Production of a Non-immunoglobulin Thyroid Stimulator by Human Lymphocytes during Mixed Culture with Human Thyroid Cells*

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Human lymphocytes from normal individuals and from patients with Graves' disease were cultured together with normal human thyroid cells in long term monolayer culture. Stimulation of thyroid cell cAMP content began on the 2nd day of co-culture and rapidly increased to reach a maximum after approximately 72 h. Cell-free culture medium obtained following the mixed culture of lymphocytes and thyroid cells contained thyroid stimulatory activity. Thyroid cells or lymphocytes cultured alone in autologous serum released little or no thyroid stimulating substance into the medium. Surprisingly, lymphocytic thyroid stimulator (LTS) was produced by lymphocytes from all 11 normal subjects tested (stimulation of target cell cAMP content to 271 ± 16% S.E. of basal values) as well as by lymphocytes from all five patients with Graves' disease (324 ± 44% S.E. of basal values). Human thyroid cells were not found to be specific activators of LTS production, in that such activation was observed with other human cell lines. In some, but not all, experiments, LTS stimulated cAMP content in target cells other than human thyroid, such as human fibroblast and dog thyroid cells. LTS was not precipitated by anti-human immunoglobulin antisera and was dialyzable and heat-stable. LTS bioactivity was extracted at pH 3.0 by 2:1 chloroform:methanol and diethyl ether. Gel filtration on Sephadex G-25 and Sephadex G-10 showed that the molecular weight of LTS was 1000 or less, and LTS co-eluted with radiolabeled prostaglandins. On paper chromatography, LTS co-migrated with prostaglandins, particularly of the E and F category. Comparison of the kinetics of LTS and prostaglandin stimulation of thyroid cell cAMP generation demonstrated close similarity in their time course of action. Indomethacin present during the mixed culture of human lymphocytes and human thyroid cells markedly reduced LTS generation, with a half-maximal inhibitory effect at approximately 10^-6 M. Chromatography of LTS on silicic acid columns provided further evidence for the prostaglandin nature of LTS and in addition, suggested that LTS is a combination of prostaglandin E and prostaglandin F. Since prostaglandins stimulate thyroid hormone secretion, it is possible that the hyperthyroidism of Graves' disease may be related to the local production of these substances by lymphocytes within the thyroid gland.

There is considerable evidence, accumulated over a period of nearly 20 years, that sera from many patients with the hyperthyroidism of Graves' disease contain thyroid-stimulating immunoglobulins (1-6). Because of the clinical association of Graves' disease with other autoimmune diseases (3), the lymphoid hyperplasia associated with Graves' disease (7), and the presence of thyroid stimulatory antibodies, there has been increasing interest in the role of the lymphocyte in Graves' disease. Several studies have demonstrated the production of a thyroid stimulator by lymphocytes from patients with Graves' disease cultured in vitro (8-13). Neutralization of this stimulatory activity by anti-human immunoglobulin antisera further strengthened the concept of the abnormal production of a thyroid stimulatory immunoglobulin in Graves' disease (9, 10, 12).

The present consensus is that lymphocytes from normal subjects, in contrast to lymphocytes from patients with Graves' disease, do not elaborate a thyroid stimulator (8-10, 12, 13). Further evidence suggests that normal human thyroid tissue, but not other human tissues, activates lymphocytes to produce a thyroid stimulator (12). This reported difference between control lymphocytes and Graves' lymphocytes in their ability to produce a thyroid stimulator, in response to normal thyroid tissue antigen or PHA, supports the observation of similar differences with respect to the generation of the lymphokines, migration inhibition factor (14-16), and cytotoxic factor (17). These studies suggest that patients with Graves' disease have an abnormal lymphocyte population.

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1 The abbreviations used are: PHA, phytohemagglutinin; LTS, lymphocytic thyroid stimulator; MEM medium, Eagle's minimum essential medium; FCS, fetal calf serum; Hepes, 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid; TSH, thyrotropin; PGE, prostaglandin E; PGA, prostaglandin A; PGB, prostaglandin B; PGF, prostaglandin F

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sensitized to normal thyroid tissue. On the other hand, there are conflicting reports in which no difference in lymphocyte sensitization to thyroid antigen (or response to phytohemagglutinin) was observed between normal subjects and subjects with Graves' disease (18-21).

The present studies were conducted to examine further the interaction between lymphocytes and human thyroid cells, using the mixed culture of these cells as an in vitro model for Graves' disease. In contrast to the previously mentioned studies utilizing thyroid homogenate, thyroid slices, or dispersed thyroid cells in short term culture, we employed human thyroid cells in long term monolayer culture. With this system it has been found that exposure of both normal human lymphocytes and Graves' lymphocytes to homologous thyroid (and non-thyroid) cells results in the formation of a dialyzable, lymphocytic thyroid stimulator. Further evidence suggests that this stimulator is a combination of prostaglandins E and F.

MATERIALS AND METHODS

Cell Cultures—Human thyroid cells were established in monolayer tissue culture by the method previously reported for dog thyroid tissue (22), except that after the thyroid slices were chopped into 100-μm square segments with a McClinn tissuechopper, 0.25% trypsin (Worthington Biochemical Corp., Freehold, N. J.) was used for cell dispersion. Trypsin was found to be more effective than collagenase, possibly because human thyroid tissue has more fibrous stroma than dog thyroid tissue and is tougher in consistency. The total period of trypsinization (with one or two changes of trypsin medium) was 45 to 90 min. Human thyroid tissue was obtained from autopsies or from pathological specimens removed at surgery, and usually consisted of surrounding a thyroid removed surgically. Dog thyroid cells were prepared as previously described (22).

Human lymphocytes from peripheral venous blood were prepared by a standard Ficoll-Hypaque technique (23) (Ficoll-Faque, Pharmacia Fine Chemicals, Inc., Piscataway, N. J.). The lymphocytes were resuspended at 1 to 2 x 10⁸ cells/ml in medium containing 20 mM Hepes, pH 7.4, and either 10% FCS or 10% autologous serum. Lymphocytes in suspension were incubated with different cells in monolayer for 66 to 72 h except when in which medium was incubated with a lymphocytes and human thyroid cells were co-cultured in medium containing 2.6 cm) and Sephadex G-10 (60 normal human thyroid cells (LIHT). Human thyroid cells were also cultured without lymphocytes (~HT). Cyclic AMP content was determined in preliminary titration experiments, to produce adequate visible precipitation with each of the antisera used. One-tenth milliliter of antiserum, LTS was added to each culture dish. After incubation for 15 min at 37°, the medium, containing most of the lymphocytes, was aspirated and the cAMP content was measured in the thyroid cell monolayer. Bracketed values for cAMP content are expressed as percent of control.

The precision of the assay was determined by measuring cAMP stimulation by lymphocytic stimulator in 10 dishes of human thyroid cells. The coefficient of variation (SD/mean) was 9.15%.

Immune Precipitation with Anti-human Immunoglobulins—In pilot experiments attempting to neutralize LTS present in medium containing 10% human serum, it was found that the addition of a relatively large volume of anti-human immunoglobulin antiserum was necessary to obtain adequate immune precipitation. This resulted in a nonspecific inhibitory effect on thyroid cell cAMP content. This was not surprising because serum is known to have an inhibitory effect on the thyroid cAMP response to TSH stimulation (26, 27). Consequently, in order to minimize the amount of added antiserum, LTS was produced in medium containing 10% FCS. Small volumes of human serum were added as carrier, as determined in preliminary titration experiments, to produce adequate visible precipitation with each of the antisera used. One-tenth milliliter of antiserum was added to 0.9 ml of medium. After a 1-h incubation at 37° and overnight incubation at 4°, the immunoprecipitate was removed by centrifugation in a Beckman microfuge at 10,000 rpm for 10 min. The clear supernatant was assayed for thyroid stimulatory activity as described above. In contrast to incubations of lymphocytes without thyroid antigen in the presence of autologous (lymphocyte donor) serum in which LTS was not produced, similar incubations conducted in the presence of heterologous serum (FCS) did on occasion result in some LTS production. However, this was relatively small and in experiments in incubations also including thyroid cells.

Sephadex Gel Filtration—Columns of Sephadex G-25 fine (95 x 2.6 cm) and Sephadex G-10 (60 x 1.6 cm) were employed. The gels were equilibrated and the samples were eluted with 50 mM NH₄HCO₃ buffer, pH 7.7. Appropriate fractions were lyophilized and redissolved in Loibovita 15 medium containing 0.5 mM 3-isobutyl-1-methyl xanthine prior to measurement of bioactivity. In preliminary experiments, LTS bioactivity was recovered in the supernatant after extraction of culture medium with 95% ethanol.

FIG. 1. Time course of thyroid stimulator generation. Human lymphocytes from a normal subject and a patient with Graves disease were cultured in 10% fetal calf serum either alone (LIHT) or together with normal human thyroid cells (LIHT). Human thyroid cells were also cultured without lymphocytes (~HT). PFA (1 μg/ml) was included in some culture dishes. At the end of the indicated culture period, 3-isobutyl-1-methyl xanthine (0.5 mM final concentration) was added to each culture dish. After incubation for 15 min at 37°, the medium, containing most of the lymphocytes, was aspirated and the cAMP content was measured in the thyroid cell monolayer. Bracketed values for cAMP content are expressed as percent of control.
Therefore, in order to concentrate LTS from relatively large volumes of culture medium for application to the medium, the cultures were first lyophilized, extracted three times with 90% ethanol, the extract was evaporated to dryness, and the sample was reconstituted in buffer.

**Paper Chromatography**—Descending paper chromatography was carried out on Whatman No. 3MM paper (27 × 53 cm) in glass chambers. Two solvent systems were used; one acid (butanol:acetic acid:water:pyridine, 15:5:12:10) and the other alkaline (butanol:ammonia:water, 10:1:5) systems. Samples (up to 0.4 ml) were applied with capillary pipettes with fire polished tips. Chromatograms were run for about 18 h at approximately 23°. Strips were eluted, the eluents were evaporated, and the residues were redissolved in chloroform. After evaporation, the residue was resuspended first in 0.2 ml of 60:40:10 benzene:ethyl acetate:methanol, and then in 0.5 ml of 60:40 benzene-ethyl acetate:methanol (Solvent 1). Tritiated prostaglandins standards were detected by eluting strips of paper placed directly into liquid scintillation vials containing 10 ml of Aquasol (New England Nuclear). Nonradioactive standards, including a variety of nucleosides and catecholamines, were detected by fluorescence under ultraviolet light or by staining with ninhydrin.

**Ether and Chloroform: Methanol Extraction—**LTS in aqueous solution was extracted twice with 10 volumes of ethyl ether or chloroform:methanol (2:1). The pH of the aqueous solution was adjusted to either pH 3.0 or 7.4 prior to extraction. After centrifugation, the ether and chloroform phases were evaporated to dryness and the aqueous phases were lyophilized. All fractions were then resuspended in medium containing 0.5 mm 3-isobutyl-1-methyl xanthine and measured for LTS bioactivity as described above.

**Silicic Acid Chromatography—**LTS was chromatographed on columns (disposable 10-ml pipettes) containing 0.5 g of silicic acid, 2×-monosuccinyl adenosine 3′:5′-monophosphate tyrosine methyl ester, PGE₁, PGE₂, adenosine, ATP, ADP, CAMP, histamine, acetylcholine, epinephrine, and isoproterenol from Sigma Chemical Co., St. Louis, Mo.; antisera for the CAMP immunoassay from Schwarz/Mann Div., Becton Dickinson and Co., Orangeburg, N. Y.; [3H]PGF₁α (specific activity 89.5 Ci/mmol) and [3H]PGF₂α (specific activity 178 Ci/mmol) from New England Nuclear, Boston, Mass.; silicic acid, 100 mesh, from Mallinckrodt Co.; PFA from Burroughs-Wellcome, Research Triangle Park, N. C.

### RESULTS

**Time Course of Thyroid Stimulator Generation during Mixed Culture of Human Lymphocytes and Human Thyroid Cells**—Lymphocytes from a normal individual and from a patient with active Graves' disease were cultured together with normal thyroid cells in monolayer, in medium containing 10% FCS for up to 108 h (Fig. 1). Some dishes of cells also contained PHA (1 μg/ml). At the end of each indicated time period, 3 isobutyl 1 methyl xanthine was added to the culture medium to achieve a final concentration of 0.5 mm. After a 15-min incubation at 37°C, the medium (the majority of lymphocytes) was aspirated and the cAMP content in the remaining cells was assayed. The 15-min incubation period was chosen on the basis of previous data regarding TSH stimulation of thyroid cell cAMP content (22). Control dishes incubated with lymphocytes alone and similarly processed contained minimal quantities of cAMP, indicating that the cAMP measured was of thyroid cell origin. After 24 h of mixed culture, very little stimulation of thyroid cell cAMP content was observed. Stimulation of cAMP content began on the 2nd day of co-culture and rapidly increased to reach a maximum after approximately 72 h. The presence of PHA initially enhanced the thyroid cell cAMP response, followed by a decline in the extent of target cell stimulation at 108 h to values below those observed with cells incubated without PHA for the same period. The results were surprisingly similar despite the different origins of the lymphocytes. Similar results were obtained when 10% human serum, rather than 10% FCS, was used in the culture medium. In further experiments, it was found that the cell-free medium obtained following the mixed culture of lymphocytes and thyroid cells contained thyroid stimulating activity. Consequently, in subsequent experiments medium was tested for the presence of stimulator after the removal of suspended lymphocytes by centrifugation, followed by the addition of the medium to freshly subcultured target cells.

**Lymphocytic Source of Thyroid Stimulator**—The preceding studies do not indicate the cellular origin of the thyroid stimulator released into the medium during the co-culture of human thyroid cells and human lymphocytes. To examine this question, normal lymphocytes were heated at 70°C for 1 h before addition to thyroid cell cultures. In contrast to cultures of thyroid cells and normal, unheated lymphocytes, the use of heated lymphocytes virtually abolished cAMP stimulating activity in the cell-free culture medium (Fig. 2). Medium from other cultures containing thyroid cells and IM-9 lymphocytes (a human leukemic cell line) did not stimulate cAMP production either. Although this suggested a lymphocytic origin for the thyroid stimulator it was possible that the generation of thyroid stimulator was dependent upon the presence of complement in the fetal calf serum and that the
complement had been destroyed during the heating procedure. It was subsequently found however that no significant difference in thyroid stimulator production was observed whether heated or unheated serum was present in the culture medium (Table I). Final proof for the lymphocytic origin of the thyroid stimulator was provided by the observation that the incubation of human lymphocytes together with a nonviable, thyroid cell particulate fraction did result in thyroid stimulator production (Table II). On this basis, the term lymphocytic thyroid stimulator was chosen to describe this factor.

**Comparison of Time Course of Stimulatory Effect of TSH and LTS**—Human thyroid cells were incubated for up to 2 h in medium containing either added TSH (200 microunits/ml) or LTS previously produced by the mixed culture of human lymphocytes and human thyroid cells for 72 h. The TSH was added to control medium incubated for the same period without either lymphocytes or thyroid cells. The concentration of TSH was chosen to produce a response of similar magnitude to that of LTS. Both TSH and LTS produced a maximal cAMP response at 10 to 15 min (Fig. 3). However, in contrast to the slow decline from maximum seen with TSH stimulation, there was an initial rapid decline in cAMP values observed with LTS.

**Comparison of Control Lymphocytes and Graves' Lymphocytes in Their Ability to Generate LTS**—In separate experiments, lymphocytes from 11 normal individuals and 5 patients with active Graves' disease were incubated with normal human thyroid cells for approximately 72 h in medium containing 10% autologous serum. LTS generation was observed with all control lymphocytes (271 ± 16% of basal cAMP values) as well as with all lymphocytes from patients with Graves' disease (324 ± 44% S.E. of basal cAMP values) (Fig. 4). These values, as determined by the unpaired t test, are not significantly different from one another (P > 0.2). Lymphocytes from both groups cultured without thyroid cells did not produce LTS. As a further control, the incubation of thyroid cells without lymphocytes for 72 h did not result in thyroid stimulating activity in the medium (data not shown).

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**Table I**

Lack of effect of heat inactivation of serum complement on generation of thyroid cell stimulator

Human lymphocytes from a normal subject were cultured for 3 days in the presence or absence of thyroid cells. The medium was then aspirated, centrifuged to remove cellular elements, and added to fresh dishes of target thyroid cells to measure thyroid cell cAMP stimulatory activity, as described under "Materials and Methods."

| 3-day co-culture | Target thyroid cell cAMP |
|------------------|--------------------------|
| Thyroid cells    | Lymphocytes | 10% fetal calf serum |
|                  |   | pmol/dish ± S.E. |
| −                | −  | 0.41 ± 0.04 |
| +                | +  | 19.95 ± 1.06 |
| +                | +  | 18.24 ± 1.58 |
| +                | Heated | 0.91 ± 0.06 |
| −                | +  | 4.92 ± 0.11 |
| −                | +  | 3.38 ± 0.04 |
| −                | Heated | 0.97 ± 0.05 |

* a Triplet dishes of cells
  b 50° for 30 min.
  c 70° for 60 min.

**Table II**

Generation of thyroid cell stimulator during incubation of lymphocytes with nonviable thyroid cell particulate fraction

Human lymphocytes from a normal subject were co-cultured for 3 days in medium containing 10% fetal calf serum either with thyroid cells in monolayer or with a thyroid cell particulate fraction. For the latter, thyroid cells were homogenized with a Polytron homogenizer, the homogenate was centrifuged at 100,000 × g for 60 min, and the pellet was resuspended in culture medium. The cell-free medium was then tested for thyroid cell cAMP stimulatory activity as described above under "Materials and Methods."

| 3-day co-culture | Target thyroid cell cAMP |
|------------------|--------------------------|
| Thyroid cells    | Lymphocytes | pmol/dish ± S.E. |
| −                | −  | 0.66 ± 0.01 |
| Monolayer        | +  | 23.97 ± 1.28 |
| Particulate      | +  | 8.55 ± 0.45 |

* a Triplet dishes of cells.
  b p < 0.001 compared to basal values.
Ability of Different Tissues to Stimulate LTS Production by Normal Human Lymphocytes — Lymphocytes from subjects without known thyroid disease were incubated for 66 to 70 h in medium containing 10% autologous serum, either alone or in mixed culture, with a variety of cells of different species and tissue origin. The cell-free media were then tested for their ability to increase cAMP content in target human thyroid cells in monolayer culture (Table III). The two animal cell lines tested, rat hepatoma (HTC) and dog thyroid, did not stimulate LTS production. In contrast, human cell lines (WI-38 lung embryo fibroblasts and HeLa cells) were potent stimulators of LTS generation. LTS production was also observed in a separate experiment using thyroid capsule fibroblasts (data not shown). The medium obtained after incubating WI-38 fibroblasts without lymphocytes did produce some stimulation of cAMP content in target human thyroid cells, but this stimulation was small relative to that seen when lymphocytes were also present. No such stimulatory effect was observed in separate experiments with medium obtained after a 3-day incubation of human thyroid cells without lymphocytes.

Effect of LTS on cAMP Content in Target Cells from Different Tissues — LTS was produced by incubating human lymphocytes in medium containing 10% autologous serum with normal thyroid cells in monolayer culture for approximately 72 h. After removal of lymphocytes by centrifugation, the lymphocyte-human thyroid medium (L/HT) was tested for its ability to increase the cAMP content in a variety of target cells. Similar results were observed in separate experiments with lymphocytes from patients with Graves' disease (Table IVA). In contrast to the stimulation of cAMP content in thyroid cells, no stimulation was seen in rat hepatoma cells and HeLa cells. In this representative experiment, only slight stimulation of cAMP content was seen in the WI-38 cells. However, much greater stimulation of WI-38 cAMP content was observed in two of the three further experiments, one of which is shown in Table IVA. These data indicate that stimulation of cAMP generation by the lymphocytic factor is not specific for the thyroid cell.

Clear evidence that human thyroid cells were stimulated by L/HT and that the target cell cAMP responses observed were not Those of "contaminating" fibroblasts was provided by the observation of the morphological transformation of polygonal thyroid cells into stellate forms (Fig. 5). A similar morphological effect occurs with TSH stimulation of thyroid cells in monolayer culture (22). Fusiform fibroblasts are not so affected.

Studies on Nature of Thyroid Stimulator Produced by Lymphocytes — Because it is presently accepted that the abnormal thyroid stimulator in Graves' disease is a 7 S immunoglobulin, attempts were made to neutralize LTS with specific anti-human immunoglobulin antisera. Anti-human IgM was tested because of the foregoing observation of LTS production by lymphocytes from normal individuals; i.e., it seemed possible that an IgM, rather than an IgG, would be produced over a 3-day period by lymphocytes not presensitized to thyroid antigen. Anti-human IgA was tested as control. Despite the formation, and removal by centrifugation, of substantial precipitates, no loss of LTS bioactivity was observed, indicating that LTS is not an immunoglobulin (Table V). Measured by radial immunodiffusion, the human IgG content in medium containing LTS was 3.4 mg/dl. After immunoprecipitation, no detectable human IgG was present (limit of sensitivity of the assay, 1 mg/dl).

Further studies indicated that LTS was dialyzable and heat stable (Table VI), confirming the nonimmunoglobulin nature of the substance. Because CAMP itself is dialyzable and heat-stable, it was important to demonstrate that LTS

### Table III

| Cell line | Lymphocytes | Target human thyroid cell cAMP contenta |
|-----------|-------------|----------------------------------------|
|           |             | Dish 1 | Dish 2 |
|           |             | pmol cAMP/mg protein |
| No cells  | −           | 13.5   | 13.9   |
| No cells  | +           | 13.2   | 14.7   |
| Rat hepatoma (HTC) | −          | 12.8   | 13.9   |
| Rat hepatoma (HTC) | +          | 13.2   | 15.0   |
| HeLa      | −           | 21.0   | 17.6   |
| HeLa      | +           | 61.5   | 81.0   |
| WI-38     | −           | 28.8   | 32.4   |
| WI-38     | +           | 81.4   | 86.3   |
| Dog thyroid | −         | 14.6   | 12.5   |
| Dog thyroid | +         | 15.6   | 16.7   |

a Individual values rather than the mean are provided to indicate the close agreement obtained in duplicate samples.
FIG. 5. The effect of human lymphocytes on thyroid cell morphology. Normal human thyroid cells were incubated at 37°C for 48 h in the absence (A) or presence (B, C, D) of normal human lymphocytes. A, human thyroid cells cultured without lymphocytes (× 250). B, the polygonal thyroid cells have undergone stellate transformation with the formation of many intertwining dendritic processes (× 250). C, in more heavily confluent cells, the presence of lymphocytes induces the formation of follicle-like spaces (× 250). D, some thyroid cells appear to attract clusters of lymphocytes to the cell body. These lymphocytes are much darker than nonadherent lymphocytes. It is not clear whether these lymphocytes are extracellular or have been phagocytosed (× 400).

TABLE V
Effect of anti-human immunoglobulin antisera on LTS activity on target human thyroid cells

| Source of Lymphocytes* | Precipitated µl | Experiment 1 Normal | Experiment 2 Normal | Experiment 3 Graves |
|------------------------|-----------------|----------------------|----------------------|----------------------|
| Control                | 0               | 15.8                 | 10.8                 | 12.8                 |
| LTS                    | 0               | 91.8                 | 73.5                 | 175.4                |
| LTS Polyvalent         | 5               | 97.3                 | 65.5                 | 142.9                |
| LTS IgG                | 5               | 97.3                 | 65.5                 | 142.9                |
| LTS IgA                | 10              | 86.5                 |                      |                      |
| LTS IgM+               | 40              | 92.5                 |                      |                      |
| Control                | 0               | 748.0                | 319.7                |                      |

* Three separately performed experiments with different human thyroid cell subcultures.

TABLE VI
Some properties of LTS

| Medium | Treatment | Target human thyroid cell cAMP content* pmol/mg protein |
|--------|-----------|--------------------------------------------------------|
| Control|           | 16.0                                                   |
| LTS‡   | Amicon filtrate‡ | 265.9                                                   |
| LTS    | Amicon concentrate | 66.4                                                   |
| LTS    | Dialyzed‡ | 23.8                                                   |
| LTS    | 80°C, 10 min | 783.3                                                   |
| Control| TSH added| 591.1                                                   |

* Mean of values from duplicate dishes of cells.
‡ Normal human lymphocytes incubated with normal thyroid cells in 10% fetal calf serum for 3 days.
Centriflo membrane cone CP-55A (50,000 molecular weight "cut-off").
‡ Dialyzed against serum-free medium, 20 volumes, 3 changes over 24 h.
 Fifty millimunits/ml.

was not an artifact resulting from the presence of cAMP in the medium to be tested, which then contaminated the target cells. Direct assay of medium containing LTS did not reveal measurable cAMP content (data not shown).

In order to examine further its size, LTS was subjected to Sephadex G-25 gel filtration (Fig. 6). LTS bioactivity was...
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retarded on the column, eluting after the B chain of insulin ($M_r = 3500$) and vitamin $B_12$ ($M_r = 1355$). This confirmed the small nature of LTS and suggested a molecular weight for the factor of 1000 or less.

Preliminary data indicating that LTS bioactivity was unaffected by prolonged treatment with pronase and was extractable at acid pH with chloroform (29) suggested that LTS was a lipid. The differential extractability of LTS bioactivity by lipid solvents was therefore examined further. At pH 3.0, LTS was extracted by 2:1 chloroform:methanol as well as by diethyl ether (Table VII). At pH 7.4, more LTS bioactivity was extracted by diethyl ether than remained in the aqueous phase. These data suggested that LTS is an unsaturated fatty acid.

Since prostaglandins are unsaturated fatty acids which stimulate thyroid cell adenylate cyclase activity (30-32), it seemed possible that LTS might be a prostaglandin. To test this hypothesis, a sample of LTS containing a tracer quantity of $[^{3}H]PGE_2$ not sufficient to cause thyroid cell stimulation was applied to a Sephadex G-10 column. LTS bioactivity eluted in the same fractions as did the radioactive prostaglandin (Fig. 7), indicating that the molecular weight of LTS was similar to that of the prostaglandins.

Further evidence suggesting that LTS is a prostaglandin was obtained using paper chromatography. After preliminary experiments, this system was chosen over thin layer chromatography because of the practical advantages of being able to apply relatively large volumes of sample and the ease of eluting strips for the subsequent bioassay of LTS. In an acid solvents system (butanol:acetic acid:water:pyridine), LTS bioactivity migrated very close to the solvent front (Fig. 8), as did the bioactivities of a number of different prostaglandins. Of the prostaglandins tested, the migration of PGE, appeared to be closest to that of LTS, however in separate experiments $PGE_2$ behaved similarly (data not shown). As judged by their migration in the same system, a number of other dialyzable substances, either proven or potential thyroid stimulators, were excluded from being LTS. These included ATP, adenosine, cAMP, ADP, histamine, acetylcholine, epineph-

Fig. 6. Sephadex G-25 (fine) gel filtration of LTS. Culture medium obtained after the mixed culture of human lymphocytes and human thyroid cells was lyophilized and then extracted with 95% ethanol. The ethanol extract was evaporated, and the residue was resuspended in 50 mM NH$_4$HCO$_3$, pH 7.7, and applied to the column which was eluted with the same buffer. Fractions were lyophilized, resuspended in Leibovitz-15 medium, pH 7.4, with 0.5 mM 3-isobutyl-1-methyl xanthine, and tested for their ability to stimulate cAMP generation in target thyroid cells.

Table VII

| Solvent                 | LTS bioactivity % basal | pH 3.0 | pH 7.4 |
|-------------------------|-------------------------|--------|--------|
| Chloroform:methanol (2:1)| Aqueous phase           | 266    | 2339   |
|                         | Organic phase           | 2284   | 402    |
|                         | Maximum (unextracted)   |         | 2652   |
| Ethyl ether             | Aqueous phase           | 206    | 504    |
|                         | Organic phase           | 2835   | 2100   |
|                         | Maximum (unextracted)   |         | 2750   |

Fig. 7. Sephadex G-10 gel filtration of a sample containing a mixture of LTS and $[^{3}H]PGE_2$. LTS was prepared, eluted, and bioassayed as described in Fig. 6.

Fig. 8. Paper chromatography of LTS and a variety of known or potential dialyzable thyroid stimulators (butanol:acetic acid:water:pyridine, 15:3:12:10). LTS and prostaglandins were bioassayed and the other compounds were visualized under ultraviolet light or after staining with ninhydrin. For the bioassay, the samples were evaporated, resuspended in Leibovitz-15 medium, pH 7.4, containing 0.5 mM 3-isobutyl-1-methyl xanthine, and measured for their ability to stimulate cAMP generation in target thyroid cells.
FIG. 9. Paper chromatography of LTS and [3H]PGE$_1$ (butanol:ethanol:2 M ammonia, 10:3:10). Paper strips 1-cm-wide were eluted and assayed for radioactivity and LTS bioactivity. Bioactivity was measured as described in Fig. 8.

FIG. 10. Time course of stimulation of cAMP generation in cultured human thyroid cells by LTS and 10$^{-6}$ M PGE$_1$. Incubations were performed at 37°C in Leibovitz-15 medium, pH 7.4, containing 0.5 mM 3-isobutyl-1-methylxanthine, and isoproterenol (Fig. 8). In an alkaline solvent system (butanol:ethanol:2 N ammonia), LTS migrated similarly to [3H]PGE$_1$ (Fig. 9). The co-migration of LTS and prostaglandins in two separate solvent systems therefore provided further evidence that LTS is a prostaglandin or a closely related substance.

In order to compare further the biological properties of LTS and prostaglandins, the time course of stimulation of thyroid cell cAMP content by LTS and PGE$_1$ was examined. Measured simultaneously, the kinetics of LTS and PGE$_1$ were similar, with maximal stimulation occurring at 10 to 15-min (Fig. 10).

Since indomethacin is a powerful inhibitor of prostaglandin synthesis, the presence of this agent during the period of mixed culture of human lymphocytes and human thyroid cells would be expected to inhibit the generation of LTS if LTS were indeed a prostaglandin. This was found to be the case. Thus, indomethacin inhibited LTS with a half-maximal inhibitory effect at approximately 10$^{-5}$ M (Fig. 11). Total inhibition of LTS generation was achieved at an indomethacin concentration of 10$^{-7}$ M. These data strongly suggested that LTS is a prostaglandin.

Finally, evidence supporting the prostaglandin-like nature of LTS was obtained by silicic acid chromatography. This method provides the additional advantage of distinguishing between three major classes of prostaglandins. Columns were initially calibrated using radiolabeled prostaglandins as standards. In confirmation of the findings of Jaffe et al. (28), [3H]PGE$_1$ eluted almost entirely in Fraction 2 as did [3H]PGF$_2$ in Fraction 3 (Table VIII). Only negligible quantities of [3H]PGE$_1$ and [3H]PGF$_2$ appeared in Fraction 1, in which PGA and PGB elute. Chromatography of LTS bioactivity on these columns demonstrated that LTS was not a single prostaglandin but was instead a combination of PGE and PGF, primarily the former (Table VIII). Although PGA and PGB were previously shown to have biological activity in our system (Fig. 8), no LTS bioactivity was present in the first fraction corresponding to PGA and PGB.

**DISCUSSION**

The present study describes the production of a thermostable, dialyzable stimulator of thyroid cell cAMP content as a result of the co-culture of peripheral human lymphocytes and human thyroid cells in monolayer. It is shown that the lymphocyte is the site of production of the stimulator. Thus, (a) thyroid cells cultured alone do not produce the stimulator, (b) heat-inactivated lymphocytes co-cultured with living thyroid cells do not generate the stimulator, and (c) viable lymphocytes co-cultured with a nonviable thyroid cell particulate fraction do produce the stimulator. We have therefore termed this factor lymphocyte thyroid stimulator (LTS). Strong evidence is provided that LTS is a mixture of prostaglandins E and F, primarily the former.
Prostaglandins are ubiquitous and their production has been described in many tissues. With regard to lymphoid cells, Ferraris and DeRubertis (33) have demonstrated that stimulation of mouse spleen cells by staphylococcal enterotoxin B and mitogens results in prostaglandin E generation. Similarly, the intravenous injection of sheep erythrocytes into mice is followed by PGF₂α synthesis within the spleen (34). To our knowledge, however, the present report is the first to indicate that antigenic stimulation can induce prostaglandin production by human peripheral lymphocytes.

There is considerable evidence that prostaglandins play a role in modulating lymphocytic function. Thus, prostaglandins have been shown to suppress the lymphocytic response to antigen stimulation with the subsequent inhibition of lymphocyte-mediated cytotoxicity (34-38). In addition to this inhibitory effect, prostaglandins may also be important mediators of the inflammatory process (38). Our data, together with those of others (33, 34), suggest that prostaglandins may, in part, mediate the effects of lymphocytes on target cells in the cell-mediated immunity process. The present findings may therefore prove to be of significance in a variety of immune and inflammatory processes such as rheumatoid arthritis.

Our data differ in a number of respects from previous data obtained following the exposure of human lymphocytes to thyroid tissue antigen (12). Thus, in the present study, (a) normal lymphocytes as well as lymphocytes from patients with Graves’ disease released a thyroid stimulating factor (LTS) into the culture medium and (b) the antigen necessary to induce the production of LTS was present not only in human thyroid cells but also in a number of other human cells. It must be emphasized, however, that these data cannot readily be compared because the stimulatory factor described by others has been shown to be an immunoglobulin (9, 10, 12), whereas the stimulatory substance demonstrated in our system is a prostaglandin.

Although our studies were directed at investigating the pathogenesis of Graves’ disease, it is unclear from the present data whether LTS plays a role in the hyperthyroidism of Graves’ disease, and if so, how this role is related to the thyroid stimulating antibodies which are of fundamental importance in the disease (40). Williams et al. (41) have suggested the possibility of a nonimmunoglobulin thyroid stimulator in Graves’ disease on the basis of studies in which dexamethasone produced a clinical improvement in hyperthyroidism faster than could be explained by the disappearance from the circulation of stimulatory immunoglobulins. A direct effect of glucocorticoids on the thyroid is unlikely in view of the evidence that glucocorticoids do not impair the thyroid response to TSH stimulation (41–45). Since certain prostaglandins are well known stimulators of thyroid tissue in vitro (30–32), and of thyroid hormone secretion in vivo (46), and since lymphocytic filtration is a histopathological feature of the thyroid in Graves’ disease (47), one consideration is that prostaglandins, produced locally within the thyroid gland, may play a role in the hyperthyroidism of Graves’ disease. Prostaglandins may produce local vascular dilatation, and it is therefore also possible that these agents may be responsible for the hypervascularity of the thyroid gland which is a feature of Graves’ disease.

Our observation of an in vitro lack of difference between control lymphocytes and Graves’ lymphocytes in their ability to produce LTS may be evidence against a role for LTS in Graves’ disease. On the other hand, there is increasing evidence that lymphocytes from healthy individuals have the potential to respond to self-antigens and that the expression of autoimmune disease is prevented by both cellular and humoral mechanisms of lymphocyte suppression (48–50). Further, there is evidence that, in vitro, the normal immunological checks and balances that exist in vivo may be disturbed, with the consequent loss of tolerance of normal lymphocytes for normal tissue antigen (51, 52). Another alternative is that cells in tissue culture may expose normally cryptic tissue antigens which are then recognized by the lymphocytes (53). Finally, it is possible that in our in vitro system humoral suppressive factors may have been diluted in that 10% autologous serum was used.

A second line of evidence against a role for LTS in the hyperthyroidism of Graves’ disease may be the fact that LTS stimulated human fibroblast cAMP accumulation in some of the present experiments and is therefore not a unique thyroid stimulator. However, a lack of thyroid specificity does not exclude a pathogenetic role for prostaglandins in Graves’ disease. Thus, since the thyroid is infiltrated with lymphocytes in this disease, thyroid stimulation may be produced by virtue of a high local concentration within the gland of a nonspecific stimulator, i.e. the specificity of the stimulation may be determined by the site of lymphocytic infiltration rather than by the specificity of the stimulatory substance.

Third, LTS may not be of importance in the pathogenesis of the hyperthyroidism of Graves’ disease because the cellular antigen responsible for the induction of LTS was not unique to human thyroid cells but was also common to a variety of human cells. It is of interest that previous studies have demonstrated that lymphocytes cultured together with retroorbital fibroblasts stimulate mucopolysaccharide synthesis by the latter, a process mimicked by cAMP and abolished by corticosteroids (54, 55). In addition, there are recent data demonstrating that PGE stimulates fibroblast mucopolysaccharide production (56). These results obtained in a different system are therefore quite similar to ours and raise the possibility that we are observing the same phenomenon. Since Werner et al. (51) have demonstrated by immunofluorescent techniques that the immune reaction in Graves’ disease appears to be limited to the thyroid tissue stroma and follicular basement membrane, it cannot be excluded that the infiltration of thyroid tissue by lymphocytes in Graves’ disease represents an immune response directed against thyroid connective tissue cells rather than the thyroid cells themselves, and the thyroid cells are stimulated as innocent bystanders. These data may in the future also provide a clue as to the relationship between the thyroidal and extrathyroidal manifestations of Graves’ disease in that these may all involve the same pathogenetic mechanism (prostaglandin generation) with the different clinical features depending upon the site and extent of lymphocytic infiltration. For the present, however, any relationship between LTS and Graves’ disease remains tenuous and speculative.

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REFERENCES
1. Adams, D. D., and Purves, H. D. (1967) Metabolism 6, 26–35
2. Krias, J. P., Pleshakov, V., and Chien, J. R. (1964) J. Clin. Endocrinol. Metab. 24, 1005–1028
3. McKenzie, J. M. (1968) Physiol. Rev. 48, 252–310
29. Rapoport, B., Pillarisetty, R. J., Herman, E. A., and Congco, E. G. (1977) Biochem. Biophys. Res. Comm. 77, 1245-1250
30. Kaneko, T., Zor, U., and Field, J. B. (1969) Science 163, 1062-1063
31. Burke, G. (1970) Am. J. Physiol. 218, 1445-1452
32. Wolff, J., and Jones, A. B. (1971) J. Biol. Chem. 246, 3939-3947
33. Ferris, V. A., and DeRubertis, F. R. (1974) J. Clin. Invest. 54, 378-386
34. Webb, D. R., and Osheroff, P. L. (1976) Proc. Natl. Acad. Sci. U. S. A. 73, 1300-1304
35. Henney, C. S., Bourgi, H. R., and Lichtenstein, L. M. (1972) J. Immunol. 108, 1529-1534
36. Strom, T. B., Deisseroth, A., Morganroth, J., Carpenter, C. B., and Merrill, J. P. (1972) Proc. Natl. Acad. Sci. U. S. A. 69, 2995-2999
37. Matus, E., Shearer, G. M., Meimmon, K. L., and Bourne, H. R. (1973) Cell Immunol. 9, 226-233
38. Strom, T. B., Carpenter, C. B., Cragoe, E. J., Norris, S., Devlin, R., and Perper, R. J. (1977) Transplant. Proc. 9, 1075-1079
39. Marx, J. L. (1972) Science 177, 780-781
40. Solomon, D. H., and Kleeman, B. A. (1977) in Advances in Internal Medicine (Stollerman, G. H., ed.) Vol. 22, pp. 273-299, Year Book Medical Publishers, Inc., Chicago
41. Williams, D. E., Chopra, I. J., Orgiess1, J., and Solomon, D. H. (1976) J. Clin. Endocrinol. Metab. 41, 354-361
42. Ingbar, S., and Freinkel, N. (1965) Metabolism 5, 652-666
43. Niccolot, J. T., Fisher, D. A., and Appleman, M. D., Jr. (1970) J. Clin. Invest. 49, 1922-1929
44. Vigneri, R., Pezzino, V., Filetti, S., Squatrito, S., Galbiati, A., and Poisla, M. (1974) J. Clin. Endocrinol. Metab. 39, 1090-1102
45. Marx, J. L. (1972) Science 177, 780-781
46. Solomon, D. H., and Kleeman, B. A. (1977) in Advances in Internal Medicine (Stollerman, G. H., ed.) Vol. 22, pp. 273-299, Year Book Medical Publishers, Inc., Chicago
47. Williams, D. E., Chopra, I. J., Orgiess1, J., and Solomon, D. H. (1976) J. Clin. Endocrinol. Metab. 41, 354-361
48. Ingbar, S., and Freinkel, N. (1965) Metabolism 5, 652-666
49. Niccolot, J. T., Fisher, D. A., and Appleman, M. D., Jr. (1970) J. Clin. Invest. 49, 1922-1929
50. Vigneri, R., Pezzino, V., Filetti, S., Squatrito, S., Galbiati, A., and Poisla, M. (1974) J. Clin. Endocrinol. Metab. 39, 1090-1102
51. Marx, J. L. (1972) Science 177, 780-781
52. Solomon, D. H., and Kleeman, B. A. (1977) in Advances in Internal Medicine (Stollerman, G. H., ed.) Vol. 22, pp. 273-299, Year Book Medical Publishers, Inc., Chicago
53. Williams, D. E., Chopra, I. J., Orgiess1, J., and Solomon, D. H. (1976) J. Clin. Endocrinol. Metab. 41, 354-361
54. Ingbar, S., and Freinkel, N. (1965) Metabolism 5, 652-666
55. Niccolot, J. T., Fisher, D. A., and Appleman, M. D., Jr. (1970) J. Clin. Invest. 49, 1922-1929
56. Vigneri, R., Pezzino, V., Filetti, S., Squatrito, S., Galbiati, A., and Poisla, M. (1974) J. Clin. Endocrinol. Metab. 39, 1090-1102
57. Marx, J. L. (1972) Science 177, 780-781
58. Solomon, D. H., and Kleeman, B. A. (1977) in Advances in Internal Medicine (Stollerman, G. H., ed.) Vol. 22, pp. 273-299, Year Book Medical Publishers, Inc., Chicago
59. Williams, D. E., Chopra, I. J., Orgiess1, J., and Solomon, D. H. (1976) J. Clin. Endocrinol. Metab. 41, 354-361
60. Ingbar, S., and Freinkel, N. (1965) Metabolism 5, 652-666
61. Niccolot, J. T., Fisher, D. A., and Appleman, M. D., Jr. (1970) J. Clin. Invest. 49, 1922-1929
62. Vigneri, R., Pezzino, V., Filetti, S., Squatrito, S., Galbiati, A., and Poisla, M. (1974) J. Clin. Endocrinol. Metab. 39, 1090-1102
63. Marx, J. L. (1972) Science 177, 780-781
64. Solomon, D. H., and Kleeman, B. A. (1977) in Advances in Internal Medicine (Stollerman, G. H., ed.) Vol. 22, pp. 273-299, Year Book Medical Publishers, Inc., Chicago
65. Williams, D. E., Chopra, I. J., Orgiess1, J., and Solomon, D. H. (1976) J. Clin. Endocrinol. Metab. 41, 354-361
66. Ingbar, S., and Freinkel, N. (1965) Metabolism 5, 652-666
67. Niccolot, J. T., Fisher, D. A., and Appleman, M. D., Jr. (1970) J. Clin. Invest. 49, 1922-1929
68. Vigneri, R., Pezzino, V., Filetti, S., Squatrito, S., Galbiati, A., and Poisla, M. (1974) J. Clin. Endocrinol. Metab. 39, 1090-1102
69. Marx, J. L. (1972) Science 177, 780-781
70. Solomon, D. H., and Kleeman, B. A. (1977) in Advances in Internal Medicine (Stollerman, G. H., ed.) Vol. 22, pp. 273-299, Year Book Medical Publishers, Inc., Chicago
71. Williams, D. E., Chopra, I. J., Orgiess1, J., and Solomon, D. H. (1976) J. Clin. Endocrinol. Metab. 41, 354-361
72. Ingbar, S., and Freinkel, N. (1965) Metabolism 5, 652-666
73. Niccolot, J. T., Fisher, D. A., and Appleman, M. D., Jr. (1970) J. Clin. Invest. 49, 1922-1929
74. Vigneri, R., Pezzino, V., Filetti, S., Squatrito, S., Galbiati, A., and Poisla, M. (1974) J. Clin. Endocrinol. Metab. 39, 1090-1102
75. Marx, J. L. (1972) Science 177, 780-781
76. Solomon, D. H., and Kleeman, B. A. (1977) in Advances in Internal Medicine (Stollerman, G. H., ed.) Vol. 22, pp. 273-299, Year Book Medical Publishers, Inc., Chicago
77. Williams, D. E., Chopra, I. J., Orgiess1, J., and Solomon, D. H. (1976) J. Clin. Endocrinol. Metab. 41, 354-361
78. Ingbar, S., and Freinkel, N. (1965) Metabolism 5, 652-666
79. Niccolot, J. T., Fisher, D. A., and Appleman, M. D., Jr. (1970) J. Clin. Invest. 49, 1922-1929
Production of a non-immunoglobulin thyroid stimulator by human lymphocytes during mixed culture with human thyroid cells.
B Rapoport, R J Pillarisetty, E A Herman, O H Clark and E G Congco

J. Biol. Chem. 1978, 253:631-640.