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A STUDY ON THE HEMOPOIETIC ACTIVITY OF HUMAN PLACENTA *IN VITRO*, IN THE PRESENCE AND ABSENCE OF STIMULATORS AND INHIBITORS OF TRANSCRIPTION AND TRANSLATION

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ABSTRACT

The hemopoietic activity of the human placenta was studied *in vitro* under various conditions in order to assess the optimal conditions of protein synthesis and colony stimulating factor (CSF) production by this tissue. The hemopoietic activity of placental tissue was assayed by the semi-solid agar medium technique. In order to obtain maximum hemopoietic activity, various stimulators were examined and it was determined that lipopolysaccharide was the most potent stimulator. Inhibitors of transcription and translation were also examined alone and in the presence of stimulators. They potently suppressed hemopoietic activity. Pulse-chase studies with the use of ^{3}H-leucine were used to determine the kinetics of protein synthesis of placental tissue *in vitro*.
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INTRODUCTION

Hemopoiesis is one of the most complex examples of multi-lineage differentiation. Pluripotent stem cells in the bone marrow give rise to large numbers of red cells, neutrophils, basophils, eosinophils, monocytes and lymphocytes. These blood cells are generally short-lived and must be continuously replenished.

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Under normal conditions *in vivo*, bone marrow is the site of hemopoiesis and other tissues have no significant hemopoietic activity. *In vitro* experiments in the past 30 years have shown that a number of glycoproteins and colony stimulating factors (CSFs) regulate the proliferation and differentiation of hemopoietic cells.[1-3]

Previous experiments have shown that human placenta is a good source of hemopoietic growth factors.[4-6] These findings and the fact that placental tissue is readily available in comparison with other human tissues, makes this tissue an ideal source for studies in this field. Hence, placenta was used as a source of human CSF and the effects of different compounds, such as stimulators and inhibitors, on the hemopoietic activity of this tissue were examined.

These studies, together with kinetic studies, provided necessary information about the ways to obtain maximum hemopoietic activity. At present, a number of recombinant hemopoietic growth factors have been produced[7-11] and are available commercially. Different stimulators such as lipopolysaccharide (LPS), concanavalin A (Con A), phytohemagglutinin (PHA), phorbol-12-myristate-13-acetate (PMA), ionomycin (Iono), and inhibitors like cycloheximide (CHX), actinomycin D (Act D), and puromycin (Pur) were used in order to obtain maximum hemopoietic activity or to examine their effects on hemopoiesis. Protein concentrations of different conditioned media and the kinetics of protein synthesis were also determined to be used as an indirect index of CSF production by the placental tissue.

MATERIALS AND METHODS

Tissue

Human placenta was acquired freshly after normal parturitions from Imam Khomeini General Hospital. Tissue samples were first rinsed with saline solution containing 0.81% glucose, pH 7.4, and then with the culture medium (0.01 g Dulbecco's Modified Eagle Medium (DMEM, GIBCO), per mL, supplemented with 0.12 mg penicillin and 0.303 mg streptomycin per mL). Tissue samples were maintained in this medium and transferred to the laboratory on ice.

Preparation of human placental conditioned medium (HPCM)

For the preparation of HPCM, 0.5 g tissue was placed in 5 mL of serum-free DMEM culture medium and minced. The minced tissue was incubated for different periods, under saturated humidity, 95% air, 7.5% CO_2 and 37°C. At the end of the incubation period the HPCM was treated as described elsewhere.[12] In short, the supernatant fluid was centrifuged at 2000 g and 4°C, heated for 30 min at 56°C, and centrifuged again as above. The supernatant was dialyzed against 40 volumes of distilled water for 48 hrs. The dialysate was centrifuged as above and, after addition of polyethylene glycol (1% final concentration), filtered through 0.45 micrometer membrane filters (Millipore). This HPCM was used without further treatment as a source of CSF in bioassay experiments.

Protein assay

Lowry's modified method[13] was used for determining the protein concentration of HPCM samples.

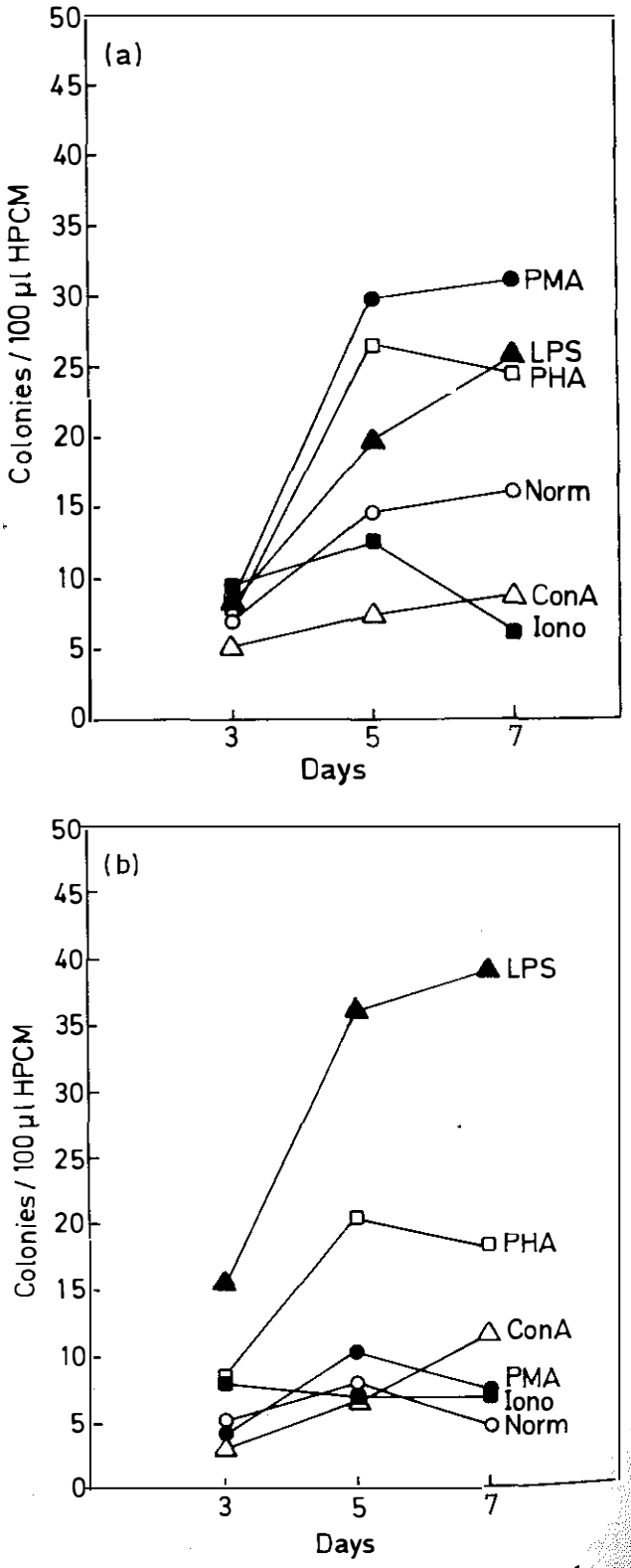

Fig. 1. Effects of different incubation periods on colony-forming capacity of placental tissue (normal and stimulated) estimated by 7(a) and 14(b) day bioassay periods.

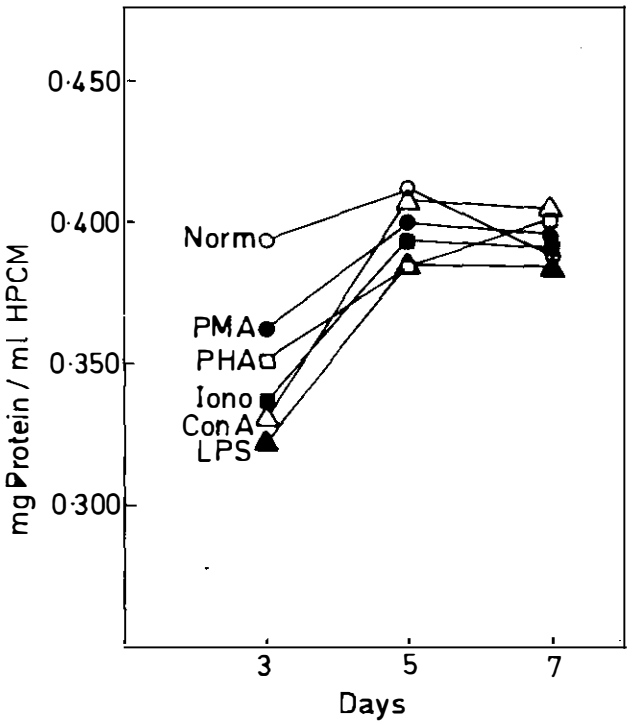

Fig. 2. Effects of different incubation periods on protein synthesis of placental tissue in the presence and absence of stimulator.

Stimulators and inhibitors

Stimulators were Con A (Sigma), PHA (GIBCO), LPS from *Salmonella typhimurium* (Sigma), Iono, and PMA (Fluka). Con A was dissolved in DMEM, LPS and PHA in distilled water and Iono and PMA in 0.2% acetone. Inhibitors were Act D, Pur, and CHX (Sigma). Act D was dissolved in ethanol and the other two in distilled water. Stimulators and inhibitors were sterilized by ultrafiltration through Millipore filters (0.45 micrometer).

Hemopoietic activity assay

HPCM prepared by the above-mentioned procedure were assayed in semi-solid agar medium.[2,3,12,25] Assays were conducted either in 35 mm petri dishes or in 96 well microplates. For petri dishes 10^5 marrow cells obtained from NIH mice were cultured in 1 mL of DMEM containing 10% HPCM, 0.3% agar, 20% fetal calf serum (FCS) and antibiotics. In microwell cultures, 10^4 marrow cells were cultured in each well containing 100 µL of DMEM containing 10% HPCM, 20% FCS, 0.3% agar and antibiotics. Placebo cultures contained no HPCM. Cultures were incubated either 7 or 14 days in 5% CO_2 at 37°C with full humidity. Hemopoietic cell colonies were stained by INT [3-(4-iodophenyl)-2-(4-nitro-phenyl)-5-phenyl(2H-tetrazolium chloride)] (Merck). Aggregates containing 40 cells or more were considered colonies. The entire plate or well was counted under a dissecting microscope. Cell aggregates containing fewer than 40 cells were considered clusters.

Kinetic studies

The kinetics of protein synthesis were studied by measuring the amount of radioactivity incorporated during incubation following the use of radioactive leucine (^{3}H-Leu, Amersham). During 5 days of incubation period, every 24 hours, the supernatant fluid from each petri dish containing placental tissue was removed and replaced with fresh culture medium supplemented with ^{3}H-Leu. The concentration of ^{3}H-Leu was 1.2 microCurie per mL. After 15 minutes the radioactive medium was collected; the tissues were rinsed with fresh nonradioactive medium and incubated in the same medium for 24 hrs. The above procedure was repeated until the termination of incubation. Radioactive samples taken after 15 minute pulses were counted in a LKB liquid scintillation counter (LKB 1219 Rackbeta).

RESULTS

Figure 1 shows the effect of different incubation times on colony formation in the presence and absence of stimulators. Figure 1-a shows the hemopoietic activity of different samples when bioassay cultures were incubated for 7 days. Samples containing PMA, LPS and PHA showed stimulatory activity in comparison with normal samples. Figure 1-b shows the effect of different incubation times of conditioned media when the bioassay cultures were incubated for 14 days. It is clear that the most significant change in activity occurred when the incubation period of conditioned media was changed from 3 to 5 days. Further increase in the incubation time either decreased the activity (PHA and Iono samples in Fig. 1-a, and PHA, PMA, and normal samples in Fig. 1-b) or had moderate effects. A placebo culture containing saline instead of HPCM was set up in every experiment. No colonies were observed in these cultures.

Total protein concentration of the conditioned media is shown in Figure 2. Maximum protein concentration was on day 5 of incubation. The difference between various samples was not statistically significant. Protein synthesis is an index of biological activity. Therefore, this observation emphasized that the tissues were biologically active after 5 days of incubation.

The effects of stimulators' concentration is shown in Fig. 3. In samples stimulated with Con A (Fig. 3-a) and PMA (Fig. 3-d) there was a negative relationship

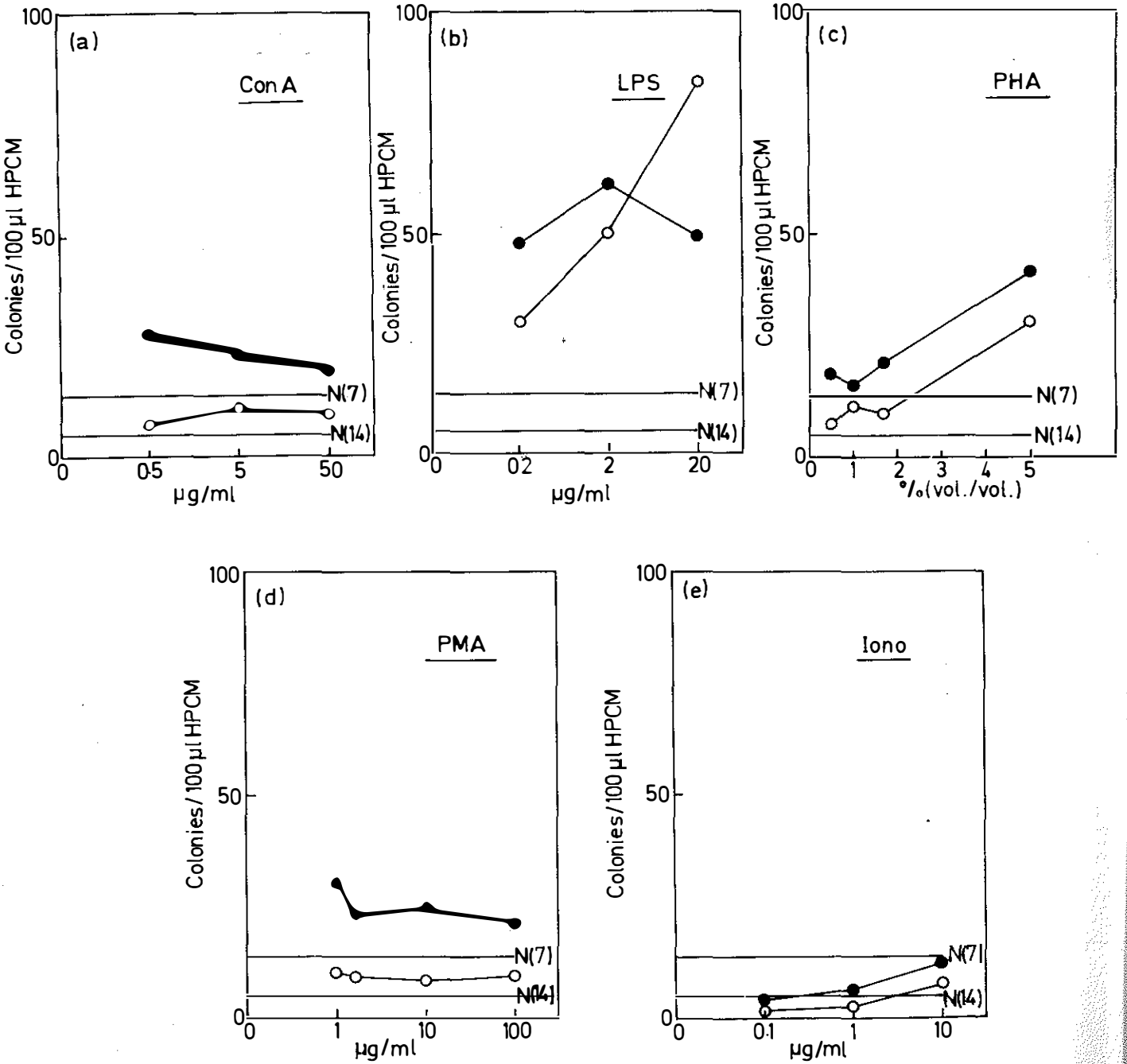

Fig. 3. Effects of different concentrations of stimulators, Con A(a), LPS(b), PHA(c), PMA(d) and Iono(e) on colony forming capacity of placental tissue, identified by 7(●) and 14(○) day bioassay periods. Horizontal lines show colony formation by normal samples in 7 (N(7)) and 14 (N(14)) day assays.

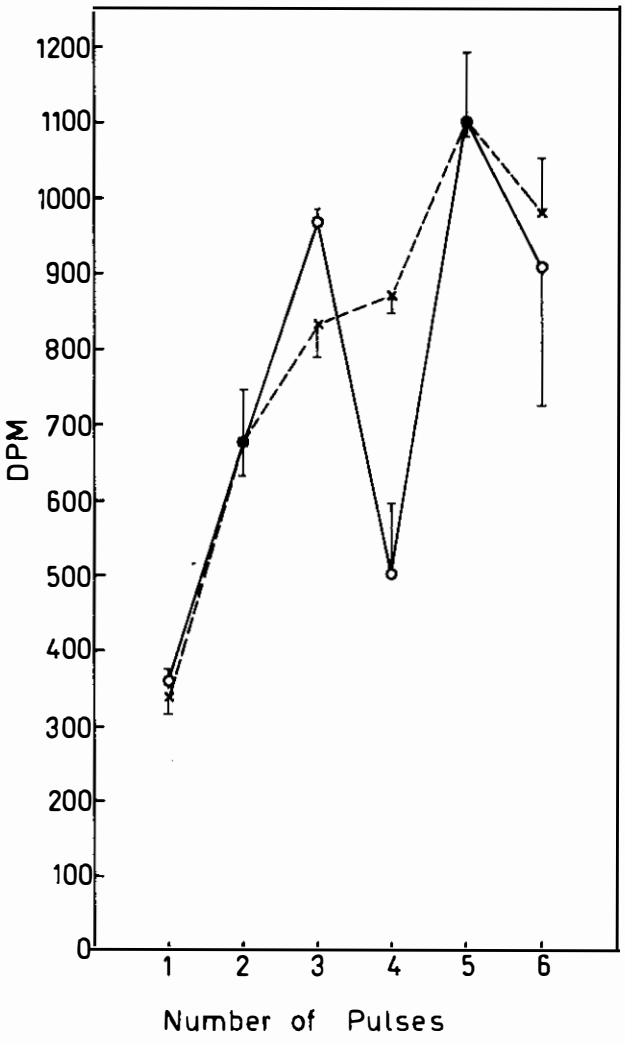

Fig. 4. Kinetic studies of protein synthesis of human placental tissue *in vitro*, stimulated by LPS, 2 micrograms per mL(o) and non-stimulated (*), with the use of ^{3}H-Leu. The radioactivity (dpm) of each pulse is plotted versus the number of that pulse. Mean ± SE of two experiments.

between the concentration of stimulator and hemopoietic activity (in the concentration ranges tested). In both cases the lowest concentrations stimulated the tissue most effectively. There was a direct relationship between the concentration of stimulator and hemopoietic activity when the other stimulators were used (i.e., LPS, PHA and Iono in Figures 3-b, 3-c and 3-e, respectively. The only exception in the latter cases was the LPS-containing samples in 7-day assays (Fig. 3-b), where there was a direct relationship between the hemopoietic activity of HPCM and the concentration of LPS up to 2 micrograms per mL, but a further increase in the concentration of LPS (to 20 micrograms per mL) decreased the hemopoietic activity; therefore the optimal concentration of LPS in 7-day assays was 2 micrograms per mL.

The effects of inhibitors (Act D, Pur, CHX) on hemopoietic activity were also investigated. These compounds, used either alone or in the presence of one of the stimulators, potently inhibited hemopoietic activity. The concentration of inhibitors was 10 micrograms per mL.

The results of kinetic studies with the use of radioactive leucine (^{3}H-Leu) are shown in Fig. 4. In this figure the radioactivity of each pulse is plotted versus the number of that pulse. It is shown that the incorporation of radioactivity, i.e., protein synthesis, gradually increased until day 5 of incubation. This was true for both LPS-stimulated and nonstimulated control samples.

DISCUSSION

Previous studies have shown that lectins such as Con A and PHA activate lymphoid cells by binding to the sugar residues at the surface of the cells.[15] Circulatory lymphoid cells that enter the placenta from the maternal side could not have considerable hemopoietic activity in comparison with the placental tissue itself, especially because the maternal blood was washed out several times before incubation. Lectins also stimulate macrophages to secrete interleukin-1 (IL-1) which in turn stimulates fibroblasts and endothelial cells to produce CSF.[15] In this study the stimulatory effect of lectins on placental tissue is presumably due to the activation of endothelial cells of placental blood vessels. Hofbauer cells which are placental tissue macrophages[16] can be targets for lectins, resulting in secretion of IL-1 afterwards.

The main secretory cells of the placenta are trophoblastic cells[17] that have been shown to produce some of the CSFs.[18] It is not known whether these cells can be stimulated by lectins or not. PMA activates protein kinase C in different cell types[19,20] and induces differentiation. However, it is not clear if there is any specific target cell for PMA in placental tissue. This enzyme triggers a phosphorylation cascade which terminates in the secretion of differentiation-inductive factors.

Our results (Fig. 1 and Fig. 3-d) demonstrate the stimulatory effects of PMA on hemopoiesis which range from mild to strong.

Ionomycin is a Ca^{2+} ionophore[21,22] that increases the intracellular concentration of Ca^{2+} in affected cells. Our study showed that ionomycin did not have any stimulatory effect on placental cells and, in most

instances, exhibited inhibitory effects on CSF production (Fig. 1 and Fig. 3-e).

As has been pointed out before, our main goal in this study was to determine the most potent factor for stimulating the production of hemopoietic growth factors in the placenta. Our results show that LPS stimulated placental tissue maximally to produce CSF *in vitro*. Studies by other investigators[23,24] have also shown that LPS stimulates its target cells to produce different biologically active compounds like CSFs, interferons, leukotrienes and prostaglandins. It will be of great value to see how and to what extent LPS induces the production of a specific cytokine in its target, a question that needs further investigation.

Since our assay method measures the cumulative effects of colony stimulating activity of conditioned medium, it is important to know during which period tissue produces the maximum amount of CSFs. Figure 4 shows the kinetic patterns of LPS-stimulated and normal (nonstimulated) tissue. Pulse number 1 refers to the beginning of the culture period. The tissue shows the minimum of protein synthesis due to the trauma imparted on the tissue. Following further incubation the tissue resumes protein synthesis and reaches a maximum (or a plateau?) on day 5 of incubation. This is in agreement with previous experiments which showed that the maximum biological activity of placental tissue was obtained after 5 days of incubation.

Inhibitors were used in this study to exclude the assumption that there may be a reserve form of CSF in the placenta. Actinomycin D is a transcription inhibitor whereas cycloheximide and puromycin are translation inhibitors. Their potent inhibitory activity indicates that there is no stored CSF in the placental tissue slices and they must be synthesized *de novo* during incubation.

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