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Dose-dependent Size Increases of Aortic Lesions following Chronic Exposure to 7,12-Dimethylbenz(*a*)anthracene[1]

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ABSTRACT

The prevalence, size, and patterns of distribution of arterial lesions (plaques) were investigated in cockerels exposed chronically to 7,12-dimethylbenz(*a*)anthracene (DMBA). Animals, from 5 to 20 weeks of age, received weekly i.m. injections of 5, 10, or 20 mg of DMBA per kg, dissolved in dimethyl sulfoxide. Control animals received weekly injections of dimethyl sulfoxide. All animals were sacrificed at 21 weeks of age. The entire aorta from each animal was cut transversely into 5-mm segments starting at the iliac trifurcation. The cross-sectional area of plaques was determined by light microscopic analysis of sections taken from the face of each segment. Plaque frequency was similar in DMBA-treated and control groups. However, mean plaque cross-sectional area was 7- to 11-fold higher for the treatment groups than for the controls. The distribution of plaque areas in both treated animals and controls was consistent with a log normal distribution. Median cross-sectional area and plaque volume index each increased in a linear fashion with DMBA dose. Small plaques were present in all groups. Large plaques were present only in DMBA-treated animals. Labeling indices of plaques, although low, were 2.3- to 26-fold higher than for underlying medial smooth muscle cells. The data indicate that the primary response to chronic DMBA exposure is a dose-dependent size increase of spontaneous aortic lesions and not the induction of new lesions.

INTRODUCTION

Proliferation of smooth muscle cells is regarded as one of the earliest cellular events in the pathogenesis of atherosclerosis (6, 8, 17, 20, 22). Experimental studies have demonstrated that atherosclerotic lesions composed largely of modified smooth muscle cells arise in animals whose arteries have been injured by chemical or mechanical treatment (9, 11, 12, 15, 21). The importance of injury as the major initiating agent in human atherosclerosis has been questioned recently. Benditt and Benditt (3) and Pearson *et al.* (14) presented evidence that atherosclerotic lesions in humans are monoclonal in origin. Benditt (2) also suggested that these plaques were benign smooth muscle cell lesions of the arterial wall. Were this suggestion correct, transforming agents including chemicals and viruses might also be expected to have atherogenic properties. Subsequently, Albert *et al.* (1) demonstrated that chickens injected weekly with either of the polycyclic aromatic hydrocarbon carcinogens, DMBA[3] or benzo(*a*)pyrene developed large, focal, fibromuscular lesions in the abdominal aorta by 20 weeks of age. This study provided the first evidence that known carcinogenic chemicals could be atherogenic as well. In addition, Fabricant *et al.* (5) showed that chickens infected with the oncogenic Marek's disease virus at 2 days of age developed occlusive, proliferative aortic lesions by 30 weeks of age. These 2 sets of results support, but do not prove, the suggestion implicit in the hypothesis of Benditt that transforming agents may be capable of initiating aortic lesions.

Spontaneous lesions have been observed in the abdominal aortas of cockerels older than 30 weeks. These lesions resemble those which occur in humans and are subject to many of the same complications (4, 13). In addition, small spontaneous lesions have been detected in the abdominal aortas of cockerels as young as 8 weeks old.[4] In order to better understand the nature of the arterial response to polycyclic aromatic hydrocarbon treatment, we undertook the DMBA dose-response experiments described here with a particular emphasis on plaque prevalence, size, and patterns of distribution.

MATERIALS AND METHODS

Fertilized eggs of a hybrid-strain white leghorn chicken (Hy-Line International, Des Moines, Iowa) were hatched, and the chickens were maintained in heated brooders until 4 weeks of age. At that time, they were sexed and banded for identification, and the males were distributed randomly into cages. Water and standard mash (Purina Co., St. Louis, Mo.) were available *ad libitum*. From 5 to 20 weeks of age, animals received weekly i.m. injections of DMBA (5, 10, or 20 mg/kg/week; Sigma Chemical Co., St. Louis, Mo.) dissolved in dimethyl sulfoxide (Fisher Chemical Co., Valley Forge, Pa.). A control group received weekly dimethyl sulfoxide injections only. There were 8 roosters in each group. All animals were sacrificed by cervical dislocation at 21 weeks of age. One hr prior to sacrifice, each animal received a single i.v. injection of [^{3}H]thymidine (specific activity, 15 Ci/mmol; 0.5 mCi/kg; Schwarz/Mann, Orangeburg, N. Y.).

Aortas were excised, rinsed with 0.9% NaCl solutions and fixed in 10% formalin. They were bisected longitudinally. One half was cut transversely into 5-mm segments beginning at the iliac trifurcation and continuing to the aortic arch. Following paraffin embedding, paired 5 μm thick sections were cut from each 5-mm segment. One slide from each pair was stained by the Verhoeff-Van Gieson procedure (10). This slide was used for the determination of plaque size. The cross-sectional area of plaques occurring in each segment was measured microscopically with a calibrated eyepiece graticule.

The second slide was processed for autoradiography (16).

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[3] The abbreviations used are: DMBA, 7,12-dimethylbenz(*a*)anthracene; LI, labeling index.

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Slides were layered with NBT-2 emulsion (Eastman Kodak, Rochester, N. Y.) and exposed for 28 days at 4°. After exposure, the slides were developed, fixed, and stained with hematoxylin and eosin. Labeling indices (number of ^{3}H-labeled cells/total number of cells × 100%) were determined for all plaque-containing sections and for selected medial sections.

The purity of the DMBA (m.p. 121.5–122.5°), which was recrystallized from acetone (25), was confirmed by gas chromatography. All solutions were prepared immediately before use.

RESULTS

Animals remained apparently healthy throughout the course of the experiment. Localized areas of necrosis were found occasionally at the injection site in the control group as well as in experimental groups. At sacrifice, gross examination of internal organs revealed no abnormalities with the exception of the reproductive system which displayed obvious deleterious effects of DMBA treatment. Testis size and weight decreased markedly as a function of DMBA dose. In nearly all DMBA-treated animals, the vasa were azoospermic. Few spermatogonia were visible in the testes of animals receiving DMBA (10 or 20 mg/kg/week). The results are consistent with those described for some rodents (7) and will be discussed in detail in another publication.[5]

Plaque Localization. Plaques were observed microscopically in the abdominal aortas almost exclusively in the first 25 mm of aorta above the iliac trifurcation. They were observed in animals from all treatment groups, including controls.

Plaques were present in 30% of the sections in control animals and in 33 to 46% of the sections from the 3 DMBA treatment groups (Table 1). These values were not significantly different from each other ($\alpha = 0.05$, χ^2 test). Plaque areas on individual sections ranged from 0.003 to 0.770 sq mm. Plaques of widely differing sizes were otherwise indistinguishable at the light microscope level (Fig. 1).

Plaque Area. Weekly DMBA injections resulted in increased plaque size in all treatment groups relative to controls. The values for mean plaque cross-sectional area per 5-mm section are presented in Table 2. The mean plaque area in each of the DMBA-treated groups increased significantly compared to that of the control group ($p \leq 0.005$, Student's t test). However, mean values for each of the treatment groups were not different from each other even at $p \leq 0.10$ (Student's t test). The upper limit on the plaque area was 10- to 12-fold higher for any group of DMBA-treated animals than for controls.

As shown in Table 2, values of median cross-sectional area also increased with DMBA dose. In all cases, the median values were lower than the corresponding means. This indicates that the cross-sectional areas of plaques were not normally distributed. When these values are replotted, the areas are shown to fit lognormal distributions. This is shown in Chart 1 where the plaque area is plotted *versus* probit units on log p paper. One straight line is drawn for the areas of all plaque-containing sections for the 3 DMBA-treated groups. A second straight line is drawn for the areas from the plaque-containing sections from control animals. In order to ascertain statistical significance in the difference between the areas of plaques in the control group and in the experimental groups, all values of plaque area were converted to log plaque area. This transformation permitted the construction of linear regression lines for each set of values. These results are presented in Chart 2. The *dashed lines* around each regression line represent the S.E. resulting from the number of observations and the variance about the regression at the level of $p = 0.05$. The regression lines for each of the DMBA-treated groups are distinct from that for the control group. There is a very small overlap of S.E.'s for the 5 mg DMBA per kg group and the control group. This occurs at

Table 1

Plaque prevalence and frequency for each treatment group

Treatment	Plaque prevalence (% of animals with plaque)	Plaque frequency (% of 5-mm segments with plaque)
Dimethyl sulfoxide	100	30[a]
DMBA		
5 mg/kg/wk	100	33
10 mg/kg/wk	100	46[a]
20 mg/kg/wk	100	42

[a] Plaque frequency was not significantly different ($\alpha = 0.05$, χ^2 test).

Table 2

Increases in plaque size following DMBA treatment

Treatment	Plaque cross-sectional area (sq mm) per 5-mm section	
	Mean ± S.E.	Median
Dimethyl sulfoxide	0.017[a] ± 0.004	0.013
DMBA		
5 mg/kg/wk	0.153[b] ± 0.034	0.036
10 mg/kg/wk	0.128[b] ± 0.031	0.060
20 mg/kg/wk	0.191[b] ± 0.035	0.143

[a] Significantly different from the mean of each DMBA treatment group at $p \leq 0.005$ (Student's t test).
[b] Not different from each other at $p \leq 0.10$ (Student's t test).

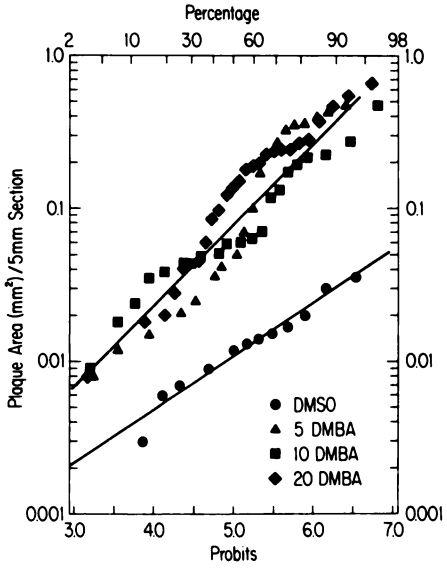

Chart 1. Lognormal distributions of plaque areas from individual aortic sections. The cross-sectional areas from each of the 82 plaque-containing sections from the 3 DMBA-treated groups and the 16 plaque-containing sections from the control group were plotted against the cumulative percentage of sections with plaque areas less than or equal to a given value (*top abscissa*). *Bottom abscissa*, probit transformation values of the percentages. These data are plotted on log p coordinates. ●, control; ▲, 5 mg DMBA per kg; ■, 10 mg DMBA per kg; ◆, 20 mg DMBA per kg.

[5] A. Penn and G. Batastini, manuscript in preparation.

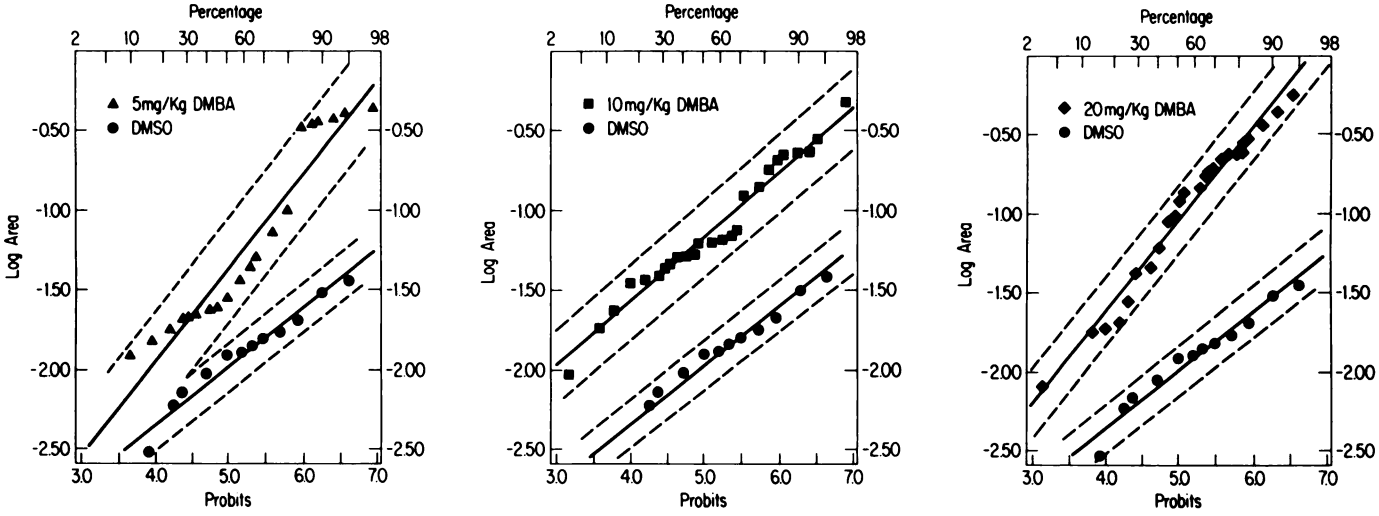

Chart 2. Correlation between log plaque area and probit values for DMBA-treated and control animals. The logarithms of the values for plaque area from Chart 1 were plotted against the probit values on linear coordinates. Linear regression lines were constructed for each set of values. - - - -, S.E. around each regression at $p = 0.05$. The slopes and correlation coefficients (r) of the individual lines are as follows:

	Slope	r
Control	0.372	0.978
DMBA		
5 mg/kg	0.603	0.963
10 mg/kg	0.420	0.966
20 mg/kg	0.599	0.983

The symbols are the same as those used in Chart 1.

a level corresponding to the smallest plaque areas in each group. Thus, at all dosage levels, DMBA treatment resulted in significantly increased plaque area.

Linear Dose Response. As shown in Chart 3, the median area values, obtained from the lognormal distributions, increased in an approximately linear fashion with DMBA dose. In addition, the plaque volume index (average number of plaque-containing sections per animal × median plaque area) increased linearly with DMBA dose.

LI. The results of LI studies in cockerels with plaques are presented in Table 3. For each animal in the 3 DMBA treatment groups, approximately 11,000 medial cells and 4,400 plaque cells were scanned for the presence of labeled cells. For each animal in the control group, approximately 9,600 medial cells and 800 plaque cells were scanned. The relatively small plaque sizes in the control group explains why fewer plaque cells per animal were scanned in the controls. Cells on the luminal surface of the intima were not included in these studies. Medial and plaque cell LI's for experimental and control groups are presented in Table 3.

Plaques displayed higher LI's than did underlying media. Plaque LI's were at least 4-fold greater than those of media in experimental groups and 2.3-fold greater in the controls. Since the media LI's were so low, high LI ratios probably represent a direct measure of plaque proliferation.

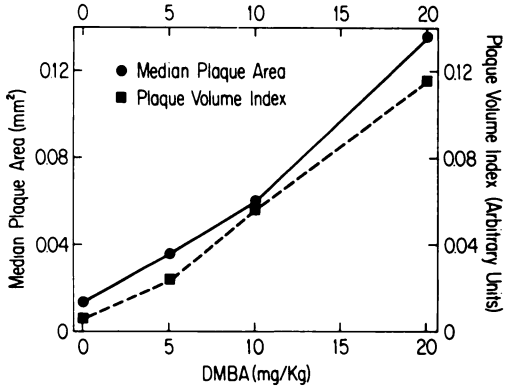

Chart 3. Linear increases in median plaque cross-sectional area and in plaque volume index with increasing DMBA dose. The median areas are those values at probit 5.0 on Chart 1.

DISCUSSION

The results demonstrate that chronic exposure to DMBA elicits dose-dependent size increases of arterial lesions in cockerels. The median cross-sectional area of plaques on individual aortic segments increased in a nearly linear fashion with DMBA dose. The plaque volume index, an approximate measure of total volume of plaque per aorta, increased similarly. In contrast to the marked increase in plaque area in DMBA-treated animals, there was only a slight increase in the percentage of aortic sections with plaques in carcinogen-treated animals compared to controls. Plaques with a small cross-sectional area were present in all groups. Plaques with a large cross-sectional area were present only in DMBA-treated animals. Lesions of widely differing cross-sectional areas appeared to be similar histologically under the light microscope. Together, these data suggest strongly that, in this system, a major effect of chronic DMBA exposure is to increase the size of spontaneous aortic lesions. Furthermore, this size increase appears to be a nonuniform response of the spontaneous lesions to DMBA exposure as indicated by the differences in slope of the linear regression lines. A uniform increase in plaque area per section would result in parallel lines. Thus, all

Table 3

LI's (LI = number of labeled cells/[total number of cells] × 100) of media and plaques

The number of labeled cells (media or plaque) is divided by the total number of cells of that type which were scanned for each group.

Treatment	Media LI	Plaque LI	Plaque LI/Media LI
Dimethyl sulfoxide[a]	0.043	0.100	2.326
DMBA[b]			
5 mg/kg/wk	0.060	0.334	5.567
10 mg/kg/wk	0.044	0.169	3.841
20 mg/kg/wk	0.021	0.538	25.619

[a] Media and plaque values are not significantly different ($\alpha = 0.05$, χ^2 test).
[b] Media and plaque values are significantly different ($\alpha = 0.05$, χ^2 test).

plaque-containing sections do not respond equally to DMBA treatment.

The possible monoclonal origin of atherosclerotic plaques has recently been the subject of much debate. Since no enzyme marker exists in chickens which is comparable to the X-linked glucose-6-phosphate dehydrogenase in humans, it is difficult to determine directly whether lesions arising in chickens are monoclonal. However, the question can be addressed indirectly by an analysis of proliferation levels of plaques appearing in 20-week-old DMBA-treated animals.

If the LI's at 20 weeks are representative of plaque cell proliferation between 4 and 20 weeks of age, then the 20-week LI's are too low to be compatible with a monoclonal origin of plaques. More than 4000 plaque cells were scanned from each animal in the DMBA treatment groups. Twelve cell doublings are necessary to produce 4000 cells from a single cell. In a 16-week treatment period, the average doubling time is 224 hr. Since

$$LI = \frac{\text{Duration of S phase} \times 0.69}{\text{Doubling time}}$$

(19), if we assume an S phase as short as 12 hr, then LI = 3.6%. The highest LI observed in any treatment group barely exceeded 0.5%.

The fact that plaque frequency in any DMBA-treated group did not differ statistically from that in controls argues against new plaques arising in response to DMBA treatment. In order for DMBA treatment to induce the formation of new lesions without increasing plaque frequency, either (*a*) a spontaneous lesion must disappear from a segment to compensate for every DMBA-associated lesion which appears or (*b*) each DMBA-induced lesion forms adjacent to but separate from a preexisting lesion and eventually overgrows it. Neither of these possibilities appears very likely.

A more reasonable possibility is that chronic DMBA administration causes preferential division of individual cells or patches of cells within the preexisting spontaneous lesions. The advantage in proliferation imparted to these cells would result in larger lesions. If all the cells that were stimulated to divide by DMBA were of the same type, the portions of the larger lesions derived from those cells would display monotypic characteristics without being monoclonal in origin. In this case, DMBA need act only as a mitogen, and thus other mitogens might also be effective. A potent naturally occurring mitogen derived from platelets has been shown to stimulate division of arterial smooth muscle cells *in vitro* (18). In addition, there is evidence that, in swine, spontaneous arterial lesions may be dormant but remain primed to divide in response to appropriate stimuli (23).

According to the monoclonal hypothesis, exposure *in vivo* to mutagens, including chemical carcinogens, should result in the formation of new monoclonal atherosclerotic plaques. Our results indicate that chronic exposure to DMBA accelerates size increases of preexisting spontaneous lesions. There is no evidence favoring the induction of new lesions by DMBA. It is possible that the preexisting lesions are monoclonal, but that issue is not related to DMBA exposure. Finally, even for those systems where plaques appear to be monoclonal, a variety of other mechanisms have been proposed to explain this phenomenon (24).

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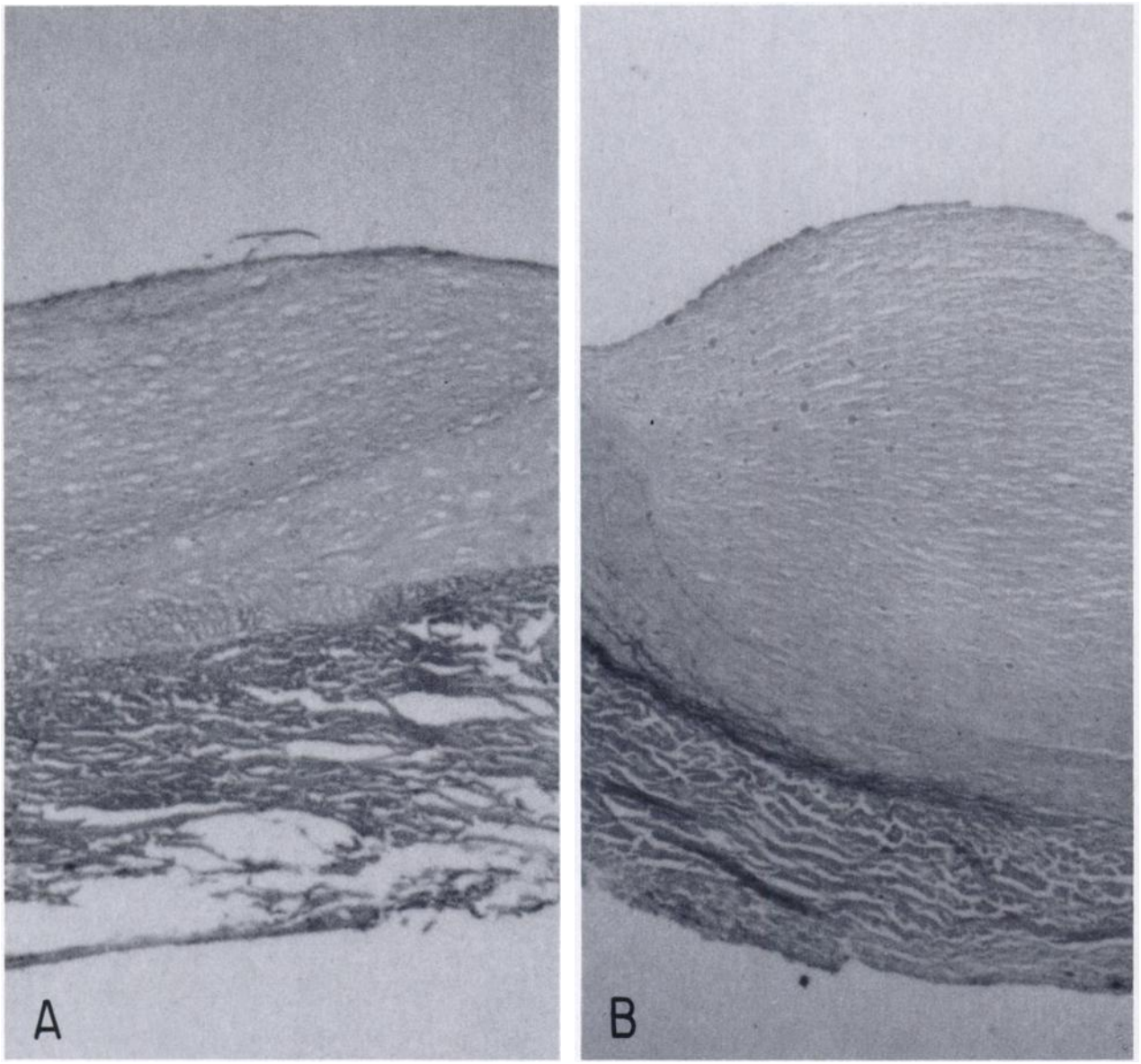

Fig. 1. Photomicrographs of lesions from dimethyl sulfoxide-treated cockerel (*A*) and DMBA-treated (20 mg/kg) cockerel (*B*). Verhoeff-Van Gieson, × 50.