Biochem. J. (1992) 285, 79–83 (Printed in Great Britain)



# A choice between glycogen and δ-crystallin accumulation is made in glial cells and not influenced by overlying neurons

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Chick-embryo neuroretinal cells convert extensively into lens under low-glucose conditions, but this transdifferentiation process is blocked by high-glucose media. We have previously observed an inverse relationship between the levels of glycogen (a marker of normal retinoglial differentiation) and of δ-crystallin (a lens marker) in such cultures. However, most of the glycogen accumulated under high-glucose conditions is apparently localized in those glial (G) cells underlying clusters of neurons (N cells). We here show that glial-enriched cultures (largely depleted of N cells) both accumulate glycogen and fail to transdifferentiate in high-glucose media. Moreover, glycogen localization in groups of glial cells is unaffected by the absence of N cells. Thus the choice between normal and foreign differentiation pathways is made autonomously within the retinoglial-cell population and is not influenced significantly by the presence or absence of N cells.

## INTRODUCTION

Previous work (Karim *et al.*, 1987) has demonstrated an inverse relationship between the levels of glycogen (a differentiated feature of retinal glial cells) and of δ-crystallin (a marker of lens conversion) in transdifferentiating cultures of chick-embryo neuroretinal (NR) cells. Glycogen is accumulated in retinal glia (Müller cells) both during normal differentiation *in vivo* (Kuwabara & Cogan, 1961; Macalh & Coimbra, 1970) and also *in vitro* during cell culture in the presence of high glucose concentrations (Karim *et al.*, 1987). In total NR cultures, high ambient glucose levels also suppress transdifferentiation into lens (de Pomerai & Gali, 1982), apparently by blocking transcription of the δ-crystallin locus (Carr & de Pomerai, 1985). In low-glucose cultures of NR derived from older (7–15-day) chick embryos (de Pomerai & Clayton, 1978), the δ-crystallin-expressing lens-like cells are derived mainly from dividing glial precursors (G cells; de Pomerai & Gali, 1981; de Pomerai *et al.*, 1984*b*; de Pomerai, 1988). This is confirmed by the observation of extensive transdifferentiation into lens in glial-enriched (GE) cultures of NR cells which have been stripped of neurons during the first few days *in vitro* by treatment with the neurotoxin chinoform–ferric chelate (Shinde & Eguchi, 1982).

Our previous study (Karim *et al.*, 1987) suggested that retinal G cells in culture might choose between normal differentiation towards the Müller-glial phenotype and a 'foreign' transdifferentiation pathway leading towards lens/pigmented-epithelium phenotypes. The former is encouraged by high-glucose and the latter by low-glucose media. *In situ* histochemical staining of high-glucose NR cultures suggests that both glycogen synthetase activity and glycogen deposits are co-localized mainly in G cells underlying clusters of neuron-like N cells (Karim *et al.*, 1987). At first sight this accords with other evidence to the effect that the normal differentiation of Müller glia depends upon interactions with overlying neurons (Linser & Moscona, 1979, 1983; de Pomerai *et al.*, 1982). This in turn implies that the N cells present in total NR cultures might co-operate with high glucose in steering G cells towards a normal Müller-glial fate, so suppressing transdifferentiation into lens. The longer survival of N cells in high-glucose cultures (de Pomerai & Gali, 1981, 1982) would also accord with this view.

However, as shown here, the presence of N cells is not obligatory for the effectiveness of the glucose block on transdifferentiation, since lens-cell production and δ-crystallin accumulation are efficiently suppressed by high-glucose media in neuron-stripped GE cultures. Moreover, the pattern of glycogen accumulation in GE cultures under low- and high-glucose conditions largely parallels that in total NR cultures. Continuous supplementation of high-glucose GE cultures with either forskolin (which stimulates adenyl cyclase and thereby promotes glycogen breakdown; Rousset *et al.*, 1985) or ouabain (which partially inhibits glucose uptake; Karim *et al.*, 1987) tends to increase δ-crystallin accumulation and decrease glycogen levels, as observed previously in total NR cultures. However, the effects on glycogen are less marked than those on δ-crystallin, suggesting that a straightforward choice between normal and foreign differentiation options is too simplistic.

## MATERIALS AND METHODS

### Materials

Fertile eggs were from Fakeeh Poultry Farm (Jeddah, Saudia Arabia). Tissue-culture media and sera were from GIBCO-Europe, (Paisley, Renfrewshire, Scotland, U.K.) and cyclic AMP assay kits from Amersham International. All other chemicals were from Sigma.

### Methods

**(i) Cell culture.** NR cells from 9-day-old chick embryos were cultured for up to 40 days as described by Karim *et al.* (1987). Cells were sown at a density of $5 \times 10^6$/ml in medium comprising Eagle's minimal essential medium with Earle's salts, 26 mM-$NaHCO_3$, 2 mM-L-glutamine, penicillin (100 i.u./ml), strepto-

Abbreviations used: N cells, retinal neurons; NR cells, neuroretinal cells; G cells, glial precursors: GE, glial-enriched; FH, FHG, FHGF and FHGO media are defined in the text; GSase, glutamine synthetase.

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mycin (100 $\mu$g/ml), 5% (v/v) horse serum and 5% (v/v) fetal-calf serum, plus extra glucose to a final concentration of 18 mM (FHG). FH medium omitted the glucose supplement (6 mM final concn.). Forskolin-treated cultures used FHGF medium, i.e. FHG containing $10^{-5}$ M-forskolin (Rousset *et al.*, 1985). Similarly, ouabain was added to $10^{-7}$ M in FHGO medium (Karim *et al.*, 1987). The protein content of all cultures was determined by the method of Lowry *et al.* (1951), with BSA as standard. Some NR cultures were stripped of > 90% of neuronal N cells by treatment with 50 $\mu$M-chinoform–ferric chelate between days 3 and 7 *in vitro* (Shinde & Eguchi, 1982). The resultant cultures are composed of monolayer sheets of epitheloid glial-like G cells (Müller glia and their precursors).

**(ii) Measurement of glycogen content.** The glycogen content of retinal cultures was determined as described previously (Rousset *et al.*, 1981; Karim *et al.*, 1987) by using the anthrone reagent. These assays were calibrated using known amounts of glucose alongside the test solutions containing glycogen. Histochemical staining for glycogen was performed as described previously (Hotchkiss, 1948; Karim *et al.*, 1987).

**(iii) Quantification of $\delta$-crystallin.** Haemagglutination-inhibition assays were performed as described previously, using a monospecific anti-$\delta$-crystallin antiserum (de Pomerai & Clayton, 1978; de Pomerai *et al.*, 1984*a*) and indicator sheep red blood cells coated with total newly-hatched-chick lens proteins (rich in $\delta$-crystallin). $\delta$-Crystallin levels in saline extracts of cultures were estimated by comparison of end points with those given by a known lens-protein standard containing 60% $\delta$-crystallin. Each data point represents the mean of at least six determinations. Standard errors are quite large, since a single-well difference in endpoint represents a two-fold dilution and thus a 2-fold difference in concentration.

**(iv) Cyclic AMP assays.** Cultures were extensively washed with phosphate-buffered saline (140 mM-NaCl/0.44 mM-$KH_2PO_4$/5.3 mM-KCl/2.5 mM-$Na_2HPO_4$/1.3 mM-$CaCl_2$/1.0 mM-$MgSO_4$, pH 7.5) before the assay procedure. Cyclic AMP levels were assayed in ethanol extracts prepared from such cultures, using an Amersham cyclic AMP assay kit according to the manufacturer's instructions (see Tovey *et al.*, 1974).

## RESULTS

As Fig. 1 shows, GE cultures produce few, if any, clusters of lens-fibre-like cells (lentoid bodies) at 35 (Fig. 1*a*) or even 50 days (not shown) in high-glucose medium, whereas parallel low-glucose GE cultures produce numerous lentoids at scattered locations across the sheet of G cells (Fig. 1*b*). This contrast is even more apparent from the data presented in Fig. 2. The levels of $\delta$-crystallin (a lens marker) accumulated in low-glucose GE cultures are some 20–25% lower than in parallel total NR cultures (possibly reflecting lower cell density), but there is a 40-fold difference in final $\delta$-crystallin levels between high- and low-glucose conditions (12–15% $\delta$-crystallin in low-glucose GE and total cultures, as against 0.2–0.3% in high-glucose cultures at 45 days). Continuous supplementation of high-glucose GE cultures with either forskolin or ouabain increases $\delta$-crystallin accumulation by some 25-fold relative to high-glucose controls (10–12% $\delta$-crystallin as against 0.4% at 50 days). This pattern for GE cultures closely follows that previously observed for total NR cultures (Karim *et al.*, 1987). These supplemented high-glucose GE cultures therefore transdifferentiate almost as extensively as low-glucose controls. The large standard errors characteristic of these assays (see the Materials and methods section) mean that none of the low-glucose or forskolin-/ouabain-supplemented high-glucose cultures are significantly different from each other, but all are significantly different from the unsupplemented high-glucose cultures ($P < 0.02$ using Student's $t$ test).

Turning to the data in Fig. 3, it appears that glycogen contents (per unit of protein) are some 25% higher in GE as compared with total NR cultures under identical high-glucose conditions at 20 and 30 days, though this difference becomes insignificant by 40 days. This may arise because N cells (which do not accumulate glycogen) remain present in the total NR cultures until at least 30 days (de Pomerai & Gali, 1982), so reducing the overall glycogen content. Under low-glucose conditions, glycogen levels decline and then reach a plateau both in GE and total NR cultures; notably N cells disappear much earlier (from 15 days onwards) in the latter case, and thus differ less markedly from their GE counterparts after 20 days. The net difference between low- and

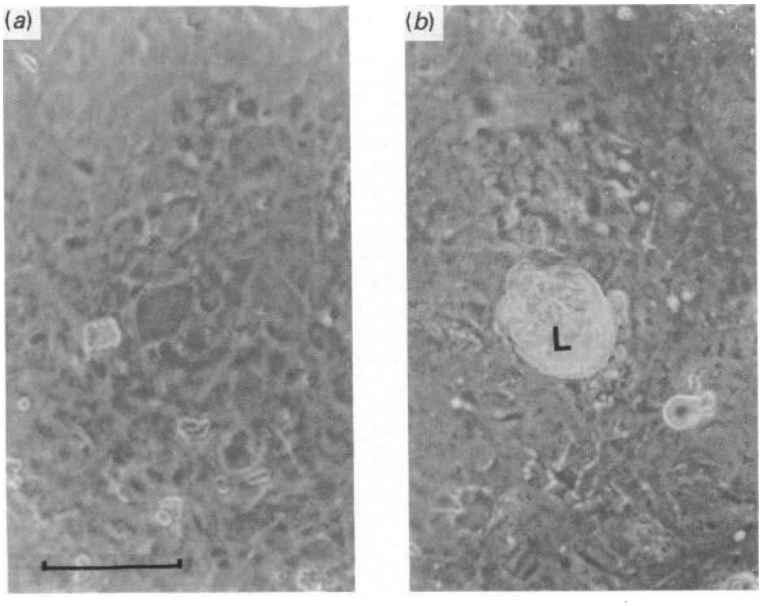


**Fig. 1. Phase-contrast photographs of glial-enriched cultures (chinoform-treated) in FHG (*a*) and FH (*b*) media after 35 days *in vitro***

Note lentoid (L) in (*b*); none could be found in FHG dishes (*a*). Clusters of neuronal cells were very sparse in both. The bar represents 100 $\mu$m.

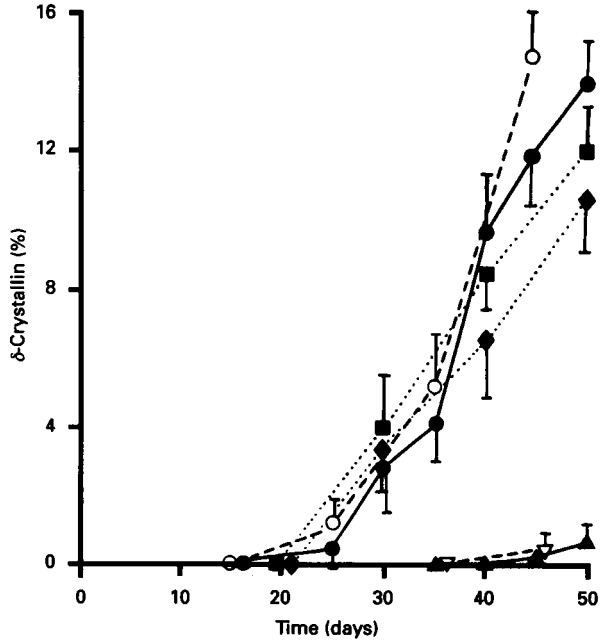


**Fig. 2. δ-Crystallin accumulation in retinal cultures**

Crystallin levels were determined by haemagglutination-inhibition assays (see the Materials and methods section). Each symbol and vertical line shows the mean ± S.E.M. from more than six such determinations. Both glial-enriched (GE; solid symbols) and total (open symbols) NR cultures were compared. Low-glucose FH medium: ○, total NR cultures; ●, GE cultures. High-glucose FHG medium: ▽, total NR cultures; ▲, GE cultures. Supplemented high-glucose FHG media: ■, GE cultures with ouabain; ◆, GE cultures with forskolin.

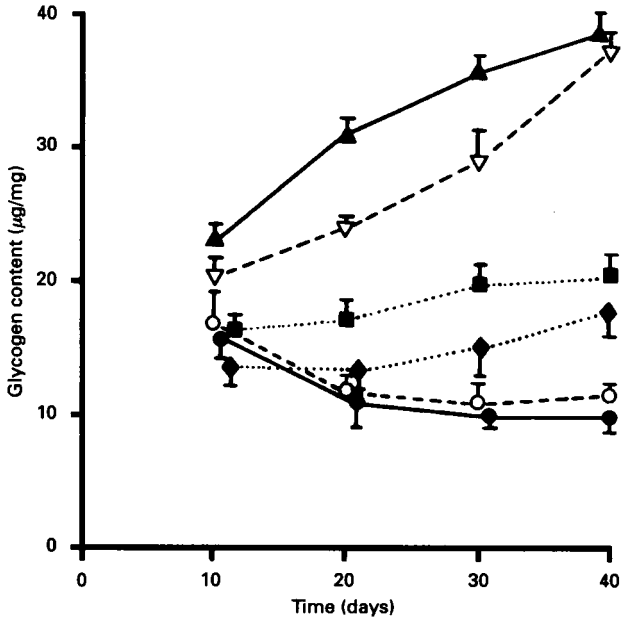


**Fig. 3. Glycogen accumulation in retinal cultures**

Glycogen was assayed as described in the Materials and methods section. Each symbol and vertical line shows the mean ± S.E.M. (bar) from four or more determinations. The key to the symbols is the same as for Fig. 2.

high-glucose conditions in terms of glycogen content is slightly greater for GE than for total NR cultures (about 3.5- as compared with 2.5-fold at 30 days, but nearly 4-fold for both types of culture at 40 days). Both ouabain and forskolin supplementation result in a marked decrease in glycogen accumulation in high-glucose GE cultures, but at all stages these glycogen contents remain substantially (30–90%) higher than in low-glucose controls. Intracellular cyclic AMP levels measured at 25 days are markedly higher in forskolin-treated cultures (14 ± 0.6 pmol/mg of soluble protein) as compared with high-glucose controls (8 ± 0.4 pmol/mg), but this difference is less marked in the case of ouabain-treated cultures (10 ± 0.2 pmol/mg); low-glucose controls contain 7 ± 0.7 pmol of cyclic AMP/mg at this same stage.

Histochemical staining for glycogen (Fig. 4) clearly demonstrates this difference between low- and high-glucose conditions in GE cultures [see Karim *et al.* (1987) for corresponding staining patterns in total NR cultures]. Both at 17 and at 28 days, glucogen staining is evident in groups of glial cells in the high-glucose cultures (Figs. 4*c* and 4*d*), but not in the corresponding low-glucose cultures (Figs. 4*a* and 4*b*). The patchy distribution of glycogen in high-glucose GE cultures is similar to that observed previously in total NR cultures, despite the absence of N cells.

## DISCUSSION

All neuroretinal and tapetal-cell types are ultimately derived from common neuroepithelial precursors constituting the inner and outer walls (respectively) of the optic cup. Such precursor cells, or their (relatively uncommitted) progeny, may initiate transdifferentiation into lens or pigment derivatives in culture; the depletion of such precursors with advancing retinal development could plausibly account for the observed decline in the extent of transdifferentiation using NR cells from older embryos (de Pomerai & Clayton, 1978). In any case, it is clear from many studies that the differentiated fates of particular neuroretinal (NR) cells are not dependent upon their ancestry, but rather upon local cell interactions. This process gives rise to an exquisitely structured tissue comprising concentric layers of different types of neuron plus radially arranged Müller glia. The differentiation of Müller glia *in vitro* has been shown to depend, at least in part, upon interactions with neighbouring neuronal cells. Thus aggregate, but not dispersed, monolayer cultures of NR cells can respond to exogenous cortisol by the induction of glutamine synthetase (GSase) in the Müller glia specifically (Linser & Moscona, 1979), suggesting that interaction with neurons is essential for the induction response within glial cells. Dense monolayer cultures of NR cells also show transient GSase induction by cortisol (de Pomerai *et al.*, 1982), but immunostaining reveals that the GSase so induced is localized specifically in glia underlying clusters of neuronal cells (Linser & Moscona, 1983). Similar patterns of staining were observed for two further markers of Müller-glial differentiation (glycogen and glycogen synthetase) in our previous study of glucose effects on NR cultures (Karim *et al.*, 1987) and were similarly interpreted.

The main conclusions of our present study contradict this neuron–glial interaction model. We find that glucose effects on cultured neuroretinal glial precursors (G cells) are not significantly mediated through the overlying neuronal (N) cells. This applies both to the high-glucose block on transdifferentiation (reflected in δ-crystallin levels; Fig. 2) and to the stimulation of glycogen accumulation by high glucose (Fig. 3). Apparently higher levels of glycogen in high-glucose GE as compared with total NR cultures probably result from the diluting effect of N cells (containing little glycogen) in the latter case. The data do not suggest a substantially higher glycogen content per G cell in total NR cultures (with N cells present) as compared with their GE counterparts under the same high-glucose conditions. The fact that glycogen contents are so similar in these two sets of

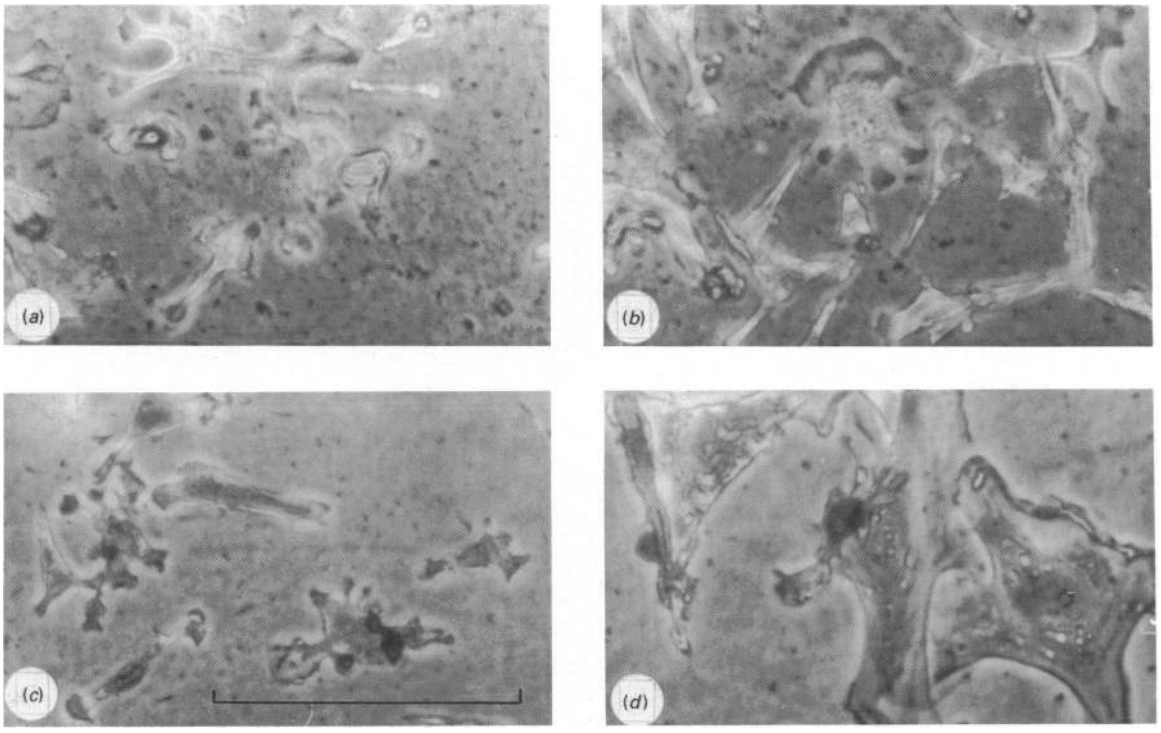


**Fig. 4. Histochemical staining for glycogen (purple coloration, appearing dark under the conditions used for black-and-white photography)**

Staining was performed as described in the Materials and methods section. GE cultures were stained at 17 days (*a* and *c*) and 28 days (*b* and *d*), comparing low-glucose medium conditions (*a* and *b*) with high-glucose conditions (*c* and *d*). The high-glucose fields selected show groups of G cells which stain intensely for glycogen (other cells stained much less intensely in these cultures); no such strongly staining cells could be detected under low-glucose conditions using an identical staining protocol. Patchy distribution of glycogen under high-glucose conditions was observed previously in total NR cultures (Karim *et al.*, 1987), but usually this occurred in G cells underlying clusters of N cells. The bar shows 100 $\mu$m.

cultures at 40 days confirms this inference, since by this stage most N cells have disappeared even from high-glucose total NR cultures. This calls into question our previous histochemical observations, which suggested much higher levels of glycogen synthetase activity and glycogen accumulation in those G cells underlying N-cell clusters in total NR cultures (Karim *et al.*, 1987). Such interactions are prerequisite for glucocorticoid induction of GSase activity in Müller glia, both in aggregate and monolayer cultures (Linser & Moscona, 1979, 1983; de Pomerai *et al.*, 1982). Overall, glycogen accumulation in groups of G cells does not depend on the continued presence of overlying N cells, as confirmed by our histochemical staining experiments (Fig. 4). Transdifferentiation into lens occurs mainly after 30 days *in vitro* (Fig. 2) and so is less likely to be influenced by N cells; however, the underlying determinative events take place mainly between 15 and 25 days, as revealed by transfer experiments between permissive and non-permissive medium conditions (de Pomerai & Gali, 1982). Our present data confirm that the presence or absence of N cells does not greatly influence the extent of $\delta$-crystallin accumulation nor the effectiveness of the high-glucose block.

The effects of two inhibitors (forskolin and ouabain) are more difficult to interpret, since both act in a relatively non-specific manner. Thus ouabain alters ion fluxes across the plasma membrane (Shinohara & Piatigorsky, 1977), as well as inhibiting glucose and other uptake processes; it was chosen in preference to more specific glucose-uptake inhibitors (such as phloretin and phlorizin) simply because NR cultures can better tolerate low concentrations of ouabain over long periods *in vitro* (Karim, 1987). Forskolin is an activator of adenyl cyclase (Rousset *et al.*, 1985), but increases in cyclic AMP levels will, of course, affect a wide variety of intracellular metabolic processes apart from glycogen breakdown. It would be rash to infer a direct link between the effects of these agents on $\delta$-crystallin and on glycogen levels in high-glucose GE cultures, even though both tend towards a low-glucose pattern (here as in total NR cultures; Karim *et al.*, 1987). However, these effects are not mediated via intracellular cyclic AMP levels, which are only slightly elevated by ouabain treatment, yet are almost doubled (as expected) by forskolin; in any case, cyclic AMP levels differ minimally between low- and high-glucose conditions (see also Karim *et al.*, 1987). A common link via glucose and glycogen remains plausible, but unproven; the use of more specific inhibitors will be required to prove the point.

The fact that the high-glucose block on transdifferentiation into lens remains equally effective in GE cultures should help to simplify future analysis of the underlying molecular events, since a single extrinsic medium factor (glucose) can be used to manipulate the pattern of differentiation and gene-expression within a single retinal cell-type (G cells). Since all G cells do not convert into lens even under optimal conditions, differences will have to be sought within the initial G-cell population. This will require markers for particular stages of Müller-cell differentiation, and some means for identifying pluripotent and/or putative stem cells within this population (see, e.g., Cotsarelis *et al.*, 1990). It remains possible that the presence of N cells during the earliest stages of culture (before chinoform treatment at 3 days) might in some way preset the future pattern of glycogen versus $\delta$-crystallin accumulation in GE cultures. However, when GE cultures are transferred from blocking (high-glucose) medium into permissive (ouabain- or forskolin-supplemented high-glucose) media and vice versa, the resultant glycogen/$\delta$-crystallin patterns are in-

fluenced mainly by the medium conditions prevailing between 15 and 25 days (cf. de Pomerai & Gali, 1982). That is, GE cultures transferred from permissive into blocking conditions at 25 days continue to transdifferentiate extensively into lens, but this is blocked if transfer is carried out at 15 days. Transfers at 15 days from blocking into permissive conditions allow transdifferentiation to proceed, but this remains blocked if transfer is deferred until 25 days (S. A. M. Karim & D. I. de Pomerai, unpublished work). Such evidence argues against any long-term influence of N cells that cannot be overridden by later medium conditions.

We thank Mr. R. Searcy for preparing the photographs and Miss J. E. Moreland for typing the manuscript.

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Received 1 July 1991/11 December 1991; accepted 18 December 1991