Prostaglandin Generation in Vitro by Peripheral Blood Mononuclear Cells in Co-culture with Human Thyroid Cells*

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Studies from a number of laboratories (1-4) have demonstrated that, following exposure to human thyroid antigen or mitogens, peripheral blood mononuclear cells from patients with Graves' disease generate thyroid-stimulating immunoglobulins. Using co-cultures of human thyroid cells and human peripheral blood mononuclear cells, we have confirmed that the latter cells release into the medium a substance which, when added to fresh cultures of human thyroid cells, stimulates cyclic adenosine 3',5'-monophosphate (cyclic AMP) generation (5). Surprisingly, however, this substance was not neutralized by anti-immunoglobulin antisera and was found to be dialyzable and relatively heat stable (5). The prostaglandin-like nature of this factor was suggested by its extraction with organic solvents at acid pH, as well as by chromatographic studies, by the time course of its biological action, and by the inhibition of its generation by low concentrations of indomethacin and corticosteroids (5). Silicic acid chromatography suggested further that the substance was primarily prostaglandin E (PGE), with a lesser amount of prostaglandin F (PGF) (5).

The objective of the present study was to establish by direct measurement that the biologically active thyroid cell stimulator described above is, in fact, a prostaglandin. Two approaches were employed. First, culture medium concentrations of three different prostaglandins were measured by specific radioimmunoassays during the co-culture of human peripheral blood mononuclear cells and human thyroid cells. Second, a comparison was made between the biological potency, with respect to thyroid cell cyclic AMP generation, of synthetic prostaglandins and endogenously produced prostaglandins as measured by radioimmunoassay. This study demonstrates that human thyroid cells can provoke immunosayable prostaglandin generation by human peripheral blood mononuclear cells. It is shown further that of the various prostaglandins produced, only PGE (primarily PGE2) is present at a biologically active concentration.

MATERIALS AND METHODS

Cell Cultures—Human thyroid cells were established in monolayer tissue culture as previously described (5, 6). The present experiments utilized a line of benign human follicular adenoma cells (19HT), established in November, 1976. These cells behave identically with normal human thyroid cells in generating thyroid stimulator activity by human peripheral blood mononuclear cells.

Human peripheral blood mononuclear cells from normal subjects were prepared by a standard Ficoll-Hypaque technique (7). We have previously reported no difference, in vitro, between normal mononuclear cells and mononuclear cells from patients with Graves' disease in their ability to generate the thyroid-stimulating substance when co-cultured with human thyroid cells (5). Mononuclear cells were co-

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cultured with human thyroid cell monolayers in Dulbecco’s modified Eagles’ medium, pH 7.4, containing 10% fetal calf serum, 2 mM glutamine, and 20 mM HEPES and nonessential amino acids, as well as 100 units of penicillin, 100 μg of neomycin, and 2.5 μg of fungizone/ml of medium. Approximately 1 x 10^6 mononuclear cells and 1 x 10^6 thyroid cells were cultured in 35-mm diameter culture dishes (Coming) containing 1.2 ml of medium. At the indicated time intervals, the incubations were ended by aspirating the medium from the thyroid cell monolayers and the mononuclear cells in the medium were removed by centrifugation at 1000 x g for 15 min. As controls, incubations were also performed with both thyroid cells and peripheral blood mononuclear cells alone.

Radioimmunoassays—Media to be assayed for prostaglandins were extracted and separated by the method of Jaffe et al. (8). Three milliliters of ethyl acetate/isopropyl alcohol 0.1 N HCl (3:3:1, v/v) was added to 0.5 ml of cell-free culture medium and the mixture vortexed vigorously. Three milliliters of 0.9% NaCl and 2 ml of ethyl acetate were then added and the mixture vortexed again. Three milliliters of the organic layer was removed and evaporated. The residue was dissolved in 0.2 ml of benzene/methyl acetate methanol (60:40:1, v/v) and 0.6 ml of benzene/methyl acetate (60:40, v/v) and then applied to silicic acid columns. Variation in the recovery rate was very small within each experiment. For example, the mean recovery rate for PGE in six replicate samples was 56 ± 3% S.D. (coefficient of variation of 6%). The recovery rate for PGF was generally higher, approximately 80%.

Radioimmunoassay of prostaglandins was performed by the double antibody technique of Levine et al. (9). PGB and PGB were measured with rabbit anti-PGB and anti-PGB antisera, respectively. For the assay of PGE, the second fraction eluted from the silicic acid column was treated with 0.5 ml of 1 N NaOH for 2 h at 37°C and then with 0.5 ml of 0.1 N HCl at 70°C to 80°C (10), and the latter was subsequently measured using the anti-PGB antisera. Cross-reactivity of PGB (and hence of PGE) with the PGB antisera was 17%.

Cross-reactivity at 50% displacement of other prostaglandins with the PGB antisera was as follows: PGE, 15%; PGD, 3%; PGE, 4%; PGE, 0.2%; and PGB, <0.2%. With the PGE antisera, at 50% displacement, the cross-reactivity of PGE was 38%; and the cross-reactivity of PGA, PGB, and PGE was <0.2%. Because of these relatively large cross-reactivities between prostaglandins of the same group differing merely by a double bond, such as between PGE, and PGE2, all data regarding radioimmunoassayable prostaglandin cross-reactivity of PGAI, PGBI, and PGE was ~0.2%. Because of the cross-reactivity of PGAI and PGBI and PGE with the PBG, antisem was 17%.

RESULTS

Time Course of Prostaglandin Generation—In previous experiments in this laboratory (5), it was observed that the culture medium obtained following thyroid cell-leukocyte co-cultures stimulated thyroid cell cyclic AMP generation. It was, therefore, of interest to determine by direct measurement how the accumulation of immunoassayable prostaglandins in the medium correlated with the time course of the appearance of bioactivity. When normal peripheral blood mononuclear cells were cultured together with human thyroid cells in monolayer for up to 7 days, progressive accumulations of radioimmunoassayable PGE, PGB, and PGF were observed in the culture media (Fig. 1). Thyroid cells cultured alone released no detectable prostaglandins into the medium. Cultures of blood mononuclear cells without thyroid cells accumulated from 25% (PGE) to 50% (PGF) of the prostaglandin concentrations observed when both cell types were cultured together. In the experiments shown (Fig. 1), the maximal PGE concentration achieved in the culture medium (3 x 10^9 M) was intermediate to the concentrations of PGF (9 x 10^9 M) and PGB (1 x 10^9 M) in the same medium. Similar relationships were observed in three separate experiments using leukocytes from other normal subjects. Maximal PGE concentrations attained in five different experiments (including the three mentioned above) were between 2 x 10^9 M and 10^7 M (mean ± S.D.; 3.8 ± 3.5 x 10^9 M). These data indicate that the time course of immunoassayable prostaglandin generation is the same as that previously reported for the appearance of thyroid cell-stimulating bioactivity (5), with the exception that an initial time lag was seen in the generation of bioactivity. It is likely that this time lag represents the time taken for sufficient PGE to accumulate in the medium to be biologically active in terms of stimulating thyroid cell cyclic AMP content. Close parallelism in the PGE radioimmunoassay was observed between dilutions of PGE standard and dilutions of material extracted from culture medium (Fig 2). This demonstrates that the measurement of endogenously produced PGE is not influenced nonspecifically by other material possibly extracted from the medium.
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FIG. 1. Time course of prostaglandin generation. Human peripheral blood mononuclear cells (L) and human thyroid cells (T) were cultured together for up to 168 h (T/L). Parallel cultures were also established with blood mononuclear cells alone (−/L) and thyroid cells alone (T/−). At the indicated time intervals, the media were assayed for PGE, PGB, and PGF concentrations by the radioimmunoassay procedure described under “Materials and Methods.” Each point represents the mean value obtained from triplicate culture dishes. The brackets indicate the S.D. from the mean.

FIG. 2. Comparison in the PGE radioimmunoassay of dilutions of an extract of culture medium and PGE1 standard. After the culture of human peripheral blood mononuclear cells together with human thyroid cells for 3 days, the culture medium was extracted and subjected to silicic acid column chromatography as described under “Materials and Methods.” The second (PGE) fraction was evaporated and resuspended in radioimmunoassay buffer. Serial dilutions (up to 64-fold) were made of this material, as well as of a PGE1 standard (7500 pg/ml, original concentration). Both were then treated with NaOH in order to convert PGE to PGB and then assayed with an anti-PGB antibody as described under “Materials and Methods.” Each point represents the mean logit value, ±S.D., as determined in triplicate samples.

FIG. 3. Effect of prostaglandins on human thyroid cell cyclic AMP content. Culture dishes of human thyroid cells in monolayer were incubated for 15 min at 37°C in Leibovitz-15 medium containing 0.5 mM 3-isobutyl-1-methylxanthine and the indicated concentrations of prostaglandins. After this incubation period, the medium was aspirated and the cellular cyclic AMP content was measured as described under “Materials and Methods.” Each point represents the mean of triplicate determinations. The shaded area represents the concentrations of endogenously produced prostaglandins measured in the medium by radioimmunoassay after thyroid cell-leukocyte co-cultures.

The biological potency of a variety of synthetic prostaglandins was tested in the same system (Fig. 3). Of the prostaglandins tested, only PGE1 and PGE2 stimulated thyroid cell cyclic AMP generation at concentrations comparable to those attained in the medium during thyroid cell-blood mononuclear...
cell co-cultures (shown by the shaded area). Thus, even at 10^{-6} M, a greater concentration than at any time measured in culture medium, prostaglandins of the A, B, and F variety were without effect on thyroid cell cyclic AMP generation. In contrast, cyclic AMP stimulation (140% of basal values) was observed at 10^{-6} M PGE, and PGE_2.

**Arachidonic Acid Incorporation into PGE during Thyroid Cell-Leukocyte Co-cultures**—In order to substantiate further the generation of PGE during thyroid cell-leukocyte co-cultures, cultures of these cells were conducted for 3 days in the presence of [^3]H]arachidonic acid. Following extraction of the medium with acid ethyl acetate and isopropyl alcohol, the radiolabeled prostaglandins were fractionated on silicic acid columns. The second fraction, containing primarily PGE, was evaporated, resuspended in methanol, and subjected to thin layer chromatography as described under "Materials and Methods." Significant generation of [^3]H]PGE was observed (Fig. 4). A much smaller quantity of PGF_2_\alpha was noted and probably represents contamination of the second silicic acid column fraction with a small amount of PGF. More surprising was the appearance of about 25% of the radioactive material together with the PGE standard. Since arachidonic acid is not a precursor of PGE_1, it is unclear whether or not this represents synthesis of PGE_1 or of another unknown metabolite. Because of the prolonged period of incubation, it is also possible that some recycling of the tritium label into a PGE_1 precursor may have occurred. Control dishes of thyroid cells cultured alone in the presence of [^3]H]arachidonic acid produced no measurable [^3]H]prostaglandins in the medium.

**Effect of Indomethacin on Prostaglandin Generation**—In previous studies in which stimulatory activity in thyroid cell-blood mononuclear cell culture medium was measured by bioassay, the presence of indomethacin during the period of cell co-culture abolished the generation of such bioactivity in the medium (5). The effect of indomethacin on medium radioimmunoassayable PGE, PGF, and PGB was, therefore, determined in similar co-cultures of human thyroid cells and peripheral blood mononuclear cells (Fig. 5). As expected, increasing concentrations of the prostaglandin synthetase inhibitor indomethacin progressively inhibited the accumulation of these prostaglandins in the medium. More important, comparison of the dose-inhibitory effect of indomethacin on PGE and bioactivity accumulation in the culture medium, previously reported by our laboratory (5), reveals half maximal inhibition of both at approximately 10^{-8} M indomethacin.

**Effect of Cortisol on Prostaglandin Generation**—Glucocorticoids are known inhibitors of prostaglandin generation in some in vitro systems (13, 14), and we have shown previously that dexamethasone inhibits the generation of medium bioactivity during human thyroid cell-human peripheral blood mononuclear cell co-cultures (15). It was, therefore, of interest to determine whether glucocorticoids would similarly inhibit medium immunoassayable prostaglandin concentrations in our system.

The presence of increasing concentrations of cortisol in the medium during cell co-cultures progressively inhibited the release of PGE, PGB, and PGF into the medium (Fig. 6). A half-maximal inhibitory effect was observed at approximately 5 \times 10^{-8} M cortisol. Endogenous cortisol in fetal calf serum was undetectable by radioimmunoassay, suggesting a free cortisol concentration in 10% fetal calf serum of no greater than 10^{-8} M. As with the preceding experiment utilizing indomethacin, the dose-inhibitory effect of cortisol on PGE accumulation in the medium was the same as that on the accumulation of thyroid cell cyclic AMP stimulatory activity in the medium. Thus, with dexamethasone, half-maximal inhibition of bioactivity occurred at approximately 10^{-9} M (15). Inhibition of bioactivity with cortisol was half-maximal at approximately 5 \times 10^{-8} M (data not shown), consistent with its lesser potency. It is noteworthy that this concentration of cortisol is within the physiological range of unbound cortisol in human serum (16).

**Studies on the Mechanism of Interaction between Thyroid Cells and Peripheral Blood Mononuclear Cells**—It was of interest to determine whether cultured human thyroid cells directly stimulated PGE generation by leukocytes, or whether they did so indirectly by releasing a stimulator into the
medium, or by making available to the leukocytes a substance such as arachidonic acid. To test this, culture medium preincubated for 3 days, either alone or together with thyroid cells, was then added for a further 3 days to mononuclear leukocyte cultures (Table I). At the end of this time, PGE bioactivity in the medium was measured. As observed previously, leukocytes cultured without thyroid cells generated a small amount of PGE bioactivity in the medium. However, this bioactivity was the same whether the medium had been previously exposed to thyroid cells (198% of basal activity) or not (214% of basal activity). These data suggest that the thyroid cells are not releasing a soluble substance into the medium which secondarily enhances mononuclear cell PGE generation.

The foregoing experiment also raised the possibility that direct contact between thyroid cells and mononuclear leukocytes was necessary for enhancement of PGE generation by the latter. This question was approached by incubating both cell types in the same culture medium in a Marbrook flask (17), separated from each other by a semipermeable membrane with a pore size (0.2 μm) small enough to exclude passage of cells, but not soluble substances. After culture for 3 days, the cell-free medium was assayed for PGE bioactivity (Table II). In control incubations in which the membrane separating the cell types was punctured, considerable enhancement of PGE generation was produced. In contrast, in those incubations in which the semipermeable membrane separating the cells was intact, medium PGE bioactivity generated was considerably less and was only slightly greater than that observed in cultures containing leukocytes alone. Examination of the thyroid cell monolayer by phase contrast microscopy at the end of the 3-day culture period indicated that a small number of leukocytes were able to traverse from one compartment to the other despite the interposing intact membrane, possibly by migrating around the borders of the membrane which was attached to the inner chamber by a rubber band. This may account, in part, for the small amount of stimulation observed. It seems likely, therefore, that direct contact between the cell types is necessary for most, if not all, of the enhancement of leukocyte PGE generation to occur.

**TABLE I**

**Evidence that thyroid cells are not releasing a soluble substance into the medium to stimulate leukocyte prostaglandin generation**

<table>
<thead>
<tr>
<th>Incubation conditions</th>
<th>Source of medium</th>
<th>Thyroid cells</th>
<th>Leukocytes</th>
<th>Medium bioactivity&lt;sup&gt;a&lt;/sup&gt; % of basal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
<td>100 ± 2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>+</td>
<td>214 ± 5&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+</td>
<td></td>
<td>856 ± 37</td>
</tr>
<tr>
<td>Thyroid cell</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>preincubation&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
<td></td>
<td>+</td>
<td>142 ± 13</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>+</td>
<td>198 ± 5&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> Three-day incubations at 37°C of normal human peripheral blood mononuclear cells (approximately 1 x 10<sup>6</sup>) and human thyroid adenoma cells (approximately 1 x 10<sup>6</sup>) in 1 ml of medium in 35-mm diameter dishes.

<sup>b</sup> Measured by the cyclic AMP response of freshly subcultured thyroid cells to cell-free medium obtained at the end of the 3-day cultures as described under "Materials and Methods." Mean ± S.E. in triplicate dishes of cells.

<sup>c</sup> Dulbecco's modified Eagles medium, pH 7.4, containing 10% fetal calf serum and 20 mM Hapes; preincubated without cells for 3 days before addition to incubations.

<sup>d</sup> The same medium as in c was preincubated for 3 days with human thyroid adenoma cells (10 ml of medium in a flask with approximately 10<sup>6</sup> thyroid cells).

**FIG. 6.** Effect of cortisol on prostaglandin generation. Human peripheral blood mononuclear cells were cultured for 3 days together with human thyroid cells in cultured medium containing the indicated concentrations of cortisol. At the end of this period, medium prostaglandin concentrations were determined by radioimmunoassay as described under "Materials and Methods." Each point is the mean of values obtained in triplicate dishes of cells. The brackets indicate S.D.

**DISCUSSION**

This study provides strong evidence that the biologically active substance(s) released by human peripheral blood mononuclear cells during co-culture with human thyroid cells (5) is PGE, primarily PGE<sub>2</sub>. First, the time course of the generation of culture medium bioactivity (5) is consistent with the observed increase in medium prostaglandin concentrations. Because medium prostaglandins can be measured by radioimmunoassay at concentrations below the threshold of bioactivity, it seems likely that the previously noted lag phase (5) in the appearance of medium bioactivity is not, in fact, a period of mononuclear cell quiescence. Prostaglandins begin to appear in the medium from the onset of culture, but bioactivity is only detected when this concentration reaches a critical threshold. Similarly, this difference in sensitivity between the bioassay and radioimmunoassay would seem to be a likely explanation as to why more radioimmunoassayable prostaglandin is released into the medium when blood mononuclear cells are cultured alone than was anticipated from the previous measurement of medium bioactivity (5). However, the observation that the generation of bioactive substance(s) is greatly

<sup>1</sup> Thyroid cell monolayer on floor of outer chamber of Marbrook two-chamber glass culture bottle; leukocytes in inner chamber immersed in the same culture medium but separated from thyroid cells by Nuclepore polycarbonate membrane (0.2 μm); 3-day incubations at 37°C as described under "Materials and Methods."

<sup>2</sup> Thyroid cell cyclic AMP response to cell-free medium at the end of the 3-day culture period, as described under "Materials and Methods."

<sup>3</sup> Control is leukocytes cultured alone in inner chamber without thyroid cells in outer chamber. Each value is the mean of closely agreeing duplicate determinations.

<sup>4</sup> Large holes made in membrane with scissors.
stimulated by the presence of human thyroid cells (5) is borne out by the present data.

A second line of evidence that the bioactive substance in the culture medium is a prostaglandin is the observation that immunosuppressible PGE concentrations in the medium approximate the concentration of exogenous, synthetic PGE necessary to produce a comparable biological effect on thyroid cell cyclic AMP generation. Thus, the PGE concentration attained in the culture medium is approximately $10^{-7}$ M, as measured by radioimmunoassay. The biological effect of endogenously produced thyroid stimulator is approximately equal to the effect of a similar concentration of synthetic PGE, namely $10^{-7}$ M. Dilutions of PGE standards and endogenously produced bioactive substance showed complete parallelism in the radioimmunoassay, demonstrating that it is PGE, and not a nonspecific substance interfering in the radioimmunoassay, that is being measured in the incubation medium. It is also apparent from the present data that even though PGF is present in the culture medium at a concentration much greater than PGE, only PGE is present at a bioactive concentration. We had previously concluded, on the basis of silicic acid separation of prostaglandins (5), that approximately 25% of medium bioactivity was produced by PGF. It now seems apparent that sufficient contamination with PGE must have occurred to account for the bioactivity seen in the PGF fraction.

The third line of evidence that prostaglandins are the stimulatory substances present in the culture medium is the remarkably similar sensitivity to glucocorticoid and indomethacin inhibition of both bioactivity and immunoassayable prostaglandin levels in the medium.

Interest is rapidly increasing in the role of prostaglandins in the modulation of the immune response (18, 19). Thus, prostaglandins have been reported to alter a variety of human lymphocytic functions including phytohemaggulatin-stimulated leukocyte inhibitory factor (20) and phytohemagglutinin-stimulated lymphocytic functions including phytohemagglutinin-stimulated lymphocytic responses (21). Ferraris and DeRubertis (22), by demonstrating prostaglandin generation by human peripheral blood mononuclear cells in the presence of mitogens, provided the first evidence that potentially regulatory prostaglandins are produced by immune cells themselves. More recently, evidence has been presented for the existence of a population of prostaglandin-producing peripheral blood mononuclear suppressor cells (23), the number or activity of which may be altered in certain disease states (24).

The present data now demonstrate that human peripheral blood mononuclear cell prostaglandin production can be stimulated by cultured thyroid cells. On the basis of previous studies (5), it seems likely that this mononuclear cell response is not specific for thyroid cells, but may be seen with other human cells as well. Because we used a homologous system, as have other investigators (2-4), it is possible that the phenomenon we observed was related to hitherto incompatibility. Against this possibility, however, is a unique experiment in which we were able to culture thyroid cells, blood mononuclear cells, and serum, all from the same individual. The generation of thyroid-stimulating bioactivity was still observed in this autologous system.\(^2\)

The mechanism by which the thyroid cells stimulate leukocyte prostaglandin generation is not known. It was previously shown that a broken thyroid cell preparation could stimulate prostaglandin production, but only by intact, viable leukocytes (5). The present study demonstrates further that the thyroid cells are not releasing a stimulator, or prostaglandin precursor such as arachidonic acid, into the culture medium which subsequently leads to enhanced leukocyte prostaglandin production. The data also suggest that direct contact between the two cell types is necessary for the expression of most, if not all, of this effect.

With respect to Graves' disease, prostaglandins are known stimulators of thyroid gland function (15-28), and peripheral blood mononuclear cells, as a potential source of prostaglandins, infiltrate the thyroid gland in this disease (29). The present study provides circumstantial evidence that prostaglandins, as local hormones, may play a role in the pathogenesis of Graves' disease. However, at the present time, this concept remains speculative and awaits direct experimental testing. Croxson et al. (30) have described a lack of effect of short term indomethacin therapy on thyroid hormone secretion in one patient with this disease. On the basis of the present data, a larger clinical study involving a longer therapeutic trial of indomethacin seems justified.

REFERENCES


\(^2\) B. Rapoport, unpublished data.
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