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Comparative Changes in Rat Liver Cytosolic Proteins by Mirex, Diethylnitrosamine, and Dimethylnitrosamine Exposure (42166)

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Abstract. Using a newborn rat model for carcinogenesis, changes in liver cytosolic proteins at three stages of tumorigenesis, on Days 21, 97, and 120, by mirex (dodecachloropentacyclo-1,3,4-metheno-2H-cyclobuta[cd]pentalene), and diethyl- and dimethylnitrosamines (DEN and DMN) were studied. Following multiple exposure to the hepatocarcinogens, groups of weanling rats were given dietary phenobarbital (PB) up to 120 days. SDS–PAGE separation of cytosolic proteins showed that at 21 days, prior to PB, two proteins of 26K and 23K mol wt were significantly induced by mirex and DMN while a high mol wt 63K protein was induced only by DEN and DMN. During the period of PB treatment up to 97 days, these proteins were well sustained at a higher level. A marked increase in 21K protein band was also observed at this point. In tumor tissues obtained from DEN and DMN rats continued on PB diet for 120 days, the high level of 63K protein was seen only in DEN and not in DMN tumor. The tumors also showed a significant reduction in 25K protein compared to 21- and 97-day groups. The presence of even lower mol wt proteins of 14–21K was seen in tumors. The early detection and further characterization of these low mol wt proteins may provide clues as to whether they are preneoplastic markers or oncogene products as speculated by other investigators. Moreover, certain similarities in the induction of cytosolic proteins by "epigenetic" and "genotoxic" carcinogens raise more interesting questions regarding the mechanisms of action of these distinct classes of carcinogens.

Recent investigations have pointed out that the hepatic carcinogenic process is associated with significant alterations in cytoplasmic polypeptides including a carcinogen-binding protein (1, 2). In hepatomas the activities of glutathione *S*-transferase (2–4), DT-diaphorase (3, 5) and aldehyde:NAD(P) oxidoreductase (6) enzymes have been shown to fluctuate considerably. A comparative analysis of the electrophoretic pattern of liver cytosolic proteins from hepatocyte nodules resulting from six different types of chemical treatment indicated the presence of certain unique polypeptides of 21,000 and 26,000 mol wt (1). In this connection, we have examined the pattern of liver cytosolic proteins at three stages of tumorigenesis in rats exposed to hepatic carcinogens: direct acting "genotoxic" carcinogens, such as diethylnitrosamine (DEN) and dimethylnitrosamine (DMN), and mirex (dodecachloropentacyclo-1,3,4-metheno-2H-cyclobuta[cd]pentalene) which is believed to be acting through "epigenetic" mechanisms (7).

Materials and Methods. Female Sprague–Dawley rats (200 g body wt) obtained from Blue Spruce Farms, Altamont, New York, were mated with males of the same strain following acclimation for 1 week in Albany Medical College Animal Research Facility. Three groups of newborn rats, (minimum nine per group) were given multiple doses of mirex in corn oil (oral gavage, 1 mg/kg; 5 doses/week), DEN (ip, 15 mg/kg; 3 doses/week), and DMN (ip, 2 mg/kg; 3 doses/week) for 21 days. Two other groups of rats were also maintained as controls receiving saline injections. All rats after weaning at Day 21 were given ground Purina Lab Chow (PLC) mixed with phenobarbital (PB, 0.05%) up to stipulated time periods, except that one control group was given only basal PLC diet without PB throughout the experiment. Three animals per group were killed at three different stages of tumor development before or after giving PB, i.e., on Days 21, 97, and 120, following birth and the livers were examined for hepatic nodules. The neonatal rat model and treatments employed here

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for tumor development were similar to that used by Peraino *et al.* (8). However, the dosing regimen and duration of treatment were as indicated above. Rats were killed by an overdose of ether, portions of liver or tumor tissue rinsed in ice-cold 0.9% NaCl, and homogenized (10% w/v) with 0.32 *M* sucrose containing 3 m*M* $MgCl_2$. Cytosolic fractions were prepared by centrifugation of 1000*g* supernatant at 105,000*g* for 2 hr in a Beckman L8-55 ultracentrifuge. Protein concentration was determined by the method of Lowry *et al.* (9). The proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) using a 5% stacker and 10% running gels according to the procedure of Laemmli (10) at pH 8.6 using a Bio-Rad high voltage system. The gels were stained with Coomassie blue B and the gel strips were scanned at 540 nm in a Beckman R-112 gel scanner. The Bio-Rad reference standard proteins used were phosphorylase *b* (92,500), bovine serum albumin (66,200), ovalbumin (45,000), carbonic anhydrase (31,000), soybean trypsin (21,500), and lysozyme (14,400) of molecular weights as indicated.

Results and Discussion. The comparative differences in the rat hepatic cytosolic protein pattern were examined during the progression of tumorigenesis, in three stages of tumor development: (a) at the initiation stage, (b) promotion stage, and (c) in the tumor. PAGE patterns of cytosolic proteins of livers at 21 days of carcinogen exposure, prior to PB treatment, are shown in Fig. 1.[3] The migration pattern of standard proteins is given in track 1. Compared with those of the control group (track 2), mirex treatment caused an enhancement threefold and twofold, respectively, in the induction of proteins of 26K and 23K mol wt (track 3). While these proteins showed no apparent induction by DEN, another protein of 63K mol wt was found markedly increased (track 4). DMN was found to induce all these proteins (63K, 26K, and 23K) in substantial levels (track 5). However, these results indicate

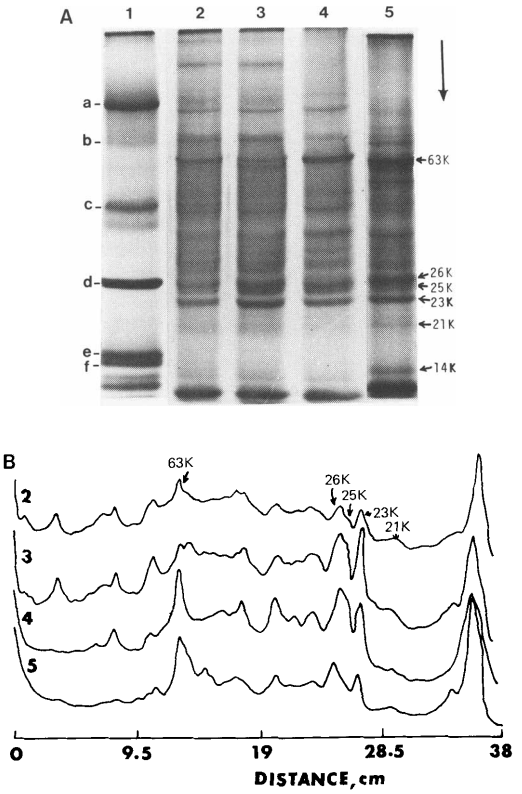

FIG. 1. SDS–PAGE patterns of liver cytosolic proteins of 21-day-old rats following mirex, diethylnitrosamine (DEN), and dimethylnitrosamine (DMN). The rats were exposed to the above carcinogens as described under Materials and Methods. At Day 21 before weanling the cytosolic fractions from liver were prepared and analyzed (50 μg protein) by electrophoresis (10% separating gel). The gel was stained with Coomassie blue B. The standard marker proteins were a, phosphorylase *b*; b, bovine serum albumin; c, ovalbumin; d, carbonic anhydrase; e, soybean trypsin; and f, lysozyme. Molecular weights of protein bands were determined in reference to the mobility of standard proteins. (A) Track—1, Marker protein; 2, control; 3, mirex-treated; 4, DEN-treated; 5, DMN-treated. (B) Densitometric scan of the gel. The numbers correspond to the tracks shown in A. The peak positions of significant proteins are indicated by their respective molecular weights.

that considerable differences exist in the pattern of protein induction by DEN and DMN during the neonatal period irrespective of similarities in their carcinogenic effects (11, 12). It is of interest to note that mirex caused the induction of two of these proteins, except a 63K protein as seen in the case of DEN and DMN. From the experiments reported by Er-

[3] Samples from all animals killed at three time periods were subjected to PAGE. The protein patterns were identical to the ones presented in Figs. 1 and 2 for various groups. Only representative gel samples are provided in the pictures.

iksson (1) and Meister and Anderson (13) it can reasonably be speculated that the proteins detected between 22K and 25K could be subunits of GSH *S*-transferases.[4] The migration of the 26K mol wt band compares well with that of a hepatic proliferation inhibitory protein isolated by McMahon (14). However, it should be noted that a 63K protein appears only in the cytosolic fraction of DEN and DMN treatments, but not with mirex. A previous study from this laboratory (15) has indicated the induction of a microsomal protein of similar mol wt (67K) in mirex-treated mice. Therefore, the 63K protein observed in the present study may have originated from microsomes since DMN or DEN-mediated damage of endoplasmic reticulum was found to facilitate the detachment of proteins from microsomal membranes (16).

The subsequent changes in the protein profile of cytosolic fractions as a result of exposure to dietary PB between 21 and 97 days were determined. Figure 2 shows the electrophoretic pattern from these groups (see tracks 2–6). Mirex + PB treatment (track 4) caused no significant induction of 63K proteins compared to control basal PLC diet (track 2) or basal diet + PB group (track 3). However, DEN + PB (track 5) and DMN + PB (track 6) animals showed a marked elevation of this protein 1.5-fold and 2-fold, respectively, as compared to the control. The level of 23K protein was more pronounced in mirex + PB and DMN + PB groups compared to other groups. Although 21K protein was present in slight amounts in all groups, its level was more enhanced in mirex + PB groups. During this period, no apparent differences were observed in the intensities of 26K protein bands between groups. The data indicate that most of these proteins which were induced by carcinogens

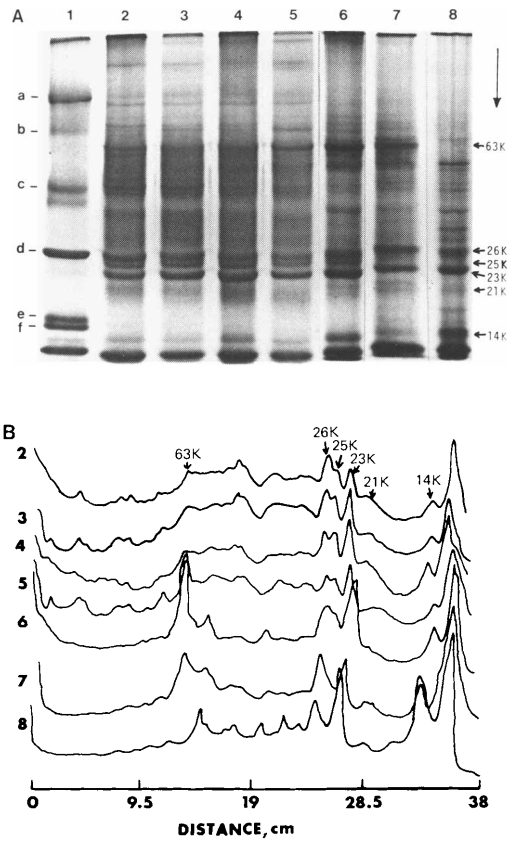

FIG. 2. SDS–PAGE patterns of liver cytosolic proteins of adult rats. The animals were initially treated with carcinogens followed by phenobarbital as described under Materials and Methods. Other details are as in Fig. 1. (A) Track—1, marker proteins; 2, control-basal diet; 3, control + PB; 4, Mirex + PB; 5, DEN + PB; 6, DMN + PB; 7, DEN-tumor; 8, DMN-tumor. (B) Densitometric scan of the gels. The numbers correspond to the tracks shown in A. The peak positions of significant proteins are indicated by their respective molecular weights.

(mirex, DEN, and DMN) at a neonatal period were sustained in the same level or even higher after prolonged exposure to PB. An important difference in the protein distribution among 21-Day and 97-Day-old animals is in the great fluctuation in the levels of 21K protein. This particular protein which was present in all neonatal groups in varying intensities, except in DEN groups, showed considerable increase following PB treatment, with a striking increase in the mirex + PB group. It is worthy to mention that the possibility of this protein as a cellular oncogene product (1) analogous

[4] Sugioka *et al.* (Cancer Res. **45**:365–378, 1985) have recently reported changes in polypeptide pattern of rat liver following 2-acetylaminofluorene, diethylnitrosamine and 3′-methyl-4-dimethylaminoazobenzene treatment for 8–12 weeks after weaning. It is interesting to note that in well-differentiated hyperplastic nodules and hepatocellular carcinoma, there was a dramatic increase in three new types of glutathione *S*-transferases of 26K mol wt each. These proteins were immunologically different and suggested to be products of gene expression relevant to malignant transformation of hepatic cells.

to the murine sarcoma virus transforming factor (17) has not been ruled out.

Tracks 7 and 8 represent the proteins present in the cytosolic fractions from the liver tumors produced by DEN and DMN, respectively, after 120 days of PB treatment. Since liver of mirex + PB animals at this period showed no tumor formation and the protein pattern was identical to that of 97 days, (track 4) electrophoretic mobility of cytosolic proteins from this group is not shown in Fig. 2. In DEN + PB tumors a high level of 63K protein, similar to DEN neonatal liver (Fig. 1), was present whereas this protein was nearly absent in DMN + PB tumors. The disappearance of this protein from DMN + PB tumor cytosolic fraction is in contrast to its abundant presence in DMN neonatal liver (Fig. 1). Although this finding is interesting, similar differential effects of DEN and DMN in producing acute hepatic lesions have been documented. In such cases DEN has been shown to be one-eighth as toxic when compared to DMN (11). More strikingly, the 25K protein was notably reduced in tumor tissues as compared to other groups of 21 and 97 days. Obvious differences also existed in tumor proteins ranging approximately 26K to 45K, although their separations and levels were more distinct in DMN + PB tumor than DEN + PB tumor. Other proteins of interest are those between 14K and 21K mol wt range whose concentrations were significantly higher in tumor tissues than in livers prior to tumor development. The former protein (14K) may correspond to the polypeptides detected by Eriksson *et al.*, (1) and Blackburn *et al.* (18).

Morphologically, the livers of 21-day-old rats showed only minimal diffuse fatty metamorphosis which is normal in young rat liver. Following PB exposure at 97 days, minimal centrolobular hepatocellular hypertrophy with large vacuolated hepatocytes were observed in these animals regardless of the "genotoxic" or "epigenetic" properties of the carcinogens used. However, at this period all liver samples except those from mirex animals, showed γ-glutamyltranspeptidase (GGT)-positive foci formation indicative of early preneoplastic lesion. GGT-positive foci development was in agreement with that reported by Peraino *et al.* (8, 19) in the newborn rat initiation–promotion model. DEN and DMN tumor tissues were histologically characterized as hepatocellular carcinoma with necrosis and focal malignant transformations. The detailed presentation of histological data is beyond the scope of this communication. However, the appearance and enhancement of the low molecular weight proteins (14–26K) are of crucial importance, especially in these three different stages of tumorigenesis supported by histological changes. It is not certain yet if such proteins described above are markers of preneoplastic or neoplastic liver. It should be pointed out that using a rabbit antibody prepared for purified placental GSH *S*-transferase subunits (21–26K) Sato *et al.* (20) has demonstrated the appearance of detectable levels of GSH *S*-transferases in preneoplastic hepatic lesions. Since these enzymes were not detectable in normal liver, the presence of these enzymes could be useful as marker proteins for preneoplasia in chemical hepatocarcinogenesis (20). Although the above proteins (14–26K) were not immunologically characterized in our study, the evidence indicates that they may possibly be characteristic subunits of GSH *S*-transferases.[4] Moreover, it is also interesting to note that despite the fact that both PB and mirex are considered to be tumor promoters (7), only the latter was found to induce low molecular weight proteins similar to those induced by DEN and DMN. This particular finding may show promise in providing a deeper insight into the mechanism of action of mirex-like chemicals.

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