Megalocytosis and Other Abnormalities Expressed during Proliferation in Regenerating Liver of Rats Treated with Methylazoxymethanol Acetate prior to Partial Hepatectomy

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SUMMARY

Rats were partially hepatectomized at various intervals after receiving a single injection of the carcinogen, methylazoxymethanol acetate. In treated rats, there was a delay and a reduction in the peak response of hepatic DNA synthesis. At 48 hr after partial hepatectomy, following completion of the first mitoses, almost all hepatocytes were enlarged (megalocytes) and many contained enlarged nuclei. Although at 7 days after the hepatectomy many megalocytes could still be found in centrilobular zones, the majority of the hepatocytes were of normal size or smaller. Abnormal anaphase and telophase figures containing chromosomal bridges and acentric fragments were found during the period of regeneration. In addition, discrete nests of small cells with increased cytoplasmic basophilia were evident. Megalocytes also appeared when rats were partially hepatectomized as late as 26 weeks after injection of the carcinogen. These results show that a single dose of methylazoxymethanol acetate can affect almost all hepatocytes and that latent effects persist for long periods of time.

INTRODUCTION

The fact that a single injection of methylazoxymethanol acetate can induce tumors of the liver, kidney, and the small and large intestine in rats (14, 16, 17) indicates that changes necessary for tumor initiation occur soon after treatment. Some of the early changes induced by the agent in liver include nucleoprotein structural alterations (7, 17), mitotic abnormalities, and induction of polyploidy (18). It is conceivable, however, that there are other early changes that are not ordinarily discernible and that may persist for long periods. We have, in fact, seen evidence for such latent effects in treated livers that have been forced to undergo intensive proliferation in response to partial hepatectomy. This report deals with the aberrant nature of the regenerative process.

MATERIALS AND METHODS

Methylazoxymethanol acetate was purchased from Schwarz/Mann, Orangeburg, N. Y. [methyl-3H]TdR (2 Ci/m mole) and orotic [6-14C]acid hydrate (12.8 mCi/m mole) were obtained from New England Nuclear, Boston, Mass. Young male SD rats 4 weeks old, CD line (Charles River Breeding Laboratories, Brookline, Mass.) were treated as described and were given food and water ad libitum.

All solutions for injection were made in 0.9% NaCl solution and were usually injected i.v. by tail vein in a volume of 10 ml/kg. Solutions of methylazoxymethanol acetate were prepared immediately before use. Animals given injections of 0.9% NaCl solution served as controls in all experiments. All partial hepatectomies were performed by 1 individual between 1 and 2 p.m. according to the method of Higgins and Anderson (8). The rats were fed Purina laboratory chow and were kept on a schedule of 12 hr light and then 12 hr darkness. DNA, RNA, and protein content; incorporation of [methyl-3H]TdR into DNA; and incorporation of orotic-[6-14C]acid hydrate into RNA were determined as previously described (16, 17).

For microscopic study, tissues were fixed in Bouin’s solution; sections were stained with hematoxylin and eosin. Mitotic indices (total number of prophase, metaphase, anaphase, and telophase mitotic figures per 1000 nuclei) were determined by scanning across liver sections, chosen at random, until having counted approximately 1000 hepatocyte nuclei per rat liver. Approximately 5000 to 8000 nuclei per liver were examined to determine the percentage of abnormal anaphase and telophase figures. Sections from at least 2 different lobes of liver were scanned for determination of both the mitotic index and the percentage of abnormal mitotic figures.

RESULTS

Microscopic Studies. We have previously reported (18) that a single tumorigenic dose of methylazoxymethanol acetate causes early inhibition of nucleic acid and of protein synthesis and hepatocyte necrosis with inflammation in rat livers. In treated rats, there was a delay and a reduction in the peak response of hepatic DNA synthesis. At 48 hr after partial hepatectomy, following completion of the first mitoses, almost all hepatocytes were enlarged (megalocytes) and many contained enlarged nuclei. Although at 7 days after the hepatectomy many megalocytes could still be found in centrilobular zones, the majority of the hepatocytes were of normal size or smaller. Abnormal anaphase and telophase figures containing chromosomal bridges and acentric fragments were found during the period of regeneration. In addition, discrete nests of small cells with increased cytoplasmic basophilia were evident. Megalocytes also appeared when rats were partially hepatectomized as late as 26 weeks after injection of the carcinogen. These results show that a single dose of methylazoxymethanol acetate can affect almost all hepatocytes and that latent effects persist for long periods of time.

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[The abbreviation used is: [methyl-3H]TdR, [methyl-3H]thymidine.]

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liver within the 1st 3 days. At the end of 1 week, necrosis and inflammation are no longer present although the residual effects of methylazoxymethanol acetate can be seen by the presence of some irregularities in size and shape of hepatocytes, of some hepatic cells containing enlarged nuclei, and of abnormal mitoses. When rats were partially hepatectomized at 7 days after the tumorigenic dose of 35 mg/kg, various abnormalities of regeneration were noted. Chart 1 lists the number of animals that were studied at various intervals after the partial hepatectomy.

The livers of those killed at 20 and 28 hr contained hepatocytes and hepatocyte nuclei larger than those of controls. By 48 hr, almost all of the hepatocytes in the livers of methylazoxymethanol acetate-treated rats were enlarged (megalocytosis) and about one-half of these contained enlarged nuclei; there was variation in both cell and nuclear size. At 72 and 96 hr, in addition to the change just described, there was also variation in the arrangement of sinusoids and plates. The contrast between the appearance of regenerating livers in control rats and that of treated rats is shown by comparing Fig. 1 with Figs. 2, 3, and 4. About one-half of the livers of treated rats contained discrete nests of cells with increased cytoplasmic basophilia (Figs. 3 and 4). These cells were smaller than the megalocytes. The extent of the changes described was maximal at 72 hr, and somewhat less thereafter. By 7 days, the megalocytes were found mainly in centrilobular zones. The majority of the hepatocytes were of normal size or slightly smaller. These small cells were diffusely arranged, unlike the discrete foci noted at 72 and 96 hr. The extent of the enlargement of hepatocytes and the number of cells so involved have no counterpart in the livers of intact rats at any time after the injection of methylazoxymethanol acetate (17, 18).

At 14 days after partial hepatectomy, the liver appeared to be normal with occasional foci of cells containing enlarged nuclei. At 3 to 4 months, in 6 of 9 rats, only an occasional enlarged hyperchromatic nucleus could be found, as previously reported in rats given methylazoxymethanol acetate without subsequent partial hepatectomy (17). In addition, each of 2 rats had a solitary focus of enlarged hepatocytes with vacuolated cytoplasm. These foci were well demarcated and did not compress the surrounding cells.

Small numbers of rats were also partially hepatectomized at 4 (4 rats), 8 (6 rats), and 26 (4 rats) weeks after receiving methylazoxymethanol acetate. As late as 26 weeks after treatment, partial hepatectomy resulted in megalocytosis; at 5 to 7 days approximately 20 to 30% of the hepatocytes were enlarged.

Megalocytosis was not observed in livers of rats that were treated with methylazoxymethanol acetate and were subsequently sham-operated.

**Mitotic Indices.** Chart 1 shows the mitotic index of hepatocytes during liver regeneration. In livers of rats treated with carcinogen this was less than that of controls at 28 hr after partial hepatectomy, was higher than controls at Day 2, and remained slightly above control values for the next 2 weeks. During the period of regeneration, mitotic figures were found in enlarged hepatocytes (Figs. 2 and 3).

**Mitotic Abnormalities.** At 28 hours after partial hepatectomy, the time of maximal mitotic activity, abnormal mitotic figures were present in the livers of 8 of 10 rats that had been treated with methylazoxymethanol acetate. The abnormalities, mainly chromosomal bridges and less frequently what appeared to beacentric fragments (18), were also observed in 3 of 4 rats at 48 hr and in each of 7 rats at 96 hr. The abnormal figures, relative to the total number of anaphase and telophase mitoses, averaged 16.2, 11.1, and 19.5% at 28, 48, and 96 hr, respectively. Of 9 control rats that were studied at 28 hr, of 4 rats at 48 hr, and of 7 rats at 96 hr only 1 animal at each time interval had a single abnormal mitosis: for the 3 groups of control rats the percentages of abnormal figures averaged 0.15, 0.7, and 0.43, respectively. Three rats were partially hepatectomized at 8 weeks after receiving methylazoxymethanol acetate. Forty-eight hr later, the livers of each rat had an average of 6.5% abnormal mitoses.

**Studies in Adult Rats.** Seven adult rats 3 months of age, in which the level of DNA synthesis and mitotic index is very low as compared to the weanling, were treated with methylazoxymethanol acetate, 35 mg/kg i.p., and were partially hepatectomized 1 week later. Rats were sacrificed either 5 or 7 days later, and in each of the 7 rats approximately two-thirds of the hepatocytes were enlarged. Four of the 7 livers of treated rats had demarcated nests of cells that were much smaller than the megalocytes; these small cells had small nuclei and increased cytoplasmic basophilia (Fig. 4). Some of the cells in the nests had vacuolated cytoplasm. In addition, about 50% of all the anaphase and telophase mitoses were abnormal.

**Actinomycin D and Partial Hepatectomy.** Actinomycin D, like methylazoxymethanol acetate, can inhibit RNA synthesis and induce alterations of nucleolar structure. However, it has not been found to be hepatocarcinogenic in rats (11). We were, therefore, interested to know whether livers of rats that had been treated with actinomycin D...
would show the aberrancies of regeneration like those described above. For this purpose, rats received a sublethal dose of actinomycin D, 0.4 mg/kg i.v. One hr after this dose, orotic acid incorporation into hepatic nRNA was inhibited by 55%, a result consistent with previous findings (6). This dose can induce nucleolar alterations in all hepatocytes (6). Four rats treated in this manner were partially hepatectomized 1 week later and were sacrificed 5 days thereafter. Examination of sections of regenerating liver from these animals did not reveal the presence of any megalocytes.

Megalocytosis and Tumor Induction. If megalocytes were precursor cells for tumors, then the induction of large numbers by partial hepatectomy might have been expected to increase the incidence of hepatic tumors. As a test for this possibility, the following brief study was carried out. Rats were treated with methylazoxymethanol acetate, 35 mg/kg, and were then partially hepatectomized 7 days later. At 8 months, none of 5 rats had developed any hyperplastic nodules or carcinomas; at 13 months, a hyperplastic nodule was found in the liver of 1 of 5 rats. At the latter time, hyperplastic nodules were also found in 3 of 7 rats that had been treated with the carcinogen and were sham-operated 7 days later.

Effects on DNA Synthesis. At 20 hr after partial hepatectomy as shown in Chart 2, \([\text{methyl-}^3\text{H}]\text{TdR}\) incorporation into hepatic DNA was significantly less in rats that had been treated with methylazoxymethanol acetate 7 days earlier \((p < 0.001)\). At 48 hr, when control values were decreasing, \([\text{methyl-}^3\text{H}]\text{TdR}\) incorporation into DNA of livers of treated rats rose to a maximum. Thereafter, the values decreased along with those of control although they were in fact significantly higher at 4 \((p < 0.02)\) and 7 \((p < 0.02)\) days. At 14 days, the rate of DNA synthesis was similar to that of controls.

The significant delay and reduction in the peak response of DNA synthesis was matched by a significant reduction in the rate of increase of total hepatic DNA. At 7 days after injection with methylazoxymethanol acetate, total hepatic DNA was similar to that in rats given 0.9% NaCl solution (see Chart 3; 0-hr values). After partial hepatectomy, the remnant contained 37% of the original amount of hepatic DNA in both groups. Within 3 days, the control rats regained their initial content of total hepatic DNA. The rate of gain in total liver DNA was significantly slower in the carcinogen-treated rats, and the prehepatectomy levels in total DNA content were not attained until about 4.5 days. At the 2nd and 3rd day after partial hepatectomy, the totals of liver DNA relative to controls were significantly different, \(p < 0.01\) and \(p < 0.05\), respectively. The increase in hepatic DNA in both groups continued beyond their prehepatectomy levels, in keeping with the continued growth of the young animals used in these studies. By 7 days, both control and carcinogen-treated animals had equivalent amounts of total hepatic DNA. Throughout the period of study, the concentration of DNA per g liver in
The concentration of DNA per g liver, however, is similar in the level of polyploidy in intact livers (18).

Carcinogen contain greater than diploid amounts of DNA. Daughter cells that arose during the regeneration period that cells in normal adult rat liver synthesize DNA and divide at tumor-inducing effects of chemical carcinogens (12). Few eddy altered. Thus, 20 hr after the hepatectomy, when DNA which develops in livers of methylazoxymethanol acetate (18) and the megalocytosis, cells are present in the regenerating liver of methylazoxy carcino gn. The response of the liver to partial hepatectomy at 7 days after treatment with methylazoxymethanol acetate is markedly altered. Thus, 20 hr after the hepatectomy, when DNA synthesis is maximal in regenerating liver of weanling rats (2), DNA synthesis in livers of rats pretreated with carcinogen is inhibited approximately 50%. The lower mitotic index seen at 28 hr is consistent with the lower rate of DNA synthesis. The reduction of total DNA at 48 hr, when almost all hepatocytes are enlarged, indicates that fewer cells are present in the regenerating liver of methylazoxy methanol acetate-treated rats than in that of control rats. The concentration of DNA per g liver, however, is similar in both the control and treated groups. Thus, it appears that a significant fraction of cells in the livers of rats treated with carcinogen contain greater than diploid amounts of DNA. In this regard methylazoxy methanol acetate can increase the level of polyplody in intact livers (18).

There are numerous studies in vivo to indicate that proliferating cells are more sensitive than resting cells to the tumor-inducing effects of chemical carcinogens (12). Few cells in normal adult rat liver synthesize DNA and divide at any given time. The fact that the necrosis induced by methylazoxy methanol acetate (18) and the megalocytosis, which develops in livers of methylazoxy methanol acetate-treated rats after partial hepatectomy, involve significant numbers of cells suggests that such changes can be induced in nonproliferating cells. These data also indicate that the daughter cells that arose during the regeneration period that followed the initial phase of necrosis in the intact liver (18) "inherited" the carcinogen-induced alteration leading to megalocytosis after partial hepatectomy. It would be of interest to determine whether a 2nd partial hepatectomy after the regenerative process elicits a similar response of extensive megalocytosis.

Megalocytes have been induced in rat liver by a variety of carcinogens, most notably the Senecio alkaloids (10). Treatment with these alkaloids results in extensive megalocytosis that persists for long periods of time. The mechanism responsible for this effect appears to be an inhibition of mitosis. In contrast, the megalocytosis induced by partial hepatectomy in methylazoxy methanol acetate-treated animals is short lived; the liver appears to be normal within 14 days after the hepatectomy. Moreover, the mitotic index is only briefly reduced in a manner that appears related to an initial decrease in the rate of DNA synthesis. In fact, mitotic figures are found in enlarged cells.

The megalocytes that appear after partial hepatectomy are probably viable cells that eventually give rise to normally appearing progeny by mitotic division. No evidence was found for necrosis of megalocytes. Moreover, animals pass through the period when almost all of the hepatocytes were involved without signs of severe intoxication or even reductions in rate of growth. In other experiments in this laboratory, rats have been partially hepatectomized, given radiolabeled thymidine 22 hr later when many hepatocytes are in the S phase of the cell cycle, and treated 2 hr later with the carcinogens N-hydroxyacetylamino fluorene or xanthine 3-N-oxide. Such treatment also results in extensive megalocytosis within the next 2 days. Thereafter, there is a slow but steady disappearance of megalocytes with few remaining at the end of 4 weeks. There was no loss of DNA-associated radioactivity during this period of time (F.S. Philips and S. S. Sternberg, personal communication).

The latent effects induced by methylazoxy methanol acetate in rat livers are long-lasting. Megalocytosis was still evident in regenerating livers of rats partially hepatectomized as late as 26 weeks after treatment with the agent. Damjanov et al. (3) reported that repair of DNA in livers of rats treated with methylazoxy methanol acetate was not complete as late as 14 days later. Other workers have demonstrated long-lasting effects of carcinogens in rat livers that are expressed when hepatic cells undergo mitosis. For example, Maini and Stich (9) have shown that, 6 months after discontinuing feeding of the carcinogen 3'-methyl-4-dimethylaminoazobenzene, partial hepatectomy elicited mitotic abnormalities in regenerating liver. X-irradiation has also been shown to induce latent effects in rat liver (1). DNA synthesis and mitosis in regenerating liver were delayed and diminished when rats were partially hepatectomized soon after X-irradiation. Many abnormal mitotic figures were observed. When rats were partially hepatectomized 8 weeks after X-irradiation, there was no delay in regeneration although abnormal mitoses were found. Mitotic abnormalities could still be elicited by partial hepatectomy even as late as 11 months after X-irradiation (13).

The presence of large numbers of megalocytes did not
result in an increased incidence of tumors. The data, although sparse, indicate that rats that are treated with carcinogen and subsequently sham-operated develop more hyperplastic nodules than did rats partially hepatectomized. The results also suggest that the nests of small cells with increased cytoplasmic basophilia observed in about one-half of the rats treated with methylazoxymethanol acetate, are not significant in tumor development. Others have found such cells in livers of rats treated with a variety of chemical carcinogens (4, 5) and have suggested that these cells may be foci for tumor development (4).

In conclusion, these studies indicate that latent effects induced by carcinogens can persist for long periods of time, that these effects become manifest during cell proliferation, and that repair of carcinogen-induced changes occurs neither rapidly nor completely in all cells. Such changes may become expressed during the slow but continuous cycle of cell renewal common to most tissues. Further studies of such latent effects may add to the understanding of the critical changes induced by carcinogens that are important in the development of tumors.

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Fig. 1. Liver section from a rat given 0.9% NaCl solution, partially hepatectomized 7 days later, and killed 96 hr after partial hepatectomy. H & E, x 400.

Fig. 2 to 4. Liver sections from rats given a single i.v. injection of methylazoxymethanol acetate, 35 mg/kg; partially hepatectomized 7 days later; and killed 96 hr after partial hepatectomy. H & E, x 400.

Fig. 2. Marked enlargement of hepatocytes, involving nucleus and cytoplasm (megalocytosis). An abnormal-appearing mitotic figure is present in 1 of these cells (upper).

Fig. 3. Center, nest of small basophilic cells; arrow, a mitotic figure in 1 of these cells (above a megalocyte in mitosis).

Fig. 4. A larger nest of basophilic cells among the megalocytes. Arrow, a mitotic figure in 1 of the basophilic cells.
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