

# Appearance of Functional Insulin Receptors during the Differentiation of Embryonal Carcinoma Cells

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**ABSTRACT** The insulin-receptor binding activity and insulin-stimulated growth response of PC13 clone 5 cells were investigated for both the embryo carcinoma (EC) and retinoic acid-induced differentiated derivatives of this cell line. Whereas the EC cell was found to have very few, if any, receptors and showed no demonstrable dependence on insulin for growth, the differentiated derivative cell expressed a large number of insulin receptors and, when challenged with the hormone, showed stimulation of both DNA synthesis and cell division. The same data were obtained for five independent PC13 clones. These results, coupled with previous observations, lend weight to the suggestion that the appearance of specific receptors for growth regulatory substances may be a manifestation of a general change in growth-regulatory mechanisms accompanying EC cell differentiation and loss of malignancy.

The proliferation of many cell types *in vitro* can be regulated by the addition of certain polypeptide hormones (1). It is known that some of these exert their effect initially by binding to specific receptors in the target cell (2) although the subsequent cellular mechanisms involved in the growth response are obscure. It has further been suggested that transformed cells have in some way lost the requirement for, or the ability to respond to, such growth-promoting polypeptides.

The murine embryonal carcinoma (EC) cell possesses many of the features of the transformed phenotype, including the ability to grow progressively and kill a suitable host, even at very low initial inocula. If EC cells are permitted to differentiate *in vitro* either in the presence of exogenous agents such as retinoic acid (3) or by plating at low density (4), they acquire certain new phenotypic properties and are markedly less malignant (5, 6). It has been shown that these differentiated progeny of EC cells also express the cell surface receptor for epidermal growth factor (EGF) and that their proliferation can be regulated by exogenous EGF (7).

These observations suggest that the differentiation of EC cells may involve a more general change in growth properties, and that the differentiated cells may respond to other growth-promoting agents. We report here that the differentiation of PC13 EC cells in the presence of retinoic acid results in the appearance of specific cell surface receptors for insulin, and that the addition of insulin to slowly proliferating differentiated EC cells results in a stimulation of both DNA synthesis and cell proliferation.

## MATERIALS AND METHODS

Porcine insulin was a gift of Professor R. E. Offord, University of Geneva. Iodine-125 (100  $\mu$ Ci/ml) and [ $^3$ H]thymidine (60 Ci/mmol) were purchased from the Radiochemical Centre, Amersham, England. Bovine serum albumin was obtained from Miles Laboratories Inc. (Stoke Poges, England), and "Aqualuma plus" scintillation fluid from LKB Instruments, Ltd., (Surrey, England). Earle's balanced salt solution (EBSS), modified Eagle's medium (MEM), and alpha modified Eagle's medium ( $\alpha$ -MEM) were purchased from Gibco Bio-cult, Paisley, Scotland. Fetal calf serum (FCS) was purchased from Sera Lab. (Crawley, U. K.). All tissue culture plastic was obtained from Sterilin (U. K.), and retinoic acid from Sigma Chemical Co. (Poole, Dorset, England). EGF and multiplication stimulating activity (MSA) were obtained from Collaborative Research Inc., Waltham, MA. Desoctapeptide insulin was a gift from Dr. K. Rose, University of Geneva.

### Cells

All experiments reported here were performed with PC13 clone 5 EC cells. EC cells were maintained in  $\alpha$ -MEM with added 10% FCS (vol/vol) on gelatinized tissue culture dishes. PC13 cells were plated for differentiation at  $5 \times 10^3$  cells/cm<sup>2</sup> in alpha medium plus 10% FCS (vol/vol) and  $10^{-6}$  M retinoic acid (added from stock,  $10^{-2}$  M, in DMSO) in nongelatinized tissue culture dishes or glass roller culture vessels. Little overt cell death was observed during this period. The cells were replated for experimentation after 6 d (by which time cell numbers had increased ~10-fold) and cultured for 2 d in alpha medium plus 10% FCS (vol/vol) before the start of the experiments.

### Cell Cloning

PC13 clones were picked as colonies growing in semisolid agar. 100 EC cells were plated in 0.5% (vol/vol) agarose in alpha medium plus 10% FCS (vol/vol) in 63 cm<sup>2</sup> bacteriological dishes. Macroscopically visible colonies were picked after 14-d growth and grown on gelatinized dishes for further analysis. The cloning efficiency under these conditions was 3.8%.

## [<sup>3</sup>H]Thymidine Uptake Assay

Cells were plated at  $5 \times 10^3$  cells per well in 96-well microtiter plates in 200  $\mu$ l of alpha medium plus 10% FCS (vol/vol). The medium was changed the following day to MEM with 0.5% FCS (vol/vol), and the hormone additions were made 48 h later. 24 h after the hormone additions, 5  $\mu$ Ci of [<sup>3</sup>H]thymidine in  $10^{-6}$  M unlabeled thymidine was added and the cells were incubated for a further 24 h. The labeling was terminated by washing the wells four times with ice-cold phosphate-buffered saline (PBS) and the cells were fixed with methanol-acetic acid (3:1 vol/vol) for 15 min, after which the fixative was removed and the cells were allowed to dry. Ice-cold 10% (vol/vol) aqueous TCA was added and the cells were incubated at 40°C for 1 h. The TCA-soluble material was removed and the cell monolayers were washed three times with cold 10% TCA (vol/vol). The residual TCA-insoluble material was dissolved in 0.2 M NaOH and, after adding to "Aqualuma plus," counted on a Rackbeta (LKB Instruments, Ltd.) scintillation counter/at an efficiency of 34%.

## Cell Numbers

Cells were plated at  $10^4$  cells per 10-cm<sup>2</sup> tissue culture dish in MEM plus 10% FCS (vol/vol). The medium was changed to the experimental regime the next day. At the termination of the experiment, the cell monolayers were washed twice in ice-cold PBS and dispersed in 0.125% trypsin (wt/vol) in 100 mM EDTA, pH 7.4. The cell numbers were determined in triplicate on a Coulter counter (model D, Coulter Electronics Inc., Hialeah, Fla.).

## Preparation of <sup>125</sup>I-Insulin

<sup>125</sup>I-labeled insulin was prepared according to the method of Freychet (8) except that maximum specific activity was restricted to 200  $\mu$ Ci/mg and separation of iodinated from damaged hormone was performed by gel filtration on Sephadex G-50 (Pharmacia Fine Chemicals, AB, Uppsala, Sweden). Radiolabeled insulin prepared in this way has previously been shown to be a homogeneous, monoiodinated derivative (8-10).

## Insulin Binding Assays

**EQUILIBRIUM BINDING:** Binding assays were performed on monolayer cultures essentially as described by Rees et al. (7) with insulin substituted for EGF. Confluent cell monolayers in 20-cm<sup>2</sup> dishes were washed three times with EBSS containing tris (10 mM) and bovine serum albumin (2% vol/vol), pH 7.6 (EBSS binding buffer). Monoiodinated <sup>125</sup>I-insulin was added to each dish in EBSS binding buffer (2 ml) and the dishes were incubated for 120 min at 22°C with intermittent agitation. Unlabeled insulin was added for competition experiments over the range  $10^{-11}$  to  $10^{-9}$  M. Nonspecific binding was calculated from the residual labeled insulin bound in the presence of  $10^{-9}$  M unlabeled insulin. At the end of the incubation a 1-ml aliquot of supernate was taken for the determination of unbound insulin, and the cell monolayers were washed three times with ice-cold EBSS binding buffer. The cells were dissolved in 0.5 M NaOH and counted in a Packard gamma-counter (Packard Instrument Co., Inc., Downers Grove, Ill.). Binding data were converted to Scatchard form and analyzed by the M-lab curve fitting program of Kahn et al. (11) for negative cooperativity. The nonspecific binding was allowed to vary in this analysis between the limits  $\pm 1\%$ . The parameters obtained from such an analysis are  $\bar{K}_e$ , the average affinity of unoccupied receptors;  $\bar{K}_f$ , the average affinity of fully occupied receptors;  $\alpha$ , the interaction factor defined as  $\bar{K}_e/\bar{K}_f$ ; and  $R_0$ , the molar receptor concentration.

**ASSOCIATION AND DISSOCIATION:** The association rate was determined using the above protocol and varying the incubation times over the range 0 to 120 min. Dissociation was measured by incubation of monolayers as above for 120 min with <sup>125</sup>I-insulin ( $10^{-11}$  M). The cell monolayers were quickly rinsed with EBSS binding buffer and then incubated in 7 ml of EBSS binding buffer for various times at 22°C. The residual radioactivity was determined as described above. The half-lives of dissociation obtained by this method may be regarded as underestimates because the dilution factor used is below that generally regarded as being optimal.

## RESULTS

### Insulin Binding

The results of insulin binding experiments to PC13 EC cells and the differentiated derivatives are shown in Fig. 1a. No significant specific binding ( $<0.2\%$ ) of <sup>125</sup>I-insulin to PC13 EC cells was observed in any of six experiments. Five subclones of

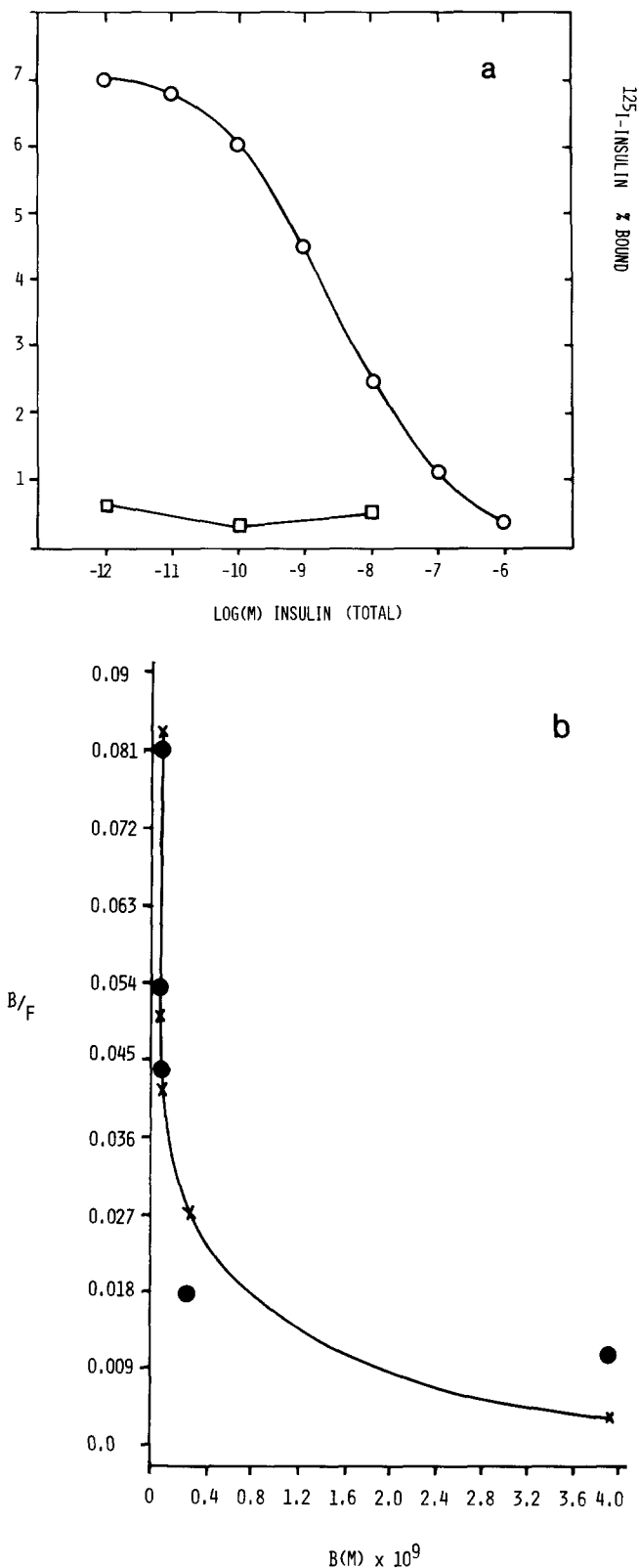


FIGURE 1 (a) The binding of <sup>125</sup>I-insulin to PC13 cells. Details of the assay procedure are as described under "equilibrium binding" in Materials and Methods. (□) binding to PC13 EC cells at  $4 \times 10^6$  per 20-cm<sup>2</sup> dish, (O) binding to PC13 EC cells after 6 d of retinoic acid treatment. Cells at  $2 \times 10^6$  per 20-cm<sup>2</sup> dish. (b) Scatchard analysis of <sup>125</sup>I-insulin binding to PC13 differentiated EC cells. Bound (B), free (F), experimental points (X), computed theoretical curve (●). The experimental curve represents the data from duplicates in three separate binding experiments.

PC13 clone 5 cells gave identical results. It would therefore appear that PC13 EC cells have few, if any, specific insulin receptors. When PC13 EC cells that have been allowed to differentiate in the presence of retinoic acid are examined, however, a significant displaceable binding of  $^{125}\text{I}$ -insulin is observed (Fig. 1a). Similar results are obtained with the differentiated progeny of five independent EC cell clones. Scatchard analysis of these binding data (Fig. 1b) shows the binding to be biphasic. This behavior is similar to that observed for specific insulin receptors in other tissues (for review, see 12). This biphasic binding curve closely fits the curve computed for the two-site negative cooperativity model by Kahn et al. (11), although the closeness of fit is not evidence per se for the existence of site-site interactions in differentiated PC13 cells. The constants computed from the fitted curve are  $K_e = 7.4 \times 10^7 \text{ LM}^{-1}$ ,  $K_f = 2.62 \times 10^{-6} \text{ LM}^{-1}$ , and  $R_o = 0.93 \text{ nmol}^{-1}$ . These constants are in the same range for those obtained for a number of other cultured cell lines (13). To establish further the nature of the observed binding, association and dissociation experiments were performed (Fig. 2a and b). Although monolayer binding conditions are not ideal for accurate measurements of association and dissociation constants, the approximate half-lives ( $t_{1/2}$  assoc = 35 min at  $22^\circ\text{C}$ ,  $t_{1/2}$  dissoc = 40 min at  $22^\circ\text{C}$ ) are again within the limits expected from insulin receptor studies in other systems (14).

The specificity of the insulin binding to differentiated PC13 cells was further examined by testing for cross-reaction with other polypeptides (Table I). Although EGF does not show any significant competition for insulin binding, MSA, which is known under some conditions to exhibit cross-reactivity with insulin receptors (15), shows slight competition as does desoctapeptide insulin, a chemically modified insulin with decreased binding activity (16).

Taken together, these results suggest that the differentiation of PC13 EC cells under the influence of retinoic acid results in the appearance of specific insulin receptors (calculated to be  $\sim 180,000$  per cell) that seem to have similar kinetic and cross-reaction properties to insulin receptors studied in other cell types. The ability to express insulin receptors on differentiation is possibly a general property of PC13 cells because each of the five separate subclones tested gave the same result (mean percent binding of EC cells, 0.15; of differentiated PC13 cells, 7.0). Thus the appearance of insulin receptors would seem to be a consequence of the differentiation of PC13 EC cells rather than the result of cell selection, unless selection is taking place during the transition itself. This latter possibility cannot definitely be eliminated. It is, however, unlikely because (a) it would require the death of about 98% of the cell population during the retinoic acid treatment (very little cell death is actually observed) and (b) it would require an average cell doubling time of  $<12$  h in any putative insulin receptor bearing population.

### Insulin Effects on Cell Proliferation

Differentiated PC13 cells slow their rates of cell division and DNA synthesis when placed in low concentrations of fetal calf serum (unpublished observations). This property provides conditions for a sensitive assay of the growth-regulatory effects of insulin and allows the measurement of a functional aspect of the insulin receptors observed in differentiated PC13 cells. We therefore examined the effect of exogenous insulin on the proliferation and DNA synthesis of differentiated PC13 cells maintained in 0.5% FCS (vol/vol).

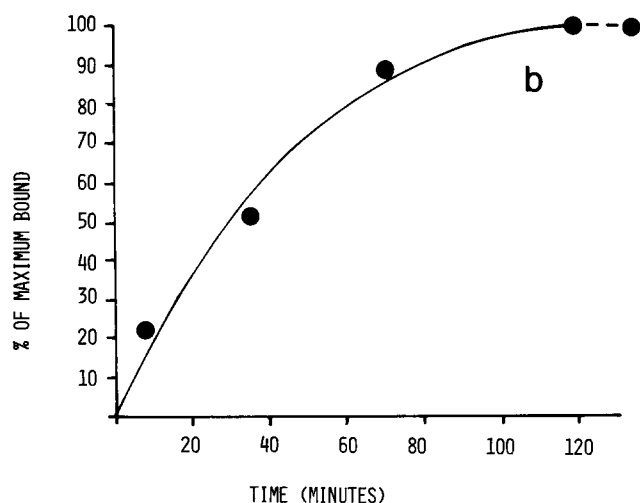
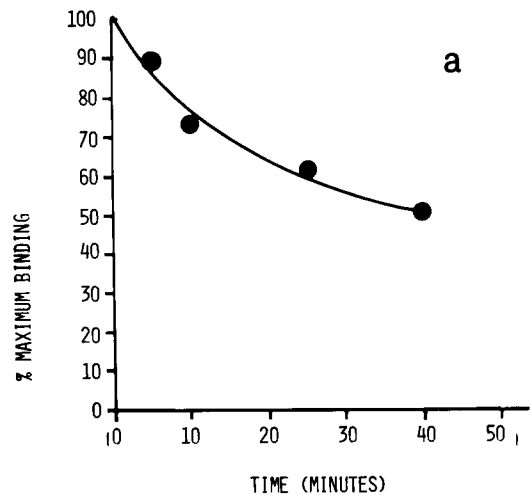


FIGURE 2 (a) Dissociation of  $^{125}\text{I}$ -insulin from PC13-differentiated EC cells. 100% of maximum bound represents 7% binding when  $[^{125}\text{I}\text{-insulin}] = 10^{-12} \text{ M}$ . (b) Association of  $^{125}\text{I}$ -insulin with PC13-differentiated EC cells. 100% of maximum bound represents 7% binding when  $[^{125}\text{I}\text{-insulin}] = 10^{-12} \text{ M}$ .

TABLE I  
Comparison of the Relative Abilities of Various Hormones to Inhibit Insulin Binding to PC13 Differentiated EC Cells

Hormone Concentration		Relative binding activity
$\mu\text{g}/\text{ml}^{-1}$		%
Insulin	2.5	100
Desoctapeptide	2.5	2
MSA	2.5	0.7
EGF	0.25	0

\* The relative binding activity is a measure of the difference in  $\text{LD}_{50}$  values of the hormones.

The addition of insulin results in a measurable dose-dependent stimulation of DNA synthesis in these cells (Fig. 3) as measured by  $[^3\text{H}]$ thymidine incorporation. The peak response is observed at insulin concentrations of  $20 \text{ ng}/\text{ml}$  ( $10^{-9} \text{ M}$ ). This response, although significant, is not as great as that observed on the addition of fresh FCS (up to 10% [vol/vol]) (Fig. 3) or other potent promoters of DNA synthesis in differ-

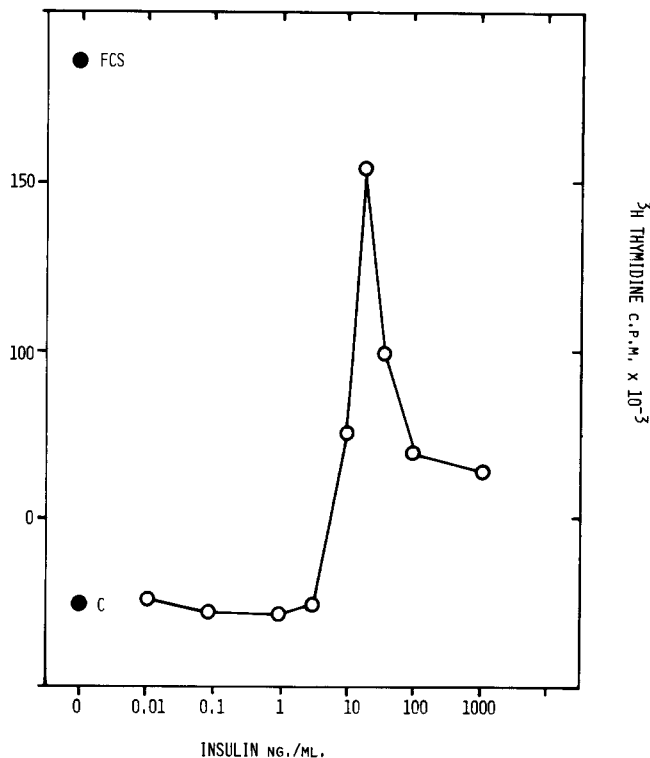


FIGURE 3 Acid insoluble [<sup>3</sup>H]thymidine incorporation into differentiated PC13 cells exposed to varying quantities of insulin (○—○); 0.5% (vol/vol) FCS (●C), and 10% (vol/vol) FCS (●FCS).

entiated PC13 cells such as EGF (7) or dexamethasone (Heath and Rees, manuscript in preparation).

To test whether the DNA synthesis response to exogenous insulin was accompanied by a concomitant increase in cell proliferation, the effect of insulin addition on differentiated PC13 cell numbers was examined (Fig. 4). An ~10-fold increase in cell numbers was observed in cultures exposed to insulin at 20 ng/ml over a 48-h period. If all the cells responded similarly, then this would correspond to the induction of about three rounds of cell division in these cells. After 48 h the division rate was observed to slow and to resemble that of the 0.5% (vol/vol) FCS control. This effect is possibly due to some form of down-regulation of the response to insulin because it was not abolished by refeeding with fresh insulin (Fig. 4).

FCS was a more potent stimulator of cell proliferation than insulin under these conditions, in that about five rounds of cell division were induced over 96 h compared to slightly more than three rounds of division for insulin over the same period.

Although the rates of initial stimulation (over 48 h) were similar for both mitogens, suggesting comparable potency, the insulin response appeared to be attenuated and this attenuation was not abolished by refeeding with insulin. By contrast, refeeding with FCS produced several further rounds of division (data not shown).

The insulin-dependent proliferation of differentiated PC13 cells is related to the appearance of specific insulin receptors because desoctapeptide insulin has little effect on cell proliferation (less than one round of cell division in 48 h) at 20 ng/ml correlating with its decreased affinity for the insulin receptor (Table I).

It is difficult to measure subtle promoters of PC13 EC cell proliferation because their rate of division cannot be readily modulated by the concentration of exogenous FCS. However,

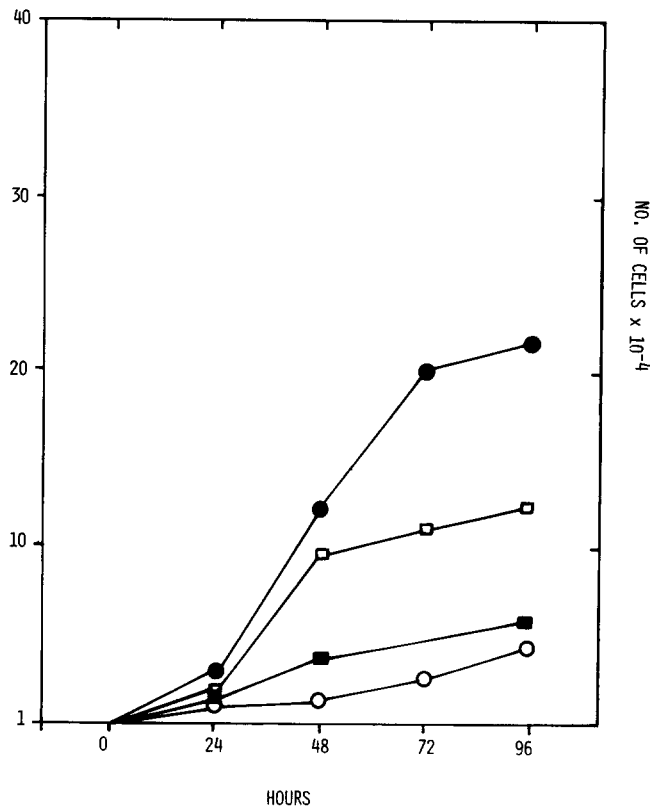


FIGURE 4 Increase of cell numbers in differentiated PC13 cells exposed to different growth conditions. (●) 10% (vol/vol) FCS; (□) 50 ng/ml<sup>-1</sup> insulin in 0.5% (vol/vol) FCS; (■) 50 ng/ml<sup>-1</sup> desoctapeptide insulin in 0.5% (vol/vol) FCS; (○) 0.5% (vol/vol) FCS. All cultures were refeed with fresh medium every 48 h.

the addition of either insulin or desoctapeptide insulin at 50 ng/ml has no significant effect on the rate of proliferation of PC13 EC cells maintained in 10% FCS (Fig. 5).

## DISCUSSION

Undifferentiated PC13 EC cells do not express measurable amounts of insulin receptors. We have presented observations that show that the differentiation of PC13 EC cells in the presence of retinoic acid leads to the expression of functional specific insulin receptors. The action of insulin upon these differentiated cells leads to a stimulation of DNA synthesis and cell proliferation.

Insulin has been reported to be a necessary component of various serum-free media developed for the *in vitro* culture of EC cell lines (17). The function of insulin in these media is, however, not clear (18). Our observations would argue that insulin cannot be acting through a specific cell surface insulin receptor in EC cells because such a receptor is apparently absent from these cells. Even if small numbers of receptors were expressed on transfer of EC cells from the FCS medium to the medium of Rizzino et al. (17), the concentration of insulin used in this latter medium (1 μg/ml) would be sufficient to down-regulate any receptors present. When insulin is used at concentrations normally sufficient to sustain growth (10<sup>-9</sup> to 10<sup>-10</sup> M) no survival effect is seen in EC cells. Our (unpublished) observations suggest that insulin can be substituted by desoctapeptide insulin in serum-free media and successfully support EC cell proliferation. It would appear that alternative explanations for the insulin requirement in the serum-free

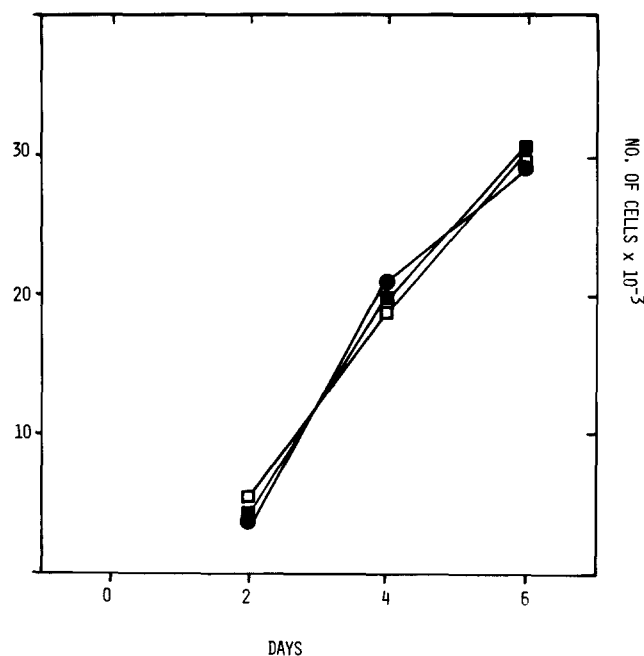


FIGURE 5 Increase of cell numbers in PC13 clone 5 EC cells exposed to different growth conditions. (●) 10% (vol/vol) FCS; (□) 50 ng/ml. insulin in 10% (vol/vol) FCS; (■) 50 ng/ml. desoctapeptide insulin in 10% (vol/vol) FCS. All cultures were refed with fresh medium every 48 h.

media for EC cells will have to be sought.

Salomon (19) has reported the presence of insulin receptors in cell cultures derived from the teratocarcinoma tumor OTT6050. The use of cloned, homogeneous, EC cell lines in our study would suggest that these cultures of Salomon's may have contained cells that resembled those derived from pure EC cells by retinoic acid treatment.

The growth-promoting action of insulin upon differentiated PC13 cells is somewhat unexpected, because, although 3T3 fibroblasts express insulin receptors (20), these 3T3 cells are not normally stimulated to proliferate by the addition of exogenous insulin (21). Insulin has, however, been reported to interact synergistically with other growth hormones in Swiss 3T3 cells to accelerate the rate of entry into S phase (for review see 22). It has also been shown that 3T3 cells can be stimulated to proliferate by insulin if the culture medium is depleted of essential nutrients. This would suggest that the mitogenic action of insulin in this case could be due to the accelerated intracellular transport of some rate-limiting nutrients (23, 24). It follows, therefore, that differentiated PC13 cells may have specific nutrient requirements for proliferation that can be supplied by insulin-stimulated transport processes.

The appearance of growth factor receptors during the differentiation of embryonal carcinoma cells may be a manifestation of a more general change in underlying growth-regulatory mechanisms accompanying this differentiation with its associated loss of malignancy. (See references 25 and 26 for examples of other EC lines that may conform to this general principle.) It is possible, for example, that apart from the appearance of specific receptors for growth-regulatory substances, there may

be an associated change in the intracellular mechanisms that regulate cell proliferation. This developmental aspect may make the *in vitro* differentiation of EC cells a useful system in which to study broader aspects of growth-regulatory mechanisms. At the very least, the differentiation of EC cells provides a suitable context in which to study the control of hormone receptor biosynthesis and expression.

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