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ALTERATIONS IN GROWTH RATE AND CELL CYCLE KINETICS OF RAT LIVER TUMOR CELLS CULTURED IN ETHANOL-CONTAINING MEDIUM

IN VITRO MODEL OF PROLIFERATIVE RESTRICTION IN RESPONSE TO ETHANOL EXPOSURE

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Abstract—Mechanisms related to the growth suppressive effect of acute ethanol exposure on liver cells were investigated using an established line of ethanol-sensitive rat hepatic tumor cells (32IIIA) and recently developed cytochemical methods for analysis of hepatocyte cell cycle kinetics. Exposure of exponentially growing 32IIIA cells to ethyl alcohol (range 10–100 mM in the growth medium) for a period of 3 days resulted in concentration-dependent decreases (4–25%) in final population density and increases (18–35%) in mean population doubling time compared to untreated cells. Viability was unaffected by ethanol exposure in the concentrations indicated and for the duration period utilized, approximating 94% under all experimental conditions. Multiparametric flow cytometric analysis revealed significant ethanol-associated differences in specific growth parameters and growth state compartments of 32IIIA hepatic tumor cell populations. Most prominent was an ethanol-associated and concentration-dependent (a) increase in the fraction of cells in the G_1 phase of the cell cycle, (b) increase in the coefficient of variation in the G_1 DNA content measurement, and (c) accumulation (in the G_1 phase) of cells with a very low mean RNA content. Increases in each of these cytochemically-defined parameters reflected increasing levels of ethanol in the growth medium. This study indicates that the effects of ethanol on cultured cells of hepatic origin are quite complex. It is concluded that the inhibition of proliferation observed during acute ethanol exposure of liver-derived 32IIIA cells *in vitro* is due to an accumulation of cells in the G_1 compartment.

Administration of Et§ to intact animals or to isolated hepatocytes significantly represses the expression of certain differentiated liver functions such as synthesis and/or secretion of plasma proteins [1–5] and impairs [^{3}H]thymidine incorporation into hepatocyte DNA [6–8]. Et-induced inhibition of cellular proliferative activities also occurs *in vitro*. Reduced DNA synthesis in primary hepatocyte cultures [9] and reductions in final population densities of *in vitro*-propagated hepatic tumor cells [10] were demonstrable during the period of Et exposure. Such Et-associated proliferative inhibition may have considerable long-term physiologic impact since it (a) might adversely affect the normal process of compensatory regeneration in response to hepatic injury [11], and (b) does not appear to be easily reversible [12].

Initial studies have indicated that some hepatic tumor cell lines respond to Et treatment with a concentration-dependent decrease in final population density, apparently due to an induced increase in mean cell cycle transit time [10]. Cultured lines of appropriate, Et-sensitive, hepatic tumor cells may be useful, therefore, in the identification of specific stages in the liver cell cycle [13, 14] which are differentially responsive to the action of Et or its metabolic by-products. Unlike primary cultures of normal hepatocytes, established tumor cell lines have certain advantages for cell cycle studies in that they represent clonal populations with known growth kinetics and Et sensitivities (e.g. Ref. 10). These properties allow for the selection of those growth conditions which provide for continuous propagation during the period of Et exposure and minimize the preparation-to-preparation heterogeneity inherent in primary cell cultures.

To investigate potential changes in hepatocyte cell cycle kinetics as a consequence of Et treatment, the established 32IIIA line of Et-sensitive rat hepatic tumor cells [10, 15–17] was utilized to probe Et-associated alterations in several growth parameters of liver-derived epithelial cells. Cell cycle compartments in control and Et-treated populations of 32IIIA cells were compared relative to the present cytochemically-defined model of the hepatocyte division cycle as illustrated in the regenerating rodent

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§ Abbreviations: AO, acridine orange; CCl_4, carbon tetrachloride; c.v., coefficient of variation; DENA, diethylnitrosamine; DX, dexamethasone; EGTA, ethyleneglycolbis (amino-ethylether) tetra-acetate; Et, ethyl alcohol; FBS, fetal bovine serum; FCM, flow cytometry; and HBSS, Hanks' balanced salt solution.

liver [13, 14]. The results form the basis of this report.

MATERIALS AND METHODS

Cell culture. The albumin-producing hepatic tumor cell line 32IIIA clone 6/2d was established from the liver tissue of an adult rat bearing a primary DENA-induced hepatocellular carcinoma [15–17]. Briefly, the liver of a DENA-treated rat was perfused *in situ* with HBSS containing 0.5 mM EGTA, pH 7.2, followed by a mixture of collagenase (0.05%) and hyaluronidase (0.1%) in HBSS. Dissociated cells were placed into culture with Ham's F-12 growth medium containing 2X amino acids, 15% FBS, and 10 μg/ml each of insulin and hydrocortisone. After 2 weeks, the medium was replaced with F-12 medium supplemented with 10% FBS and 10^{-6} M DX for long-term culture. A clonal isolate (32IIIA) from this parental culture was shown previously to exhibit morphologic and functional properties consistent with a parenchymal origin [15–17] and growth restriction in response to Et [10].

Multiparameter flow cytometry. 32IIIA cells were harvested from monolayer culture by treatment with 0.05% trypsin/0.02% EDTA in Ca^{2+}- and Mg^{2+}-free HBSS for 10 min at 37°. The trypsin reaction was stopped by addition of growth medium containing FBS, and the released cells were collected by centrifugation at 900 *g*. Cells were stained for FCM quantitation of cellular DNA and RNA content with AO (Polysciences, Inc., Warrington, PA) under acid conditions. As described previously [13, 14], 0.2 ml of cell suspension (at 10^6 cells/ml) was mixed with 0.4 ml of 0.08 N HCl, 0.15 M NaCl, 0.1% (v/v) Triton X-100 for 30 sec followed by addition of 1.2 ml of 0.2 M Na_2HPO_4/0.1 M citric acid buffer (pH 6.0) containing 1 mM EDTA-Na, 0.15 M NaCl, and 6μg AO per ml [13, 14, 18, 19]. Immediately thereafter, the stained cells were analyzed in a 488 nm argon ion laser-equipped FC 200 Cytofluorograf interfaced to a Nova 1220 minicomputer for simultaneous measurement of the DNA and RNA content of individual cells [13, 14]. Treatment with Triton X-100 at low pH increases cell permeability to AO while nucleic acids remain insoluble [20]. Addition of AO in the presence of chelating agents (citrate, EDTA) results in denaturation of any double-stranded RNA which then fluoresces red (600–650 nm), while DNA, which remains in its native double helical form, intercalates the dye thereby emitting light in the green region of the spectrum (515–570 nm). Under the present staining conditions, green fluorescence is proportional to DNA content [21], whereas red fluorescence intensity (after appropriate subtraction of nonspecific signal) is stoichiometric for RNA [22]. The metachromatic red fluorescence (RNA) emission ($F > 600$, measured in a band from 600 to 650) and green fluorescence (DNA) emission (F_{530}, from 515 to 570) from each cell were separated by optical filters and measured by separate photomultipliers; the integrated values were stored in computer memory for analysis as described [13, 14]. Contour plots (representing the distribution of cellular DNA and RNA contents within a population) were computer-constructed [14] using measurements obtained on 10^4 cells/sample.

Identification of hepatocyte cell cycle compartments. Liver regeneration was induced in adult male mice (strain C57BL/6) by intraperitoneal injection of CCl_4 (0.5 ml/kg body wt) [13, 14]. Fifty-six hours after inoculation of the hepatonecrotic agent, hepatocytes were isolated [23] and stained with AO for FCM [13, 14]. Two-parameter (cellular DNA and RNA content) contour plots were computer-constructed [13] to illustrate the various cytochemically-defined subcompartments (G_{1Q}, G_{1A}, G_{1B}, S, and G_2 + M) of the hepatocyte cell cycle.

RESULTS

Exposure of exponentially growing 32IIIA cells to culture medium containing Et (concentration range:

Table 1. Effect of ethanol concentration on final population density and population doubling times in cultures of 32IIIA rat hepatic tumor cells

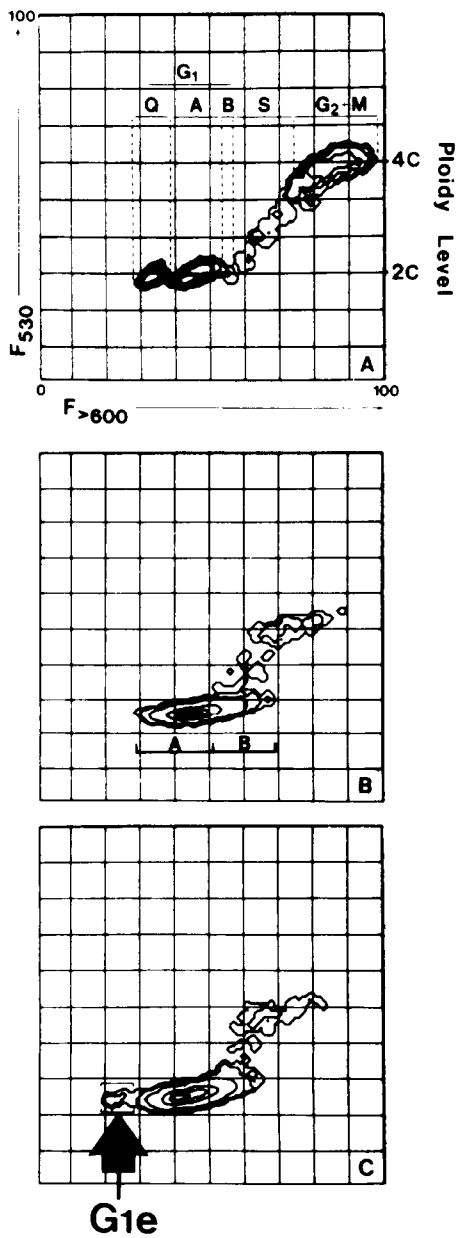
	Final population density† (% of control)			Population doubling time‡ (in hr)		
	Ethanol concentration (mM)			Ethanol concentration (mM)		
Experiment*	10	50	100	0	50	100
1	96.7 ± 1.8	82.0 ± 2.2	73.3 ± 1.5	25.8 ± 0.8	29.5 ± 3.3	33.6 ± 5.5
2	95.5 ± 2.3	86.6 ± 2.1	77.6 ± 1.9	24.9 ± 1.0	30.3 ± 2.4	34.8 ± 4.9

* Equivalent numbers of 32IIIA clone 6/2d hepatic tumor cells were pipetted into 60 mm Petri dishes each containing 5 ml of Ham's F-12 growth medium containing 10% fetal bovine serum. After 2 days, the medium was replaced with control medium or medium to which ethanol was added in the concentrations indicated. Exposure was for 3 days with changing of the test medium every 24 hr.

† Cells were trypsinized into suspension on the final (3rd) day of exposure to ethanol and counted with a hemacytometer. Final population density is expressed as percent of control (i.e. non-ethanol-treated cultures) based on mean ± standard deviation of at least triplicate cell counts made on each of two to three cultures per ethanol concentration.

‡ Hemacytometer counts of cells trypsinized into suspension were done at subsequent 24-hr intervals after initial change-over to control or ethanol-containing medium. Regression lines were constructed based on observed cell numbers (mean ± standard deviation of at least triplicate determinations on each of two to three cultures per time point) during the test period, and the population doubling times were calculated therefrom.

10–100 mM) resulted in concentration-dependent decreases in final population density and increases in mean population doubling time (Table 1). It can be calculated from these data that the doubling time in cultures of 50 and 100 mM Et-treated cells was increased by 17.7 and 34.6%, respectively, relative to control values. These changes occurred without an attendant loss in cell viability (as determined by standard Trypan Blue dye exclusion assay) either during or at the termination of the 3-day Et exposure period. Percentages of viable adherent cells observed over the duration of exposure typically ranged as follows: 0 mM Et, 95.1 ± 4.9%; 10 mM, 93.2 ± 6.1%; 50 mM, 95.5 ± 2.2%; and 100 mM, 93.8 ± 5.6%. Reductions in culture density as a consequence of Et exposure, thus, were not due to death within the adherent cell population.



The Et-associated increase in population doubling times, in the absence of demonstrable loss in cell viability, suggested that 32IIIA cell cycle transitions may be altered during the period of Et exposure. Two-parameter contour plots (illustrating the distribution of cellular RNA and DNA contents within each population) were computer-constructed using FCM data in order to evaluate the affect of Et on various 32IIIA cell cycle compartments. Comparisons were made relative to the current cytochemically-defined model of the hepatocyte cell cycle as best illustrated in the regenerating rodent liver (Fig. 1A) (see also refs. 13 and 14). This construct is identical in its delineation of hepatocyte division cycle substates to that determined by available binding site interaction of AO with liver cell nuclear RNA and DNA of the mouse [13, 14] and rat [13]. This model depicts several key aspects of the liver cell cycle (Fig. 1A). G_{1Q} cells are characterized as having a diploid (2C) DNA content with the lowest mean RNA distribution; these cells do not have an associated S (DNA synthesizing) phase and are presumed to comprise a quiescent (Q) cell population, as defined by Tsanev [24]. G_{1A} and G_{1B} cells in Fig. 1A are the epithelial counterparts of the G_1 subcompartments indentified in exponentially growing cultures of mouse fibroblasts and erythroleukemia cells [18, 25, 26]. G_{1A} and G_{1B} are functionally distinct substates of the G_1 phase. G_{1A} cells cannot directly enter the DNA synthetic (S) phase regardless of their residence time in G_{1A} (period of indeterminate duration) [27]. Transition from G_{1A} to the deterministic G_{1B} substate, and subsequent entrance into S phase, is characterized by a significant increase in mean total cellular [18] or nuclear [13, 14] RNA content.

Using FCM data (Fig. 1A) as reference, several obvious differences were apparent between the RNA–DNA contour plots of regenerating hepatocytes (Fig. 1A) and exponentially growing 32IIIA cells (Fig. 1B). Relative to adult hepatocytes under-

Fig. 1. Computer-generated two-parameter contour plots illustrating the green (F_{530}; DNA content) and red ($F > 600$; RNA content) fluorescence emission per cell for 10,000 individual cells (per sample) obtained from (A) the liver tissue of carbon tetrachloride-treated (0.5 ml/kg body wt) C57BL/6 male mice, (B) exponential phase 32IIIA cultures, and (C) 50 mM ethanol-treated 32IIIA cultures. (A) The positions of the G_{1Q}, G_{1A}, G_{1B}, S, and $G_2 + M$ phase populations are indicated on the contour plot of regenerating liver cells as are the positions of the 2C and 4C DNA content cells. The 2C DNA content (diploid G_1) population was localized with the use of mouse splenic lymphocytes. The G_1 substates were classified as G_{1Q}, G_{1A}, and G_{1B} in accord with the functional criteria defined previously [13, 14]. (B) Log phase 32IIIA hepatic tumor cells; obvious is the absence of a G_{1Q} substate, an expansion of the range of cellular RNA contents in the $G_{1A\text{-}B}$ compartment, and an increase in the fraction of G_{1B} cells in the $G_{1A\text{-}B}$ population. (C) Fifty millimolar ethanol-treated cells. The position of the G_{1e} subpopulation is indicated by brackets; evident also is the shift to lower F_{530} values with an associated increase in the variation of this measurement. These parameters are developed to a lesser or greater extent in 10 mM and 100 mM ethanol-treated cells respectively.

Table 2. Distribution of G_1 substates in regenerating liver cells and in log phase cultures of 32IIIA hepatic tumor cells

Source	G_1 substate* (% of total G_1 compartment)		
	G_{1O}	G_{1A}	G_{1B}
Regenerating liver†	47.5 ± 14.3	41.9 ± 10.1	10.7 ± 4.3
Log phase 32IIIA cells‡	0	66.8 ± 5.9	32.8 ± 7.1

* Computer-calculated from two-parameter frequency histograms (F_{530}, DNA content; F > 600, RNA content) [13, 14] obtained by flow cytometric analysis of acridine orange-stained liver cells. Values represent the mean ± standard deviation of data obtained in three to six experiments.

† Liver cells were isolated 56 hr after intraperitoneal inoculation of C57BL/6 male mice (age range: 5–10 weeks post partum) with 0.5 ml CCl_4/kg body wt [13].

‡ Harvested from culture with trypsin/EDTA solution.

going compensatory regeneration, untreated log phase hepatic tumor cells were distinguished by (a) the absence of a defined G_{1O} substate, (b) a marked expansion (as indicated by the increased range of cellular RNA contents) for the entire $G_{1A,B}$ compartment (spanning over 40 channels as compared to just 20 channels for regenerating liver cells) and (c) a significant increase in the fraction of G_{1B} cells in the $G_{1A,B}$ compartment (Table 2). These data indicate that 49.1% of the total $G_{1A,B}$ compartment in log phase 32IIIA cultures resided in the G_{1B} substate, whereas only 24.8% of $G_{1A,B}$ cells in the regenerating liver were in G_{1B}.

Exposure of 32IIIA cells to Et in the growth medium for a 3-day period was associated with a concentration-dependent shift (evident in all cell cycle compartments) to lower F_{530} values relative to log phase cells (Tables 3 and 4). More obvious in Et-treated populations, however, was an accumulation of cells in G_1, an attendant increase in the c.v. of the G_1 DNA content measurement, and the development of a new population of 2C cells characterized as having RNA contents lower than the G_{1A} substate

Table 3. Ethanol-associated alterations in the acridine orange-staining characteristics of the G_1 compartment of 32IIIA hepatic tumor cells

Ethanol concn* (mM)	DNA parameters of G_1 compartment cells†		
	Peak	Median	Mean
0	25.5 ± 0.7	26.0 ± 0.1	25.7 ± 0.4
10	24.5 ± 0.7	25.5 ± 0.6	24.8 ± 0.8
50	24.0 ± 1.4	24.0 ± 1.4	23.6 ± 1.1
100	21.0 ± 0.5	21.5 ± 0.7	20.7 ± 0.5

* 32IIIA clone 6/2d cells were cultured in control or ethanol-containing growth medium as described in the legend to Table 1.

† F_{530} emission values in arbitrary units; values represent the mean ± standard deviation of measurements on two to three cultures per ethanol concentration.

Table 4. Ethanol-associated alterations in the acridine orange-staining characteristics of the S and G_2 + M phase compartments of 32IIIA hepatic tumor cells

Ethanol concn* (mM)	Mean F_{530} (DNA) emission†	
	S phase	G_2 + M phase
0	39.3 ± 0.4	50.5 ± 0.3
10	37.8 ± 0.4	49.0 ± 1.1
50	36.8 ± 1.1	48.2 ± 0.5
100	32.5 ± 1.3	43.2 ± 0.7

* 32IIIA clone 6/2d cells were cultured in control or ethanol-containing growth medium as described in the legend to Table 1.

† F_{530} emission values in arbitrary units; values represent the mean ± standard deviation of measurements on two to three cultures per ethanol concentration.

in control cultures (Table 5; Fig. 1C). Such low RNA-containing 2C cells were not resolvable in control 32IIIA cultures. This subpopulation was designated "G_{1c}" to indicate that G_1 cells of this phenotype were associated with *in vitro* exposure to Et. The G_{1c} subpopulation was clearly comprised of intact cells and did not represent nuclei-containing cellular fragments (perhaps produced during the course of Et exposure) since this substate lacked an associated S phase (evident in FCM measurements of isolated liver cell nuclei [e.g. Refs 13 and 14]) and was resistant to a DNase treatment prior to AO addition (which eliminates signal due to naked nuclei or nuclei-containing cytoplasmic fragments, but not intact cells). Moreover, the two-step staining procedure used in this study (detergent permeabilization prior to addition of AO staining buffer) effectively eliminates staining of nuclei surrounded by fragments of cytoplasm due to the detergent instability of such structures in the unfixed state.

Table 5. Alterations in composition of the G_1 compartment of 32IIIA hepatic tumor cells cultured in the presence of various concentrations of ethyl alcohol

Ethanol concn* (mM)	Percentage of		DNA content measurement
	G_1 cells†,‡	G_{1c} cells†,§	Coefficient of variation
0	59.5 ± 1.5	0	6.5 ± 0.1
10	60.8 ± 0.4	7.9 ± 1.3	6.6 ± 0.2
50	64.0 ± 2.3	9.8 ± 2.5	7.6 ± 0.4
100	68.1 ± 1.3	11.1 ± 1.9	9.9 ± 1.9

* 32IIIA clone 6/2d cells were cultured in control or ethanol-containing growth medium as described in the legend to Table 1.

† Determined by flow cytometry of acridine orange-stained cells obtained from cultures following a 3-day exposure to control or ethyl alcohol-containing medium. Values represent the mean ± standard deviation of measurements on two to three cultures per ethanol concentration.

‡ Percent of total population.

§ Percent of G_1 population.

¶ Computer-calculated from DNA frequency histograms of the isolated 2C DNA content G_1 compartment.

Table 6. Effect of ethanol exposure on the percentage of S and G_2 + M cells in 32IIIA hepatic tumor cell cultures

Ethanol concn* (mM)	Percentage of cells in	
	S phase†	G_2 ± M phase†
0–10‡	29.0 ± 1.9	14.2 ± 2.0
50–100‡	22.9 ± 1.2	8.1 ± 2.1

* 32IIIA clone 6/2d cells were cultured in a control of ethanol-containing growth as described in the legend to Table 1.

† Determined by flow cytometry of acridine orange-stained cells obtained from cultures following a 3-day exposure to control or ethyl alcohol-containing medium. Values represent the mean ± standard deviation of measurements on four to six cultures per group.

‡ Since there was no significant difference in the S and G_2 ± M parameters between control (0 mM) and 10 mM ethanol-treated cells nor between 50 and 100 mM ethanol-treated cultures, the 0 and 10 mM measurements were combined, as were the 50 and 100 mM measurements, for analyses between the two groups.

Although more difficult to calculate because of the Et-associated general decrease in overall cell staining and increase in c.v. of the G_1 compartment DNA measurement, it was apparent that accumulation of 32IIIA cells in G_1 as a consequence of Et treatment was reflected in a decrease in the percentage of S and G_2 + M cells within a culture. No significant changes in the fraction of S and G_2 + M cells were observed between control (0 mM Et) and 10 mM cultures or between 50 and 100 mM cultures. Comparisons between the two groups (0 + 10 mM vs 50 + 100 mM), however, revealed considerable differences in the two parameters (Table 6).

DISCUSSION

The culture protocol that was used of Et concentrations up to 100 mM and a duration of exposure of 72 hr: (a) does not adversely affect cell viability nor produce obvious cytopathic changes in 32IIIA hepatic tumor cells [10], (b) saturates (at levels of 50 mM and above) cellular enzyme systems involved in Et oxidation [1], and (c) maximally inhibits hormone-stimulated DNA synthesis in primary cultures or normal rat hepatocytes [9]. The question of whether the observed results were due directly to Et or to its oxidative byproduct, acetaldehyde, was not addressed in the present study. In addition to reductions in final population density, however, these same culture conditions also result in increased cellular protein content and reduced secretion of albumin in 32IIIA cells during the period of Et exposure [10]. These Et-associated effects are also seen in acetaldehyde-containing growth medium and are prevented by simultaneous addition of pyrazole [10]. It is likely, therefore, that at least some alterations observed in the parameters measured previously (population density, albumin secretion, cellular protein content) [10] and in the present study were induced by products of ethanol metabolism.

Hepatic regeneration, as measured by incorporation of [^{3}H]thymidine, appears to be inhibited by acute and chronic ethanol exposure [6–8, 11] and is reflected in a diminished stimulation of ornithine decarboxylase activity [7]. These data suggest that the ability of hepatocytes to enter S phase is impaired as a consequence of ethanol treatment. *In vitro*, the Et-associated increase in population doubling time of 32IIIA cells, as measured by regression analysis of mean cell counts and viability estimates over the 3-day exposure period, was not due to cell loss through death. The lowered mean cellular RNA content observed during the period of Et exposure (due to generation of the G_{1e} subpopulation) directly reflects an Et-associated accumulation of cells in G_1 phase (Table 5). Mechanistically, this may be attributed to delays in transition from the indeterministic G_{1A} to the deterministic G_{1B} substates or from G_{1e} to the cycling $G_{1A,B}$ compartment. These observations do not exclude the possibility that G_{1e} may be, in fact, a quiescent (G_{1Q}-like or at least a slowly cycling) substate induced as a consequence of Et exposure. Since such growth state transitions are normally associated with an increase in cellular RNA (13, 14, 18), interference with this process may greatly prolong total G_1 residence time. The present data are compatible with previous observations implicating Et in the transcriptional inhibition of hepatic RNA synthesis in the regenerating rodent liver [28]. The mechanisms underlying this change (decrease) in RNA content are not known. Et, or its metabolic by-products, may influence chromatin structure (as suggested by the decrease in overall F_{530} emission and the increased c.v. of the G_1 DNA content measurement), thereby potentially altering genomic level events necessary for proper cell cycle progression. While it is recognized that such spectral changes may result from Et-modified AO-nucleic acid interaction [29], decreased liver cell RNA content during Et exposure has now been observed using both cytochemical and biochemical [28] methods. The accompanying decrease in F_{530} emission, suggestive of a reduction in cellular DNA content (or at least reduced DNA–AO interaction) in Et-treated as compared to control cells, was not unexpected since Et was shown previously to reduce the amount of fluorometrically-determined DNA in primary cultures of rat hepatocytes [9].

It remains to be determined whether even clonal hepatic tumor cell lines, like 32IIIA and other derived sublines thereof (which exhibit a spectrum of differentiated liver properties [17]), respond homogeneously to Et-initiated growth restriction. The bimodality in the G_1 RNA profile of Et-treated cells (due to the generation of the G_{1e} subpopulation) may be indicative of heterogeneity in growth response. Models consistent with the observed population densities and doubling times can be constructed assuming a heterogeneous response (within a culture) to Et. In such response, low numbers of cells (approximately equal in abundance to the G_{1e} population) might leave (or be greatly prolonged) in the cycling pool following each cell division. Alternatively, the general inhibition of hepatic regeneration which accompanies Et administration *in vivo* [6–8, 11] is suggestive of a more uniform population-wide effect. Current work is focused on the use of synchronized cell cultures to measure changes in the dur-

ation of the two pre-DNA synthetic G_1 phases (G_{1A} and G_{1B}) in individual 32IIIA cells as a function of ethanol concentration. Such studies may define more precisely that G_1 substate most sensitive to Et exposure.

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