Phenotypic and Karyotypic Changes Induced in Cultured Rat Hepatic Epithelial Cells That Express the "Oval" Cell Phenotype by Exposure to N-Methyl-N'-nitro-N-nitrosoguanidine

MING-SOUND TSAO, MD, JOE W. GRISHAM, MD, KAREN G. NELSON, PhD, and JUDITH D. SMITH, BSc

From the Department of Pathology, University of North Carolina, School of Medicine, Chapel Hill, North Carolina

A diploid population of cultured rat hepatic epithelial cells that expresses the "oval" cell phenotype was exposed briefly and repetitively to N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), and the effect on more than 20 phenotypic properties was evaluated during the neoplastic transformation of the population. MNNG treatments of this hepatic epithelial cell population resulted in a progressively increasing phenotypic alteration and heterogeneity including changes in specific activities of several cellular enzymes and expression of isozymes, synthetic functions, and various *in vitro* growth properties. Changes in phenotypic expression were clustered episodically and were associated with major karyotypic changes. The development of increasing phenotypic heterogeneity preceding and accompanying tumorigenicity in cultured liver epithelial cells *in vitro* and the specific phenotypes that occur resemble superficially the pattern of phenotypic changes that occur in hepatocytes during chemical hepatocarcinogenesis *in vivo*. The results of this study provide the basis for future investigations to further elucidate the mechanistic and linkage relationship between specific pretumorigenic and paratumorigenic phenotypes and tumorigenicity. (Am J Pathol 1985, 118:306–315)

PRODUCTION of hepatocellular carcinoma in rats is a prolonged process characterized by the reproducible occurrence of discrete populations of liver cells that express altered phenotypic properties.[1–3] Included among the populations of phenotypically altered cells that appear in the liver during chemically induced carcinogenesis are "oval" cells[4] and foci and nodules of altered hepatocytes. It has been hypothesized that one or more of these new populations is the precursor to hepatocellular carcinoma[1,5] and represents preneoplastically altered cells progressing to cancer. Although this provocative hypothesis has stimulated many studies, it has not been possible to establish unequivocally that any of these altered populations are precursors of hepatocellular cancer. A major handicap to the tracing of cell lineages leading to hepatocellular carcinoma has been the inability to analyze clonally the *in vivo* process. Although various types of cells can be isolated from livers of chemically treated rats,[6–8] and some of these isolated cells can be transplanted into recipient animals, where they grow to form lesions,[6,9–11] a lesion cannot be identified as a clone arising from a single transplanted cell. Several hundred thousand to several million genetically heterogeneous cells must be transplanted to yield a graft of liver tissue, and this situation prevents a clonally based tracing of the process of neoplastic progression in the chemically treated rat liver *in vivo*.

It appears possible that the clonal analysis of the development of liver cancer may be accomplished with the use of cultured liver cells *in vitro*. Although mature hepatocytes cannot yet be continuously propagated in

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Address reprint requests to J. W. Grisham, MD, Professor and Chair, Department of Pathology 228H, University of North Carolina, Chapel Hill, NC 27514.

culture,[12] they now can be made to cycle one or more times in culture[13,14] Further manipulations of culture conditions may make it possible to maintain cycling hepatocytes *in vitro* for long periods, to clone them, and to establish clonal subpopulations. Although it is not yet possible to perform this study with hepatocytes, it is possible to use cultured "oval" cells for this purpose.[15] We recently have reported the isolation in culture and characterization of a diploid epithelial cell line (WB-F344) derived from normal rat liver that expresses the phenotypic properties of "oval" cells, including the synthesis of both albumin and alpha fetoprotein and the expression of selected enzymes and isozymes.[16] In this paper we report that the exposure of these cells in culture to the directly active carcinogenic chemical N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) induces them to express altered phenotypic properties and karyotypic changes that resemble those that occur in liver cells of carcinogen-treated rats *in vivo* prior to the acquisition of tumorigenicity.[17,18] Some of the tumors that occur after back-transplantation of tumorigenic cells into syngeneic hosts resemble hepatocellular carcinomas. This paper characterizes the development of phenotypic alterations and tumorigenicity of WB-F344 rat hepatic epithelial cell populations by treatment with MNNG.

Materials and Methods

Chemicals

Imemzo tissue culture medium was obtained from Associated Biomedic Systems, Inc., and fetal bovine serum was obtained from GIBCO. Sheep anti-rat α-fetoprotein antibody was obtained from Nordic Immunological Reagents. Goat anti-rat albumin antibody and rabbit anti-human fibronectin antibodies were obtained from Cappel Laboratories. The Vectastain ABC kit was from Vector Laboratories. Unless stated, all other chemicals were obtained from Sigma Chemical Company.

Tissue Culture

WB-F344 is a propagable rat epithelial cell line isolated in our laboratory from the liver of an adult male Fischer-344 rat. The detailed growth, kinetics, and the karyotypic, histochemical, immunocytochemical and isozymic characteristics of this epithelial cell line have been presented elsewhere.[16] Methods for assessments and analyses of karyotype, cellular DNA content by flow cytometry, histochemistry, immunocytochemistry, and growth in low calcium medium or in soft agar were identical to those previously described.[16,19]

Exposure of Cell Populations to MNNG

WB cells were exposed to MNNG in a final concentration of 5 μg MNNG/ml culture medium. MNNG was weighed and dissolved in reagent grade acetone. A sufficient volume of MNNG solution in acetone was added to complete culture medium to yield a final concentration of 5 μg/ml. Medium containing MNNG was left in contact with cells for 24 hours; because of the short biologic half-life of this chemical, actual exposure was limited to about 90 minutes.[20] The concentration of acetone in culture medium was always less than 1%. Acetone vehicle controls (1% acetone) in complete culture medium were carried in parallel, as were controls maintained in complete culture medium without additives.

Cell populations were exposed to MNNG during logarithmic growth. Following each exposure, treated populations were allowed to recover from MNNG-induced toxicity and to reach confluence, following which they were split as noted above. One dish was retreated when it entered logarithmic growth, and other dishes were used to generate populations for immediate phenotypic analysis and for freezing for future studies.

Acetone vehicle and basal medium controls were passaged at intervals that caused them to undergo approximately the same number of population doublings as did the MNNG-treated cultures. Control cultures were evaluated for phenotypic properties in parallel to MNNG-treated cultures.

Assessment of Phenotypic Properties

Selected phenotypic properties were assessed in populations of treated and control cultures after each exposure to MNNG. Phenotypic analysis other than enzymatic studies usually occurred within 3–6 population doublings following the last exposure to MNNG (and in similarly passage control cultures).

Enzymatic Studies

Preparation of Enzyme Extracts

Frozen cell lines were thawed simultaneously and cultured in parallel. Enzyme extracts were prepared routinely from cultures 1 to 2 days after they had reached confluence. For each test sample, two plates of cells were harvested by trypsinization and pooled, washed three times with cold PBS, and suspended in 0.1–0.2 ml of

0.1 M Tris-HCl buffer, pH 7.4, containing 1 mM EDTA and 5 mM 2-mercaptoethanol. Cells were lysed by three freeze–thaw cycles and centrifuged at 100,000*g* for 1 hour, and the supernatant was used for measurement of the activities of soluble cytoplasmic enzymes (NADH-diaphorase, aldolase, glucose-6-phosphate dehydrogenase, and pyruvate kinase). The pellet was resuspended in 1% Triton X and incubated at 0–4 C for 2–4 days. After centrifugation for 30 minutes at 10,000*g*, the supernatant was collected and used for measuring the activities of γ-glutamyl transpeptidase and alkaline phosphatase.

For extraction of lactate dehydrogenase (LDH), cells were lysed in distilled water for 30 minutes at room temperature and subsequently reconstituted with an equal volume of a solution containing 25 mM sucrose and 0.15 M NaCl. Freezing was not used because it may inactivate the muscle form of LDH isoenzyme.[21]

NADH-diaphorase was assayed by following the reduction of 2,6-dichlorophenol indophenol (DCPIP) spectroscopically at 600 nm.[22] The reaction mixture was composed of 1.4 ml of 0.2M Tris-HCl buffer, pH 8.5, containing 0.05 ml of 2.4 mM DCPIP, 0.1 ml of 28.2 mM NADH, and 10 μl of enzyme extract. The enzyme reaction was optimal at pH 8.5, rather than at pH 7.5.[23] Activity of the enzyme was calculated from the initial rate of decrease in absorbance at 600 nm with the use of a millimolar extinction coefficient of 18.2 (Sigma Chemical Co. data sheet). One unit was defined as micromoles of DCPIP reduced per minute at room temperature (22 ± 1 C).

Pyruvate kinase was assayed according to the method of Bucher and Pfleiderer.[24] The reaction mixture contained 50 mM Tris-HCl buffer, pH 7.5, 10 mM $MgCl_2$, 150 mM KCl, 0.25 mM NADH, 2 mM ADP, 1 unit/ml lactate dehydrogenase, and 3 mM phosphoenolpyruvate. One unit is defined as oxidation of 1 μmol NADH per minute at 37 C.

Glucose-6-phosphate dehydrogenase (G6PD), aldolase, γ-glutamyl transpeptidase, and *lactate dehydrogenase* were assayed with Sigma's enzyme assay kits nos. 345-UV, 752, 545, and 340-UV, respectively. One unit of activity of each of these enzymes represents the utilization of 1 μmol of substrate or the formation of 1 μmol of product at 37 C.

Alkaline phosphatase was assayed according to the method of Lowry et al.[25] One unit measures the hydrolysis of 1 μmole of *p*-nitrophenol per minute at 37 C.

Protein was measured in cytoplasmic enzyme extracts by the method of Bradford[26] with the use of the Bio-Rad protein assay kit (Bio-Rad Laboratories). For membranous enzymes solubilized in 1% Triton X, the method of Bramhall et al[27] was used. Bovine serum albumin was used as reference protein.

Methods for the separation of aldolase, hexokinase, and lactate dehydrogenase isozymes have been described previously.[16] Electrophoresis of phosphofructokinase was carried out in glycyl-glycine buffer according to Kemp.[28]

Analysis of Tumorigenicity

Cultured cells were dispersed by 0.25% trypsin in Ca^{2+}–Mg^{2+}-free Hanks' solution and then washed twice in Hanks' salt solution. One million cells in 0.1 ml were inoculated subcutaneously into the abdominal wall of each of several 1-day-old Fischer 344 rats of both sexes. Tumor-bearing rats were killed when neoplasms were about 1–2 cm in diameter. Rats in which tumors could not be palpated were killed 16 months after inoculation of cells. Tissues at the site of inoculation (including obvious tumors) were examined histologically.

Results

From the parental population of WB hepatic epithelial cells, we have developed 33 sublines, based on the number of repeated exposures to MNNG or to acetone vehicle and on the number of population doublings. Sublines WB-5-1 to WB-5-11 were exposed to 5 μg MNNG/ml of culture medium 1–11 times. Control sublines WB-A-1 to WB-A-11 were exposed to basal culture medium containing acetone vehicle (less than 1% acetone). Sublines WB-0-1 to WB-0-11 were doubling controls which were subcultured in basal medium at appropriate intervals so that they went through the same number of transfers and approximately the same number of population doublings as did the MNNG-treated populations. No significant differences in the parameters studied were discerned between WB-A and WB-0 subpopulations. In addition, we have studied a cell line (WB-5-11-T) reestablished in culture from one of the tumors produced by subcutaneously inoculating a day-old Fischer-344 rat with 10^6 WB-5-11 cells.

Growth Characteristics

Cell populations treated with MNNG demonstrated a decrease in doubling time, which paralleled that of the multiply subcultured WB-0 and WB-A cells. In similar fashion, colony formation increased progressively with repetitive subcultures or treatments with MNNG, and values between the two did not differ significantly (Figure 1). The saturation density did not change significantly in populations of either control or MNNG-treated cells during approximately 35 population doublings and 11 consecutive treatments with MNNG.

Cell populations treated repetitively with MNNG (WB-5) showed improved growth in calcium-deficient

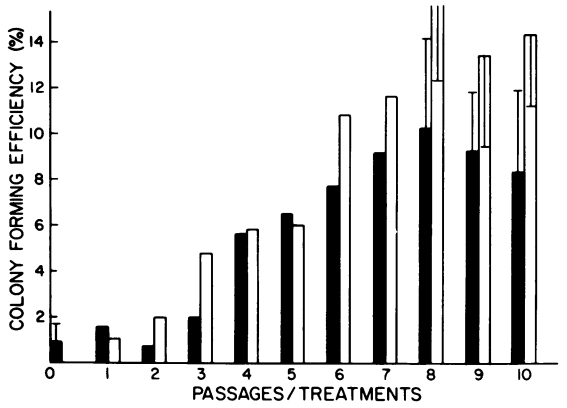

Figure 1—Colony-forming efficiency of control WB cells (*black bars*) and of Wb cells treated up to 10 times with 5 μg MNNG/ml as described in the text (*open bars*). Mean colony-forming efficiencies did not differ significantly (mean ± SD).

medium after 6–8 treatments, and the population treated 11 times with MNNG (WB-5-11) grew in calcium-poor medium (0.02 mM) with 55% to 85% of the efficiency as in calcium-rich (2.0 mM) medium. Control populations (WB-0 and WB-A) demonstrated no significant improvement in their ability to grow in calcium-poor medium. The tumor-derived line (WB-5-11-T) grew equally well in medium containing either high or low levels of calcium.

As were the WB-0 and WB-A cells, MNNG-treated populations were unable to form colonies in soft agar prior to treatment 11 (WB-5-11), when a few tiny colonies estimated to contain fewer than 50 cells were seen. In contrast, the tumor-derived line (WB-5-11-T) formed progressively enlarging colonies in soft agar with relatively high efficiency (>10%).

Tumorigenicity

When tested within five population doublings after treatment with MNNG, only the population of cells treated 11 times with MNNG (WB-5-11) was tumorigenic. When 10^6 cells of this population were inoculated subcutaneously into each of 61 1-day-old Fischer-344 rats, in 21 (34%) progressively enlarging tumors developed during the next 16 months. The latent period before the appearance of the first tumor was 10 months. Neoplasms were poorly differentiated tumors resembling hepatoblastomas, hepatocellular carcinomas, or biliary adenocarcinomas. Control populations (WB-0-

Table 1—Chromosomal Characteristics of WB Cells Treated Multiply With MNNG

Cell line	Euploid (Diploid and tetraploid)	Aneuploid	
		Paradiploid	Paratetraploid
WB-0-0	94% ± 5%	6% ± 5%	0
WB-5-1	61	36	5
WB-5-2	63	24	13
WB-5-3	60	25	15
WB-5-4	66	22	16
WB-5-5	42	15	43
WB-5-7	50	16	44
WB-5-9	48	15	45
WB-5-10	28	18	54
WB-5-11	18	33	59
WB-5-11-T	2	–	98

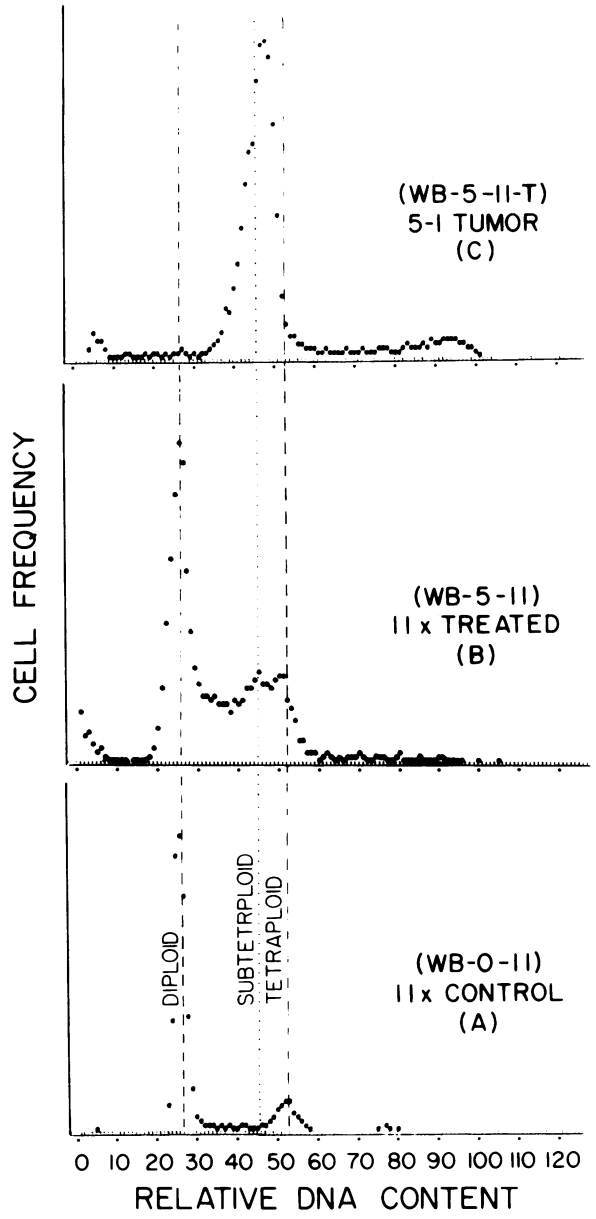

Figure 2—Flow cytofluorometric tracing of DNA/cell in populations of WB cells treated 11 times with 5 μg MNNG/ml (**B**) and a control population passaged for a similar number of population doublings in culture (**A**). Also shown is the tracing from a population of cells (WB-5-11-T) reestablished in culture from a tumor formed from WB-5-11 cells (**C**). MNNG treatment appears to cause the development of a subtetraploid clone in the WB-5-11 population (which is lacking in the controls). Cells in the tumor produced by WB-5-11 cells are all subtetraploid (WB-5-11-T), indicating that the subtetraploid clone was able to grow selectively *in vivo*.

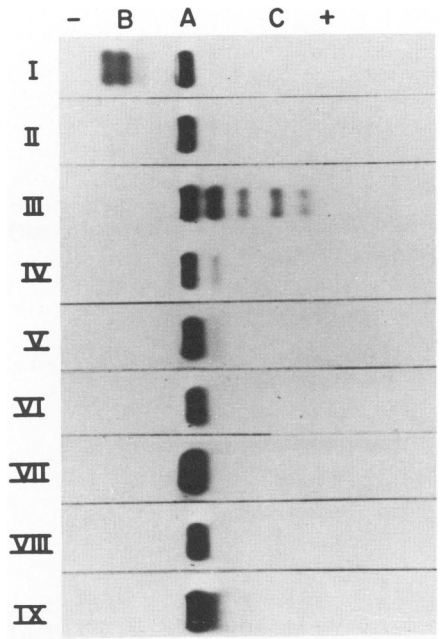

Figure 3—Electrophoretic patterns of aldolase isozymes. Panels contained extracts from the following tissues and culture populations: I, fetal rat liver; II, WB-0 cells; III, rat brain; IV, WB-5-2 cells; V, WB-5-5 cells; VI, WB-5-6 cells; VII, WB-5-7 cells; VIII, WB-5-11 cells; IX, WB-5-11-T cells. The letters A, B, and C refer to designated isozymes, which form as multimers. Multimers A_3C, A_2C_2, AC_3, and C_4 can be seen in brain, WB-5-0, WB-5-2, and WB-5-5 populations show weak A_3C bands, which are lacking in more extensively treated populations.

11 and WB-A-11) subcultured for equivalent population doublings *in vitro* were not tumorigenic even when 10^7 cells were transplanted into 1-day-old Fischer-344 rats.

DNA Cytofluorometry and Karyotypic Analysis

WB-0 cells were karyotypically diploid. By DNA cytofluorometry both the diploid (G_1) and tetraploid (G_2) peaks demonstrated slight but perceptible widening during multiple treatments with MNNG. Karyotypic analyses of the same populations indicated that widening of diploid and tetraploid peaks resulted from increased proportions of cells that contained hypodiploid and hyperdiploid and hypotetraploid and hypertetraploid numbers of chromosomes (Table 1). Data in Table 1 also indicate that major aneuploid shifts occurred after treatments 1, 2, 5, and 11. In the population of cells treated 11 times with MNNG (WB-5-11), DNA cytofluorometry showed a distinct hypotetraploid peak (Figure 2), which was corroborated by karyotypic analysis of the same population. That the hypotetraploid cells of the WB-5-11 population were responsible for tumor formation is suggested by the finding that the WB-5-11-T population, derived from a tumor produced *in vivo* by transplantation of WB-5-11 cells, was composed totally of subtetraploid cells.

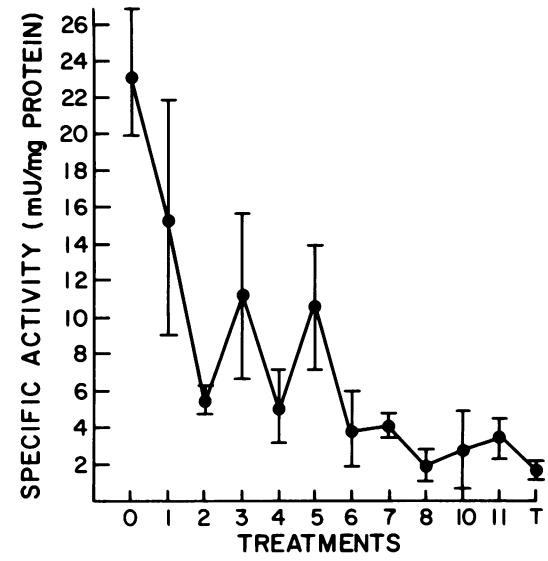

Figure 4—Specific activity of alkaline phosphatase in WB cells multiply treated with MNNG. *T* indicates cells of the tumor-derived line (WB-5-11-T). *Vertical bars* indicate standard deviation.

Nonenzymatic Cellular Products

As did the WB-0 and WB-A cells, all MNNG-treated populations continued to be positive for both albumin and α-fetoprotein. Cells derived from a tumor (WB-5-11-T) contained neither albumin nor α-fetoprotein. In contrast, multiply treated populations (WB-5-7 to WB-5-11) developed the capacity to store putative glycogen (PAS-positive, diastase-resistant material), and cells of the tumor-derived line (WB-5-11-T) contained large amounts of putative glycogen.

Expression of intracellular cytokeratin fibers was maintained in WB cells after multiple treatments with MNNG, as well as in the tumor-derived population. Fibronectin continued to be found extracellularly in MNNG-treated populations, but in multiply-treated

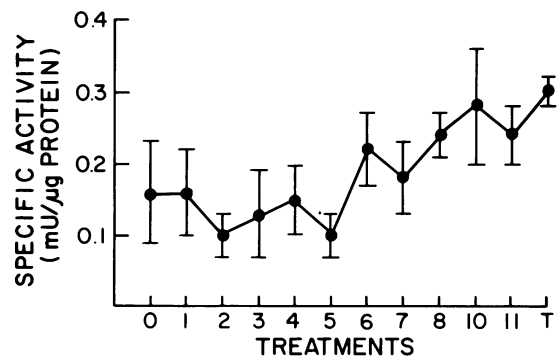

Figure 5—Activity of glucose-6-phosphate dehydrogenase in WB cells multiply treated with MNNG. *T* indicates cells of the tumor-derived line (WB-5-11-T). *Vertical bars* indicate standard deviations.

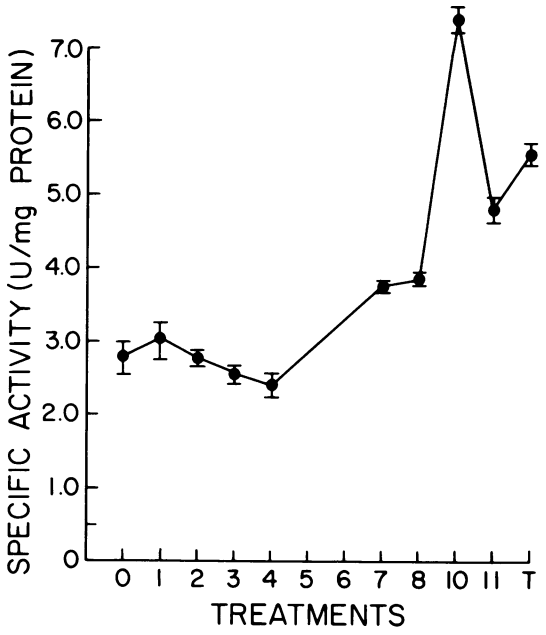

Figure 6—Activity of LDH in WB cells multiply treated with MNNG. *T* indicates cells of the tumor-derived line (WB-5-11-T). *Vertical bars* indicate standard deviations.

populations extracellular fibronectin was focally deficient. Foci of cells in the population treated 11 times with MNNG (WB-5-11) lacked extracellular fibronectin. Cells of the tumor-derived line (WB-5-11-T) failed to produce extracellular fibronectin.

Enzymes and Isozymes

No significant alterations in enzyme activities were noted in control populations (WB-0 and WB-A) during approximately 20 subcultures. Of the enzymes studied, *aldolase* was the only one whose activity was not affected by treatment with MNNG. However, modest changes did occur in the expression of aldolase isozymes. The expression of A and A_3C isozymes was maintained in control cultures and in MNNG-treated cultures through six consecutive treatments, after which the type C isozyme was lost (Figure 3). The type B isozyme, characteristic of adult liver,[29] was never seen. *Alkaline phosphatase* decreased markedly in activity after only two or three treatments, reaching a nadir by six treatments and remaining low for the rest of the study period (Figure 4).

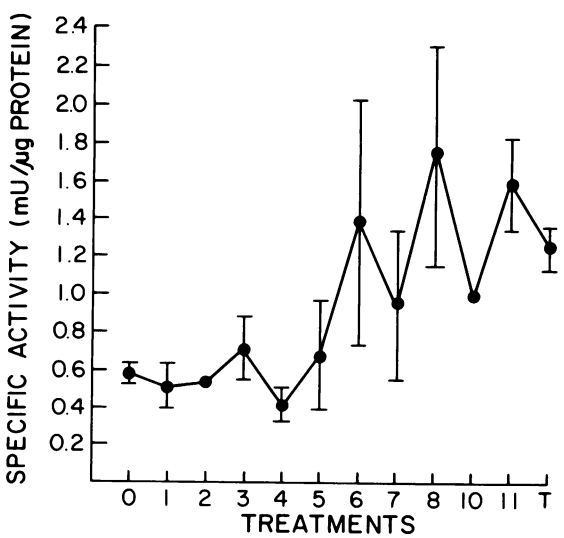

Figure 7—Activity of NADH-diaphorase in WB cells multiply treated with MNNG. *T* indicates cells of the tumor-derived line (WB-5-11-T). *Vertical bars* indicate standard deviations.

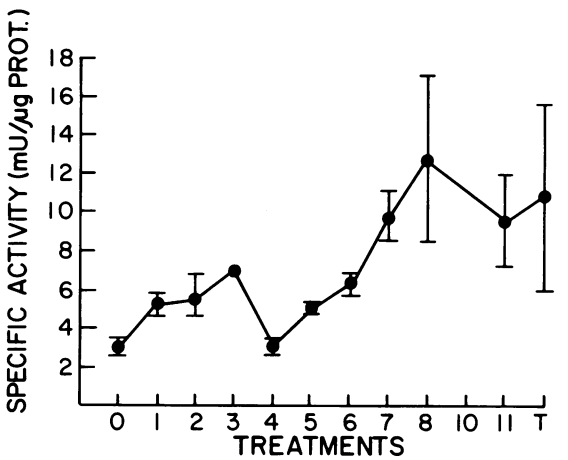

Figure 8—Activity of pyruvate kinase in WB cells multiply treated with MNNG. *T* indicates cells of the tumor-derived line (WB-5-11-T). *Vertical bars* indicate standard deviations.

Glucose-6-phosphate dehydrogenase (Figure 5), *lactate dehydrogenase*, (Figure 6), *NADH-diaphorase* (Figure 7), and *pyruvate kinase* (Figure 8) all increased in specific activity and/or showed modified isozyme patterns in populations of treated cells between Treatments 5 and 7. The results of the study of hexokinase isozymes are shown in Table 2. WB cells expressed only the Type I and II isozymes, Type I being the predominant form. Populations of WB cells treated more than six times with MNNG showed an increased fraction of Type II isozyme. In addition, WB-5-11-T cells expressed a small but inconstant amount of Type III isozyme. Type IV isozyme was never expressed. The distribution of lactate dehydrogenase isozymes in WB cells is shown in Table 3. After two treatments with MNNG, populations of cells showed sharply increased fractions of the M_4 isozyme. In populations of WB cells treated more than 6 times with MNNG, phosphofructokinase showed an extra, slower-moving band, in addition to the two bands found in all control cell populations (Figure 9).

The specific activity of *γ-glutamyl transpeptidase* remained low in all control and MNNG-treated cell popu-

Table 2—Distribution of Hexokinase Isoenzymes in Rat Tissues and Hepatic Epithelial Cells in Culture

Cell line	Percent of total hexokinase activity			
	H_I	H_{II}	H_{III}	$H_{IV}(G)$
WB-0	82%	18%	0%	0%
WB-5-1	83%	17%	0%	0%
WB-5-2	74%	26%	0%	0%
WB-5-3	79%	21%	0%	0%
WB-5-4	75%	25%	0%	0%
WB-5-5	71%	29%	0%	0%
WB-5-6	51%	49%	0%	0%
WB-5-7	62%	38%	0%	0%
WB-5-8	71%	29%	0%	0%
WB-5-10	60%	40%	0%	0%
WB-5-11	73%	27%	0%	0%
WB-5-11-T	44%	53%	3%	0%

H, hexokinase; G, glucokinase. Values are averages of two to four separate samples.

lations through 11 consecutive treatments (Figure 10). However, the tumor-derived cell line (WB-5-11-T) expressed a sharply increased activity of this enzyme (Figure 10). Gamma-glutamyl transpeptidase was not expressed histochemically in parental or multiply passaged WB cells (WB-0 and WB-A) when the reaction time was 5 minutes and only faintly when the reaction time was increased to 30 minutes. Under these same conditions, MNNG-treated populations (WB-5) also showed only occasional weakly positive cells. However, after 11 consecutive treatments (WB-5-11), a few cells were seen that stained intensely for γ-glutamyl transpeptidase after even a 5-minute reaction. Histochemical staining of colonies arising from single cells indicated that 9% ± 1% of the cells in the WB-5-11 population were histochemically positive for γ-glutamyl transpeptidase (5-minute reaction time). In contrast, over 75% of the cells were positive in the WB-5-11-T line derived from a tumor produced from the WB-5-11 population.

Discussion

This study demonstrates that repeated brief treatments with MNNG induce progressively altered phenotypic expression in a population of cultured diploid hepatic epithelial cells that express the "oval" cell phenotype. Phenotypic alterations emerge in a pattern which appears to be related to the number of treatments with MNNG and can be categorized into early (expressed by the fourth treatment), intermediate (expressed between the fifth and eighth treatments), and late (expressed after the ninth treatment) stages (Table 4). These clusters of phenotypic changes are temporally correlated with major changes in karyotype, and the pattern resembles that occurring in the focally altered hepatocytes of rats undergoing chemically induced carcinogenesis *in vivo*.[1]

Chemical hepatocarcinogenesis in rats is a multistep process characterized by the sequential emergence of altered islands, foci, neoplastic nodules, and carcinomas. Early lesions such as islands, foci, and nodules are characterized by altered enzyme histochemical staining properties, such as diminished activity (negative markers) of glucose-6-phosphatase and ATPase or enhanced activity (positive markers) of GGT, DT-diaphorase, G6PD, glyceraldehyde-3-phosphate dehydrogenase and epoxide hydrolase.[18,19] Elevation of specific activities of pyruvate kinase in hyperplastic nodules also has been reported.[30,31] This study suggests that the enzymatic alterations produced during *in vivo* studies of rat hepatocarcinogenesis can be reproduced *in vitro* by treatment of rat liver epithelial cells with MNNG. Elevated specific activities of NADH-diaphorase, G6PD, pyruvate kinase, and GGT preceded or accompanied the emergence of tumorigenicity after multiple carcinogen treatments. These phenotypic changes were seen in populations of cells which received more than six carcinogen exposures, whereas the specific activity of alkaline phosphatase was depressed after only one to two treatments. The latter enzyme is a sensitive marker of the action of MNNG on this rat liver epithelial cell line.

Specific activity of LDH in hyperplastic nodules of rat livers during carcinogenesis has not been reported, but elevation in LDH activity concomitant with an in-

Table 3—Distribution of Lactic Dehydrogenase Isoenzymes in Rat Liver and Hepatic Epithelial Cells in Culture

Cell line	$LDH_5(M_4)$	$LDH_4(M_3H)$	$LDH_3(M_2H_2)$	$LDH_2(MH_3)$	$LDH_1(H_4)$	Ratio 5/4
WB-0	39.5	37.8	19.7	3.0	–	1.1
WB-5-1	51.8	36.6	11.7	–	–	1.4
WB-5-2	57.1	33.2	9.7	–	–	1.7
WB-5-3	54.2	35.4	11.3	–	–	1.6
WB-5-4	58.6	26.3	15.1	–	–	2.2
WB-5-7	72.9	27.1	–	–	–	2.7
WB-5-8	54.0	36.0	10.0	–	–	1.5
WB-5-10	75.9	22.5	1.6	–	–	3.4
WB-5-11	78.5	21.5	–	–	–	3.7
WB-5-11-T	57.4	29.5	11.1	2.0	–	2.0

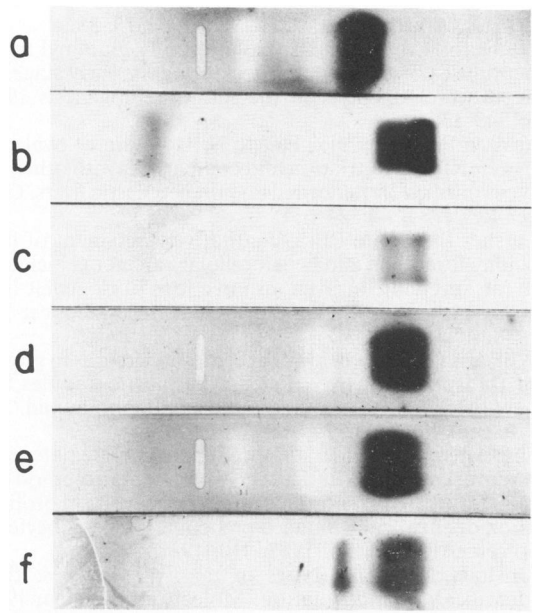

Figure 9—Electrophoretic patterns of phosphofructokinase isozymes. Panels contained extracts from the following tissues and culture populations: **A**, rat skeletal muscle; **B**, rat adult liver; **C**, rat fetal liver; **D**, WB-0 cells; **E**, WB-5-1 cells; **F**, WB-5-6 cells. Two bands, which are distinct from the muscle band, are seen in rat liver. WB cells show the liver bands. Six treatments with MNNG cause the appearance of a muscle band in WB cells.

crease in "muscle"-type LDH isozyme expression in rat and human hepatocellular carcinoma as well as tumors of other human organs have been documented amply.[32,33] Unlike G6PD, pyruvate kinase, and LDH, the

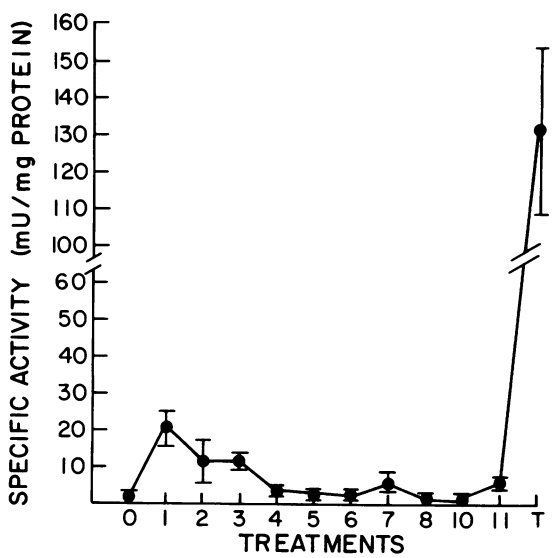

Figure 10—Activity of γ-glutamyl transpeptidase in WB cells multiply treated with MNNG. *T* indicates cells of the tumor-derived line (WB-5-11-T). When reacted for 5 minutes, no histochemically GGT-positive cells could be discerned in WB populations prior to WB-5-11. The latter population contained 9% ± 1% cells that reacted strongly for GGT histochemically. More than 75% of the cells of the tumor-derived line (WB-5-11-T) were histochemically strongly positive for GGT. *Vertical bars* indicate standard deviations.

Table 4—Summary of Phenotypic Changes in Populations of WB Cells Treated Multiply With MNNG

Chronology	MNNG treatments	Phenotype
Early	1–3	Paradiploid aneuploidy Decreased alkaline phosphatase
Intermediate	5–7	Paradiploid and hypotetraploid aneuploidy Increased NADH-diaphorase Increased pyruvate kinase Increased glucose-6-phosphate dehydrogenase Increased hexokinase isozyme 2 Loss of aldolase isozyme C Appearance of new phosphofructokinase isozyme
Late	9–11	Hypotetraploid aneuploidy GGT-positive cells Increased LDH isozyme 5 Glycogen storage in some cells Focally absent extracellular fibronectin Ability to grow in Ca^{2+}-poor medium Ability to grow in soft agar Tumorigenic in isogeneic rats

specific activity of aldolase was not changed by multiple carcinogen treatments. Aldolase specific activity also is generally not significantly elevated in malignant tumors,[34] possibly because it is not a key regulatory enzyme in the metabolic pathway.

In addition to the biochemical or enzymatic changes, rat liver epithelial cells also developed an aneuploid subpopulation of cells following multiple carcinogen treatments. This change also has been documented in hepatocytes initiated in rat livers during chemical carcinogenesis.[35] The development of aneuploidy may constitute a critical step in neoplastic transformation.[36]

Multiple exposures of cells to carcinogen induced increasing phenotypic alterations, and the final tumorigenic population produced following 11 treatments with MNNG demonstrated the most marked changes in the phenotypes. The enzymatic and isozymatic changes observed were not the result of multiple subcultures only, because enzymatic assays were made repeatedly during 11 or more passages of control cultures (WB-0 and WB-A) and systematic alterations of activities were not observed. It is also unlikely that the enzyme changes observed represent the result of selection of cells by carcinogen treatments, because the treatments were briefly episodic rather than prolonged.

Neoplastic lesions in rat livers have been induced by single carcinogen treatment or continuous carcinogen exposure in the diet.[37] Mechanistic studies of carcinogenesis and neoplastic progression can be performed only in a system involving initiation by a single carcinogen treatment, because continuous or multiple treat-

ments by carcinogen may result in multiple and random episodes of genomic damage and thereby make studies to trace sequential progression impossible.[38] Previously reported studies of carcinogenic transformation in rat liver epithelial cells in culture have been performed only by multiple or continuous carcinogen treatments,[39-43] because neoplastic transformation after a single *in vitro* treatment with a chemical carcinogen has not been accomplished. Nevertheless, it is possible to trace the connection between specific phenotypes and tumorigenicity by evaluating the clonal segregation of tumorigenicity and the chosen phenotype. Using this strategy, we already have shown that the ability of cells to grow in Ca^{2+}-poor medium, a putatively neoplastic phenotypic property, does not segregate with tumorigenicity.[19]

The *in vivo* and *in vitro* studies differ significantly in one important aspect. Cells in foci or neoplastic nodules or chemically treated rat livers are morphologically similar to hepatocytes and may be derived from preexisting hepatocytes. Rat liver epithelial cells in culture are phenotypically very similar to the "oval" or bile ductular cells. It should be noted that some investigators posit that chemically induced hepatocellular carcinomas in rats may be derived from "oval" cells.[5] In any event, the results reported here are useful because they demonstrate a phenotypic parallelism between the *in vivo* and *in vitro* studies. Studies with cell culture systems can complement the *in vivo* mechanistic studies of rat hepatocarcinogenesis because of the relative ease with which cultured cells can be manipulated and with which clonal subpopulations that express a specific phenotypic expression can be selected. Such clonal studies are important in establishing firmly whether phenotypic changes observed during neoplastic or tumor progression are mechanistically or sequentially linked to the induction of tumorigenic phenotype.

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