[CANCER RESEARCH 40, 3680–3685, October 1980]
0008-5472/80/0040-0000$02.00

Oral Contraceptive Steroids as Promoters of Hepatocarcinogenesis in Female Sprague-Dawley Rats[1]

James D. Yager, Jr.,[2] and Rosemary Yager

Department of Pathology, Dartmouth Medical School, Hanover, New Hampshire 03755

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 → 0.05% phenobarbital, DEN → basal diet, H_2O → phenobarbital, and H_2O → 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 HCA[3] 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).

Received April 14, 1980; accepted July 10, 1980.

[1] This work was supported by Grant CA 23916, awarded by the National Cancer Institute, Department of Health, Education, and Welfare. A preliminary report of this work was published previously (34).

[2] To whom requests for reprints should be addressed.

[3] The abbreviations used are: HCA, hepatocellular adenoma; M+N, mestranol plus norethynodrel; PB, phenobarbital; γGT, γ-glutamyl transpeptidase; DEN, diethylnitrosamine; H & E, hematoxylin and eosin; DEN → M+N, diethylnitrosamine-treated mestranol- plus norethynodrel-containing diet; H_2O → M+N, H_2O-treated mestranol- plus norethynodrel-containing diet; DEN → N, diethylnitrosamine-treated norethynodrel-containing diet; DEN → M, diethylnitrosamine-treated mestranol-containing diet; DEN → PB, diethylnitrosamine-treated phenobarbital-containing diet; H_2O → PB, H_2O-treated, phenobarbital-containing diet; DEN → basal, diethylnitrosamine-treated basal diet.

Downloaded from http://aacrjournals.org/cancerres/article-pdf/40/10/3680/2406963/cr0400103680.pdf by guest on 04 May 2025

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-ethinyl estradiol 3-methyl ether) and norethynodrel (Chem. Abstr. No. 68-23-5, 17α-ethinyl-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 ether 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, 158 ± 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 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.

Table 1

Treatment groups

Female Sprague-Dawley-derived rats were adapted to basal diet and housing conditions for 14 days and then subjected to partial hepatectomy at 158 ± 12 g body weight. Twenty-four hr later, they were intubated with either H_2O or DEN (5 mg/kg body weight) and, 24 hr after that, transferred to the test diets containing the compounds indicated. There were 15 animals/group; 5 animals/group were killed 4 months, and the remainder were killed 9 months after the DEN treatment.

A. Controls	
	$H_2O \rightarrow$ M + N
	$H_2O \rightarrow$ PB
B. DEN-treated	
	DEN → M
	DEN → N
	DEN → M + N
	DEN → basal
	DEN → PB

γGT Determination. At the time of kill, the livers were removed, and 1 slice from each of the 4 remaining lobes was frozen in liquid N_2 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 the 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

Downloaded from http://aacrjournals.org/cancerres/article-pdf/40/10/3680/2406963/cr0400103680.pdf by guest on 04 May 2025

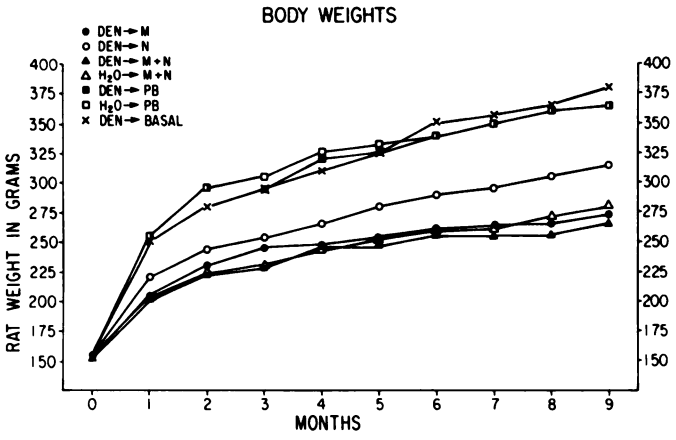

Chart 1. Body weight curves for the 7 groups of animals over the course of the experiment. Initially, there were 15 animals/group. Five animals/group were killed after 4 months, and the remainder were killed after 9 months. The animals were weighed weekly, but only monthly weights are shown.

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, H_2O → 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 H_2O → M+N group was greater ($p < 0.05$) than in Groups DEN → N, DEN → PB, DEN → M, and H_2O → 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 γGT 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 γGT 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 γGT foci than did either the H_2O → PB (Chart 2; $p < 0.001$) or DEN → basal group (Chart 2; Table 2). Animals in Group H_2O → M+N had more foci than did those in groups H_2O → 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 γGT foci. The groups DEN → M and DEN → M+N contained more γGT foci than did the H_2O → 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 H_2O → 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 γGT

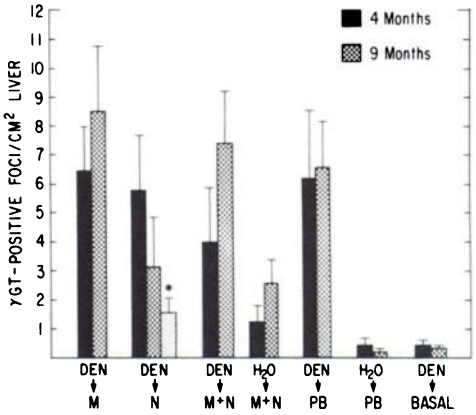

Chart 2. γGT foci/sq cm in livers of animals from the 7 treatment groups. The number of γGT-positive lesions was determined as described in "Materials and Methods." *Columns*, mean, *bars*, S.E. There were 5 animals/group at 4 months and 10 animals/group at 9 months. *Stippled column*, (*), the mean for 9 of the 10 animals in the DEN → N group at 9 months. The animal excluded in calculating this mean was the only one in the entire experiment that developed an hepatocellular carcinoma. The liver also had numerous γGT foci (18/sq cm).

Table 2

Significance of differences in γGT foci/sq cm liver among selected experimental groups

The rank sum test was used to determine these levels of significance. However, realizing the problems associated with making multiple individual comparisons between groups, we also conducted a Newman-Keuls analysis of variance. This test confirmed the significance of the differences indicated with at least $p < 0.05$.

	p for selected group comparisons[a]			
	H_2O → M + N		DEN → basal	
Group	4 mos.	9 mos.	4 mos	9 mos.
DEN → M	<0.05	<0.01	<0.01	<0.001
DEN → N	NS[b]	NS	<0.05	NS
DEN → M + N	NS	<0.05	NS	<0.001
DEN → PB	<0.05	NS	<0.01	<0.001
H_2O → M + N			NS	<0.001

[a] See Chart 2 for the actual numbers of γGT foci/sq cm liver in all experimental groups.

[b] NS, not significant; $p > 0.05$.

Downloaded from http://aacrjournals.org/cancerres/article-pdf/40/10/3680/2406963/cr0400103680.pdf by guest on 04 May 2025

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 were easily distinguishable. However, in 5 of the 70 animals killed at 9 months, the diffuse staining was intense. Upon decoding of the slides after counting, the percentage of animals in each group that showed diffuse γGT staining was determined. None of the animals on control or PB diets showed diffuse γGT staining. However, diffuse γGT staining was detected in 80% of the animals in group DEN → M and 40% of the animals in Groups DEN → M+N and H_2O → M+N; diffuse γGT staining was undetectable in Group DEN → N. We then determined the γGT enzyme activity in livers of animals from the various treatment groups (Table 3). These data indicate that γGT activity tended to be elevated in livers of animals receiving diet containing mestranol. On a per g liver basis, animals ingesting basal diet or diet containing PB or norethynodrel had significantly less γGT activity than did those ingesting mestranol-containing diet ($p < 0.05$). Animals ingesting basal and PB-containing diet also had significantly less γGT activity than did those in the DEN → M+N group ($p < 0.05$). Basically, the same significant differences were seen when the γGT activity was expressed as units/mg protein.

The total γGT activities reflect activity due to both γGT foci and the diffuse γGT staining. However, the majority of the difference in total activity probably reflects the diffuse staining. Both the DEN → M and DEN → PB groups had similar numbers of γGT foci (Chart 2) but significantly different γGT activities, whereas the animals ingesting mestranol showed diffuse γGT staining, and those on PB diet did not. In addition, in looking at the data from individual animals from the groups fed mestranol-containing diet, it appeared that the level of γGT activity correlated with the degree of diffuse staining (data not shown). Thus, feeding diet containing mestranol but not norethynodrel results in the appearance of γGT in a subpopulation of hepatocytes. The γGT-positive foci were not restricted to such 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/

Table 3

γGT activity in homogenates of treated rats

Group	mg protein/g liver	Units γGT[a]/g liver	Units γGT/mg protein
DEN → M	174 ± 11[b]	1689 ± 693	9.60 ± 3.40
DEN → N	181 ± 7	1028 ± 178[c]	5.71 ± 1.05[c, d]
DEN → M + N	177 ± 6	1499 ± 575	8.50 ± 3.25
H_2O → M + N	171 ± 10	1300 ± 258	7.68 ± 2.00
DEN → PB	176 ± 7	1066 ± 192[c, d]	6.05 ± 1.05[c]
H_2O → PB	168 ± 12	1040 ± 134[c, d]	6.22 ± 0.82[c, d]
DEN → basal	169 ± 11	910 ± 249[c, d]	5.37 ± 1.25[c, d]

[a] One unit of γGT activity is the activity of enzyme which converts 1 μmol of substrate in 1 min at standard conditions.
[b] Mean ± S.D. for 10 animals/group at 9 mos.
[c] Significantly less than DEN → M; $p < 0.05$.
[d] Significantly less than DEN → M + N; $p < 0.05$.

Table 4

Incidence and number of hepatocellular lesions detected by histopathological evaluation

All sections were taken from 1 lobe of the liver and stained with H&E. Each group had 10 animals.

	Foci					
Groups	Clear cell	Eosinophilic (ground glass)	Basophilic	Mixed	Neoplastic nodules	Hepatocellular carcinomas
DEN → M	10[a] (2.4)[b]	2 (2.5)	7 (2.0)	9 (5.4)	0	0
DEN → N	5 (1.6)	2 (3.0)	1 (1.0)	6 (2.3)	0	1(1.0)
DEN → M + N	9 (2.3)	2 (1.5)	6 (1.8)	8 (5.0)	3 (1.0)	0
DEN → PB	0	4 (1.8)	2 (1.0)	4 (2.3)	0	0
H_2O → M + N	3 (2.3)	2 (1.0)	2 (1.5)	10 (3.9)	0	0
H_2O → PB	0	1 (1.0)	0	2 (1.0)	0	0
DEN → basal	0	0	1 (1.0)	4 (2.0)	0	0

[a] Number of animals in group with at least 1 lesion.
[b] Numbers in parentheses, lesions per lesion-bearing rat.

Downloaded from http://aacrjournals.org/cancerres/article-pdf/40/10/3680/2406963/cr0400103680.pdf by guest on 04 May 2025

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 γGT-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 $H_2O \rightarrow M+N$, 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 γGT 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 H_2O 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/million for nonusers or short-term (<24 months) users of oral contraceptives in women 16 to 30 and 31 to 44 years old, 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.

ACKNOWLEDGMENTS

The authors wish to thank Dr. Theodore Colton of the Norris Cotton Cancer Center at the Dartmouth Medical School for his help in the statistical evaluation of the data; Dr. Daniel S. Longnecker for his help in the histopathological evaluation; and Drs. B. D. Roebuck, D. S. Longnecker, and T. Gindhart for valuable discussion.

REFERENCES

1. Ansari, S. R., and Adharmi, U. M. Effect of antifertility drugs (norethinodrone and mestranol) on the bone marrow chromosomes of albino rat (*Rattus: Norvegicus*). Indian J. Hered. *9:* 7–9, 1977.
2. Armitage, P. Comparison of several groups. *In:* Statistical Methods in Medical Research, pp. 205–207. New York: John Wiley & Sons, Inc., 1971.
3. Baum, J. K., Holtz, F., Bookstein, J. J., and Klein, E. W. Possible association between benign hepatomas and oral contraceptives. Lancet, *2:* 926–929, 1973.
4. Berenblum, I. The cocarcinogenic action of croton resin. Cancer Res., *1:* 44–48, 1941.
5. Cayama, E., Tsuda, H., Sarma, D. S. R., and Farber, E. Initiation of chemical carcinogenesis requires cell proliferation. Nature (Lond.), *275:* 60–62, 1978.
6. Colton, T. Nonparametric methods. *In:* Statistics in Medicine, pp. 221–223. Boston: Little, Brown & Co., 1974.
7. Craddock, V. M. Liver carcinomas induced in rats by single administration of dimethylnitrosamine after partial hepatectomy. J. Natl. Cancer Inst., *47:* 899–905, 1971.
8. Edmondson, H. A., Henderson, B., and Benton, B. Liver-cell adenomas associated with use of oral contraceptives. N. Engl. J. Med., *294:* 470–472, 1976.
9. Edmondson, H. A., Reynolds, T. B., Henderson, B., and Benton, B. Regression of liver cell adenomas associated with oral contraceptives. Ann. Intern Med., *86:* 180–182, 1977.
10. Friedwald, W. F., and Rous, P. The initiating and promoting elements in tumor production. J. Exp. Med., *80:* 101–125, 1944.
11. Gindhart, T. D. Liver tumors and oral contraceptives: pathology and pathogenesis. Ann. Clin. Lab. Sci., *8:* 443–446, 1978.
12. Goldfarb, S. Sex hormones and hepatic neoplasia. Cancer Res., *36:* 2584–2588, 1978.
13. Hartree, E. F. Determination of protein: a modification of the Lowry method that gives a linear photometric response. Anal. Biochem., *48:* 422–427, 1972.
14. Hatcher, R. A., Stewart, G. K., Stewart, F., Guest, F., Stewart, D. W., and Jones, S. A. Combined oral contraceptives. *In:* Contraceptive Technology 1980–1981, Rev. Ed. 10, p. 26. New York: Irvington Publishers, Inc., 1980.
15. Higgins, G. M., and Anderson, R. M. Experimental pathology of the liver: restoration of the liver of the white rat following partial surgical removal. Arch. Pathol., *12:* 186–202, 1931.
16. International Agency for Research on Cancer. IARC Monograph on the Evaluation of Carcinogenic Risk of Chemicals to Man, Vol. 6, pp. 87–97. Lyons, France: International for Research on Cancer, 1974.
17. International Agency for Research on Cancer. IARC Monograph on the Evaluation of Carcinogenic Risk of Chemicals to Man, vol. 6, pp. 191–200. Lyons, France: International Agency for Research on Cancer, 1974.
18. Lang, R., and Redmann, U. Non-mutagenicity of some sex hormones in the Ames *Salmonella*/microsome mutagenicity test. Mutat. Res., *67:* 361–365, 1979.
19. Neuberger, J., Nunnerly, H. B., Davis, M., Portmann, B., Laws, J. W., and Williams, R. Oral contraceptive-associated liver tumors: occurrence of malignancy and difficulties in diagnosis. Lancet, *1:* 273–276, 1980.
20. Peraino, C., Fry, R. J. M., and Staffeldt, E. Reduction and enhancement by phenobarbital of hepatocarcinogenesis induced in the rat by 2-acetylaminofluorene. Cancer Res., *31:* 1506–1512, 1971.
21. Peraino, C., Fry, R. J. M., Staffeldt, E., and Christopher, J. P. Comparative enhancing effects of phenobarbital, amobarbital, diphenylhydantoin, and dichlorodiphenyl trichloroethane on 2-acetylaminofluorene-induced hepatic tumorigenesis in the rat. Cancer Res., *35:* 2884–2890, 1975.
22. Peraino, C., Fry, R. J. M., Staffeldt, E., and Kisieleski, W. E. Effects of varying the exposure to phenobarbital on its enhancement of 2-acetylaminofluorene-induced hepatic tumorigenesis in the rat. Cancer Res., *33:* 2701–2705, 1973.
23. Pitot, H. C., Barsness, L., Goldsworthy, T., and Kitagawa, T. Biochemical characterization of stages of hepatocarcinogenesis after a single dose of diethylnitrosamine. Nature (Lond.), *271:* 456–458, 1978.
24. Rooks, J. B., Ory, H. W., Ishak, K. G., Strauss, L. T., Greenspan, J. R., Paganini, A., and Tyler, C. W., Jr. Epidemiology of hepatocellular adenoma: the role of oral contraceptive use. J. Am. Med. Assoc., *242:* 644–648, 1979.
25. Rutenberg, A. M., Kim, H., Fischbein, J. W., Hanker, J. S., Wasserkrug, H. L., and Seligman, A. M. Histochemical and ultrastructural demonstration of γ-glutamyltranspeptidase activity. J. Histochem. Cytochem., *17:* 517–526, 1969.

Downloaded from http://aacrjournals.org/cancerres/article-pdf/40/10/3680/2406963/cr0400103680.pdf by guest on 04 May 2025

26. Scherer, E., and Emmelot, P. Kinetics of induction and growth of precancerous liver-cell foci, and liver tumor formation by diethylnitrosamine in the rat. Eur. J. Cancer, *11:* 689–696, 1975.
27. Sherlock, S. Hepatic tumors and sex hormones. *In:* H. Remmer, H. M. Bolt, P. Bannasch, and H. Popper (eds.), Primary Liver Tumors. Proceedings of the 25th Falk Symposium, pp. 201–212. Baltimore: University Park Press, 1978.
28. Solt, D. B., Medline, A., and Farber, E. Rapid emergence of carcinogen-induced hyperplastic lesions in a new model for the sequential analysis of liver carcinogenesis. Am. J. Pathol. *88:* 595–618, 1977.
29. Squire, R. A., and Levith, M. H. Report of a workshop on classification of specific hepatocellular lesions in rats. Cancer Res., *35:* 3214–3223, 1975.
30. Szasz, G. A kinetic photometric method for serum γ-glutamyl transpeptidase. Clin. Chem., *15:* 124–136, 1969.
31. Taper, H. S. The effect of estradiol-17-phenylpropionate and estradiol benzoate on *N*-nitrosomorpholine-induced liver carcinogenesis in ovariectomized female rats. Cancer (Phila.), *42:* 462–467, 1978.
32. Vana, J., Murphy, G. P., Aronof, B. L., and Baker, H. W. Survey of primary liver tumors and oral contraceptives. J. Toxicol. Environ Health, *5:* 255–273, 1979.
33. Wogan, G. N., and Newberne, P. M. Dose-response characteristics of aflatoxin B_1 carcinogenesis in the rat. Cancer Res., *27:* 2370–2376, 1967.
34. Yager, J. D., and Yager, R. Promotion of hepatocarcinogenesis by oral contraceptive steroids. Proc. Am. Assoc. Cancer Res., *21:* 76, 1980.

Downloaded from http://aacrjournals.org/cancerres/article-pdf/40/10/3680/2406963/cr0400103680.pdf by guest on 04 May 2025