Oral Contraceptive Steroids as Promoters of Hepatocarcinogenesis in Female Sprague-Dawley Rats

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ABSTRACT

A number of reports have described the occurrence of liver cell adenomas in women using oral contraceptives. Circumstantial evidence derived from human and early experimental animal data, together with the recent report of Taper [Cancer (Phila.), 42: 462-467, 1978], suggests that oral contraceptive steroids may be liver tumor promoters. The objective of this study was to determine whether the feeding of two commonly used oral contraceptive steroids, mestranol and norethynodrel, can promote diethylnitrosamine (DEN)-initiated hepatocarcinogenesis. Female Sprague-Dawley rats were partially hepatectomized and intubated with either water or DEN (5 mg/kg body weight) 24 hr later. Twenty-four hr after carcinogen treatment, the animals were separated into seven groups (15 rats/group). The treatment groups were as follows: DEN → mestranol, DEN → norethynodrel, DEN → mestranol plus norethynodrel, DEN → M + N; phenobarbital, DEN → basal diet, H2O → phenobarbital, and H2O → mestranol plus norethynodrel. Steroid consumption was mestranol = 0.02 to 0.03 mg/kg body weight/day and norethynodrel = 0.5 to 0.75 mg/kg/day. These doses are equivalent to 10 to 15 times the human dose. Five animals from each group were killed after 4 months and ten after 9 months. Histochemically detectable γ-glutamyl transpeptidase positive foci, together with hematoxylin and eosin-detectable lesions and γ-glutamyl transpeptidase enzyme activity were scored in each liver. At both kill times, DEN-initiated animals fed diets containing mestranol, mestranol plus norethynodrel and phenobarbital had significantly greater numbers of γ-glutamyl transpeptidase foci (p < 0.05) than did the controls. In addition, DEN-initiated animals fed diets containing mestranol or mestranol plus norethynodrel had greater numbers of basophilic foci than did animals in the other groups. These results suggest that the oral contraceptive steroid mestranol is a promoter of the appearance of putative precursor lesions of hepatocarcinogenesis.

INTRODUCTION

Since the paper of Baum et al. (3), a number of reports have indicated that liver adenomas and, in a few cases, hepatocellular carcinomas may appear in women using oral contraceptives (8, 9, 11, 12, 19, 24, 27, 32). Rooks et al. (24) reported the results of a case-control study of HCA3 in 79 female patients and in 220 age- and neighborhood-matched controls. These investigators concluded that increasing duration of oral contraceptive use increases the risk of developing HCA, a result which supported the earlier finding of Edmondson et al. (8). Rooks et al. (24) also observed a positive correlation between incidence and hormonal potency of the oral contraceptive but were unable to determine whether this was related to the estrogen or progestin component. Two synthetic estrogens are currently widely used in oral contraceptives in the United States, mestranol and ethinyl estradiol. Rooks et al. (24) were unable to demonstrate a difference in relative risk between these 2 estrogens. However, Edmondson et al. (8) found that HCA was more frequently found in patients using oral contraceptive preparations containing mestranol.

Most of the HCA's appear to be benign neoplasms, and several cases have been reported where the HCA's have regressed following cessation of oral contraceptive use after diagnosis (9, 27). Therefore, it appears that, at least in some instances, the effects of the oral contraceptives are reversible. Experimental animal studies conducted during evaluation of the safety of oral contraceptives revealed that they induced neoplasms in several tissues (see Refs. 16 and 17 for references). Hepatomas were reported to occur in mice, and benign liver cell neoplasms were observed in rats fed M + N. These are the estrogen and progestin used in the oral contraceptive known as Enovid E (14).

Friedwald and Rous (10) and Berenblum (4) were the first investigators to demonstrate that experimental carcinogenesis in skin can be separated into 2 stages, initiation and promotion. Peraino et al. (20-22) presented the first definitive evidence that hepatocarcinogenesis also consists of at least 2 stages. In those studies, initiation was accomplished by a short period of feeding 2-acetylaminofluorene, and promotion was accomplished by subsequent prolonged feeding of PB. More recent studies have resulted in the development of protocols where hepatocarcinogenesis can be initiated by a single low-dose treatment with a carcinogen administered 24 hr after surgical partial hepatectomy, a time when hepatocyte DNA synthesis is maximal (5, 7, 23, 26). Subsequent feeding of liver tumor promoters results in enhanced tumor formation (23). In addition, several histochemical techniques have been used to reveal early putative preneoplastic foci or nodules of hepatocytes. Among these phenotypic markers is γGT (28).

In general, sex hormones and their synthetic analogs are not mutagenic in the Ames Salmonella-microsome mutagenicity test (18). While data on the mutagenicity of mestranol have not been reported, mestranol fails to cause chromosomal aberrations in bone marrow cells of albino rats (1).
A review of the data on the carcinogenicity in animals and humans and on the mutagenicity of oral contraceptive steroids suggested that at least some of these steroids were acting as tumor promoters in liver. A recent study by Taper (31) confirmed this notion for an oral contraceptive preparation widely used in Europe. We set out to conduct experiments to test the liver tumor-promoting activity of 2 common oral contraceptive components, mestranol and norethynodrel.

**MATERIALS AND METHODS**

**Chemicals.** Mestranol (Chem. Abstr. No. 72-33-3, 17α-ethyl-17β-styrenodiol-3-methyl ether) and norethynodrel (Chem. Abstr. No. 68-23-5, 17α-ethyl-17β-hydroxy-5(10)-estren-3-one) were obtained from Sigma Chemical Co. (St. Louis, Mo.). DEN was obtained from Eastman Organic Chemicals (Rochester, N. Y.). All other chemicals were either from Sigma or were ordered through Fisher Scientific (Pittsburgh, Pa.) and were of reagent grade.

**Animal Diets.** All animal diets were obtained from Teklad (Madison, Wis.) and fed in the form of agar gels as described by Wogan and Newberne (33). The basal control diet contained 30% protein (Teklad Diet No. TD 78324). The 0.05% PB diet was mixed by Teklad whereas the other test diets were mixed at Dartmouth. Basal diet minus the 5% cottonseed oil was obtained from Teklad. The oral contraceptive steroids were dissolved in a small volume of ethanol and mixed with cottonseed oil, which was then added to this diet.

**Animals and Treatment Groups.** One hundred five 40-day-old female Sprague-Dawley-derived rats (Charles River Breeding Laboratories, Inc., Wilmington, Mass.) were housed individually in wire-bottomed cages under controlled conditions of temperature, humidity, and lighting. Food and distilled water were available ad libitum.

Upon arrival, the animals were fed basal control diet and allowed to adapt to their new environment for approximately 2 weeks. At 7 weeks of age, 156 ± 12 g (S.D.) body weight, the animals were subjected to a surgical partial (two-thirds) hepatectomy (15). Twenty-four hr later, the animals were intubated with either water or DEN (5 mg/kg body weight). Twenty-four hr after intubation, the animals were transferred to the various treatment groups shown in Table 1, with 15 animals/group. Food consumption data were obtained from each rat on a weekly basis during the first 3 months of the experiment and then monthly thereafter. Body weights were determined weekly. Five animals from each group were killed 4 months after the initial DEN treatment, and the remaining 10 were killed 9 months after initial treatment.

**Mestranol, Norethynodrel, and PB Doses.** Mestranol and norethynodrel were added to the diet at levels sufficient to provide the rats with 10 to 15 times the human dose as calculated for a 50-kg human ingesting the oral contraceptive pill, Enovid E (14). Thus, based on food consumption and body weight determinations, the rats ingested mestranol at 0.02 to 0.03 mg per kg body weight per day) and norethynodrel at 0.5 to 0.75 mg per kg body weight per day. The mestranol and norethynodrel: mestranol ratio is 25:1, which reflects that found in Enovid E (14). The range of dose levels was due to variations in food consumption. The amount of the steroid added to the diet was adjusted as necessary to maintain the doses in the ranges indicated. The estrus cycle of 3 rats from each group was monitored once per month during the first 3 months of the experiment. Animals ingesting diets containing mestranol, norethynodrel, or M + N fluctuated between diestrus and proestrus with no estrus being detected. Rats fed basal or PB diet had 4- to 5-day estrus cycles. Rats fed the 0.05% PB ingested between 19.4 and 27.4 mg per kg body weight per day.

**xGT Determination.** At the time of kill, the livers were removed, and 1 slice from each of the 4 remaining lobes was frozen in liquid N2 at —70°. One slice from the large right lobe was fixed in Susa’s and subsequently processed for standard H & E staining and histopathological analysis. The remainder of each liver was also frozen and stored at —70°. For γGT histochemical analysis, 5-μm cryostat sections were mounted on glass slides and stored frozen until stained. One section from each lobe was stained for γGT by the procedure of Rutenberg et al. (25). The number of γGT-positive foci present on each cryostat section was determined independently by 2 individuals. Discrepancies in the counts were resolved by simultaneous counting of the problem slides. The slides were coded so that the treatment was unknown to the individuals while the γGT foci were being scored. The area of liver tissue on each slide was then determined by projection of the slide on a calibrated piece of paper. The total number of foci and the total tissue area counted for each rat were determined and expressed as γGT-positive foci/sq cm tissue counted. These values for all rats in each group were summed, and the S.D. was determined. Biochemical determinations of γGT activity were done on liver homogenates by the method of Szasz (30). Protein determinations were done by the method of Hartree (13).

**Statistical Analyses.** Data were subjected initially to a one-way analysis of variance. Multiple comparisons among group means were performed using the Neuman-Keuls procedure (2). All results reported as significant using this procedure are at least at the 5% level of significance. For the data shown in Chart 2 in which comparisons between 2 groups were conducted and summarized in Table 2, we used the Wilcoxon rank sum test, since there was some indication of lack of normality of distribution among the data (6). In those instances where we used both parametric and nonparametric statistical methods of analysis, there were no substantive differences in the results.

**RESULTS**

Chart 1 shows the body weight curves for the 7 groups of
animals over the course of the experiment. Animals on control or PB-containing diets initially gained weight at a faster rate than did animals receiving diets containing mestranol, norethynodrel, or M + N. However, between the first and second months of the experiment, the rate of weight gain in control and PB-treated animals decreased. From that point on, animals in all groups appeared to undergo a gradual but similar rate of increase in body weight. At 4 months, the animals in groups DEN —> M + N, H2O —> M + N, and DEN —> N weighed significantly less (p < 0.05) than did control or PB animals. Animals in Group DEN —> M weighed significantly less (p < 0.05) than did those in the PB groups but not less than did the control animals. At 9 months, all animals being fed a steroid-containing diet weighed less than did those on PB or control diets (p < 0.05). In addition, animals in Group DEN —> M + N weighed less (p < 0.05) than did those in Group DEN —> N. These results show that the presence of mestranol, norethynodrel, and M + N in the diets caused a decrease in animal body weights.

The liver weights per 100 g body weight were calculated at both kill times (data not shown). No significant differences (p > 0.05) were detected among liver sizes at 4 months. However, at 9 months, the liver weights per 100 g body weight in the groups receiving PB, mestranol, norethynodrel, and M + N were greater (p < 0.05) than they were in controls. These results show that feeding of PB or steroids resulted in an increase in liver size. In addition, liver size in the H2O —> M + N group was greater (p < 0.05) than in Groups DEN —> N, DEN —> PB, DEN —> M, and H2O —> PB. With PB, increased liver size is known to be due to both hyperplasia and hypertrophy. However, little is known regarding the mechanism(s) by which the gonadal steroids induce liver growth.

Chart 2 shows the number of yGT foci per sq cm liver at both kill times. Table 2 shows the levels of significance, as determined by the rank sum test, for comparison of selected experimental groups. The relationships among the different groups are very similar at both kill times. In several instances, differences among groups which were not apparent or were less significant at 4 months became significant or increased in significance at 9 months. This could have been due to the longer time and/or to the greater number of animals at the 9-month kill. In one instance, a difference that was significant at 4 months failed to show significance at 9 months (DEN —> N versus DEN —> basal). One rat in the 9-month DEN —> N group contained a large number of yGT foci and an hepatocellular carcinoma. Exclusion of this rat from calculation of the mean number of foci for the group both decreased the mean and reduced the S.E. (Chart 2). Further discussion of the data concerns only the results obtained at the 9-month kill.

The positive control for this experiment was the DEN —> PB group which contained significantly more yGT foci than did either the H2O —> PB (Chart 2; p < 0.001) or DEN —> basal group (Chart 2; Table 2). Animals in Group H2O —> M + N had more foci than did those in groups H2O —> PB (Chart 2; p < 0.001) and DEN —> basal (Chart 2; Table 2). This suggests that, in the absence of DEN pretreatment, either mestranol or norethynodrel, or both together, is more potent than PB in inducing yGT foci. The groups DEN —> M and DEN —> M + N contained more yGT foci than did the H2O —> M + N and DEN —> basal groups (Chart 2; Table 2). The number of foci in the DEN —> N group (with or without the carcinoma-bearing rat) was not significantly different from that in the H2O —> M + N and DEN —> basal groups. In addition, there was no difference (p > 0.05) between the number of foci in the DEN —> M and DEN —> M + N groups (Chart 2). These results suggest that mestranol alone is able to enhance the appearance of DEN-initiated yGT foci.

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**Table 2**

<table>
<thead>
<tr>
<th>Group</th>
<th>4 mos.</th>
<th>9 mos.</th>
<th>4 mos.</th>
<th>9 mos.</th>
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<td>&lt;0.01</td>
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<tr>
<td>DEN —&gt; N</td>
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<td>&lt;0.05</td>
<td>NS</td>
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<tr>
<td>DEN —&gt; M + N</td>
<td>&lt;0.05</td>
<td>NS</td>
<td>&lt;0.01</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>DEN —&gt; PB</td>
<td>&lt;0.05</td>
<td>&lt;0.01</td>
<td>&lt;0.001</td>
<td>NS</td>
</tr>
<tr>
<td>H2O —&gt; M + N</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

* See Chart 2 for the actual numbers of yGT foci/sq cm liver in all experimental groups.
* NS, not significant; p > 0.05.
foci and that norethynodrel alone is only weakly able to do so. There is no additive effect apparent between mestranol and norethynodrel, and it appears that the enhancing effect of M+N may be due entirely to mestranol alone. It should be remembered that norethynodrel is present at 25 times the level of mestranol. In addition, there is no difference (p > 0.05) in the number of γGT foci among Groups DEN → M, DEN → M+N, and DEN → PB (Chart 2). PB is present at 0.05% in the diet, and the rats ingest between 19 and 27 mg/day, whereas they ingest only between 0.02 and 0.03 mg mestranol/day. Thus, it would appear that, on a weight basis, mestranol may be a much more potent enhancer of the appearance of γGT foci. Of course, a definitive test of this awaits experiments comparing these 2 compounds at equal doses.

In determining the number of γGT foci, it was evident that some treatments had caused a diffuse γGT-positive staining that appeared to radiate from the portal areas. No distinct cellular alterations following such patterns were clearly evident in H & E sections from the same animal. In most cases, the diffuse staining was mild to moderate in extent, and γGT foci and areas, but seemed instead to be randomly distributed in the liver lobules. Random distribution was also seen in the DEN → PB animals.

The H & E sections were analyzed for the presence of several types of hepatocellular lesions which appear during the course of hepatocarcinogenesis (29). The data obtained are shown in Table 4. In general, the animals in Groups DEN → M and DEN → M+N had a greater incidence and number of hepatocellular lesions detected by H & E staining. It is thought by some that the basophilic foci or areas have the greatest significance with regard to the development of neoplasms (29). Both the DEN → M and DEN → M+N animals had the highest incidence of basophilic foci. Only one animal developed an hepatocellular carcinoma, and it was in the DEN → N group. The liver of this animal also had a large number of γGT-positive foci. However, since the 9 other rats in this group had low numbers of γGT and basophilic foci and since no animal in any other group developed a carcinoma, it was felt that perhaps the animal with the carcinoma was exceptional and that, in reality, norethynodrel is only a weak promoter. The results presented in Table 4 indicate that the total number of advanced hepatocellular lesions (neoplastic nodules and carcinomas) was very low. This was most probably due to the low initiating dose of DEN (5 mg/
kg) coupled with the relatively short duration of the experiment. Future studies will use a higher dose of DEN in order to provide more latent initiated cells for promotion.

**DISCUSSION**

The results obtained in this study clearly show that mestranol, alone and together with norethynodrel, is able to enhance the appearance of putative preneoplastic lesions as represented by yGT-positive foci or populations of hepatocytes. The experimental protocol used in this study was designed to test whether these 2 contraceptive steroids are promoters in the 2-stage model of hepatocarcinogenesis. The results suggest that mestranol should be classified as a promoter. However, in Group H2O → M+N, the number of foci detected was greater than that in the control groups. PB is a known promoter of hepatocarcinogenesis (20), and the low number of yGT foci appearing in livers of animals previously untreated with an initiator but fed PB suggests that it has little, if any, ability to act as a complete carcinogen (i.e., initiate and promote). On the other hand, the greater number of putative preneoplastic foci in the animals given H2O and then fed M+N suggests that perhaps mestranol or norethynodrel, or both together, has some ability to act as a complete carcinogen. Since the data suggest that the numbers of foci in the DEN → M and DEN → M+N groups are not different, it appears that norethynodrel adds little or nothing to the response observed. Thus, it may be that mestranol alone has some ability to act both as a weak initiator and as a promoter. An experiment is currently underway to test whether mestranol has any detectable ability to initiate hepatocarcinogenesis. Goldfarb (12) has suggested possible mechanisms by which synthetic gonadal steroid hormones might act as initiating agents.

This paper represents the second report of data showing that oral contraceptive steroids can act as promoters of experimental hepatocarcinogenesis in rats. Recently, Taper (31) tested estradiol-17-phenylpropionate and estradiol benzoate, 2 steroids used in oral contraceptive preparations in Europe, for their ability to promote N-nitrosomorpholine-initiated hepatocarcinogenesis. The results of his study showed that these contraceptive steroids promoted the appearance of basophilic foci, liver nodules, and hepatocellular carcinomas as detected 300 days after the beginning of the carcinogen (initiator) treatment. Benign and malignant tumors were also seen in other organs.

The recent report by Rooks et al. (24) on the epidemiology of hepatocellular adenoma in oral contraceptive users estimated that HCA develops at annual rates of about 1.0 and 1.3/100,000 for nonusers or short-term (<24 months) users of oral contraceptives, respectively. Applying risk estimates derived from their study to this base line, Rooks et al. (24) estimate that, for long-term users of low potency oral contraceptives, the annual incidence of HCA will be 3.4/100,000. Both this study and that of Taper (31) have demonstrated that these agents may be acting as tumor promoters. Thus, it is conceivable that, as women enter the work force at an increasing rate and perhaps become occupationally exposed to potential hepatocellular initiators, the incidence could continue to increase. This problem deserves careful observation and more experimental study to determine possible mechanisms for reducing or preventing the promoting activity of oral contraceptive steroids.

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