STIMULATION OF DNA SYNTHESIS AND CELL DIVISION IN A CHEMICALLY DEFINED MEDIUM: EFFECT OF EPIDERMAL GROWTH FACTOR, INSULIN AND VITAMIN B_{12} ON RESTING CULTURES OF 3T6 CELLS

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Received September 15, 1976

SUMMARY Addition of Epidermal Growth Factor, Insulin and Vitamin B_{12} in completely serum free medium to cultures of 3T6 cells arrested in the G_1/G_0 phase of the cell cycle stimulates DNA synthesis in 80-90% of the cell population. Cell division occurs 48-72 hours after factor addition. Because the peptides are active at low levels and interact synergistically, this model system offers a powerful tool for elucidating mechanisms of growth regulation which might have a physiological relevance.

Many mammalian cells become arrested in G_1/G_0 or A state (1) when the culture medium becomes depleted of growth factors. Addition of serum to such cultures stimulates a complex array of biochemical events (2,3) culminating in DNA synthesis and cell division (3-5). The analysis of the biochemical events and control mechanisms implicated in the stimulation of growth requires pure growth-promoting molecules. EGF*, a polypeptide of molecular weight 6045 (6) promotes growth of a variety of cells (7) including fibroblasts in cell culture (8-12). However, to stimulate cell division in culture, EGF requires the presence of serum or BSA (8-12); the factors provided by serum or BSA are unknown and of importance because their requirement prevents the study of the proliferative response under completely defined conditions.

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*Abbreviations: EGF Epidermal Growth Factor. BSA Bovine Serum Albumin FCS Fetal Calf Serum. PBS Phosphate-buffered Saline (pH 7.2) DEM Dulbecco's Modified Eagle's Medium. L.I. Labelling Index. PCA Perchloric Acid

Here we describe a system in which EGF stimulates DNA synthesis and cell division in 3T6 cells resting in G_1/G_0 in the complete absence of serum. We found that insulin and vitamin B_{12} replace the requirement for any exogenously added serum in these cells. Because this model system uses completely chemically defined molecules, it provides a powerful experimental method for elucidating the biochemical mechanisms involved in cell proliferation.

MATERIALS AND METHODS

Cell Culture In preliminary experiments EGF was tested as a mitogen in several cultured cell lines in DEM and in DEM supplemented with Waymouth Medium (13). DNA synthesis was found in the supplemented medium and it was particularly marked in 3T6 cells. In these cells vitamin B_{12} was the only component of Waymouth medium required. Ascorbic acid had no activity in this system (12).

Stock cultures of 3T6 cells (14) were grown in DEM supplemented with 10% FCS, 100 units ml^{-1} penicillin and 100 ng ml^{-1} streptomycin in a humidified atmosphere of 10% CO_2 and air at 37°. Cultures were kept sub-confluent and passaged regularly at three day intervals. For experimental purposes cells were plated at approximately 10^5 cells per 33mm dish (Nunc) in 0.5% serum DEM, and kept for at least one week; they were then washed twice in DEM and left in serum free medium for two days prior to use.

Experimental Techniques Cells were radioactively labelled with [^{3}H] methyl thymidine at 0.5 uCi ml^{-1}, 2.5 uM for 24 hours, and incorporation into acid insoluble material measured by scintillation counting as previously described (15). The percentage of nuclei incorporating [^{3}H] thymidine (labelling index) was determined by exposing parallel cultures to the isotope (4 uCi ml^{-1}, 0.2 uM) for the same time period and fixing cells for autoradiography.

For determination of the total amount of DNA, cultures were trypsinised, washed twice with PBS and extracted twice with 0.75 ml 0.5N PCA at 77° for 20 minutes. The two extracts were pooled and aliquots assayed for DNA by the diphenylamine reaction (16).

Cell number determinations were made in triplicate cultures; 4 aliquots of cell suspension from each culture were counted on a Coulter Counter.

Factors and Reagents Mouse EGF was a kind gift from Dr. S. Cohen. Standard solutions of EGF containing 500 ng ml^{-1} factor in a 50 ug ml^{-1} solution of BSA were prepared from a stock solution of pure EGF (100 ug ml^{-1}). The final concentration of BSA in the medium did not exceed 2.5 ug ml^{-1}. Controls in which BSA was omitted showed no difference in level of stimulation when tested with the factor combination. Insulin (26 I.U. mg^{-1}, Sigma) was freshly dissolved for each experiment. Crystalline vitamin B_{12} was obtained from Sigma. [^{3}H] thymidine was from the Radiochemical Centre, Amersham.

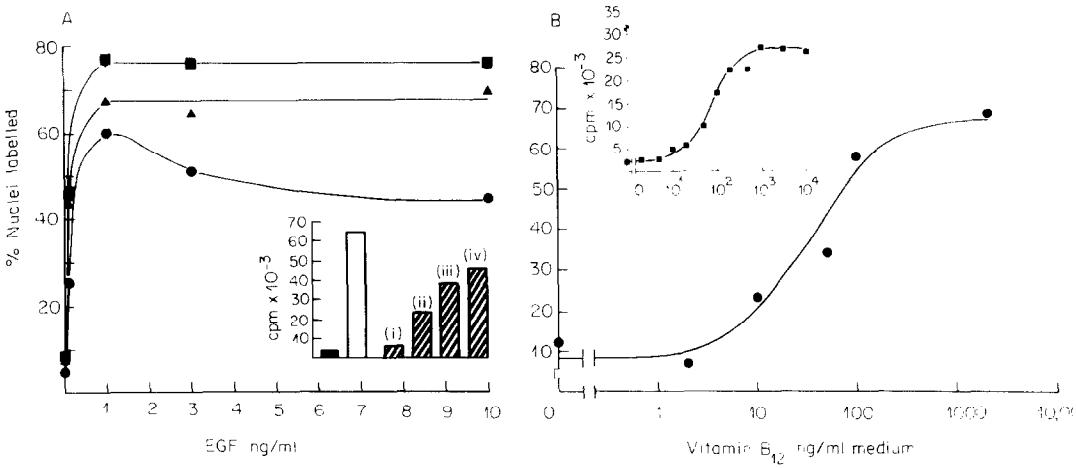

Fig. 1 Stimulation of DNA synthesis by EGF, insulin and vitamin B_{12}. Panel A Effect of a range of concentrations of EGF on the labelling index, in the presence and absence of three levels of insulin. Insulin concentrations (ng ml^{-1}): 0 (●–●); 10 (▲–▲); 50 and 100 (■–■). Insert shows a parallel experiment in which thymidine incorporation was measured. Additions to vitamin B_{12}-DEM are as follows: solid bar, none; open bar, 10% serum; cross hatched bars, (i) 100 ng ml^{-1} insulin (ii) 10 ng ml^{-1} EGF (iii) 10 ng ml^{-1} EGF 10 ng ml^{-1} insulin (iv) 10 ng ml^{-1} EGF, 100 ng ml^{-1} insulin. Vitamin B_{12} concentration was 400 ng ml^{-1} in both autoradiography and thymidine incorporation experiments, and all points represent the means of two separate determinations. Panel B Effect of different concentrations of vitamin B_{12} on the labelling index at a constant level of EGF and insulin (5 ng ml^{-1} and 50 ng ml^{-1} respectively). DEM alone (□). Insert shows a parallel experiment in which thymidine incorporation was measured. All points represent the means of two separate determinations. 10% serum, for comparison (◪).

RESULTS

Fig. 1A shows that EGF is a potent stimulator of DNA synthesis in 3T6 cells. Low levels of insulin, which per se only slightly stimulate DNA synthesis potentiate the effect of EGF significantly. The factors stimulate DNA synthesis only in the presence of vitamin B_{12}, which when added to DEM enhances the proliferative response in a dose dependent manner (Fig. 1B). Similar results were obtained whether the labelling index or total $[^3H]$ thymidine incorporation was measured (inserts Fig. 1A 1B). Throughout at least 30 experiments, the factor combination always stimulated

Table 1

Effect of EGF, insulin and vitamin B_{12} on total cell DNA levels

Incubation with factors (10 ng ml^{-1} EGF, 100 ng ml^{-1} insulin and 500 ng ml^{-1} vitamin B_{12}) was carried out over a 48 hour period. For each determination, 4 cultures were pooled and assayed for DNA as described in materials and methods.

Addition to vitamin B_{12}-DEM	DNA per culture (ug/dish)
None	11.5
2% fetal calf serum	34.8
EGF, insulin	26.9

DNA synthesis, though the contribution of each component (enhancement by vitamin B_{12} or synergistic action of insulin) varied in different experiments.

Direct measurement of the amount of DNA per culture shows that the factors stimulate net DNA synthesis rather than change the specific activity of the thymidine pools. Total DNA per culture doubles after 48 hours incubation with the factor combination (Table 1).

The stimulation induced by the factors strongly depends on cell density; it decreases when the cell density increases (Results not shown). Optimal response for DNA synthesis and growth (see below) is obtained when cells are at a density of 2×10^4 cells/cm^2.

Fig. 2 shows the time course of DNA synthesis after the addition of different combinations of the factors to sparse cultures of 3T6 cells arrested in G_1/G_0 phase of the cell cycle. Neither the addition of vitamin B_{12} nor the peptides by themselves stimulates a substantial increase in

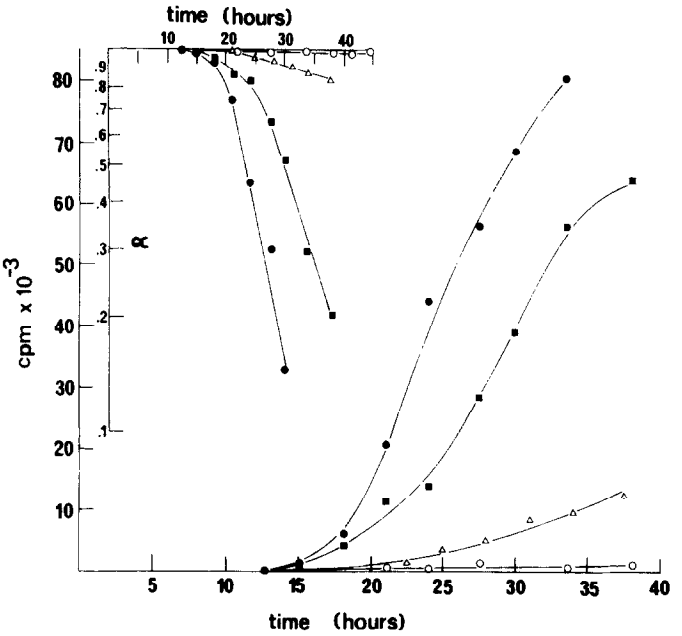

Fig. 2 Effect of various combinations of factors on cumulative $^{3}[H]$ thymidine incorporation as a function of time. Factor combinations: 10 ng ml^{-1} EGF, 100 ng ml^{-1} insulin, 400 ng ml^{-1} vitamin B_{12} (■–■); 10 ng ml^{-1} EGF, 100 ng ml^{-1} insulin (Δ–Δ); 400 ng ml^{-1} vitamin B_{12} (O–O). Serum (●–●).. Factors and label were added at 0 hours. At three hourly intervals, pairs of dishes from each group were fixed and processed for scintillation counting. Insert shows time-course data plotted as an α-curve (a semi-logarithmic plot of the proportion of cells remaining in the A-state against time). The levelling off of the serum curve after 33 hours was taken as equivalent to 100% labelling of the culture ($\alpha \rightarrow 0$). From this figure, the proportion of the cells that had not yet entered S (α) was calculated.

cumulative $[^{3}H]$ thymidine incorporation. In contrast, the combination of the factors dramatically stimulates DNA synthesis after a lag (G_1) of approximately 15 hours. Addition of whole serum stimulates DNA synthesis after an approximately similar time lag. When the time course data is replotted as described by Smith and Martin (1) the slope of the straight line reflects the probablity of any cell leaving the A state (G_1/G_0) and entering S. The results (Fig. 2, insert) show that EGF, insulin and vitamin B_{12} stimulate a dramatic increase in this slope, which approaches that seen upon addition of serum.

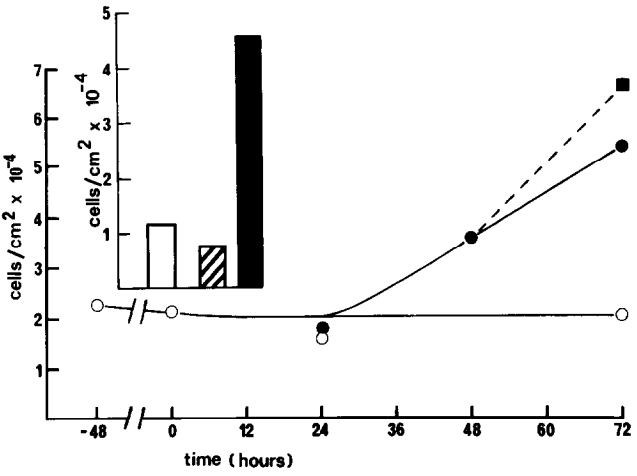

Fig. 3 Increase in cell number in cultures over three days following stimulation by the factor combination. EGF, insulin and vitamin B_{12} concentrations were 10, 100 and 500 ng ml^{-1} respectively. Additions to vitamin B_{12}-DEM: None (O-O); EGF and insulin (●-●); 3% serum added at 48 hours (—■). Insert shows a similar growth experiment carried out over six days. Additions to vitamin B_{12}-DEM: cross hatched bar, none; solid bar, EGF and insulin (concentrations as in main figure). Open bar represents original cell number.

Cultures stimulated to enter the S phase by the factors at the optimal cell density double in cell number 48-72 hours after factor addition (Fig. 3). This experiment clearly shows that the stimulated cells are able to traverse a complete cell cycle. An incubation for six days results in a five fold increase in cell number, which in a homogeneous population represents at least two divisions for each cell (Insert Fig. 3). Exposure to factors for several days changes the morphological appearance of the culture which would suggest that other factors are required for prolonged incubation. Indeed, whether this procedure allows serial passage of cells is under current investigation.

DISCUSSION

The present experiments describe a model system for studying the stimulation of DNA synthesis in cells arrested in G_1/G_0 in a chemically

defined system. This system combines a) the vitamin B_{12}-supplemented DEM medium b) two pure polypeptides of known sequence, EGF and insulin, and c) the use of 3T6 cells which are able to survive, respond to peptide factors and accumulate in G_1/G_0 in the complete absence of serum. At optimal conditions, most of the cells of the population are stimulated to enter S and divide with a kinetic rate comparable to that of whole serum.

The levels of EGF that stimulate the entry of cells into S are similar to those reported in mouse serum (8) and therefore physiological. Insulin acts synergistically when it is also at low levels. Because the peptides are active within the concentration range at which they interact with specific surface receptors (17,18) it seems likely that the modulation of cell proliferation by low levels of several interacting peptide factors may represent a flexible mechanism for the fine regulation of growth of individual cells in the intact animal.

Our results show that most of the mitogenic functions of serum can be replaced by two polypeptide factors, in a medium that provides all the essential low molecular weight components. This model system provides a powerful tool of elucidating the biochemical events that occur between recognition of a defined proliferative 'signal' at the cell surface, and the stimulation of DNA synthesis.

ACKNOWLEDGEMENTS

We thank Drs. Henry Bourne, Ivan Diamond and Leon Heppel for their helpful comments in the preparation of this manuscript.

REFERENCES

1. Smith, J.A. and Martin, L. (1973) Proc. Natl. Acad. Sci. U.S.A. 70 1263-1267
2. Rozengurt, E. Early Membrane Changes in Growth Stimulation in "Surface Properties of Normal and Neoplastic Cells" (Ed. R. Hynes) J. Wiley & Sons, Sussex, England (in press)

3. Pardee, A.B. and Rozengurt, E. (1975) in "Biochemistry of Cell Walls and Membranes. pp. 155-165 (Ed. C.F. Fox) Medical and Technical Publishing Co., London.
4. Todaro, G.J., Matsuija, Y., Bloom, S., Robbins, A. and Green, H. (1967) in "Growth Regulating Substances for Animal Cells in Culture" (Eds. Defendi, V. & Stoker, M.) Wistar Institute Press, Philadelphia, U.S.A. pp. 87-98
5. Holley, R.W. (1975) Nature 258 487-490
6. Savage, Jr., C.R., Inagami, T. and Cohen, S. (1972) J.Biol. Chem. 247 7612-7621
7. Cohen, S. and Savage, Jr., C.R. (1974) Recent Prog. Horm. Res. 30 551-574
8. Carpenter, G. and Cohen, S. (1975) J. Cell. Physiol. 88 227-238
9. Hollenberg, M.D. and Cuatrecasas, P. (1973) Proc.Natl. Acad. Sci. U.S.A 70 2964-2968
10. Rose, S.P., Pruss, R.M. and Herschman, H.R. (1975) J.Cell. Physiol. 86 593-598
11. Westermark, B. (1976) Biochem. Biophys. Res. Comm. 69 304-310
12. Lembach, K.J. (1976) Proc.Natl. Acad. Sci. U.S.A. 73 183-187
13. Waymouth, C. (1959) J.Nat. Cancer Inst. 22 1003-1017
14. Todaro, G.J. and Green, H. (1963) J.Cell Biol. 17 299-313
15. Rozengurt, E. and Heppel, L.A. (1975) Proc. Natl. Acad. Sci. U.S.A. 72 4492-4495
16. Burton, K. (1968) in "Methods in Enzymology" XII B pp. 163-166 (Eds. Grossman, L., and Moldave, K.) Academic Press, New York
17. Carpenter, G., Lembach, K.J., Morrison, M.M. and Cohen, S. (1975) J. Biol. Chem. 250 4297-4304
18. Hollenberg, M.D. and Cuatrecasas, P. (1975) J. Biol. Chem. 250 3845-3853