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Lentoidogenesis from neural retina cells in culture is affected by interactive relationships between different cell types

By SHIN TAKAGI[1], HISATO KONDOH[1], KAZUYA NOMURA[2] AND T. S. OKADA[1]

From the Institute for Biophysics, Faculty of Science, University of Kyoto

SUMMARY

By centrifugation in a Percoll gradient, two cell fractions were separated from cell populations harvested from 8-day cultures of neural retina cells of 3·5-day-old quail embryos. The heavy (H-) fraction contained mostly N-cells, which are considered to be putative neuronal cells, while the light (L-) fraction contained both E-cells, putative retinal glial cells, and N-cells. Determination of choline acetyltransferase activity in both fractions suggested that this enzyme is predominantly localised in N-cells. After replating the separated L-fraction for further culturing, frequent lentoidogenesis occurred from clusters of N-cells which, though few in number, were included in this fraction. Addition of H-fraction to L-fraction cells caused a significant increase in lentoidogenesis up to a ratio of N- to E-cells of 3: 1. However, addition of excess H-fraction cells beyond this ratio inhibited lens differentiation. This difference in the expression of lens phenotypes resulting from the different ratios of H- and L-fraction was confirmed by monitoring the level of δ-crystallin in cultures. These results are discussed in the light of interactive relationships between N- and E-cells in the transdifferentiation of neural cells into lens in cell culture.

INTRODUCTION

Vertebrate embryonic neural retina (NR) can transdifferentiate into lens cells, when dissociated and cultured *in vitro* (cf. reviews by Okada, 1976, 1980*a*; Clayton, 1979). This phenomenon is especially prominent in early chick or quail embryos such as 3·5 days of incubation (Araki & Okada, 1977, 1978; Nomura & Okada, 1979). *In ovo*, however, lens cells are derived exclusively from their ancestral cells in the lens placode, and never from NR. The abnormal pathway of differentiation of NR into lens cells observed in culture is regarded as a remnant of the potential of some non-lens ocular tissues to regenerate lens in some amphibians (see Yamada, 1977). The study of this dramatic switch of major differentiation is advantageous for elucidating the mechanisms of determination and stabilization of differentiated states in general.

[1] *Authors' address:* Institute for Biophysics, Faculty of Science, University of Kyoto, Sakyo-ku, Kyoto 606, Japan.
[2] *Author's address:* Institute of Biology, Faculty of Science, Kyushu University, Higashi-ku, Fukuoka 812, Japan.

NR cells during the early stages of culture comprise at least two distinctive cell types, putative neuronal cell (N-cells) often with axon-like processes of various lengths, and epithelial cells (E-cells) representing retinal glial cells (Nomura, Takagi & Okada, 1980). Previously, we reported observations to suggest the N-cell origin of lens cells appearing in the later stages of culture (Okada, Yasuda & Nomura, 1979; Yasuda *et al.*, 1982). However, it seems certain that E-cells transdifferentiate into lens cells in NR cultures (Okada, 1977). Evidence for the transdifferentiation of lens from N-cells remains to be demonstrated. In a previous paper, we reported a method to separate two different cell populations, the one mostly consisting of N-cells and the other of E-cells, by fractionating the cells harvested from early NR cultures (Yasuda *et al.*, 1982). We now attempt to examine how N-cells actually take part in lens differentiation by recombining the separated fractions in different ratios for further culturing. The results suggest lentoidogenesis (Moscona & Degenstein, 1980) may occur from N-cells and also indicate that cellular interactions may occur between N- and E-cells in the process of transdifferentiation.

MATERIALS AND METHODS

Primary culture

Pure pieces of NR were obtained from Japanese quail embryos at 3·5 days of incubation, corresponding to Hamburger-Hamilton's stage 21 in chick development, as described by Araki and Okada (1978), and were dissociated by the treatment with 1 mM EDTA in Ca- and Mg-free Hanks' saline. Into each plastic culture dish of 35 mm diameter with a collagen-coated substrate (Nomura & Okada, 1979), 2×10^6 cells were inoculated. The culture medium was Eagle's minimum essential medium (Nissui) supplemented with 10 % foetal calf serum (Gibco) and 0·2 mM ascorbic acid (Itoh, 1976).

Separation of different types of cells

Cultures were washed with Hanks' saline, treated with 0·05 % collagenase (Amano) in Hanks' saline at 37 °C for 30 min, washed with HEM (Ca-free Hanks' saline buffered with 10 mM-HEPES containing 1 mM-EGTA and 1 mM-$MgCl_2$). After treating washed cultures with 0·25 % trypsin (Difco) in HEM at 37 °C for 20 min, a half volume of the culture medium was added and a single-cell suspension was obtained by gentle pipetting. Cells were pelleted and resuspended in HEM containing 0·05 % deoxyribonuclease I (Sigma) and 0·01 % pokeweed trypsin inhibitor (Sigma). A cell suspension of 2·4 ml was mixed in a polyallomer tube (Beckman) with 1·6 ml of 100 % Percoll which was made by mixing 9 parts of Percoll (Pharmacia) with 1 part of 10× concentrated Puck's saline G, and centrifuged at 13 000 ***g*** for 90 min at 15 °C in a Beckman Sw50-1 rotor. After centrifugation, cell fractions were collected either dropwise by puncturing the bottom of the tube or from the side of the tube by drawing each visible

band into a sterile syringe through 22-gauge needles. Buoyant density of the fractions was determined using marker beads (Pharmacia). Each fraction was diluted with 10 volumes of HEM, and cells were pelleted by centrifugation at 1500 *g* for 10 min and resuspended in an appropriate medium (see later).

Culture of separated cells

A mixture of fresh medium and the medium from the original culture (conditioned medium) in the ratio of 1: 1 was used to replate the separated cells into a collagen-coated culture well with a diameter of 15 mm. To culture recombined cell populations, the cells were mixed before replating.

Other methods

δ-crystallin was quantitated by the haemagglutination inhibition assay (Evans, Steel & Arthur, 1974), as described by de Pomerai & Clayton (1980). The activity of CAT (choline acetyl-transferase, EC.2.3.1.6.) was measured as described previously (Crisanti-Combes, Pessac & Calothy, 1978; Nomura *et al.*, 1980). Protein concentration was determined according to Lowry, Rosebrough, Farr & Randle (1951), using bovine serum albumin as a standard. Staining of cultures with merocyanine 540 and indirect immunofluorescent staining of δ-crystallin were carried out according to Yasuda *et al.* (1982) and Okada, Itoh, Watanabe & Eguchi (1975).

RESULTS

Primary cultures

In stationary cultures of dissociated NR cells of 3·5-day-old quail embryos, putative neuronal cells (N-cells) and underlying epithelial cells (E-cells) became distinct during the first week and lentoidogenesis began in 10-day-old cultures. Many lentoid bodies (LB) were formed in the locations where clumps of N-cells had been found in earlier culture stages, and often they were observed to originate directly from the latter (see Fig. 1). Thus the general features of transdifferentiation of dissociated quail embryonic NR cells into lens in culture, were similar to those repeatedly demonstrated with the corresponding chick embryonic material (Araki & Okada, 1977, 1978; Nomura *et al.*, 1980; Yasuda *et al.*, 1982).

Properties of separated NR cell fractions

Cell fractionation was achieved using cells harvested from 8-day cultures, in which the activity of CAT was at a peak and thus the expression of neuronal phenotypes was maximal, while lentoidogenesis had not yet started (Nomura *et al.*, 1980). A suspension of dissociated cells in Percoll was centrifuged to generate the gradient and to separate the cells according to their buoyant density. The cells

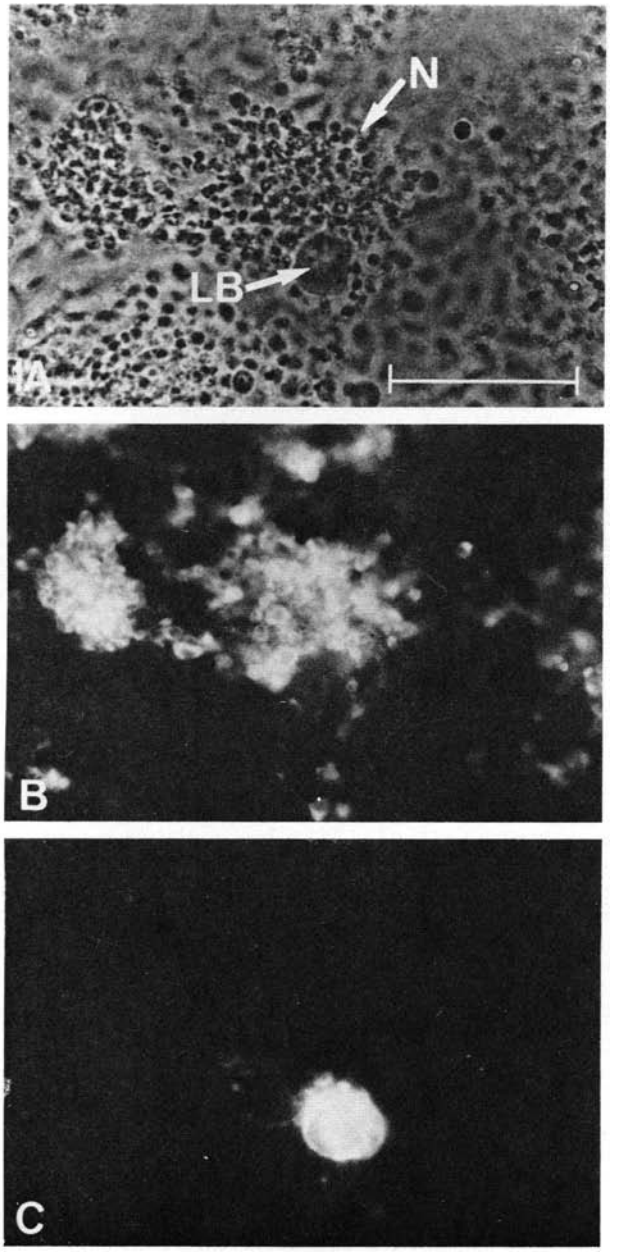

Fig. 1. The differentiation of LB amidst a clump of N-cells in 12-day culture. (A) A phase-contrast micrograph of a living culture. (B) A fluorescent micrograph of the same field as shown in A, after staining with merocyanine-540. (C) An immunofluorescent micrograph of the same field as shown in A and B, fixed and indirectly stained with anti-δ-crystallin. Note fluorescence only in N-cells in B, while in LB in C. LB; lentoid body: N; N-cells. Bar represents 100 μm.

were mainly located in two visible bands formed near the top and the bottom of a gradient, with density of 1·04 g/cm^3 and 1·08 g/cm^3, respectively (Fig. 2). The cell fraction forming the upper band is designated as L (light), while that forming the lower band is designated as H (heavy).

The H-fraction consisted exclusively of small cells (average 7 μm in diameter) probably representing the N-cells in cell cultures *in situ*, while the L-fraction (average 10 μm in diameter) contained many larger cells (between 15 μm and 25 μm in diameter), probably representing E-cells, together with small cells as well (Fig. 3).

In order to assess neuronal characteristics in the separated cell populations, the level of CAT activity in each fraction was determined (Table 1). H-fraction had two-fold higher specific activity of CAT than L-fraction. This difference was expected, if the enzyme is present only among small cells of each fraction. In a previous paper, we reported a similar distribution of CAT activity between two fractions separated from chick NR cultures (Yasuda *et al.*, 1982).

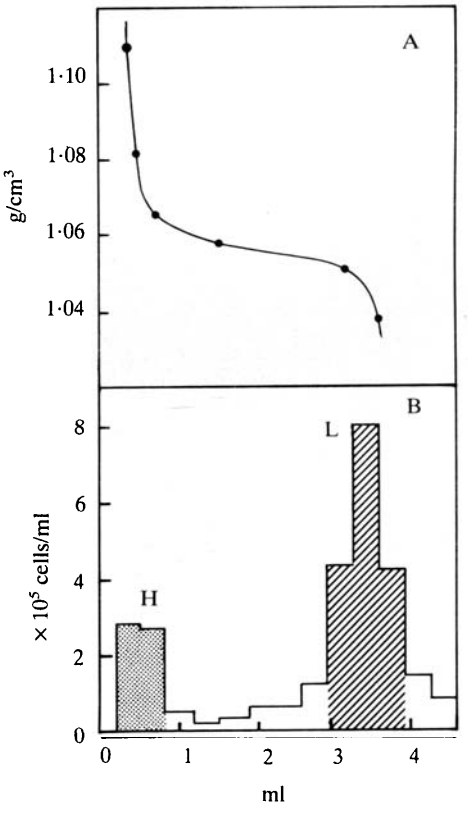

Fig. 2. Separation of two cell fractions, H and L, from dissociated cells of 8-day NR cultures in Percoll. Abscissa; volume from the bottom of the tube. Ordinate; buoyant density (in A) and cell concentrations (in B). Shaded area of H and L indicate the cell populations taken as two different fractions.

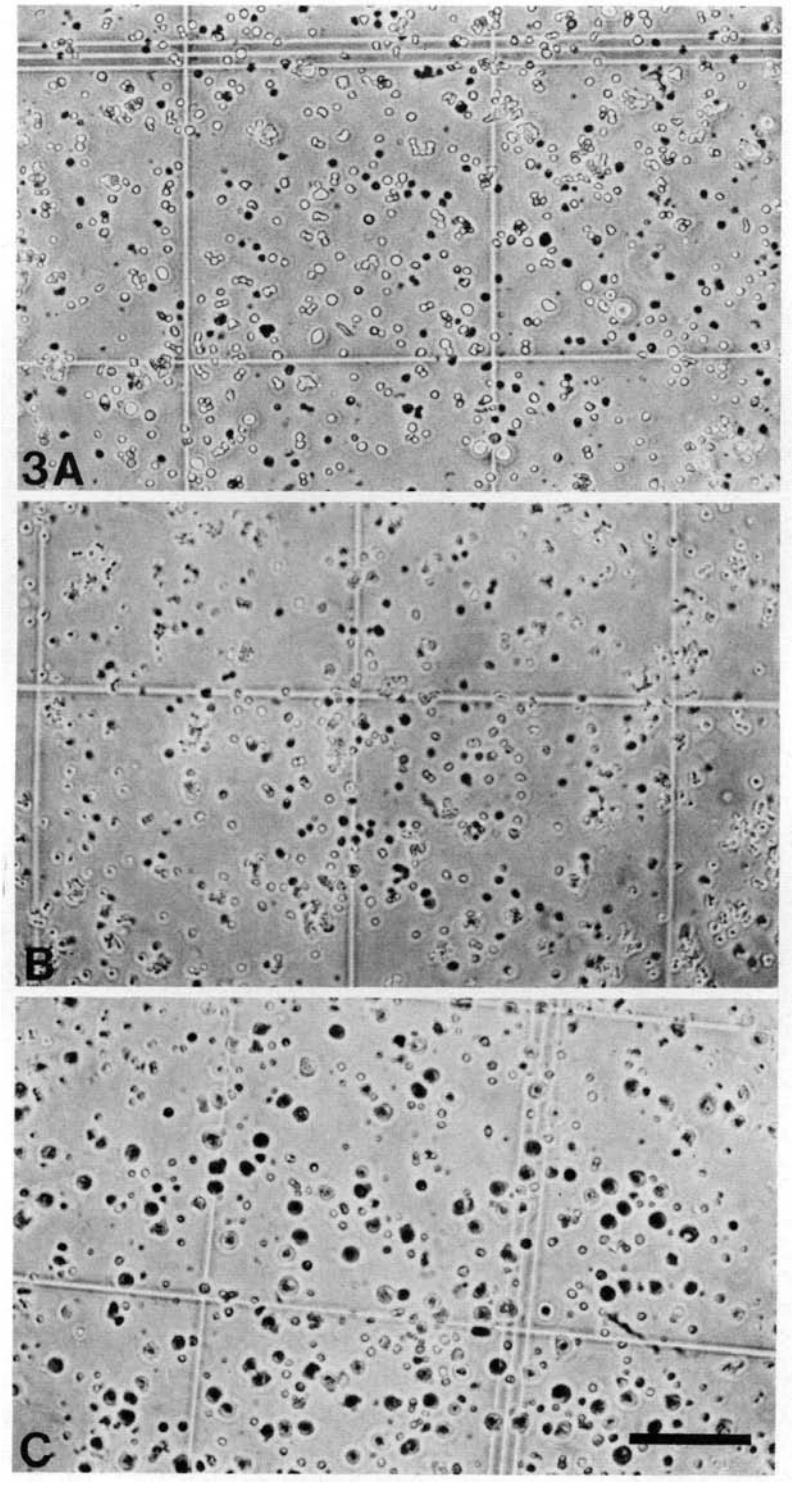

Fig. 3

Table 1. *Some properties of two different cell fractions separated from 8-day cultures of 3·5-day embryonic quail NR*

Experiment	Fraction*	Relative† yield (%)	Average cell diameter (μm)	Trypan blue exclusion	CAT activity (pmol acetylcholine/min/mg protein)
	U	100	–	–	64
I	H	20	7	95	84
	L	60	10	80	36
	U	100	–	–	66
II	H	60	–	–	78
	L	20	–	–	42

* U; unfractionated, H; H(heavy)-fraction, L; L(light)-fraction.
† This was subjected to considerable experimental variations reflecting the extent of differentiation of N-cells in primary cultures. These experiments represent two extreme cases with the lowest and the highest H to L ratio.

Cultures of cells of separated fractions

The majority of cells in separated fractions excluded trypan blue (Table 1), indicating that most cells remained viable after centrifugation in Percoll under our conditions. When replating them separately, cells of H-fraction (Hf-cells) and of L-fraction (Lf-cells) behaved quite differently. Within 24 h after replating Lf-cells E-cells adhered to the culture substrate and N-cells attached on top of them. Thereafter, flattened E-cells grew and established a confluent cell sheet, while N-cells, though well maintained for the first week, gradually decreased in number (Figs. 4A and 5A). Lentoidogenesis started 5 days after replating. Some foci of pigmented epithelial cells were also formed from this time point on.

By contrast, most Hf-cells remained unattached after replating and were gradually lost from cultures over several days. A few clusters of N-cells appeared to attach onto culture dishes. However, a careful examination revealed that this attachment was not direct to the culture substrate, but indirect via anchorage to E-cells, which were still included in H-fraction. These observations indicate that N-cells by themselves fail to adhere to the culture substrate, and they require E-cells for their anchorage and survival. These very sparsely attached clumps detached within several days and were lost from cultures. Thus, under present culture conditions, it was not possible to maintain cultures of Hf-cells alone long enough to examine the occurrence of transdifferentiation.

Cultures of recombined cell populations

In primary cultures, lentoid bodies (LBs) appeared very frequently from

Fig. 3. Micrographs of a cell suspension of 8-day cultures before fractionation (A), of H-fraction (B) and of L-fraction (C). Bar represents 100 μm.

small- to middle-sized clumps of N-cells (Okada, 1980*a*; Yasuda *et al.*, 1982). This observation suggests that LBs might originate from N-cell clumps directly, or from E-cells closely associated with the clumps (i.e. able to interact with the latter). To determine whether any interactive relationships occur between N-cells and E-cells during the transdifferentiation, and also to elucidate cell-type origin of lens cells in transdifferentiating cultures of NR cells, we mixed Hf-cells with Lf-cells to obtain cultures with different numbers of N-cells over a constant number of E-cells. To 5×10^6 Lf-cells, 0, 5, 15 and 30×10^6 Hf-cells were added. The third combination corresponded to the ratio in 8-day primary cultures at the time of harvesting cells for fractionation.

Figure 4 shows the recombined cultures 2 days after replating. Because half of the cells in L-fraction were small, the cultures of Lf-cells alone should contain roughly an equal number of E- and N-cells. When 5×10^6 Hf-cells were added, N-cells were distributed more densely over a sheet of E-cells. With further addition of Hf-cells, the whole surface of the E-cell sheet was completely covered with N-cells. In all cultures, N-cells were distributed evenly over the culture surface, though the distribution of N-cells was uneven in primary cultures.

LB started to appear after 5 days in recombined cultures and gradually increased in number. Although substantial lentoidogenesis occurred in cultures of Lf-cells alone, addition of an equal number of Hf-cells resulted in more extensive production of LB (Figs. 5A, B). Daily observations disclosed that many of the LBs were apparently derived directly from clumps of N-cells by the same process as described in primary cultures of chick embryonic NR cells (Okada, 1980*b*; Yasuda *et al.*, 1982). Some supporting evidence for this claim (in the quail NR system) might be helpful. Further addition of Hf-cells inhibited lentoidogenesis in cultures and few LBs were found after a total of 28 days' culturing (Figs. 5C, D). These results were the same throughout three culture batches each started from a different inoculum.

The level of δ-crystallin in cultures was measured 28 days after the initiation of recombined cultures. The results confirmed our observations of living cultures. As shown in Table 2, the δ-crystallin level was maximal in cultures where Hf- and Lf-cells were present in a ratio of 1: 1, while it became much lower in the presence of excess Hf-cells.

It is possible that the suppression of lens differentiation with excess N-cells in the previous series of experiments could simply be due to too high cell density. Thus, in the next series, we mixed Hf-cells with Lf-cells in different ratios so as

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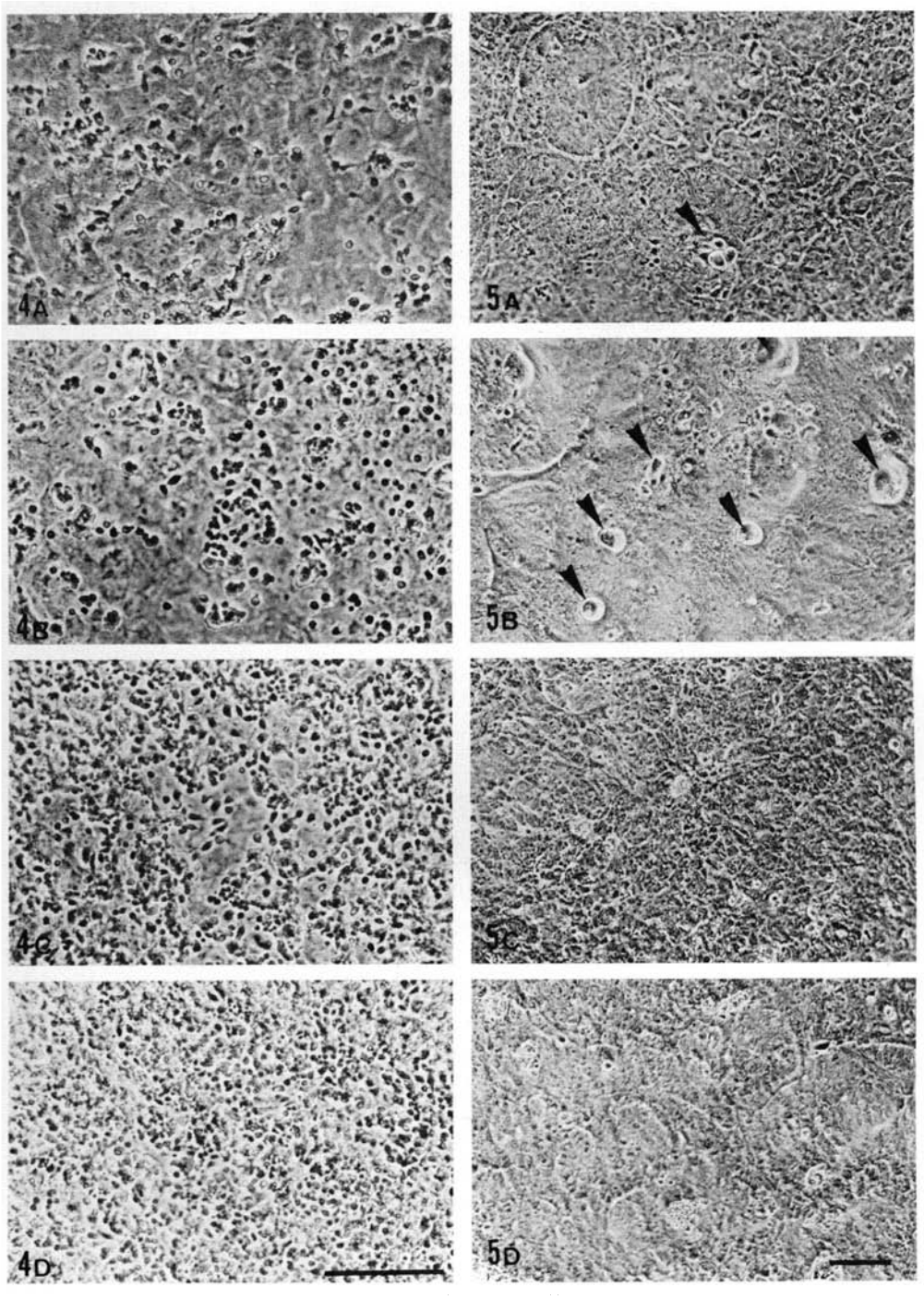
Fig. 4. Living cultures after recombination of different numbers of Hf-cells added to 5×10^6 Lf-cells, 4 days after replating. (A) no addition of Hf-cells. (B) addition of 5×10^6 Hf-cells. (C) addition of 15×10^6 Hf-cells. (D) addition of 30×10^6 Hf-cells. Bar represents 100 μm.

Fig. 5. Living cultures after recombination of different numbers of Hf-cells added to 5×10^6 Lf-cells, 20 days after replating. A, B, C and D correspond to those of Fig. 4. Arrows indicate LB. Bar represents 100 μm.



Figs. 4 & 5

Table 2. *The effect of adding different numbers of Hf-cells to a constant number of Lf-cells on lens differentiation*

Initial ratio*		δ-crystallin content†	
H to L	N to E	μg/plate	% soluble protein
0:1	1:1	20	7
1:1	3:1	80	20
3:1	7:1	0·6	0·5
6:1	13:1	0·3	0·2

* The number of Lf-cells was 5×10^6 cells/plate. L-fraction contained roughly an equal number of N- and E-cells in this particular experiment. H; H-fraction. L; L-fraction. N; N-cells. E; E-cells.

† Determined after a total 36 days' culturing, i.e. 28 days after replating.

Table 3. *Suppression of lens differentiation by high N- and E-cell ratios*

Initial ratio*		δ-crystallin content† (μg/plate)
H to L	N to E	
0:4	2:1	100
1:3	3:1	70
2:2	5:1	20

* The total number of cells was 2×10^6 cells/plate. L-fraction in this particular experiment contained N- and E-cells in the ratio of 2 to 1.

† Determined after a total 27 days' culturing, i.e. 19 days after replating.

to maintain the total number of replating cells constant. Cultures were terminated 2 weeks after replating and the level of δ-crystallin was determined (Table 3). With the increase of Hf- to Lf-cell ratios, the δ-crystallin level decreased. This result indicates that a high N- to E-cell ratio caused an inhibition of lens differentiation. Thus, it seems that an optimal N- to E-cell ratio exists for transdifferentiation of lens from NR cells in culture.

The formation of foci of pigmented epithelial cells occurred in most cultures. However, there was little difference in this respect following recombination of different ratios of the two cell fractions.

DISCUSSION

In a previous paper, we suggested that lentoidogenesis from clumps of N-cells with neuronal characteristics may occur in NR cultures from chick embryos (Okada *et al.*, 1979; Yasuda *et al.*, 1982). To demonstrate more directly that N-cells are precursors of lens cells in transdifferentiating cultures, we attempted to

isolate N-cells from the epithelial cell population and to culture them to examine their differentiation. To this end, we fractionated cell populations from cultures at early stages by isopycnic centrifugation in Percoll. As we previously reported for chick cells (Yasuda *et al.*, 1982), N-cells in cultures of 3·5-day-old embryonic NR of quail were isolated nearly free from E-cells, and the isolated cells were mostly viable. This successful fractionation was achieved by the use of EGTA, instead of EDTA, as a chelating agent and by protecting cells with Mg^{2+} to increase viability. The use of EGTA is based on the fact that a calcium-dependent mechanism is essential for cell adhesion between NR cells, while Mg^{2+} ions have little significance in this respect (Takeichi, Ozaki, Tokunaga & Okada, 1979). Sheffield (1982) also reported a successful fractionation of different cell types from freshly dissociated cells of 14-day-old chick embryonic NR.

However, it has not been possible yet to maintain cultures of the separated H-fraction alone for long enough to follow their ability to transdifferentiate in the present culture conditions. Therefore, this separated fraction was recombined with L-fraction cells in different ratios and cultured to assay the degree of expression of the lens phenotype. The extent of lens differentiation was greatly (though only quantitatively) affected by different ratios of recombination. Addition of H-fraction to L-fraction cells up to an initial N: E cell ratio of 3: 1 resulted in an increase in lentoidogenesis. This result could be attributed either to lens differentiation from the added N-cells (H-fraction), or else to cell interactions between N- and E-cells. However, an increase in the proportion of N-cells beyond this ratio greatly inhibited lentoidogenesis.

So far a number of factors have been shown to affect the process of transdifferentiation from NR cells into lens cells; for instance, the type of culture medium (Araki & Okada, 1978; Agata *et al.*, 1980), the type of serum used for culturing (de Pomerai & Gali, 1981*a*,*b*), supplements to the standard media such as hormones (de Pomerai & Clayton, 1980) and ascorbic acid (Itoh, 1976), the initial cell density etc. (see review by Okada, 1980*a*). In addition, the present results showing inhibition of lentoidogenesis by excessive N-cells suggest that interactions between N- and E-cells may affect the transdifferentiation of lens from NR cells in culture.

It should be remembered that aggregates of freshly dissociated NR cells cultured on a gyratory shaker never transdifferentiate, while they maintain some characteristic neuronal phenotypes even after culture periods long enough to allow transdifferentiation to occur in sister cell populations maintained in stationary culture (Okada *et al.*, submitted). A disruption of native cell–cell interactions, topographically as well as functionally, can be a cue to cause a major switch of differentiation of NR cells into the lens direction (Moscona & Degestein, 1980; Okada *et al.*, 1982). As shown repeatedly in NR cultures, there is a gradual decrease in the numbers of N-cells with time prior to transdifferentiation (Nomura *et al.*, 1980). Therefore, it is speculated that by replating recombined cell populations with a high ratio of H-fraction (rich in N-cells), conditions

similar to the initial stage of culturing or to aggregate cultures are recovered and native cell interactions in intact NR tissue can be regained, at least partially. With a drop in the ratio of N- to E-cells below a critical level, an altered system of cellular interactions starts to operate between these two cell types, which leads to a switch of both N- and E-cells into the pathway of lens differentiation.

There are several examples indicating a critical role for cellular interactions between different cell types within the nervous tissues for cell differentiation. The differentiation of pigment cells occurs when chick embryo ganglia are cultivated in a stationary culture so as to disrupt native topographical relationships between constituent cells of the ganglia *in situ* (Nicholas & Weston, 1977*a*,*b*). The fact that the expression of glial cell-specific phenotypes depends on the interaction of their precursor cells with neighbouring neuronal cells was shown in cultures of both spinal ganglion and NR (Linser & Moscona, 1979; Holton & Weston, 1982*a*,*b*). The present results suggest a phenomenon belonging to the same category as these examples.

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