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Stimulation of DNA synthesis in carcinogen-induced diploid hepatocytes *in vitro*

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Diploid hepatocytes induced by a combination of diethylnitrosamine and 2-acetylaminofluorene were isolated and separated from polyploid hepatocytes by centrifugal elutriation. The diploid and polyploid cell fractions were ~90% pure and contained between 1 and 1.5 × 10^7 cells. When kept in monolayer cultures both cell populations responded to the mitogenic effect of EGF and insulin. However, the percentage of labelled nuclei was higher in predominantly diploid compared to predominantly tetraploid hepatocyte cultures at all epidermal growth factor (EGF) concentrations used in this study. At 10 ng EGF/ml and 10 mU insulin/ml the labelling index was twice as high in the diploid liver cells. Further work is required to show the relevance of the stronger response of the diploid cell fraction to mitogens in the process of carcinogenesis.

Introduction

During the developmental growth of rat liver, an increase in ploidy of hepatocytes takes place (1,2). The neonatal liver is diploid, but the portion of diploid, tetraploid and octaploid hepatocytes in the adult animal amounts to 4–10%, 70–80% and 5–20% respectively (1,3,4). The functional significance of polyploidization is still not known. It has been proposed that this process represents an irreversible aspect of hepatocellular differentiation (1,2). Sequential treatment of partially hepatectomized rats with diethylnitrosamine (DEN*, 1 × 50 mg/kg body wt) and 2-acetylaminofluorene (2-AAF, 0.02% in the diet for 4 weeks) causes the emergence of diploid liver cells (3); after termination of the treatment >40% of the hepatocytes are diploid. These newly arising diploid liver cells most likely contain the precursor cells for liver carcinomas; because neoplastic nodules and carcinomas induced by the protocol contain mostly diploid cells as compared with the surrounding hepatocytes, which are predominantly polyploid (5). A role of diploid preneoplastic hepatocytes as precursors of hepatocellular tumor cells is also suggested by theoretical considerations. A diploid genome maintains a higher risk of further carcinogenic progression than a polyploid genome.

During the stepwise transformation of a normal cell to a malignant cell there is a change in the control of proliferation. The basis of this change can be alterations in the requirement, reception and transduction of growth signals including autocrine mechanisms (6–10) as well as a deficient growth inhibition (11). Because there is very limited information on growth control of pre-stages of tumor formation, the present study isolated carcinogen-induced diploid hepatocytes by means of centrifugal elutriation and examined the stimulation of DNA synthesis by epidermal growth factor (EGF) and insulin *in vitro*.

*Abbreviations: DEN, diethylnitrosamine; 2-AAF, 2-acetylaminofluorene; EGF, epidermal growth factor; PVP, polyvinylpyrrolidone; fraction I, elutriated cell fraction containing predominantly diploid hepatocytes; fraction III, elutriated cell fraction containing predominantly polyploid hepatocytes; LI, labelling index.

Materials and methods

Animals

Male Wistar rats (4 weeks old, 70–80 g body wt), from the GSF breeding colonies, were subjected to a two-thirds hepatectomy; 20 h later they received DEN (1 × 50 mg, Serva, Heidelberg, FRG) by gastric intubation. After 1 week the animals were fed a standard pellet diet (Altromin, Lage, FRG) containing 0.02% 2-AAF for a period of 5 weeks.

Media

The medium used for centrifugal elutriation contained: NaCl (137 mM), KCl (5.6 mM), $MgSO_4 \cdot 7H_2O$ (1 mM), $CaCl_2 \cdot 2H_2O$ (1 mM), KH_2PO_4 (0.9 mM), $Na_2HPO_4 \cdot 2H_2O$ (2.1 mM), glucose (5 mM), MEM amino acids and vitamins (Biochrom KG, Berlin, FRG), HEPES (20 mM), polyvinylpyrrolidone (PVP, average mol. wt 40000; Sigma, Deisenhofen, FRG) (1%) and 100 μg DNase I/ml (bovine pancreas 3500 U/mg, Boehringer, Mannheim, FRG), pH 7.4.

Modified Waymouth MB 752/1 is fortified with some nutrients and trace elements and some components have been adjusted to more physiological values: $NaHCO_3$ (25 mM), KCl (5.4 mM), D-glucose (6 mM), D-galactose (3 mM), L-lactate (0.7 mM), L-pyruvate (0.08 mM), choline chloride (0.2 mM), *i*-inositol (30 μM), vitamin B_{12} (0.4 μM), ethanolamine (16.4 μM), L-alanine (0.34 mM), L-glutamic acid (1 mM), L-glutamine (2 mM), L-serine (0.25 mM), $CuSO_4 \cdot 5H_2O$ (5 nM), $ZnSO_4 \cdot 7H_2O$ (1.5 μM), $FeSO_4 \cdot 7H_2O$ (1 μM), $MnCl_2 \cdot 4H_2O$ (0.5 nM), $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ (0 5 nM), $NiSO_4 \cdot 6H_2O$ (0.25 nM), H_2SeO_3 (60 nM), $Na_3VO_4 \cdot H_2O$ (2.5 nM), albumin (1%) gentamycin (50 mg/l). To inhibit the growth of non-parenchymal liver cells, arginine was omitted and replaced by 0.3 mM ornithine (12).

Isolation and centrifugal elutriation of liver cells

Liver cells were isolated by collagenase perfusion as described recently except that no second collagenase treatment was performed prior to centrifugal elutriation (4). Viability of the cells was determined with 0.4% trypan blue; >75% of the hepatocytes excluded the dye.

Vital hepatocytes (100 × 10^6) were loaded in the Beckman JE-6B rotor (standard elutriation chamber) at a flow rate of 17 ml/min, 1700 r.p.m. and 10°C. The elutriation medium used (see Media) contained PVP and DNase to protect the cells, improve the flow conditions and prevent cell aggregation during elutriation. Debris and dead cells were eluted at 26 ml/min. Fractions I–III were obtained by increasing the flow to 33, 50 and 60 ml/min and finally fraction IV by setting the rotor speed to 300 r p.m.; in the latter fraction many cell aggregates were eluted. The volumes used to elute dead cells and fractions I–IV were 250, 200, 250, 200 and 100 ml, respectively. Fractions I and III contain predominantly diploid and polyploid hepatocytes respectively.

Control experiments studied the effect of elutriation on replicative DNA synthesis. Vital hepatocytes 100 × 10^6 were centrifuged for 25 min at 1700 r.p.m., and then all cells were eluted by increasing the flow to 60 ml/min and decreasing the r.p.m. to 300. Elutriation of the hepatocytes for 25 min had a small decreasing effect on replicative DNA synthesis, [^{3}H]thymidine incorporation amounted to 88 ± 4% of that determined in control cells (mean ± SD of three separate experiments). To exclude an effect of different elutriation times we changed the protocol and isolated diploid and polyploid hepatocytes in two separate elutriation runs. Diploid hepatocytes were obtained as described above. In order to shorten the time required to isolate polyploid hepatocytes during the second run, the volume to eluate dead cells was decreased from 250 to 230 and fraction I (diploid cell fraction) was omitted. As a result both diploid and polyploid hepatocytes were isolated in similar times (25 min). The 'diploid' and 'polyploid' cell fractions were concentrated by low-speed centrifugation and resuspended in modified Waymouth MB 752/1 (see Media). Trypan blue exclusion was 80 and 90% in diploid and tetraploid hepatocytes respectively.

Flow cytometric analysis

DNA content of hepatocytes in the parent cell suspension and the elutriated cell fractions were analysed using a FACS analyser (Becton Dickinson, Heidelberg,

FRG). Flow cytometry and correction of the data was performed as recently described (4).

Primary cultures of adult hepatocytes

Hepatocytes were cultured on Falcon Petri dishes (35 × 10 mm, Becton Dickinson) or Lab-Tek 2 chamber Permanox slides (Nunc, Wiesbaden, FRG) in modified Waymouth MB 752/1 medium at 37°C in a humidified atmosphere of 5% CO_2 in air. The dishes were coated with rat-tail collagen according to Gebhardt and Jung (13). Viable hepatocytes (5.1 × 10^4/cm^2) of the parent cell suspension and the diploid cell fraction respectively, and 3.4 × 10^4/cm^2 viable hepatocytes of the polyploid cell fraction, were plated in 1 ml medium per dish, which contained dexamethasone (10^{-7} M), EGF (10 ng/ml) and insulin (10 mU/ml). At the two cell densities used, diploid and polyploid hepatocytes covered almost similar areas of the dishes. It is known that the density of membrane contacts is a major determinant for the stimulation of DNA synthesis in hepatocyte cultures, i.e. with lower cell densities there is an increase in the mitogenic response to EGF (14). Thus, the density of the diploid hepatocytes, which have only about half the volume of polyploid hepatocytes, had to be increased so that they cover similar areas of the dishes as the polyploid cell fraction and accordingly form a similar degree of cell membrane contacts.

In the presence of the three hormones, hepatocytes readily attach to the dishes and serum can be omitted. The presence of EGF and high insulin concentrations for only 2 h does not stimulate the DNA synthesis of hepatocytes (data not shown). After 2 h of incubation the medium was replaced by a medium without EGF and with a lower insulin concentration (0.1 mU/ml).

Assays of DNA synthesis and labelling index of hepatocytes

Three different protocols have been used, for details see legends to Figures 2–4. Basically, 44 or 48 h after plating, the medium was changed and DNA synthesis was stimulated by addition of EGF (10 ng/ml; Collaborative Research Inc. Renner, Dannstadt, FRG) and insulin (10 mU/ml; bovine pancreas 24 IU/mg, Sigma) in the presence of dexamethasone (10^{-7} M). Subsequently [^{3}H]thymidine ([methyl-^{3}H]thymidine, sp act. 5 Ci/mol, Amersham Buchler, Braunschweig, FRG) was added after 6 or 13 h respectively. Cell culture was then continued in the presence or absence of EGF and high insulin concentrations for another 24 or 14 h respectively.

For the determination of the labelling index the Permanox slides were washed twice with PBS and the cultures were fixed with acetone (−20°C) for 2 min. Autoradiography was performed as described by Roßberger and Andrae (15). In the pulse chase experiments (Figure 3), DNA synthesis was not assessed by autoradiography but rather by determining the radioactivity incorporated into the DNA. After washing the monolayer twice with ice-cold PBS the reaction was terminated by the addition of PBS (Ca^{2+} and Mg^{2+} free) containing 5 M urea and 1 mM EDTA. Because hepatocytes also catabolize [^{3}H]thymidine and incorporate the radioactive label in molecules other than DNA (16–18), the DNA was purified according to Stout *et al.* (19) prior to the determination of [^{3}H]thymidine incorporation. The radioactivity incorporated into the DNA was determined by liquid scintillation counting and the amount of DNA was quantitated fluorimetrically using diaminobenzoic acid (20).

Results

Separation of diploid and polyploid hepatocytes by means of centrifugal elutriation

Sequential treatment of two-thirds hepatectomized Wistar rats with DEN and 2-AAF induces the emergence of a diploid hepatocyte population (Table I). In order to study this carcinogen-induced cell population in more detail we established an elutriation procedure that allowed us to isolate relatively large quantities of diploid and polyploid hepatocytes at high purity. Figure 1 shows DNA fluorescence histograms of the parent hepatocyte suspension and of the various elutriated cell fractions. While the parent cell suspension (Figure 1a) contained almost equal amounts of diploid and tetraploid hepatocytes, fraction I contained mostly diploid (Figure 1b) and fraction III (Figure 1d) predominantly tetraploid hepatocytes. Based on the demonstrated purity of the elutriated fractions (Table II), fraction I will be referred to as the diploid fraction and fraction III will be referred to as the polyploid fraction. The cell yield in the two fractions, determined after concentration by low-speed centrifugation, varied between 1 and 1.5 × 10^7 hepatocytes.

Table I. Ploidy distribution of hepatocytes obtained from rats after partial hepatectomy and treatment with carcinogens

Treatment[a]	Ploidy (% of hepatocytes)			
	2c	4c	8c	*n*
PH	8 ± 1	74 ± 3	18 ± 3	(3)
+ DEN	6 ± 1	69 ± 2	25 ± 1	(3)
+ 2-AAF	27 ± 5	63 ± 3	10 ± 3	(3)
+ DEN + 2-AAF	44 ± 5	52 ± 3	4 ± 2	(7)

[a]PH, partial hepatectomy; DEN, diethylnitrosamine, 50 mg/kg body wt; 2-AAF, 2-acetylaminofluorene, 0.02% in the diet for 5 weeks. Cell isolation and analysis of the ploidy distribution was carried out by flow cytometry 8–11 weeks after the treatment of the animals. The values are means ± SD of 3–7 animals (*n*).

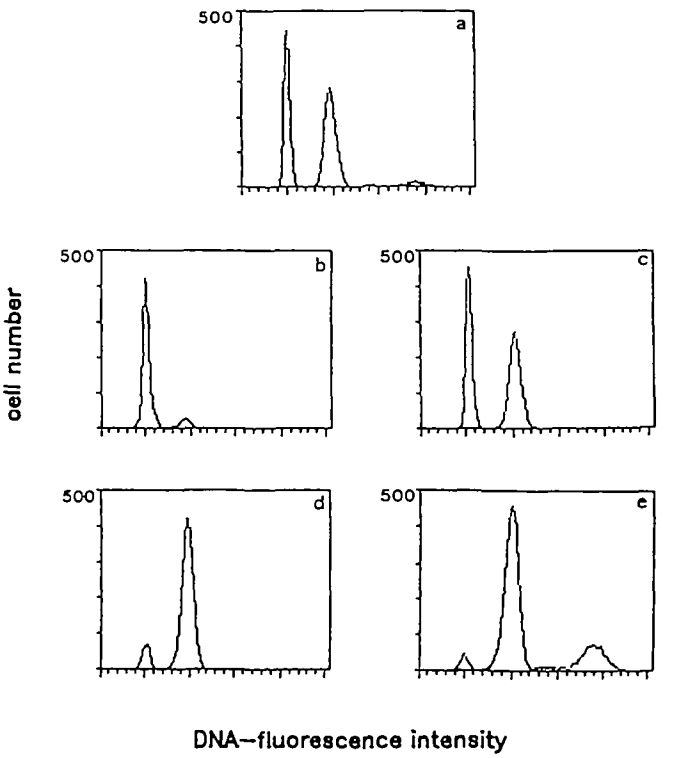

Fig. 1. DNA fluorescence histograms of the parent hepatocytes and hepatocyte fractions after centrifugal elutriation. Isolated hepatocytes were subjected to centrifugal elutriation and DNA was quantitated by flow cytometry as described in Materials and methods. Abscissa, propidium iodide DNA fluorescence (arbitrary units, same for all fractions); ordinate, cell numbers. Parent cell suspension (a), fractions I (b), II (c), III (d) and IV (e).

Table II. Centrifugal elutriation of hepatocytes from carcinogen-treated rats: ploidy distribution of the parent cell suspension and of the 'diploid' (fraction I) and 'polyploid' (fraction III) cell fractions

Hepatocytes[a]	Ploidy (% of hepatocytes)		
	2c	4c	8c
Parent cell suspension	42 ± 4	53 ± 4	4 ± 1
Fraction I	89 ± 4	11 ± 4	0
Fraction III	13 ± 1	85 ± 2	2 ± 1

[a]The data represent the ploidy distributions of the hepatocyte fractions used in the studies shown in Figures 2–4. Values represent the means ± SD of five different cell isolations.

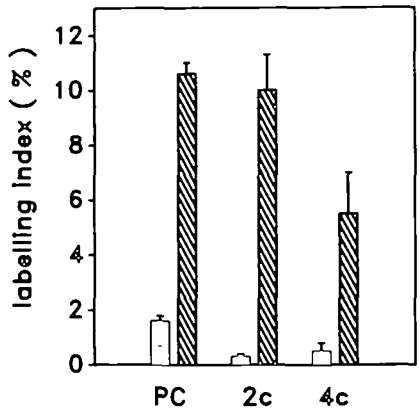

Fig. 2. Stimulation of DNA synthesis in parent hepatocytes and isolated diploid and polyploid hepatocytes. Liver cells were isolated 8–14 weeks after the treatment of the animals and predominantly diploid and polyploid hepatocyte fractions were obtained by centrifugal elutriation. Enrichment of diploid and polyploid hepatocytes in the respective elutriated fractions is shown in Table II. Cell culture was performed as described in Materials and methods; 48 h after plating the medium was changed and DNA synthesis stimulated with EGF (10 ng/ml) and insulin (10 mU/ml). Subsequently, the cells were washed twice and cultivation was continued for another 14 h in the presence of insulin (0.1 mU/ml), dexamethasone (10^{-7} M) and [^{3}H]thymidine (5 μCi/2 nmol). PC, parent cell suspension; 2c and 4c, predominantly diploid and polyploid cell fractions respectively. Closed bars, cultures in the absence of EGF/insulin; hatched bars, cultures in the presence of EGF/insulin. The values represent the mean (± SD) of three different cell preparations, each experiment was performed in duplicate. Significance was determined using the paired Student's t-test: 4c versus 2c, $P \leq 0.002$; 4c versus PC, $P \leq 0.001$.

Stimulation of DNA synthesis

Isolated diploid and polyploid hepatocytes were cultured as monolayers and DNA synthesis was stimulated. Figure 2 shows the response to EGF and insulin. The labelling index (LI) in predominantly diploid hepatocyte cultures was twice as high as that of predominantly polyploid cultures. There was no difference in the LI between predominantly diploid cultures and cultures of the parent cell suspension. The difference in the extent of mitogenic stimulation of diploid and polyploid hepatocytes, however, could be due to the fact that different times are required for the transition from the G_0/G_1 to the S phase. We therefore exposed cultures to EGF and high insulin concentration during the period from 48 to 61 h after plating, then rates of DNA synthesis were measured with a series of 2 h exposures to [^{3}H]thymidine during the following 14 h period. The results show (Figure 3) that the onset and duration of the S phase in diploid and polyploid hepatocytes are similar and thus may not account for the observed differences in DNA synthesis.

Finally, the dependence of DNA synthesis on the concentration of EGF was determined (Figure 4). Using EGF concentrations ranging from 0.5 to 25 ng/ml, the predominantly diploid or tretraploid cultures showed a similar dependence on the growth factor. However, at all concentrations the LI was higher in diploid than in tetraploid hepatocytes. The higher LIs in this experiment compared to those of Figure 2 are most likely due to the prolonged presence of the mitogens and the radiolabel (see Materials and methods).

Discussion

The present study describes the isolation of carcinogen-induced diploid hepatocytes and shows that replicative DNA synthesis stimulated by EGF and insulin is 2-fold higher in the diploid compared to the polyploid cell fraction *in vitro*. The development

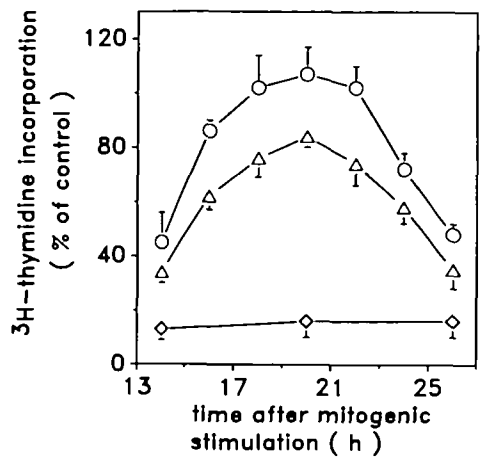

Fig. 3. Time course of [^{3}H]thymidine incorporation into the DNA of diploid and polyploid hepatocytes. Liver cells were isolated 8–14 weeks after treatment of the animals and predominantly diploid and polyploid hepatocyte fractions were obtained by centrifugal elutriation. Enrichment of diploid and polyploid cell fractions is shown in Table II. Cell culture was performed as described in Materials and methods; 48 h after plating the medium was changed and DNA synthesis stimulated with EGF (10 ng/ml) and insulin (10 mU/ml) in the presence of dexamethasone (10^{-7} M) for a further 13 h. Subsequently, the cells were washed twice and cultivation was continued in the presence of insulin (0.1 mU/ml) and dexamethasone (10^{-7} M). In the following 14 h pulse labelling was performed. The cultures were labelled with [^{3}H]thymidine (10 μCi/7 nmol) every 2 h for 2h. Stimulation of [^{3}H]thymidine incorporation in diploid (○) and polyploid (△) hepatocytes by EGF and insulin. In the absence of the mitogens [^{3}H]thymidine incorporation in diploid and tetraploid cells did not differ significantly and therefore only the data were pooled into a single value for the non mitogenic treated hepatocytes for clarity (◇). The values represent the mean (± SD) of four different cell preparations; each experiment was performed in triplicate. The values are expressed as percent of the maximal [^{3}H]thymidine incorporation of elutriated but unfractionated liver cells. For this hepatocytes (100×10^6 viable cells) were loaded to the rotor and centrifuged at 1700 r.p.m. After 25 min all cells were eluted by increasing the flow to 60 ml/min and decreasing the r.p.m. to 300. The maximal DNA synthesis of the unfractionated cells was set as 100% and amounted to 4450 ± 670 c.p.m./μg DNA.

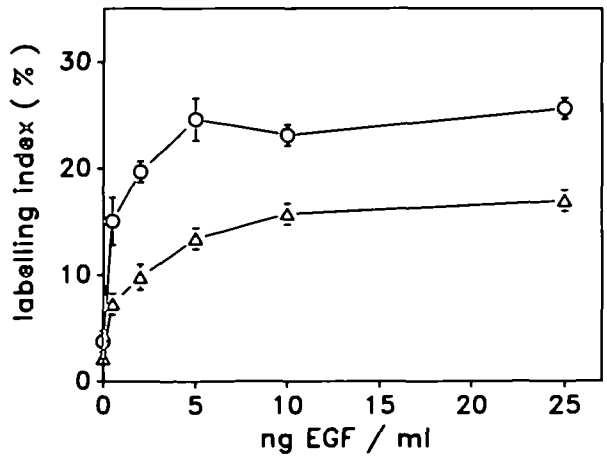

Fig. 4. EGF dependence of DNA synthesis in isolated diploid and polyploid hepatocytes. Liver cells were isolated 10 weeks after treatment of the animals and predominantly diploid and polyploid hepatocyte fractions were obtained by centrifugal elutriation. Enrichment of diploid (○) and polyploid (△) cell fractions is shown in Table II; cell culture was performed as described in Materials and methods; 44 h after plating the medium was renewed and DNA synthesis stimulated with EGF (0.5–25 ng/ml) and insulin (10 mU/ml) in the presence of dexamethasone (10^{-7} M). After 6 h [^{3}H]thymidine (5 μCi/2 nmol) was added (no medium change) and the cells cultured for another 24 h. In this protocol the mitogens were continuously present during the last 30 h of culture. The values represent the average and range of duplicate determinations.

of diploid hepatocytes after treatment of partially hepatectomized rats with DEN and 2-AAF (Table I) is in accordance with recent published studies (3,21–23). The only substantial difference is that Seglen and co-workers (23) report a significant diploidization by treating partially hepatectomized rats with DEN while we found a slight increase in octaploid hepatocytes. We cannot explain this difference. However, other studies do indicate a polyploidizing effect of nitrosamines (24–29).

Schwarze *et al.* (30) have also isolated carcinogen-induced diploid hepatocytes by centrifugal elutriation, by using the Beckman JE-10X rotor, which has a considerably larger cell separation chamber than the JE-6B rotor used in our studies. Because the cell separation chambers differ in size, the conditions of separation (flow, r.p.m.) of the two elutriation systems are not compatible. Consequently, a new elutriation protocol had to be established for the present study. The purity of the diploid and tetraploid hepatocyte fractions achieved with this protocol was similar to that obtained by Schwarze *et al.* (30).

Elutriated hepatocytes in monolayer cultures responded to mitogenic stimuli. EGF and insulin were chosen as mitogens because of their suggested involvement in liver growth after partial hepatectomy (31–33). EGF and insulin have also been shown to stimulate replicative DNA synthesis in monolayer cultures of rat hepatocytes (34–36). The concentration dependence of the stimulation of DNA synthesis by EGF is similar in diploid and polyploid hepatocytes. However, carcinogen-induced diploid hepatocytes showed a higher response to the mitogens EGF and insulin than polyploid liver cells at all concentrations (Figures 2 and 4).

This difference is not due to either differential viability of diploid and polyploid liver cells or to contamination with non-parenchymal cells. Despite a somewhat lower viability in terms of trypan blue exclusion (see Materials and methods), diploid hepatocytes show a higher DNA synthesis. On the other hand, contamination with non-parenchymal liver cells is very unlikely since they are considerably smaller than diploid hepatocytes and thus elute prior to the diploid liver cell fraction during centrifugation. The volume of the largest non-parenchymal cell type, the oval cells, is 13 μm (37), while that of the diploid hepatocytes is $\sim$17.3 μm (30). Moreover a culture medium was used that prevents growth of non-parenchymal liver cells (see Materials and methods) and the cultures only contained cells of hepatocellular appearance.

Interestingly Schwarze *et al.* (3) reported a higher percentage of cells in S phase in nodules, which are predominantly diploid, compared to the surrounding tissue. Moreover, they also found a positive correlation between the degree of diploidy in nodules and their rate of proliferation (5). At present we cannot decide whether a higher proliferation activity is an inherent property of diploid compared to polyploid hepatocytes, or if it is a distinct characteristic of carcinogen-induced diploid hepatocyte populations. There are only a few studies on the stimulation of DNA replication in hepatocytes of various ploidy classes. Brodsky and Uryvaeva (2) showed that in untreated mice diploid and polyploid hepatocytes respond to a similar extent to partial hepatectomy. However, the onset of the S phase was delayed in polyploid hepatocytes (2) while the duration of the S phase was similar in both hepatocyte populations (38). In the present study, carcinogen-induced diploid hepatocytes required similar times for passing G_0/G_1 and for the S phase *in vitro* (Figure 3). In view of the fact that the duration of the lag phase after partial hepatectomy depends on the localization of the hepatocytes in the liver acinus (39) and the reports that diploid hepatocytes are not randomly distributed in the acinus (cf. 1), the significance of the observed differences in diploid and polyploid liver cells is not clear.

In the present study the parent cells responded equally well to EGF and insulin as the elutriated diploid cell fraction. In view of the fact that the parent cell suspension contains only $\sim$44% diploid hepatocytes one would have expected a lower LI. The lack of a difference between the LIs of the parent cell suspension and the diploid cell fraction cannot be due to elutriation, since we determined in preliminary experiments that the elutriation for 25 min decreased DNA synthesis by only 10%. Apparently, co-operative effects are likely to occur between the two cell types when kept in monolayer cultures. Recently it has been shown that hepatocytes express transforming growth factor α *in vivo* after partial hepatectomy (40) and that nodules and carcinomas induced by DEN and 2-AAF were positive for the growth factor bombesine (41). It is tempting to speculate that autocrine and paracrine mechanisms may take place (9), but future work must address this issue.

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