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Relationship of Colonic Mucosal Background to Neoplastic Proliferative Activity in Dimethylhydrazine-treated Mice[1]

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ABSTRACT

Proliferative activity of background and neoplastic colonic mucosa was examined following five months of weekly injections of 1,2-dimethylhydrazine (20 mg/kg) and one or four months of rest to determine whether previously reported changes may result from an acute or chronic effect of dimethylhydrazine and whether differences exist between stages of neoplasia. To determine whether neoplasia is responsive to a proliferative stimulus, 1,2-dimethylhydrazine dihydrochloride-treated mice were inoculated with *Citrobacter freundii*. The labeling index and the proliferative zone increased in background mucosa after one month; whereas after four months labeling index, proliferative zone and crypt heights increased, but the mitotic index decreased. There was a positive linear correlation between advancing tumor grade and increasing tumor labeling index and mitotic index. Background labeling index, even when elevated by *C. freundii* inoculation, had no effect upon tumor labeling index. Mitotic index diminished in background and neoplastic mucosa following prolonged rest and increased in both following *C. freundii* inoculation. These studies show that 1,2-dimethylhydrazine dihydrochloride causes long-term changes in background mucosa that are apart from a reparative response to cytotoxicity. As tumors progress, labeling index and mitotic index increase, suggesting a multistage process of evolution.

INTRODUCTION

The pathophysiology of colorectal cancer has been interpreted to a large extent from rodent carcinogenesis models, particularly those utilizing DMH.[2] High or repetitive doses of DMH result in a spectrum of antecedent and neoplastic changes analogous to those seen in humans. The antecedent mucosa prior to DMH tumor induction or the transitional mucosa between existing tumors may manifest cell-proliferative kinetics which resemble the mucosa of patients with proliferative bowel diseases that predispose to colorectal cancer and the transitional mucosa of patients between polyps or tumors (2, 11, 17, 18, 21). Sequential morphogenesis studies have shown a continuous spectrum of apparent progression from early foci of altered cells involving a single crypt to overt invasive carcinomas (5, 10, 14, 20, 23, 28), resembling the spectrum of lesions seen in patients with familial polyposis and cancer (8, 22).

There is a close association between exaggerated mucosal proliferation and colorectal carcinogenesis, both clinically and experimentally (5, 24, 32). This relationship has been explored with a mouse model system which incorporates reversible mucosal hyperplasia induced by a variant of *Citrobacter freundii* and DMH carcinogenesis (2–5). These studies have shown that hyperplasia influences the early stages of carcinogenesis by reducing the lag period for the appearance of neoplastic lesions, increasing their number, and allowing their induction with single subthreshold doses of DMH. The cell kinetics of benign hyperplasia, including labeling of surface epithelium, mirrors the kinetics of antecedent or transitional mucosa. This suggests that these changes are nonspecific and may be indicative of a mucosal response to injury rather than impending neoplasia.

The purpose of the present study was to clarify several areas of uncertainty in DMH carcinogenesis: (*a*) the proliferative kinetic parameters of background mucosa following a standard multiple weekly DMH regimen were examined before and after a prolonged rest period to determine whether the changes reported by others represent a reparative response to the acute cytotoxic effect of DMH or a more stable, chronic effect of the carcinogen; (*b*) the LI and MI were examined to determine if differences exist between stages of neoplastic progression; (*c*) neoplastic lesions were subjected to *C. freundii* to determine whether they are autonomous or responsive to an exogenous proliferative stimulus. These aspects of colorectal carcinogenesis have heretofore not been explored.

MATERIALS AND METHODS

NIH Swiss [N:(S)] mice were obtained and maintained as described previously (2). The room was kept at approximately 22° with a 12-hr-on (7 a.m. to 7 p.m.), 12-hr-off light cycle. The diet utilized throughout the experiment was from a single production lot of Purina Laboratory chow to minimize dietary variables (6) between treatment groups. Diet was kept at 4° until use.

Seventy mice, 5 weeks of age (one-half male, one-half female), were consecutively enumerated by ear punch, then assigned to treatment groups, and subsequently selected for necropsy using a table of random numbers. One week after the establishment of cohorts, 39 mice were given weekly injections of freshly prepared DMH (Matheson, Coleman and Bell, Norwood, Ohio), 20 mg/kg s.c. in 0.1 ml 0.001 M EDTA, for 5 months, and 31 mice were treated similarly with EDTA alone.

After 1 month of rest (23 weeks after onset of DMH/EDTA treatment), 13 DMH-treated mice and 18 EDTA-treated mice were each given an i.p. injection of 1 μCi [^{3}H]dThd (specific activity, 50 Ci/mmol; Schwarz/Mann, Orangeburg, N. Y.) per g body weight in 0.1 ml sterile water. All [^{3}H]dThd for the entire experiment was obtained from a single 10-ml vial and administered between 10 a.m. and 3 p.m. Mice were killed with ether

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[2] The abbreviations used are: DMH, 1,2-dimethylhydrazine dihydrochloride; LI, labeling index, MI, mitotic index; [^{3}H]dThd, [*methyl*-^{3}H]thymidine.

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1 hr after [^{3}H]dThd administration. Colons were processed for histology as described (3). Three 5-μm sections of tissue were obtained from each of 3 different levels of the paraffin block from each mouse (9 sections/mouse). Deparaffinized sections were processed for autoradiography (2) in 2 separate batches, one for each phase of the experiment (1 and 4 months rest). DMH-induced neoplastic lesions in the descending colon were graded and tabulated on a histological basis of 1 to 4 (5), with Grade 1 lesions corresponding to focal atypia involving a single crypt and Grade 4 lesions corresponding to early carcinomata with submucosal invasion. MI was calculated by counting the fraction of cells in early prophase to late telophase (15) among the cell population within 10 vertically complete crypts of background descending colonic mucosa (minimum 250 cells), or the cell population in each grade 1 to 4 DMH lesion (Grade 1, 30 to 90 cells; Grade 2, 70 to 200 cells; Grades 3 and 4, 300 to 400 cells). Data were derived from larger tumors by counting equal fractions from all segments of the tumor to minimize bias due to regional differences that have been noted in large tumors (24, 31). LI was calculated by counting the fraction of labeled cells among the same cell population examined for MI. Cells with 2 or more grains over the nucleus were considered labeled. Background labeling was 1 grain/cell or less (26). The average crypt cell column height (number cells along one side of the crypt from midpoint of the crypt base to the crypt neck) and proliferative zone (the basal fraction of the crypt occupied by labeled cells) were determined for each mouse among the same background mucosal crypts utilized for LI and MI determination. Three mice in the DMH-treated group had no neoplastic lesions. Following exclusion of these mice and those that did not label or survive treatment, 10 DMH-treated and 17 EDTA-treated mice were utilized.

The remaining 29 mice were allowed to rest for 4 months after cessation of DMH-EDTA treatment (34 weeks after onset of DMH-EDTA treatment) and then assigned to 3 treatment groups. Nine DMH-treated mice were inoculated p.o. with a broth culture of *C. freundii* (6) to induce mucosal hyperplasia of the descending colon. Nine DMH-treated mice and 11 EDTA-treated mice received sterile broth. Sixteen days later, when the mucosal hyperplasia induced by *C. freundi* should be most severe (4), all mice were given [^{3}H]dThd and processed as in the first part of the experiment. Following exclusion of mice due to mortality, no tumor development or ineffective labeling, 5 DMH-*C. freundii*-treated, 7 DMH-treated, and 11 EDTA-treated mice were utilized.

Tabulated data for background and neoplastic mucosa were evaluated statistically using Student's paired and unpaired *t* tests, linear regression, and linear correlation analysis.

RESULTS

In comparison to the mucosa of EDTA-treated controls, the background mucosa of DMH-treated mice following a 1-month rest period exhibited no differences in the mean crypt cell column height or MI ($p < 0.95$), but the proliferative zone and LI were elevated ($p \leq 0.01$ and $p \leq 0.001$, respectively) (Table 1). In DMH-treated mice, labeling of well-differentiated surface epithelial cells of background mucosa was found, but mitoses were never observed in this location. Other changes, including leukocytic infiltration, resembled those described previously (5). Following the 4-month rest period, a number of changes were evident in background epithelium. Despite maintaining identical husbandry conditions and diet, the mucosa of the older EDTA-treated mice had a lower proliferative zone height ($p \leq 0.001$) and LI ($p \leq 0.001$) than did the EDTA-treated mice in the first part of the experiment, but no differences were detectable in crypt cell column height or MI (Table 1). DMH-treated mice that were rested for 4 months had elevated crypt cell column height, proliferative zone, and LI but diminished MI of background mucosa compared to EDTA-treated controls ($p \leq 0.001$, 0.001, 0.001, and 0.05, respectively). Crypts were elongated, but their upper reaches and surface mucosa were populated by well-differentiated epithelial cells. Labeling among surface epithelium of background mucosa was increased compared to the DMH-treated mice rested for 1 month, but no mitotic figures were visible. When the proliferative stimulus of *C. freundii* was added to DMH-treated mice, the crypt cell column height, proliferative zone, LI, and MI of background mucosa were all elevated above that of DMH-treated mice without *C. freundii* ($p \leq 0.01$, 0.05, 0.001, and 0.001, respectively) (Table 1).

Table 1

Proliferative parameters of background mucosa of the descending colon among treatment groups

Treatment	Crypt cell column ht	Proliferative zone (%)	LI (%)	MI (%)
Part I[a]				
EDTA	26.7 ± 6.8[b]	39.2 ± 5.7	17.2 ± 2.5	5.5 ± 1.3
DMH-EDTA	27.3 ± 4.8	45.2 ± 4.6	23.8 ± 4.6	5.6 ± 1.6
Part II[c]				
EDTA	28.3 ± 3.0	29.7 ± 4.6	8.2 ± 1.9	4.8 ± 2.1
DMH-EDTA	38.5 ± 5.8	74.2 ± 8.1	27.0 ± 4.0	2.8 ± 0.7
DMH-EDTA + *C. freundii*	52.6 ± 6.6	83.6 ± 4.4	37.8 ± 2.0	5.1 ± 0.6

[a] One month following completion of 5 months of DMH-EDTA or EDTA injections.
[b] Mean ± S.E.
[c] Four months following completion of 5 months of DMH-EDTA or EDTA injections and 16 days after p.o. *C. freundii* or sterile broth inoculation.

Grades 1 through 4 neoplastic lesions were found among the DMH-treated mice following the 1-month rest period, with a high degree of correlation between lesion grade and increasing lesion MI and LI [MI correlation coefficient $r = 0.72$, $p \leq 0.01$, and LI $r = 0.29$, $p \leq 0.01$, $n = 56$, Chart 1]. When the MI of each DMH Lesion was compared to the MI of its homologous background mucosa using a Student's paired *t* test, Grades 2, 3, and 4 had MI higher than that of background ($p \leq 0.01$, 0.001, and 0.01, respectively). Grade 1 lesions had a MI lower than that of background ($p \leq 0.01$). When LI was similarly analyzed, Grades, 2, 3, and 4 lesions had LI higher than that of background ($p \leq 0.01$, 0.001, and 0.05, respectively). No difference was detected between the LI of Grade 1 lesions and background ($p < 0.95$).

Like DMH-treated mice in the first experiment, there was a positive correlation between DMH lesion grade and MI or LI among DMH-treated mice following the 4-month rest period (Chart 1; MI $r = 0.63$, LI $r = 0.41$, $p \leq 0.01$, $n = 56$). The MI of each DMH neoplastic lesion was compared to the MI of its homologous background mucosa using a Student's paired *t* test. Grades 1, 2, and 3 had MI higher than that of background ($p \leq 0.01$, 0.001, and 0.001, respectively). The LI of each Grade 1 lesion was not detectably elevated above homologous background mucosa ($p < 0.95$), but the LI of each Grade 2

and each Grade 3 lesion was higher than that of homologous background ($p \leq 0.001$). There was also a positive correlation between DMH lesion grade and MI or LI among DMH-*C. freundii*-treated mice (Chart 1; MI $r = 0.72$, $p \leq 0.01$, LI $r = 0.26$, $p \leq 0.05$, $n = 22$). Despite the elevated MI of hyperplastic background mucosa in DMH-*C. freundii*-treated mice, the MI of Grades 2 and 3 lesions ($p \leq 0.05$ and 0.01, respectively) but not Grade 1 lesions ($p < 0.95$) were greater than those of homologous background. The LI of hyperplastic background mucosa of DMH-*C. freundii*-treated mice was elevated, thus negating differences between neoplastic and nonneoplastic mucosa which existed without *C. freundii*. The LI of each Grade 1 lesion was significantly lower than homologous background mucosa ($p \leq 0.05$), but no differences were detected with Grades 2 and 3 lesions ($p < 0.95$). No obvious differences in LI or MI existed among each lesion grade of the different treatment groups (Table 2).

Correlation analysis was performed between the MI and LI slopes of Grades 1 to 4 neoplastic lesions of different treatment groups (Chart 2). The MI slope of mice treated with DMH and

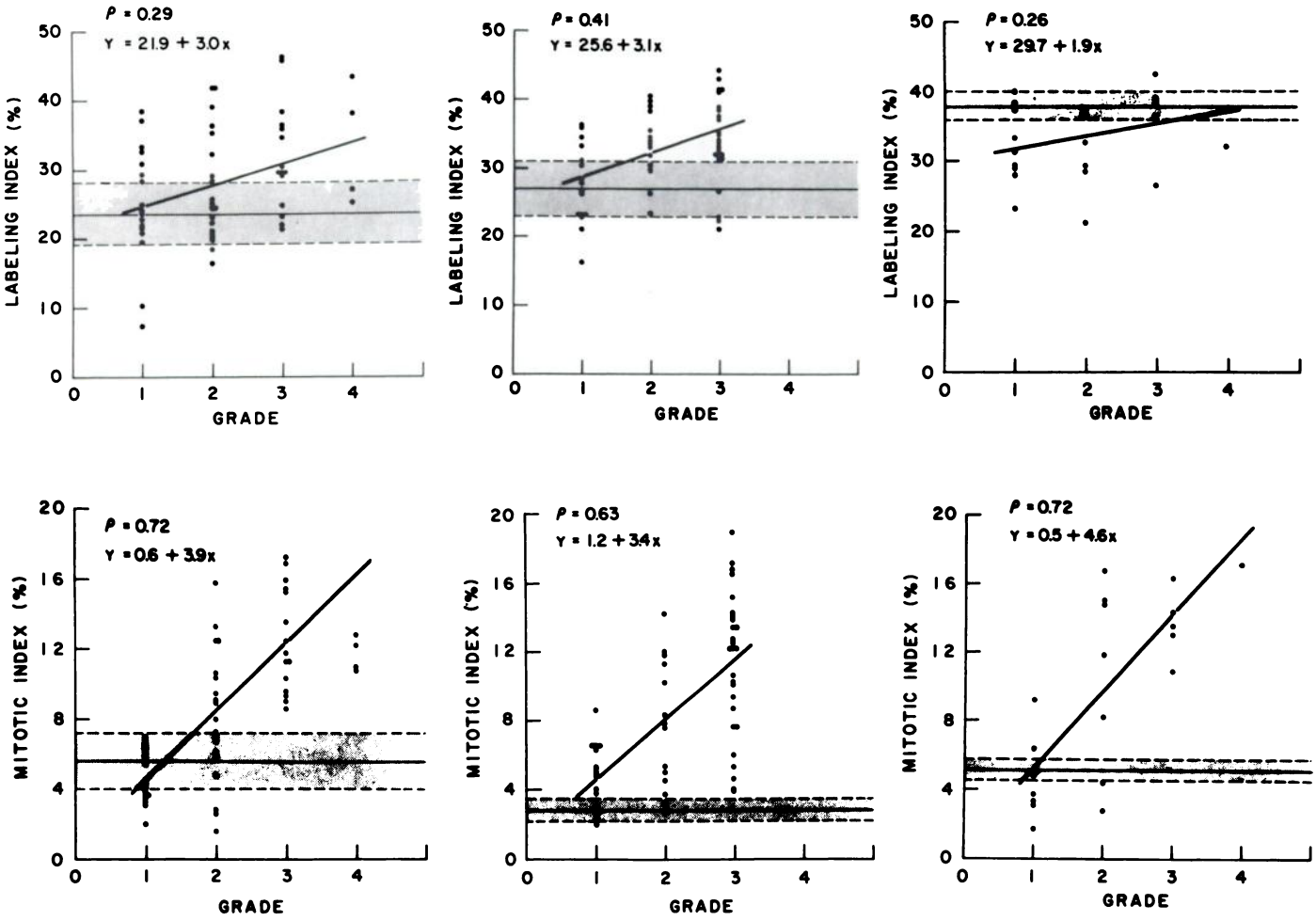

Chart 1. LI (*top*) and MI (*bottom*) of descending colonic background and neoplastic mucosa following DMH treatment. *Shaded areas*, mean (——) ± S.E. (- - -) of background; *points*, values of individual neoplastic foci. Formulas and *r* values are given for each regression line. *Top* and *bottom*, mice 1 month after cessation of DMH treatment. *Middle*, changes in mice following 4 months of rest. *Right*, changes in mice 4 months after cessation of DMH treatment and 16 days after *C. freundii* inoculation (hyperplasia).

Table 2

LI and MI of DMH neoplastic lesions among treatment groups

	Grade 1		Grade 2		Grade 3		Grade 4	
Treatment	LI	MI	LI	MI	LI	MI	LI	MI
Part I[a]								
DMH-EDTA	25.2 ± 8.4[b]	4.7 ± 1.5	27.5 ± 8.0	8.2 ± 3.7	31.4 ± 7.9	12.6 ± 3.0	33.6 ± 8.9	11.8 ± 1.0
Part II[c]								
DMH-EDTA	27.7 ± 5.8	4.7 ± 1.9	33.8 ± 5.0	7.7 ± 3.7	34.1 ± 6.1	11.6 ± 4.0		
DMH-EDTA + *C. freundii*	32.4 ± 5.5	4.8 ± 2.3	31.8 ± 5.9	10.6 ± 5.5	36.6 ± 6.0	13.7 ± 2.0	32.0	17.2

[a] One month following completion of 5 months of weekly DMH-EDTA injections.
[b] Mean ± S.E.
[c] Four months following completion of 5 months of weekly DMH-EDTA injections and 16 days after p.o. *C. freundii* or sterile broth inoculation.

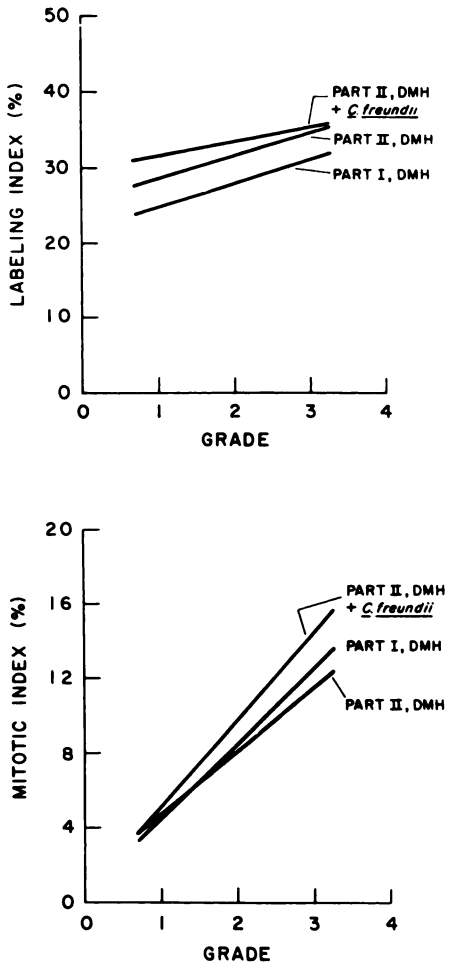

Chart 2. Linear correlation summary of tumor LI and MI slopes from Chart 1. *Part I*, DMH from mice 1 month following cessation of DMH. *Part II*, DMH from mice rested for 4 months following cessation of DMH; *Part II*, DMH plus *C. freundii* from mice rested for 4 months following cessation of DMH and 16 days after *C. freundii* inoculation. No differences in LI slope occurred among treatment groups. Part II DMH mice had significantly less incline to their MI slope compared to Part I DMH, and *C. freundii* treatment (Part II, DMH plus *C. freundii*) resulted in a significantly steeper slope than that of Part II DMH.

rested for 1 month was steeper than the MI slope of DMH-treated mice rested for 4 months ($p \leq 0.001$). DMH-treated mice inoculated with *C. freundii* had a significantly steeper MI slope than did DMH-treated mice without the proliferative stimulus of *C. freundii* ($p \leq 0.001$). No differences were detectable between the LI slopes of these treatment groups ($p < 0.95$).

DISCUSSION

DMH treatment resulted in a persistent alteration of background colonic mucosa beyond a reparative response to the acute cytotoxic effect of the drug. Multiple doses of DMH have been shown to cause increased LI and MI, expansion of the proliferative compartment, elongation of the crypt cell column, and surface labeling (10, 12, 19, 20, 23, 25, 27–29, 31). These changes subside within 2 weeks when only single doses of DMH are given (9, 13), suggesting a reversible reparative response to DMH damage. They resemble changes (including surface labeling) seen in benign reversible hyperplasia (2, 4). On the other hand, these alterations also mimic the mucosa of patients with diseases that predispose to colorectal cancer and the transitional mucosa of patients with colorectal polyps and carcinomas (2, 7, 13, 17, 18, 21, 24). Therefore, considerable significance has been placed on these changes as indicative of preneoplastic alteration. In this study, multiple DMH treatments caused expansion of the proliferative zone, surface labeling, increased LI, and elongation of the crypt cell column even after a prolonged (4-month) rest period and are thus apart from a reparative response to cytotoxicity.

The inconsonant relationship of LI to MI in DMH-treated background mucosa may reflect a fundamental change in the cell populations in response to DMH. Following prolonged rest (4 months), DMH treatment resulted in significant elevation of crypt height, proliferative zone, and LI but a decrease in MI as compared to controls. A larger population of cells appeared to be synthesizing DNA but not dividing, as compared to normal. In support of this, well-differentiated cells in surface mucosa were labeled, but mitotic activity was never found in this area. In contrast, labeled surface mucosal cells are undifferentiated and mitotically active in severe hyperplasia (2, 4). An age effect was also observed among controls. Older EDTA control mice had significantly lower proliferative zones and LI compared to the younger control group, corresponding with the diminished intestinal cell renewal observed in aging mice (16).

Despite the categoric classification criteria used in this study based upon lesion size, there was a significant positive correlation of LI and MI with advancing grades of neoplasia. Rodent tumors have a continuous spectrum with no obvious qualitative distinctions between stages. Because of this, several workers (20, 24) consider most rodent tumors carcinomas from their outset as focal atypia. Classification of neoplastic lesions was therefore simply performed by size, avoiding controversies in terminology. If the spectrum of neoplastic lesions represent different stages of sequential development, then there appears to be a progressive increase in LI and MI as these lesions evolve.

The LI slope of neoplastic lesions did not differ significantly after rest, despite increased background LI activity. In the presence of hyperplasia, background LI rose significantly higher than in Grade 1 lesions without modification of the Grade 1 lesions themselves. This observation supports the contention that Grade 1 lesions, or focal atypia, display autonomy similar to neoplasia, despite their potential reversibility (3).

In contrast to LI, the MI slope was slightly less acute following rest from DMH treatment. MI of background mucosa was also decreased at this time but increased when stimulated by *C. freundii*. *C. freundii* inoculation caused a modest rise in the MI slope of neoplastic lesions. Although hyperplasia seems to influence the MI of neoplastic lesions, it does not seem to have any appreciable long-term effect upon the progression or severity of neoplasia (5).

Previous studies have indicated that mucosal hyperplasia has a profound effect upon early carcinogenesis (3, 5), but results of this study show that it has little effect upon existing neoplasia. Hyperplasia, analogous to tumor promoters which induce proliferation (30), has been shown to reduce the lag period for the appearance of carcinogen-induced neoplastic lesions and to allow their induction with single, subthreshold doses of carcinogen. The findings that focal atypia, although they display autonomy, may be reversible (3) and that there is a progressive increase in LI and MI as tumors evolve negate the concept that carcinomas arise *de novo* and support a

multistage process (1) which is not readily visible on simply a histological basis.

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