. ( $\mu\text{g/ml}$ medium)	Incidence of reactive cultures		Culture response
				%
—	—	1/5	0/5	10
THF	20*	3/5	4/5	70
Theophylline	0.001	4/5	5/5	90

\*  $\mu\text{g}$  protein per 1 ml culture.

not require antigenic stimulation to induce competence since syngeneic preparations of THF restores competence to spleen cells before their exposure to antigenic stimulation (8). It was, therefore, of interest to find out whether substances that increase intracellular levels of cAMP will also restore immunological competence to lymphoid cells in the absence of the allogeneic stimulation provided by the GvH assay. To test this hypothesis, the in vitro GvH response of spleen cells from NTx C57BL/6 mice was assayed after preincubation with theophylline. The cells were incubated for 30 min at 37°C with no addition, with THF, or with theophylline. After incubation the cells were washed and tested in the GvH assay. The results are shown in Table IV. It can be seen that both THF and theophylline activate spleen cells of NTx mice prior to their exposure to allogeneic antigens. However the increase in intracellular cAMP in-



TABLE IV  
*In Vitro* GvH Response of Spleen Cells Obtained from NTx C57BL/6 Mice after Preincubation with THF or Theophylline\*

Substance tested	Concn. ( $\mu\text{g/ml}$ of medium)	Incidence of reactive cultures			Culture response
					%
—	—	0/5	0/5	1/5	7
THF	20 $\dagger$	3/5	3/5	4/5	67
Theophylline	0.001	4/5	5/5	4/5	87

\* Cells were incubated for 30 min at 37°C in E.M. or E.M. with THF, or with theophylline. After incubation, the cells were washed in O.C.M. and  $1 \times 10^6$  live cells tested in the GvH assay.

$\dagger$   $\mu\text{g}$  protein per 1 ml culture.

duced by THF, or theophylline, or by incubation of cells with D-B cAMP does not provoke by itself a nonspecific enlargement of spleen fragments in the *in vitro* assay. This is shown in the following experiment: spleen cells from NTx C57BL/6 mice were incubated with THF, with theophylline, or with D-B cAMP for 30 min. The cells were then washed and exposed to spleen fragments obtained from newborn syngeneic instead of allogeneic mice. From the results shown in Table V, it can be seen that no induction of *in vitro* splenomegaly occurred under such conditions.

The experiments presented so far suggest that the first event in the induction of competence in a noncompetent lymphoid cell population is characterized by a rapid rise in intracellular cAMP levels after stimulation of adenylyl cyclase activity. To determine whether this is necessary we proceeded to test whether inhibition of activation of adenylyl cyclase prevents the cells from acquiring competence. Since antagonistic agents to THF are yet unknown, we chose to stimulate adenylyl cyclase in lymphocytes by PGE<sub>2</sub> (31, 32), and to test the effect of its antagonist, flufenamic acid (33, footnote 3), on the induction of immune competence by PGE<sub>2</sub>.

The results presented in Table VI show that PGE<sub>2</sub> increases by five-fold the level of cAMP in thymus cells obtained from C57BL/6 mice. On the other hand, flufenamic acid completely inhibits the rise in intracellular cAMP when added together with PGE<sub>2</sub> to thymus cells suspensions. Flufenamic acid by itself does not appear to enhance either synthesis or breakdown of intracellular cAMP in thymocytes.

The effect of PGE<sub>2</sub> and its antagonist flufenamic acid on the induction of immune competence in spleen cells obtained from NTx mice was then tested. Cells were incubated with THF, with PGE<sub>2</sub> with PGE<sub>2</sub>, and flufenamic acid, or

<sup>3</sup> Zor, V., S. Bauminger, S. A. Lamprecht, Y. Koch, P. Chobsieng, and H. R. Lindner. 1973. Stimulation of cyclic AMP production in the rat ovary by luteinizing hormone: independence of prostaglandin mediation. *Prostaglandins*. In press.

TABLE V

*The Effect of THF, Theophylline, or D-B cAMP on the Induction of In Vitro Splenomegaly in Spleen Fragments from C57BL/6 Mice by Spleen Cells from Syngeneic NTx Mice\**

Substance tested	Concn. ( $\mu\text{g}/\text{ml}$ of medium)	Incidence of reactive cultures		Culture response
				%
---	---	0/5	1/5	10
THF	20 $\ddagger$	1/5	0/5	10
Theophylline	0.001	0/5	1/5	10
D-B cAMP	0.001	0/5	0/5	0

\* Cells were incubated in E.M. for 30 min at 37°C with no addition, with THF, with theophylline, or with D-B cAMP. After incubation the cells were washed in O.C.M. and  $1 \times 10^6$  live cells tested in the assay.

$\ddagger$   $\mu\text{g}$  protein per 1 ml culture.

TABLE VI

*The Effects of PGE<sub>2</sub> and of Flufenamic Acid on Intracellular cAMP Content of Thymus Cells from C57BL/6 Mice*

Substance tested (concn.)	cAMP	
	pmoles/ $50 \times 10^6$ thymocytes*	
	Exp. 1	Exp. 2
---	17	18
THF (50 $\mu\text{g}$ protein/ml)	30	34
PGE <sub>2</sub> (5 $\mu\text{g}/\text{ml}$ )	97	98
PGE <sub>2</sub> (5 $\mu\text{g}/\text{ml}$ ) + flufenamic acid (75 $\mu\text{g}/\text{ml}$ )	17	13
Flufenamic acid (75 $\mu\text{g}/\text{ml}$ )	15	18

\*  $25 \times 10^6$  cells/ml were incubated in E.M. for 5 min at 37°C. Reaction initiated by addition of test materials.

with flufenamic acid only. Control cell suspensions were incubated with no substance added to the medium. After 30 min of incubation the cells were washed and tested for immune competence in the in vitro GvH assay. The results are shown in Table VII. It can again be observed that the noncompetent spleen cell population from NTx mice acquired immunological competence following preincubation with THF. Similarly, preincubation with PGE<sub>2</sub> also induces the deficient spleen cell population of NTx mice to react in the GvH assay. However, preincubation of these cells with both PGE<sub>2</sub> and flufenamic acid abolished the induction of immunological competence. To discard the possibility that flufenamic acid may block the effect of PGE<sub>2</sub> by some nonspecific toxic phenomenon, immunologically competent normal spleen cells from C57BL/6 mice were preincubated under the same conditions with and without flufenamic acid and subsequently tested for immune competence. The results indicate that no impairment of the immune competence of normal spleen cells was caused by flufenamic acid (Table VII).

TABLE VII  
*The Induction of GvH Response in Spleen Cells of NTx C57BL/6 Mice by Incubation with PGE<sub>2</sub> and Inhibition of the Effect by Flufenamic Acid*

Substance tested (concn.)	Source of spleen cells*	Incidence of reactive cultures			Culture response
					%
—	NTx	0/5	0/5		0
THF (50 µg protein/ml)	NTx	4/5	3/5		70
PGE <sub>2</sub> (5 µg/ml)	NTx	5/5	4/5	4/5	87
PGE <sub>2</sub> (5 µg/ml) + flufenamic acid (75 µg/ml)	NTx	1/5	0/5	1/5	13
Flufenamic acid (75 µg/ml)	NTx	1/5	0/5		10
—	Intact	4/5	5/5	5/5	93
Flufenamic acid (75 µg/ml)	Intact	4/5	5/5	5/5	93

\*  $25 \times 10^6$  spleen cells obtained from either NTx or intact mice were incubated with or without the substances tested in 1 ml E.M. for 30 min at 37°C. After incubation the cells were washed and tested in the GvH assay.

Thus, induction of adenylyl cyclase and a rise in cellular levels of cAMP are necessary events for acquisition of competence by the noncompetent spleen cells of NTx mice.

#### DISCUSSION

The experiments presented here were aimed at the understanding of the mechanism by which THF increases the competence of spleen cells from NTx mice. The results indicate that the endowment of competence to these cells by THF is mediated via a rapid increase in cellular levels of cAMP. It has been previously demonstrated that Poly(A:U) induces competence in spleen cells from NTx mice when tested in vitro for response to SRBC or in vivo for homograft rejection (20) and to stimulate adenylyl cyclase activity in lymphocytes (28). We have now shown in addition that Poly(A:U) induces in adequate concentrations the capacity of spleen cells from NTx mice to react in an in vitro GvH test. We have also presented evidence that D-B cAMP and substances known to increase cellular cAMP levels in lymphocytes by different mechanisms such as PGE<sub>2</sub> and theophylline induce competence in lymphocytes tested in the in vitro GvH assay used here. The increase in cellular cAMP is apparently a necessary requirement for the induction of competence, since it could be shown that when the increase in intracellular cAMP induced by PGE<sub>2</sub> is blocked by its antagonistic agent flufenamic acid, PGE<sub>2</sub> loses its ability to induce competence in spleen cells of NTx mice. Moreover the kinetic data showing the rapid elevation of cellular cAMP by THF indicate that this event is probably the first in the sequence of events leading to the induction of competence.

The possibility that THF exerts its effect as a consequence of contaminating amounts of Poly(A:U) or Poly(A:U)-like homoribopolymers was excluded since THF preparations did not lose their activity after degradation by RNase

(Kook and Trainin, unpublished observations). The preparations also contained no detectable amounts of cAMP.

Previous observations suggest that an active substance is released by the reticular epithelial cells of the thymus medulla (34–36) and is present in the blood in measurable small quantities (37). The present results indicate that induction of competence by THF involves activation of adenyl cyclase and a rise in cellular cAMP levels. We also observed that D-B cAMP and theophylline mimic the effect of THF. Consistent with the postulates established by Sutherland to define hormonal action mediated by cAMP (38), our findings permit the classification of TFH as a thymus hormone. Hormones which exert their effect via cAMP exhibit a dose response curve with a sharp peak in activity and with decreased activity at higher dosages (39, 40). In agreement with this, we found that the induction of competence in the in vitro GvH reaction is dose dependent on cAMP levels. Thus both D-B cAMP and Poly(A:U) exhibit a dose-response pattern, where optimal concentrations produce GvH response which was inhibited by higher concentrations. Similar observations not presented here were made in our laboratory for different THF concentrations. The range for induction of in vitro GvH response was found to be 20–80  $\mu\text{g}$  protein per ml of THF.

As regards the target cell population, the data shown indicate that THF activates adenyl cyclase in thymus-derived and not in bone marrow-derived spleen lymphocytes. It is interesting to note that Poly(A:U) has also been reported to exert its enhancing effects on cell mediated immunity, and antibody production on thymus-derived cells only and not on bone marrow-derived cells or macrophages (21, 25, 41, 42). These observations are in agreement with previous findings in our laboratory (9–11) which suggest that THF exerts its immunoenhancing effect on T-cell populations. On the other hand, it has been reported that  $\theta$ -negative bone marrow or spleen cells may acquire some physicochemical properties of thymus-derived cell populations following interactions with thymus extracts (43–45).

Previous experiments from this laboratory in which spleen cells from NTx mice were preincubated with THF demonstrated that THF induces competence in the noncompetent spleen cell population before their exposure to foreign antigen (8). Moreover, THF itself does not provide antigenic stimulation since it has been previously shown that the THF preparations from syngeneic source will also induce immune competence in spleen cells from NTx mice (8). Likewise, the present results (Table V) demonstrate that GvH response will not take place unless cells which were preincubated with THF were subsequently exposed to allogeneic antigens.

In conclusion, it appears that induction of adenyl cyclase and a rise in cellular levels of cAMP are early events necessary for acquisition of competence by the noncompetent spleen cells of NTx mice. This competence is acquired in the absence of antigenic stimulation and is induced by THF.

## SUMMARY

Experiments reported here were performed to understand the mechanism by which THF increases the immunocompetence of spleen cells from NTx mice.

Dibutyryl cAMP or substances which increase intracellular levels of cAMP in lymphocytes such as Poly(A:U), theophylline, or PGE<sub>2</sub> were shown to mimic the effect of THF and confer reactivity in an in vitro GvH response to spleen cells from NTx mice. Flufenamic acid, an antagonist to PGE<sub>2</sub>, was shown to inhibit the induction of competence by this substance. It was found that THF induces competence by activating membranal adenyl cyclase which leads to a rise in intracellular cAMP in thymus-derived cells only. These biochemical changes occur before antigenic stimulation and are unrelated to antigenic challenge.

These findings indicate that THF exerts its effect via cAMP and are in agreement with the concepts which permit to classify THF as a thymus hormone.

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