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Mitogenic response of rat lung and tracheal epithelial cells in monolayer primary cultures—Modulation of TNF-α expression

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Abstract. Epithelial cells isolated from rat lung and trachea were grown on monolayers and their response to a number of hormones and growth factors were studied. Maximum proliferative response in serum containing media was observed when insulin, cholera toxin and cortisol were present together. However, these additives when present independently showed a marginal response. The synergism, due to these factors in promoting growth was seen very early in culture (day 4) as shown by thymidine labelling studies, On examining the indices of early mitogenesis, such as the expression of *c-myc,* our data suggests that these factors stimulate the expression of *c-myc* within 4 h. With respect to expression of TNF-α mRNA, this study suggests a possible modulation of TNF-α expression in response to these mitogens that stimulate proliferation maximally. Whether this expression of TNF-α by these epithelial cells is due to a maximal proliferative stimulus and/or is an early step in the cascade of intracellular signalling events is to be investigated in detail.

Keywords. Growth factors; cell regulation; TNF-α expression; cell signalling.

1. Introduction

In vitro systems for the propagation of epithelial cells from specific tissues are important in understanding the complexities of growth regulation. Recent developments in tissue culture methodologies have made it easier to isolate specific cell types from an organ. The addition of specific growth factors and hormones to the culture medium enhances the growth and functional capabilities of such cells *in vitro.* Thus, the development of a primary culture of epithelial cells isolated from the lung and trachea will help in understanding the normal regulatory controls of proliferation *in vitro* and also to understand the role of pulmonary defense systems. Several mitogens that regulate the proliferation of epithelial cells have been identified and predominantly the role of epidermal growth factor (EGF) and cortisol have been studied in detail (Yang *et al* 1987). Glucocorticoids elicit an array of physiological responses in target tissues and cultured cells *via* the modulation of gene expression and regulation of growth. Glucocorticoids have also been shown to enhance the EGF binding on specific cells. In addition they have also been reported to either potentiate or antagonize insulin stimulated macromolecular synthesis in differentiation (Osborne *et al* 1979). EGF another potent mitogen operates *via* specific binding sites (Christine *et al* 1985). Epithelial cells from mammary glands

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of rat, mouse and human are stimulated to proliferate both by EGF and cortisol (Yang *et al* 1987).

The prime target for the action of environmental pollutants are the epithelial cells of the lung and trachea. The availability of such a system will also help to understand the interactions of alveolar macrophages and the epithelial cells in the lung and trachea during either an infection or exposure to environmental pollutants. Enhanced production of several immunomodulators by alveolar macrophages in response to an infection has been reported (Dubois *et al* 1989; Strieter *et al* 1989; Hunninghake *et al* 1979). However, whether this enhanced production of growth factors, due to a prolonged inflammatory response will interfere with the normal regulatory controls of epithelial cells in the lung or trachea need to be examined. The role of TNF-α, one of the immunomodulators released from alveolar macrophages has been elucidated in detail (Dubois *et al* 1989; Strieter *et al* 1989). TNF-α is a cytolytic and cytotoxic agent in many transformed cell lines *in vitro,* but has no effect on normal embryo fibroblast (Sugarman *et al* 1985). TNF-α is also a mitogenic agent and shows a variety of biological effects like modulation of growth and differentiation (Carswell *et al* 1975). With the epithelial cells of the lung and trachea no evidence has yet been presented to demonstrate the modulation of TNF-α as a response to mitogenic stimuli unlike *c fos* and *c-myc* whose role in early mitogenic stimuli has been well studied (Curran 1984; Blanchard *et al* 1985). In this paper we report the development of a primary culture system for lung and tracheal epithelial cells and possible role of TNF-α expression on epithelial cells of the lung on exposure of these cells to specific mitogens. Since with trachea, the number of cells obtained after dissociation is minimal, it was difficult to set up hybridization experiments. Whether this expression of TNF-α is growth related and/or is through the modulation of early genes like *c-myc* and TNF-α, which may further trigger the various steps of signal transduction has been investigated,

2. Materials and methods

2.1 *Chemicals and reagents*

Growth factors and hormones like EGF, cholera toxin (CT), hydrocortisone (F) and fetal calf serum were purchased from Sigma Chemicals Co., Missouri, USA. The stock solutions were freshly prepared and stored at –20°C. RPMI 1640, DME/F12 used were purchased from Gibco, USA.

2.2 *Plasmids*

The probe for TNF-α was obtained from Glaxo, USA. It contains 457 bp *Pst*I-*Bam*HI fragment derived from the human genomic clone inserted into the vector pGEM. The probe for *c-myc* was obtained from Leicester University, UK, It contains 600 bp *Sal*I-*Pst*I fragment derived from 9 kb *Eco*RI-*Hind*III human genomic clone inserted into pBR 322 (name of the construct is pGC *myc*).

2.3 *Media and supplements*

The basal medium was 1 : 1 mixture of DME/F12 with Hepes (10 mM) and $NaHCO_3$ (1·2 g/1) containing 10% FCS and insulin (10 μg/ml). The additives CT (10 ng/ml), EGF (10 ng/ml), and cortisol (0·5 μg/ml) were added in various combinations to the basal medium.

2.4 *Tissue source*

Lung and tracheal tissues were dissected from 3-4 months old Wistar rats.

2.5 *Cell dissociation*

Cell culture procedures followed were adapted from protocol previously described for isolation of breast epithelial cell cultures (Richards *et al* 1983; Yang *et al* 1986, 1987). Briefly, the lung and trachea were dissected from healthy rats. They were minced finely and subjected to enzymatic dissociation in RPMI 1640 containing 0·25% collagenase and few drops of 0·04% DNase at 37°C in a water bath shaker set for gentle shaking. The contents of the flask were examined at frequent intervals under the phase contrast microscope to visualize whether the residual stroma around the epithelial clumps dissociated. When most of the stroma had dissociated, the samples were removed, the cells were washed twice with medium to remove collagenase and were pelleted gently at 1000 rpm. The dissociation was completed in about 2 h. To prevent cell clumping a few drops of DNase was added and the cells were layered on preformed Percoll gradients and spun at 1500 rpm for 15 min. The epithelial band was collected, washed and counted in a haemocytometer.

2.6 *Primary cell culture*

The dissociated epithelial cells were seeded at a density of $1{\cdot}5 \times 10^4$ cells per well in 6 well plates. DME/F 12 with 10% FCS containing the different growth factors and hormones in relevant concentrations were added either singly or in combinations. The cells were incubated at 37°C in humidified 5% CO_2 atmosphere for the period described in the experiment. The extent of proliferation was assayed by quantitation of DNA by fluorometric method as described by Hinegardner (1971).

2.7 *Mitogenic effect of growth factors and hormones*

In order to study the mitogenic effect of insulin, CT, EGF, and cortisol on lung and tracheal epithelial cells, the cells were seeded in six well plates and the different additives were added either singly or in combinations in serum containing DME/F12 as the basal medium. The cultures were maintained for the required period of the experiment and terminated by treating the cells with 70% ethanol. Before DNA quantitation the plates were dried and the reaction was set up in the tissue culture plates. Diaminobenzoic acid (DABA) 0·1 g/ml solution in distilled water was prepared fresh. The solution (0·4 ml) was added to each well and

incubated at 55–60°C for 1 h in a water bath. Two ml of 1 N HCl was added to each well and the readings were taken in a fluorometer. The mitogenic effect of the different growth factors and hormones were identified.

2.8 *[^{3}H]thymidine labelling of cells*

The primary monolayer cultures of lung and tracheal cells were incubated in respective media conditions and labelled with [^{3}H]thymidine at a concentration of 2 μCi/well. The cultures were terminated at the end of 2, 5, 8 and 12 days. The cells were pelleted, radioactive media was gently aspirated and cells were washed with 10% and 5% TCA sequentially. To the pellet 0·3 ml of 0·1 N NaOH and 0·0025% SDS were added for solubilization. Thymidine incorporation counts were taken in the LKB 1211 RACKBETA liquid scintillation counter and were expressed as cpm/mg protein.

2.9 *Detection of early gene expression of TNF-α and c-myc*

In order to identify the early mitogenic signals like the expression of *c-myc,* and TNF-α under the influence of mitogens that stimulate proliferation, cells were seeded at high density in 48 well plates or 96 well plates, exposed to the specific combinations of growth factors and hormones, cultures were terminated at respective periods and the total RNA was extracted from cells using acid guanidinium thiocyanate -phenol-chloroform procedure (Chomzynski and Sacchi 1987) and screened both by dot and northern hybridization. The denatured RNA was bound to Genescreen (NEN Research Products) hybridization membrane. For northern, RNA was fractionated through 1% agarose-2·2 M formaldehyde gel and transferred to Genescreen hybridization membrane by capillary blot. Hybridizations were performed according to Sambrook *et al* (1989) using nick translated ^{32}P-labelled probes.

2.10 *Time course analysis of mRNA expression*

The cells isolated from the lung and trachea were seeded in 6 well plates and treated with the combination that would stimulate proliferation. The cultures were terminated at various time points (0 min, 30 min, 60 min, 2 h, 4 h, 10 h, 24 h, 48 h, 72 h and 5 days) and total RNA was extracted (Chomzynski and Sacchi 1987).

2.11 *Morphology of cells*

Morphology of the cells was determined by phase contrast microscope and the macrophage contamination was identified using esterase staining.

3. Results

3.1 *Growth stimulation*

Epithelial cells isolated from rat lung and tracheal tissue were separated by percoll gradient centrifugation. The separated cells were then grown in different combinations

of growth media. Various additives were added to basal medium of DME/F 12 containing insulin and 10% FCS. From epithelial cells isolated from trachea, after 2–3 days the viable cells attached to the substratum and began to spread. The rate of spreading was related to the combination that was mitogenic. The cells isolated from lung tissue predominantly grew as suspension cultures. Earlier studies with epithelial cells isolated from mammary glands of rats and mice were found to proliferate under the influence of EGF, F, CT (Yang and Nandi 1983; Nandi *et al* 1984). Hence, we initiated the present study to evaluate whether any of the known mitogens would stimulate the proliferation of lung and tracheal cells. In order to determine the mitogenic response of the growth factors and hormones that would stimulate maximal growth in culture, the mitogens were tested either singly or in combinations as described in §2. When either EGF or cortisol was present with CT in the basal medium, maximal growth was observed. However, in the absence of CT the growth stimulation was only partial, a similar response was observed both for the lung and tracheal cells (figure 1). The presence of EGF together with cortisol and CT did not evoke any additional mitogenic effect.

The optimum concentrations of EGF and cortisol that were used in the above experiment was determined after a dose response study with each of them independently (data not shown). At higher concentrations of cortisol toxicity on the cells was observed, while concentrations of EGF above the optimum did not have any enhanced effect on proliferation. In the rapidly proliferating medium (I, CT, F), the cultures attained confluency within 10 to 14 days after which the cells begin to show signs of senescence and keratinization.

3.2 *Morphological studies*

Morphological analysis of the cells under the phase contrast microscope showed typical epithelial-like colonies in the cells isolated from trachea. The cells isolated from the lungs seem to exist as a mixed population which grew as suspension cultures (figure 2). Characterization of the cells using epithelial cell markers is in progress. Morphology of the cells indicate that they were predominantly epithelial in origin and the cells in culture in I, CT, F medium retained their epithelial morphology even after 14 days in culture.

3.3 *Growth kinetics by [^{3}H]thymidine incorporation*

To study the cell growth, freshly dissociated cells were seeded at a density of 1×10^6 cells/well. After an initial lag phase, the cells entered the multiplication phase and DNA synthesis began. Cells retained the proliferative capacity up to 14 days in culture after which stationary phase was reached. Further period of culture induced the cells to differentiate terminally.

Continuous labelling of cells with [^{3}H]thymidine also gave similar results (figure 3). It was found that the maximal incorporation of the label was at day 4 of culture in the medium supplemented with I, CT, F in the presence of 10% serum, while in the absence of CT, the incorporation of the label diminishes by about 50%.

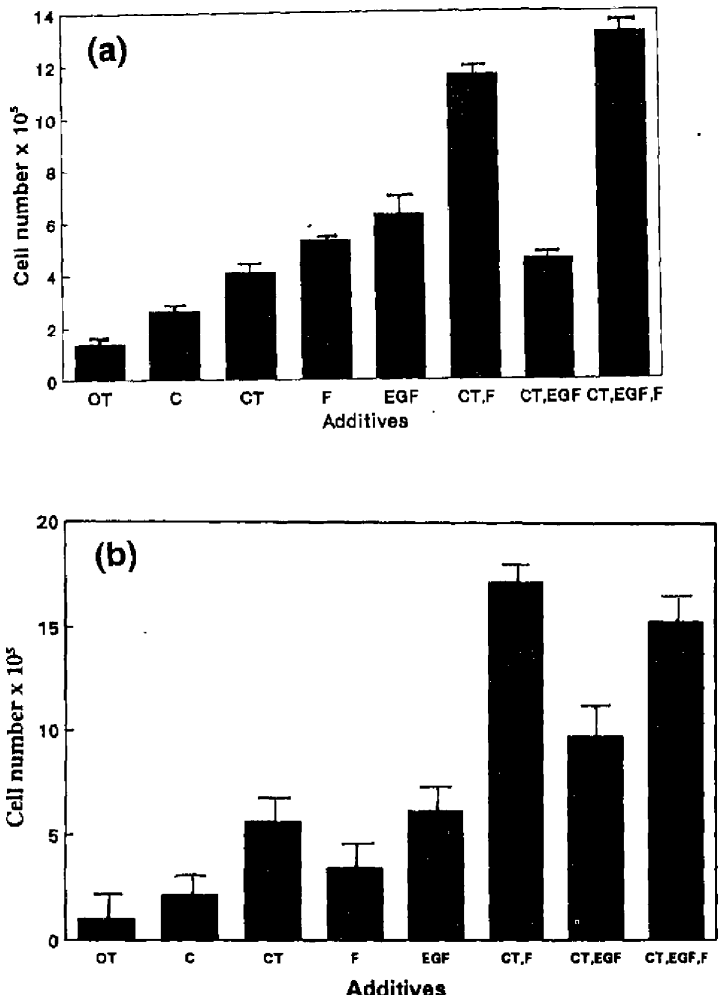

Figure 1. Effect of exogenous growth factors on lung **(a)** and tracheal **(b)** epithelial cells in culture. The basal medium was DME/F12 +10% FCS +1. Culture period was 14 days. OT refers to the initial cell number, C refers to control with basal medium, CT is cholera toxin, F is cortisol, EGF is epidermal growth factor. Total DNA content of the cells was measured by di amino benzoic acid method and extrapolated to cell number.

3.4 *Early gene induction*

The pattern of mRNA expression of early response genes were also studied in the epithelial cells isolated from lung. The cells were treated with different concentrations of hormones and growth factors that were used for the growth studies and determined to have mitogenic effect. Similar combinations were used to evaluate the mRNA expression of *c-myc* and TNF-α.

3.5 *Time course analysis*

Time course study of the expression of *c-myc* and TNF-α mRNA suggest that

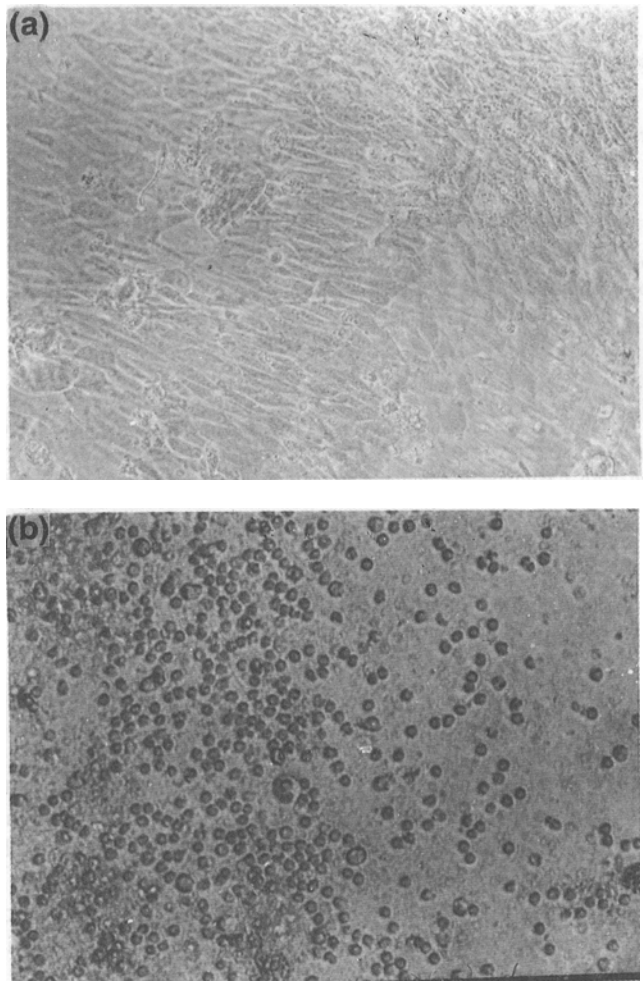

Figure 2. (a) Primary monolayer culture of tracheal epithelial cells at day 14. The epithelial morphology of the cells are maintained. The medium used was DME/F12+ 10% FCS containing I, CT, F. (b) Primary monolayer culture of lung epithelial cells at day 14, indicates a mixed population of cells. The cells were grown under similar condition.

c-myc was expressed within 4 h in the proliferative condition (I, CT, F), while in the non-proliferative conditions, expression of *c-myc* was not observed. In the rapidly proliferating media (I, CT, F) the TNF-α mRNA expression was observed from 2h and shows maximum signal at 10 h, after which the signal begins to diminish when studied up to 50 h (figure 4).

3.6 *Synergistic effect of the additives on expression of TNF-α and c-myc*

In order to establish whether the expression of TNF-α and *c-myc* in these cells were related to the presence of either CT or F the experimental data suggested

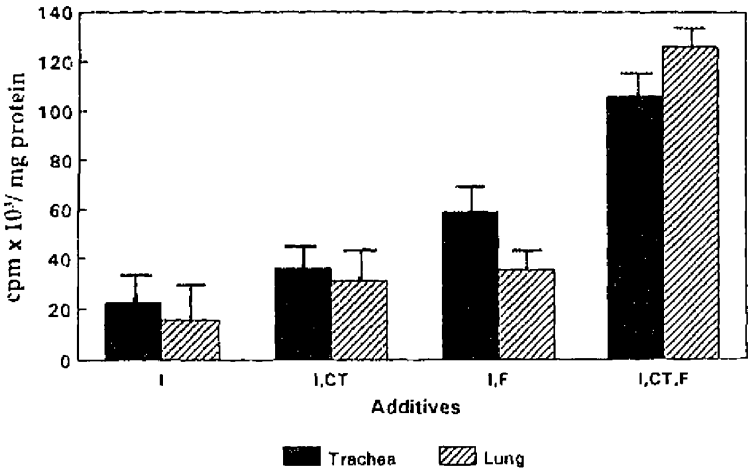

Figure 3. Effect of exogenous growth factors on lung and tracheal epithelial cell proliferation. The medium used was DME/F12+ 10% FCS. The culture period was 4 days. The culture was labelled with [^{3}H]thymidine and the incorporation expressed as cpm/mg protein.

that none of the additives *viz.,* I, CT and F when present singly were capable of eliciting an enhanced expression of both *c-myc* and TNF-α. However, when I, CT, and F were present together a dramatic increase in the expression of both *c-myc* and TNF-α was found (figure 5).

4. Discussion

Epithelial cells isolated from rat lung and tracheal tissues were cultured in serum containing media as monolayers and the effect of different mitogens were tested either singly or in different combinations, specifically to understand the modulation of normal growth regulation. The different mitogens were tested both for long term stimulation of growth and also to understand the intricate steps involved in early mitogenesis. A consistent observation of our study was the strong mitogenic signal when CT and F were present together in the basal medium. This effect was observed both with the lung and tracheal epithelial cells.

Cortisol has been reported to have growth promoting effects, increase in colony number and thymidine incorporation of cells and increase in thymidine uptake with specific cell types (Taylor *et al* 1977; Gaffney and Pigott 1978a, b). On deletion of CT a 50% decrease in growth stimulation was noted. EGF which is a strong mitogen of epithelial cells isolated from fibroadenomas and normal breast epithelial cells did not show any profound mitogenic effect either singly or in combination with CT, F (figure 1). This observation is identical with results obtained with normal human breast epithelial cells when grown as monolayers (Yang *et al* 1986). Attempts to delete serum or reduce the concentration of serum in order to evaluate the effect of these mitogens on monolayer cultures is in progress.

Morphological examination of the cells both after initial isolation and after prolonged period in culture for a maximum of 14 days suggested that most of them are predominantly epithelial in origin. Further studies to subtype the cells using immunocytochemistry is in progress with the aim to delineate the specific

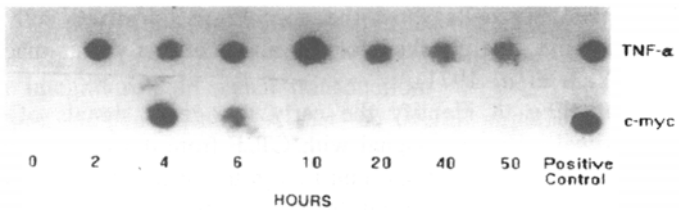

Figure 4. Time course analysis of early gene induction in lung epithelial cells. Total RNA was extracted and mRNA expression of TNF-α and *c-myc* were examined under the influence of various mitogens.

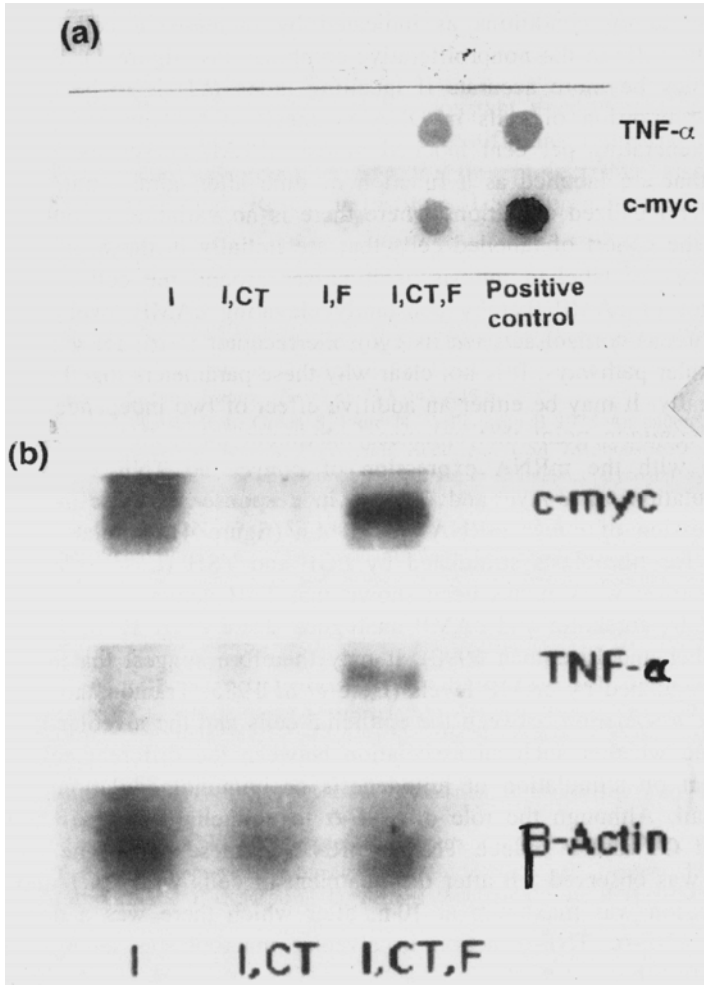

Figure 5. (a) Synergistic effect of additives on expression of TNF-α and *c-myc* in lung epithelial cells. Total RNA was extracted and mRNA expression of TNF-α and *c-myc* were examined under the influence of various mitogens, (b) Northern blot showing the expression of TNF-α (10h), *c-myc* (4 h) and β-actin.

nature of the epithelial cells proliferating under these culture conditions. Under phase contrast microscope the tracheal cells have an epithelial like morphology (figure 2a) while the lung cells show the appearance of mixed cell type (figure 2b). The cell preparation was checked for the absence of alveolar macrophages by esterase staining (Yam *et al* 1971).

[^{3}H]thymidine labelling to identify the early mitogenic signals of proliferation also indicate a maximal mitogenic signal with CT, F from day 4 onwards suggesting that these two factors may play an important role in triggering the various intracellular signalling pathways which would result in an overall increase in cell number. In our studies, the factors when present independently the mitogenic stimuli was marginally observed probably due to the priming of the cells with some of the factors already present in serum. Although the cells were exposed to thymidine over the duration of culture, the incorporation was reflective of the overall proliferation under the stimulatory conditions as indicated by increase in cell number when compared to the cells in the nonproliferative combinations (figure 1). Pulse labelling experiments, may be more accurate if labelling index (LI) is to be calculated. LI represent the proportion of cells in DNA synthesis at the time of [^{3}H]thymidine exposure. In generating per cent labelled mitosis (PLM) curves the percentage of mitotic cells that are labelled as a function of time after administration of [^{3}H] is recorded. Under idealized conditions where there is no variation in duration of cell cycle phases, the cohort of labelled cells that are initially in the S-phase, generates successive waves of labelled mitosis as it passes around the cell cycle. CT is a known activator of cAMP thereby constantly elevating cAMP levels in cell lines (Gill 1977) whereas cortisol acts *via* its cytosolic receptor to trigger varying changes in the intracellular pathways. It is not clear why these parameters together synergisize growth maximally. It may be either an additive effect of two independent parameters merging at a common point that enhances maximum intracellular signalling.

Our results with the mRNA expression of *c-myc* and TNF-α also suggest a possible modulation of *c-myc* and TNF-α in response to these mitogens. The maximal expression of *c-myc* mRNA was at 4h (figure 4). Similar findings have been reported for fibroblasts stimulated by EGF and TSH (Luscher and Eisenman 1990). In an earlier' work it has been shown that TSH activation of *c-myc* mRNA was mimicked by forskolin and cAMP analogues (Dere *et al* 1985; Tramontano *et al* 1986; Luscher and Eisenman 1990). It may therefore suggest that *c-myc* mRNA expression is regulated by cAMP levels (Dere *et al* 1985; Tramontano *et al* 1986). In lung a close association between the epithelial cells and the alveolar macrophages is observed and whether such an association between the different cell types may have any effect on stimulation of mitogenesis or immunomodulation needs to be studied in detail. Although the role of TNF-α in epithelial cells is not very clear it appears that CT and F induce TNF-α mRNA in these cells. The induction of TNF-α signal was observed 2 h after the treatment of cells with I, CT and F (figure 4). The expression was maximum at 10 h, after which there was a decline in the expression of TNF-α. TNF-α action in alveolar macrophages as a mitogen and immunomodulator has been studied in detail (Dubois *et al* 1989; Strieter *et al* 1989). Our results also suggest that the expression of TNF-α in these epithelial cells could be related to early mitogenic signals. The modulation of *c-myc* and TNF-α by CT and F could also suggest that these intracellular signals may be related to stimulation of cAMP or possible activation of an effector molecule like

G protein or phosphorylation of specific proteins. In lung cells, the expression of both TNF-α and *c-myc* mRNAs appeared to be triggered by factors that stimulated mitogenesis and this expression was not observed under nonproliferative conditions. It may be suggest that both *c-myc* and TNF-α may behave as transcriptional factors and play an integral role in signal transduction.

In conclusion our studies suggest that the overall stimulation of proliferation by CT and F on lung epithelial cells may be modulated by the induction of early genes *viz., c-myc* and TNF-α in these cells when exposed to a mitogenic stimulus. The inability of either CT and F to independently stimulate proliferation or early mitogenic signals may indicate that these mitogenic parameters operate through different routes of signal transduction and may ultimately trigger the intracellular signal pathways for proliferation.

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