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Daniel S. Longnecker
Dartmouth College

B D. Roebuck
Dartmouth College

Thomas J. Curphey
Dartmouth College

Evelyne Lhoste
Dartmouth College

Charles I. Coon
Dartmouth College

See next page for additional authors

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Effects of Corn Oil and Benzyl Acetate on Number and Size of Azaserine-Induced Foci in the Pancreas of LEW and F344 Rats

by Daniel S. Longnecker,* B. D. Roebuck,† Thomas J. Curphey,* Evelyne Lhoste,* Charles I. Coon,* and Denise MacMillan†

The response of LEW and F344 strain rats to the pancreatic carcinogen azaserine was compared using the size and number of azaserine-induced acidophilic acinar cell foci and nodules as parameters in a 4-month experiment. A second experiment compared the effect of corn oil intake by gavage and dietary routes on the growth of azaserine-induced pancreatic lesions in LEW rats. A third experiment tested the activity of benzyl acetate in regard to its ability to induce acinar cell foci or to promote the growth of such foci in azaserine-treated rats. The results showed that equivalent doses of azaserine induce two to seven times more foci in LEW than in F344 rats, and that LEW rats have a higher incidence of "spontaneous" foci than F344 rats. Azaserine-treated LEW rats that were given 5 mL corn oil/kg body weight 5 days per week by gavage developed more acinar cell foci than rats fed a basal diet (chow). Addition of an equivalent amount of corn oil to chow had a similar effect of enhancing the development of foci. Rats of neither strain developed acinar cell foci when benzyl acetate was given by gavage or in the diet nor was there evidence that benzyl acetate has a significant effect on the development of foci in azaserine-treated rats. These studies also demonstrate that the azaserine/rat model of pancreatic carcinogenesis which was developed in LEW rats can be adapted for use with F344 rats.

Introduction

Benzyl acetate and other compounds have recently been evaluated for carcinogenic potential in chronic bioassay studies conducted through the National Toxicology Program in which the test compounds have been given by gavage using corn oil as a vehicle (*1*). Review of data for vehicle control groups from several such studies has shown that the incidence of focal acinar cell hyperplasia and adenomas of acinar cells was increased in comparison with untreated control groups (*2*). This observation has raised questions about the marginally significant increased incidence of such lesions in the pancreases of animals that received benzyl acetate dissolved in corn oil and led to the studies reported here. Goals of these studies were: to compare the response of LEW and F344 strain rats to the pancreatic carcinogen azaserine in order to adapt the well-characterized azaserine model for use with the F344 strain; to compare the effect of giving corn oil by gavage with the feeding of corn oil added to the food in regard to enhancement of the growth of azaserine-induced foci in the pancreas; and to evaluate the ability of benzyl acetate either to initiate foci of atypical acinar cell hyperplasia or to enhance the growth of such foci in the pancreas.

We have used a short-term model for these studies. The number and/or size of two-dimensional transections of foci and nodules of atypical acinar cells have previously been used as indicators of pancreatic carcinogenesis (*3,4*). We have adopted the convention of referring to small acinar cell lesions, i.e., those that are less than 1 mm in diameter, as foci. Lesions in the size range of 1 to 3 mm are referred to as nodules. Many nodules are visible grossly. The quantitative stereologic methods that have been applied to the study of hepatic foci (*5,6*) have recently been used to quantitate the number and size of azaserine-induced pancreatic foci and nodules (*7–9*). We have used these techniques in the present study. Two phenotypically different populations of foci have been described (*7,10*). The acidophilic but not the ba-

*Department of Pathology, Dartmouth Medical School, Hanover, NH 03756.
†Department of Pharmacology and Toxicology, Dartmouth Medical School, Hanover, NH 03756.

sophilic foci appear to have a strong growth potential. Accordingly, we have focused primarily on data for the acidophilic lesions in this report.

Materials and Methods

Animals

Male Lewis (LEW) or F344 rats (Charles River Breeding Laboratories, Inc., Kingston, NY, or Simonsen Laboratories, NIH Colony, Gilroy, CA) were obtained as nursing litters. The dams were fed *ad libitum* a purified control diet, AIN-76A (AIN) (Teklad, Madison, WI), without the antioxidant ethoxyquin (*11*) or NIH 07 chow (Ziegler Brothers, Inc., Gardners, PA). The pups were weaned at 21 to 25 (LEW) or 28 (F344) days of age and food and deionized water were available *ad libitum* thereafter. The rats were housed individually in suspended, wire-mesh bottom cages (experiment 2) or in pairs in polycarbonate shoebox cages on aspen bedding (experiments 1 and 3). Rats were weighed weekly.

Carcinogen, Diets and Gavage Treatments

Azaserine (Calbiochem-Behring Corp., LaJolla, CA), 3 mg/mL, was dissolved in 0.9% NaCl solution and injected intraperitoneally. Three independent experiments were performed.

Experiment 1 was a dose response comparison of azaserine-treated LEW and F344 rats. The azaserine doses are shown in Table 1. The azaserine solution was diluted to 0.3 or 1 mg/mL for injection into rats receiving 3 or 10 mg/kg. The rats were fed AIN diet throughout.

Experiment 2 was designed to compare a group given oil by gavage with groups fed three different levels of oil in the diet. All rats were LEW strain and treated once with 30 mg azaserine/kg at 14 days of age. Treatment groups are shown in Table 2. The oil-gavage group was given 5 mL/kg of corn oil (Best Foods, Englewood Cliffs, NJ) via 18-gauge, 3-inch stainless steel feeding needles (Popper and Sons, Inc., New Hyde Park, NY) 5 days/week to match the schedule commonly used in National Toxicology Program (NTP) bioassay studies (*2*). All groups were fed powdered chow diet, or chow with corn oil added. Chow was used to duplicate the conditions of NTP studies. The amount of corn oil added to the diet in the "oil-low" group was varied weekly so that the intake of oil in the diet by this group closely matched the total oil intake from both diet and gavage of the oil-gavage group. Food consumption was measured by weighing individual diet dishes for one 24-hr period weekly in both groups, and the calculation of fat intake was based upon the consumption and the fat content of each lot of chow. The fat content of the four lots of diets used were 5.4, 5.9, 6.1, and 6.4%. Corn oil was added to these diets so that the total fat in the oil-low diet varied from 9.4 to 12.5% during the course of the experiment.

Experiment 3 compared the incidence, number and size of acinar cell foci in LEW and F344 rats treated with benzyl acetate (BA) and/or azaserine as indicated in Tables 3 and 4. LEW rats received one 30 mg azaserine/kg injection at 14 days of age, and F344 rats received 2 such injections at 14 and 21 days of age.

Tricaprylin was used as a vehicle for BA in the "oil-gavage" group until all rats weighed 200 g. Thereafter, the BA, 500 mg/kg/day, 5 days/week, was given by gavage without vehicle, and an equivalent volume of deionized water was given by gavage to the vehicle control group. We found no evidence of esophageal or gastric irritation in a pilot gavage study using neat BA. Rats were weaned to the appropriate diet and gavage treatments were started on the day after weaning.

BA was added to the diet by dissolving it in corn oil. This solution was then added to the remaining components of the AIN diet to achieve a final concentration of 5% corn oil in the diet and a calculated level of 0.9% BA. This diet was pelleted by using a pasta maker (*12*) or a sausage-making attachment for the Kitchenaid model 5SS mixer (Hobart Corporation, Troy, OH). Preliminary studies showed a high degree of retention of

Table 1. Comparison of LEW and F344 rats in regard to induction of acinar cell foci by azaserine.

				Parameters (mean ± S.E.) for acidophilic foci				
				Observed data		Calculated data		
Strain	Dose, mg/kg[a]	*N*	Pancreas, g	Number[b]	Area, $mm^2 \times 100$	No./cm^3	Diameter, μm	Volume %
LEW	1	10	1.29 ± 0.03	1.3 ± 0.3	23.6 ± 7.0	c	c	0.12 ± 0.03
	3	10	1.25 ± 0.05	3.4 ± 0.6	14.6 ± 2.2	32.4 ± 7.6	540 ± 59	0.20 ± 0.05
	10	9	1.36 ± 0.05	30.9 ± 4.0	15.5 ± 1.3	198.6 ± 25.7[d]	513 ± 20	1.50 ± 0.17[d]
F344	3	11	0.93 ± 0.03	1.8 ± 0.1	15.2 ± 2.3	c	c	0.15 ± 0.05
	10	12	0.95 ± 0.04	6.3 ± 1.1	12.5 ± 0.9	74.0 ± 15.7[d]	485 ± 38	0.41 ± 0.08[d]
	30	12	0.94 ± 0.03	14.1 ± 2.6	18.0 ± 1.1	115.1 ± 25.1	563 ± 26	1.07 ± 0.18
	30 × 2[e]	9	1.18 ± 0.05	33.1 ± 4.1	10.8 ± 1.3	417.8 ± 38.1	385 ± 20	1.74 ± 0.29

[a] Data are shown as mean ± standard error. There were no significant differences in body weight between rats that were given different doses of azaserine in the same strain. The mean weight for all LEW rats was 474 ± 3.3 g and for all F344 rats was 318 ± 4.5 g.

[b] The number reported is the count for the tail portion of the pancreas.

[c] The number of foci observed was too few to allow calculation of three-dimensional data.

[d] The values in LEW rats are significantly larger than in F344 rats (*t* test, $p < 0.001$).

[e] Azaserine was given twice at 2 and 3 weeks of age. In all other groups a single azaserine treatment was given at age 2 weeks.

Table 2. Effect of dietary fat or corn oil by gavage on growth of acinar cell foci in Lewis rats.[a]

				Parameters (mean ± SE) for acidophilic foci				
				Observed data		Calculated data		
Group	*N*	Body wt., g	Pancreas, g[b]	Number[c]	Area, $mm^2 \times 100$	No./cm^3	Diameter, μm	Volume %
Chow	10	403 ± 5.7	1.20 ± 0.05	14.1 ± 2.5	10.0 ± 0.9	154 ± 23	431 ± 28	0.616 ± 0.107
Oil-gavage	10	413 ± 4.0	1.21 ± 0.03	19.6 ± 2.9	10.2 ± 0.8	232 ± 32	420 ± 24	0.995 ± 0.168
Oil-low	9	440 ± 7.5	1.24 ± 0.04	16.1 ± 3.7	9.6 ± 0.7	199 ± 39	417 ± 20	0.798 ± 0.175
Oil-high	10	473 ± 8.7	1.26 ± 0.03	27.8 ± 3.5	12.2 ± 0.9	321 ± 36[d]	443 ± 19	1.719 ± 0.213[d]

[a] Data are given as mean ± standard error. Gavage treatment or diets with added oil were started 4 days after weaning.
[b] There is no significant difference between groups (*t*-test).
[c] The number reported is the count for the tail portion of the pancreas.
[d] $p < 0.05$ when compared to chow group (analysis of variance).

BA in a diet prepared in this way. There was negligible loss of BA from diet that was stored in a closed container in a freezer. Diet kept at room temperature in an open container for 72 hr lost only 18% of the BA originally present. Accordingly, rats were fed fresh diet every other day. Estimates of food consumption were used to calculate the intake of BA by dietary vs. gavage routes. At the level of 0.9% BA in the diet, we calculated that the LEW rats fed the BA-containing diet received about 57% more BA than the gavaged group, and that BA-fed F344 rats received 31% more BA than the gavaged group.

Autopsy and Histologic Evaluation

Rats were autopsied four months after azaserine treatment (experiments 1 and 2) or weaning (experiment 3). The entire pancreas was excised, fixed *in toto* in Susa's fixative or 10% phosphate buffered formalin, and completely embedded by a standardized method for routine histology. The sections, stained with hematoxylin and eosin, were examined by light microscopy. The pancreas was divided into two portions—head and tail—by a transection near the superior mesenteric artery. The tail (splenic) section was used for quantitative microscopy. Azaserine-induced atypical acinar cell foci or nodules, henceforth called foci, were identified and classified as acidophilic or basophilic in accord with the criteria of Rao et al. (*10*) and Roebuck et al. (*7*). The focal transections are generally elliptical. Only foci with ten or more nuclei in the plane of transection were counted. This corresponds to a minimum transectional diamater of approximately 0.1 mm. The area of pancreatic focal transections and the area of the tissue sections were measured with an X,Y-digitizer (GTCO Corp., Rockville, MD). From 85-260 (F344) or 130-516 mm^2 (LEW) of pancreatic tissue from each rat was screened for foci. From the observed number and area of the focal transections, the mean number and size of the foci were determined by the quantitative stereological methods of Pugh et al. (*6*). Details of the application of these methods to pancreatic foci are published (*7*).

Statistical comparisons of the data were done by the χ^2-test, Student's *t*-test, linear regression or by an analysis of variance followed by the Newman-Keuls test.

Results

The results of quantitative stereological analyses of pancreatic tissue sections are shown in Tables 1–4. The observed data of the number and size of the focal tran-

Table 3. Effect of benzyl acetate (BA) on the incidence and size of pancreatic acinar cell foci in azaserine-treated and non-carcinogen-treated F344 rats.[a]

					Parameters (mean ± SE) for acidophilic foci				
					Observed data		Calculated data		
Group #	Treatment	Body wt., g	Pancreas, g	Number[b]	Area, $mm^2 \times 100$	No./cm^3	Diameter, μg	Volume %
	No carcinogen								
1	AIN diet only	379 ± 12.2	0.98 ± 0.02	0	—	—	—	—
2	Vehicle/gavage	363 ± 6.2	0.96 ± 0.03	0	—	—	—	—
3	BA/gavage	359 ± 7.3	0.95 ± 0.03	0	—	—	—	—
4	0.9% BA/diet	382 ± 17.4	1.05 ± 0.06	0	—	—	—	—
	Azaserine-treated								
5	AIN diet only	363 ± 17.1	1.04 ± 0.04	16.8 ± 3	8.55 ± 1.4	326 ± 47	354 ± 26	1.07 ± 0.27
6	Vehicle/gavage	352 ± 12.2	0.87 ± 0.08	12.2 ± 2	9.82 ± 1.6	253 ± 52	383 ± 30	0.86 ± 0.16
7	BA/gavage	321 ± 8.1	0.94 ± 0.03	13.2 ± 2	9.39 ± 2.0	348 ± 99	341 ± 45	0.93 ± 0.22
8	0.9% BA/diet	345 ± 11.7	1.04 ± 0.03	19.2 ± 2	9.58 ± 1.8	367 ± 37	371 ± 42	1.33 ± 0.37

[a] There were 5 rats in groups 1-2 and 4-8, and 4 rats in group 3.
[b] The number reported is the count for the tail portion of the pancreas.

Table 4. Effect of benzyl acetate on the incidence and size of pancreatic acinar cell foci in azaserine-treated and non-carcinogen-treated LEW rats.[a]

Group				Parameters (mean ± SE) for acidophilic foci				
				Observed data		Calculated data		
#	Treatment	Body wt., g	Pancreas, g	Number[b]	Area, $mm^2 \times 100$	No./cm^3	Diameter, μg	Volume %
	No carcinogen							
1	AIN diet only	469 ± 7.2	1.11 ± 0.03	1	—	—	—	—
2	Vehicle/gavage	452 ± 4.5	1.05 ± 0.05	1	—	—	—	—
3	BA/gavage	431 ± 7.5	1.08 ± 0.05	1	—	—	—	—
4	0.9% BA/diet	441 ± 7.2	1.14 ± 0.03	1	—	—	—	—
	Azaserine-treated							
5	AIN diet only	479 ± 11.9	1.28 ± 0.03	47 ± 6	9.66 ± 1.1	814 ± 126	335 ± 39	2.60 ± 0.50
6	Vehicle/gavage	471 ± 9.5	1.20 ± 0.07	48 ± 3	13.59 ± 2.6	696 ± 71	402 ± 33	3.99 ± 1.24
7	BA/gavage	440 ± 6.6	1.08 ± 0.07	46 ± 4	8.81 ± 1.0	896 ± 54	336 ± 37	2.62 ± 0.39
8	0.9% BA/diet	463 ± 11.6	1.25 ± 0.03	48 ± 9	12.39 ± 1.4	766 ± 108	377 ± 20	3.78 ± 0.83

[a] There are 5 rats in groups 1-4 and 6-8 and 4 rats in group 5.
[b] The number reported is the count for the tail portion of the pancreas.

sections are presented to convey the type of data collected. It is, however, inappropriate to use these data for statistical analysis, as they represent random sections through foci (transections) and not actual diameters of the foci themselves. The mean number of transections observed is influenced by the size of the foci; thus, the two-dimensional data may not accurately represent the actual number and size of the foci. Therefore, all statistical analyses are based upon the calculated volumetric data, i.e., number of foci per cubic centimeter, mean focal diameter, and the volume percent of the pancreas that is occupied by foci. The volume percent is a measurement analogous to tumor burden.

The data of Table 1 demonstrate that LEW rats are more sensitive to induction of acinar cell foci than F344 rats, as has been reported (*4*). The present study provides a more precise comparison over a range of several doses of azaserine. On the basis of these data, we concluded that two doses of 30 mg/kg given at 14 and 21 days of age would be adequate for quantitative morphometric analysis of foci in F344 rats. It is of interest that focal size was similar in the two strains.

The data of Table 2 demonstrate that the development of foci is enhanced in azaserine-treated rats that are fed chow diets with added corn oil, or given corn oil by gavage at the level that has been used routinely in the NTP bioassays. Although both the calculated number of foci per cubic centimeter and the volume percent of pancreas occupied by foci were larger in the oil-gavage group than in the oil-low group, the numbers were not significantly different, suggesting that total oil intake is more important than the mode of intake. Over the range of 5 to 15% total dietary fat there appears to be a dose-response correlation of oil intake with number of acinar cell foci per cubic centimeter and with volume percent of pancreas occupied by foci. When compared to chow-fed animals, both parameters are higher in animals fed the intermediate oil levels by either gavage or diet and are higher still in the animals receiving the highest oil levels. Analysis by linear regression shows that this trend is highly significant ($p < 0.01$).

The data of Tables 3 and 4 again demonstrate that LEW rats are more sensitive than F344 rats to the induction of foci by azaserine. There is no evidence that BA induced foci or affected pancreatic growth in non-azaserine-treated rats of either rat strain when given by either gavage or dietary routes. The incidence of animals with acinar cell foci in non-carcinogen-treated LEW rats was 30% and virtually zero in F344 rats. The purpose of giving BA to azaserine-treated rats was to see if the number or size of foci would be affected (increased), and if administration by the two routes would have a similar effect. The results for several parameters are inconsistent in the two strains, and no significant differences were noted by analysis of variance using rat strain, benzyl acetate, and gavage as factors.

A four-way analysis of variance of data in Tables 3 and 4 for body weight, with rat strain, carcinogen, benzyl acetate, and gavage as factors indicated that rats given benzyl acetate by either route were smaller than their controls ($p = 0.0004$); that rats given either benzyl acetate or vehicle by gavage were smaller than their non-gavaged controls ($p = 0.0013$); and that azaserine-treated F344 rats were smaller and LEW rats larger than their controls ($p = 0.0001$).

Discussion

These experiments demonstrate that quantitative stereological analysis of atypical acinar cell foci in the azaserine/rat model offers an approach for quantitative comparisons of factors that influence early steps in pancreatic carcinogenesis. The data clearly demonstrate a positive correlation of number of foci per cubic centimeter with dose of azaserine over a noncytotoxic (nonlethal) range, a positive correlation of number of foci per cubic centimeter and of volume percent of pancreas occupied by foci with increasing levels of dietary fat in the range of 5–15%, and a consistent difference in the response of two rat strains to comparable doses of azaserine.

We failed to show any effect of benzyl acetate on the

rat pancreas that suggests that it can initiate carcinogenesis in this organ. Further studies will be required to evaluate whether benzyl acetate can serve as a promoter of carcinogenesis in the pancreas. The data for non-azaserine-treated rats of experiment 3 also suggest that the incidence and number of "spontaneous" acinar cell foci and nodules is lower in F344 than in LEW rats. The incidence among LEW rats was similar to that seen in 4-to-5-month-old noncarcinogen-treated rats in previous experiments.

We have previously demonstrated that two synthetic retinoids decreased the number and size of acidophilic but not basophilic foci (*8*). Similarly, we have reported that food restriction in this short-term model suppressed the growth (number and mean size) of acidophilic but not the basophilic foci as compared to *ad libitum* fed controls (*13*). A diet of 20% unsaturated fat stimulated the growth as shown by an increase in both number and mean size of acidophilic but not basophilic foci (*9*). The results in our current studies are generally consistent with the previous observations. Acidophilic foci and nodules were more numerous than basophilic foci in all azaserine-treated rats. Basophilic foci were induced by azaserine in a dose-dependent fashion in both rat strains in experiment 1, but their number and growth was not significantly altered by corn oil intake in experiment 2, nor by benzyl acetate intake in experiment 3. Admittedly, the classification of focal lesions as acidophilic or basophilic involves somewhat arbitrary judgment in a low percentage of cases. Bias was minimized by examining slides without knowledge of treatment or strain.

These studies have provided data that have served as a basis for planning long-term *in vivo* studies of the effects of corn oil and benzyl acetate on the pancreas of F344 rats. Both agents will be evaluated in regard to their ability to act as promoters when added to the diets of azaserine-treated F344 rats.

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