The Role of the Adrenal in Toxicity in Rats Caused by Dimethylbenzanthracene

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SUMMARY

The damage to the adrenal glands of rats that occurs after the administration of dimethylbenzanthracene (DMBA) is preceded by a severe, often lethal, generalized toxicity and bone marrow destruction. Protection of the adrenal glands is afforded by administration of methylcholanthrene (3-MC), small doses of DMBA, or other substances, if administered 24 hours prior to the toxic dose of DMBA. The fact that 3-MC protects against generalized toxicity in adrenalectomized or intact rats shows that death following DMBA is not a function of adrenal necrosis. The administration of DMBA directly into the adrenal glands, so as to circumvent the generalized toxicity and bone marrow destruction, fails to cause extensive adrenal necrosis.

The thrombosis of the medullary sinusoids that is frequently seen in damaged adrenals seems to be a result of the adrenal lesion, not its cause, and occurs even after heparinization. Serial sections of the major vasculature of adrenals that had undergone complete necrosis failed to reveal thrombosis.

A distinct, heretofore unreported lesion of the adrenal medulla occurs after administration of large doses of DMBA; it may appear before evidence of cortical necrosis is seen.

INTRODUCTION

The adrenal necrosis that develops in rats after oral administration of appropriate doses of 7,12-dimethylbenzanthracene (DMBA) occurs only in the presence of a functioning pituitary gland that elaborates adrenocorticotropic hormone and in adrenals that produce corticoids (7). 3-Methylcholanthrene (3-MC), other chemicals, and even small doses of DMBA, if given 24 hours prior to the administration of the toxic dose of DMBA, prevents the adrenal necrosis (4). As a result, it has been inferred that DMBA or one of its metabolites (3) causes a fatal adrenal crisis. The emphasis on the supposed direct relationship between DMBA and adrenal damage has obscured the fact that the administration of DMBA results first in a severe generalized toxic reaction, followed by complete destruction of the bone marrow, each of which, as will be shown below, is sufficient to kill the rat in the absence of adrenal necrosis.

Thus, the evidence does not indicate that DMBA causes adrenal necrosis, but rather that adrenal necrosis is one of the complications that follow the administration of DMBA and, in fact, be a reaction secondary to the severe tissue damage that it causes. The animals die several days after receiving DMBA, indicating that the toxic process is quite complex and may involve intermediaries.

In order to investigate this mechanism, we have studied the pathology of the lesion and performed experiments designed to evaluate the role of the adrenals in relation to DMBA and the generalized toxicity that evolves.

MATERIALS AND METHODS

Male Wistar rats were used for all experiments, except that female Wistar rats were used in those studies in which DMBA was instilled directly into the adrenals. Unless indicated otherwise, the single oral dose of DMBA was 50 mg/100 gm body weight; it was 10 mg per rat for methylcholanthrene. Hydrocortisone was given in doses of 25 mg twice daily subcutaneously, and the dose of heparin was adjusted to prolong the clotting time past 45 minutes.

Adrenalectomy and procedures involving the adrenals were performed through a midline abdominal incision (8).

DMBA and 3-MC (secured from Distillation Products, Rochester, New York) were administered dissolved in olive oil via a metal esophageal catheter. 3-MC, when used, was administered 24 hours prior to DMBA.

In experiments in which DMBA was applied directly to adrenals, the animals were sacrificed 4 days after the DMBA treatment; the adrenals were removed for histologic study, bone marrow smears were made, and other organs were examined grossly.

Rats were maintained in groups of 4 or 5 in double-sized cages and fed Lab Blox and water ad libitum. Adrenalectomized rats were maintained on isotonic saline.

In survival experiments, animals were permitted to die, and information concerning morphologic changes were derived from parallel groups in which animals were sacrificed at predetermined intervals.

RESULTS

Clinical Course of Rats Given DMBA

Male Wistar-derived rats, given doses of DMBA up to 30 mg/100 gm body weight, were slightly more resistant than females to generalized toxicity and adrenal necrosis; at higher doses, these differences between the sexes were not apparent.
Doses of 30 mg/100 gm body weight killed 50-75 percent of the rats within 4 days, whereas 50 mg/100 gm body weight approximated a LD$_{100}$ by the 4th day. The animals became listless, the hair ruffled, diarrhea developed, and secretions coated the nares. Methylcholanthrene, given 24 hours prior to DMBA (50 mg/100 gm body weight) diminished the intensity of the acute toxic reaction, prolonged life (Table 3), and prevented adrenal necrosis. Although bone marrow damage appeared to be somewhat less than with DMBA alone, it was severe, and many of these rats died within 14 days, possibly of intercurrent infection. Reseeding of the marrow from residual foci that escaped damage may permit recovery.

**Pathologic Changes**

Bone marrow damage preceded adrenal changes and was observed within the first 24 hours. It was characterized by progressive hypocellularity, hemorrhage into the marrow space, and finally aplasia.

Initial damage to the adrenals was observed under the microscope between 24 and 72 hours after administration of DMBA and became more severe, proceeding to necrosis, during the ensuing 48 hours. Although larger doses increased the severity of the lesion, they did not shorten the latent period. With lower doses (30 mg/100 gm body weight) the lesion was usually restricted to the inner two zones of the cortex, with the medulla and zona glomerulosa escaping serious damage. The initial changes occurred in the zona reticularis and were characterized by fragmentation of the nuclei of the innermost cells. This was followed by congestion, then hemorrhage into the zona fasciculata and reticularis, and ultimately, infarctoid necrosis.

With higher doses, thrombi appeared in the medullary sinusoids. Fig. 1 reveals early fibrillar thrombi found in the dilated medullary veins of a male rat 48 hours after administration of DMBA (80 mg/100 gm body weight). The cortex was congested and contained areas of hemorrhage. Fig. 2 demonstrates a mature thrombus in the medullary vein of a male rat 3 days after a DMBA dose of 50 mg/100 gm body weight. There was infarctoid necrosis of the zona fasciculata.

In addition, animals sacrificed 48 hours after receiving DMBA at 30-80 mg/100 gm body weight showed pathology of the medulla prior to significant anatomic alterations in the adrenal cortex.

This change in the medulla is morphologically analogous to the cortical lesions described by Rich (10) in man after acute infection and is characterized by the appearance of either vacuolization or fibrillar material that develops between the cells of the medullary cord, forcing them to the periphery. Vacuolization of the central area results in the formation of a pseudotubule, and hemorrhage into these areas may occur (Fig. 3); conceivably, this could result in the eventual incorporation of these zones in the vascular network.

**The Effect of Heparin on Thrombus Formation in the Adrenal after DMBA**

The presence of thrombi in the medullary sinusoids suggests that the cortical necrosis, which when fully developed is infarctoid in appearance, might indeed be an infarct caused by vascular occlusion due to thrombus. However, serial sections of the major adrenal vasculature failed to disclose thrombi in any of the vessels. In addition we performed an experiment in which male rats were heparinized until the coagulation time approached infinity (no clotting at 45 minutes) and then administered DMBA. Prolongation of coagulation time to 45 minutes failed to prevent mortality, adrenal necrosis, or bone marrow damage, and thrombi could still be found in the medullary sinusoids (Tables 1, 2). The thrombi may result from the release of a thromboplastic material from the necrotic cortical cells in sufficient concentration to overcome the effect of heparin. Another possibility is that damaged medullary cells might form a nidus on which thrombi propagate.

**The Effect of DMBA Introduced Directly into the Adrenal Glands**

When DMBA is administered orally, its direct effect on the adrenals cannot be assessed because of the interposition of intermediary events, such as digestion, absorption, the possible formation of degradation products, tissue toxicity, and bone marrow damage. To circumvent interference by these phenomena, we attempted to evaluate the effect of direct application of DMBA into the adrenals. All rats were sacrificed four days after surgery; each rat served as its own control.

**Experiment 1.** The instillation of 0.01-0.05 ml of DMBA in olive oil (100 mg/ml) beneath the capsule of the left adrenal and olive oil alone to the right of female rats.

**Experiment 2.** The instillation of a cotton thread soaked in DMBA in olive oil (100 mg/ml) into the left adrenal and a cotton thread soaked in olive oil alone in the right adrenal of female rats.

**Experiment 3.** The introduction of DMBA crystal into the left adrenal and 3-MC crystals into the right adrenal of female rats.

In none of the rats in which the adrenals were treated directly with carcinogens, was there evidence of generalized toxicity or bone marrow damage. There was no evidence of sig-

### Table 1

<table>
<thead>
<tr>
<th>Day</th>
<th>DMBA (single dose, 50 mg/100 gm body wt.)</th>
<th>DMBA (single dose, 50 mg/100 gm body wt.) and heparin (250 units, twice each day)</th>
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<tbody>
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<td>6/9</td>
</tr>
<tr>
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<td>2/9</td>
<td>4/9</td>
</tr>
<tr>
<td>3</td>
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<table>
<thead>
<tr>
<th>Day</th>
<th>DMBA (single dose, 40 mg/100 gm body wt.)</th>
<th>DMBA (single dose, 40 mg/100 gm body wt.) and heparin (135 units, twice each day)</th>
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<td>6/8</td>
</tr>
<tr>
<td>2</td>
<td>3/8</td>
<td>4/8</td>
</tr>
<tr>
<td>3</td>
<td>0/8</td>
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Survival of rats on 7,12-dimethylbenzanthracene (DMBA) and heparin.
Table 2

<table>
<thead>
<tr>
<th>Rat #</th>
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<td>1</td>
<td>Sacrificed</td>
</tr>
<tr>
<td>10</td>
<td>2</td>
<td>Sacrificed</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>Died</td>
</tr>
<tr>
<td>12</td>
<td>4</td>
<td>Sacrificed</td>
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</tbody>
</table>

Adrenal pathology in male rats given 7,12-dimethylbenzanthracene (DMBA) (single dose, 30 mg/100 gm body weight) and heparin (125 units, twice daily).

Table 3

<table>
<thead>
<tr>
<th>Adrenalectomy</th>
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<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
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<tr>
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<td></td>
<td>X</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Hydrocortisone</td>
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<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
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<tr>
<td>Dimethylbenzanthracene</td>
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<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Day after single dose of DMBA</th>
<th>Survivors/number of animals treated</th>
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</thead>
<tbody>
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</tr>
<tr>
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<tr>
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<td>4</td>
<td>7/19 12/15 8/17 11/15 18/21 1/21 1/15 2/21</td>
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<tr>
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<td>7/19 11/15 5/17 10/15 17/21 1/21 1/15 1/15</td>
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<td>6</td>
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<tr>
<td>7</td>
<td>6/19 7/15 2/17 7/15 15/21 2/21 1/15 1/15</td>
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</table>

The effect of adrenalectomy, methylcholanthrene, and hydrocortisone in various combinations on the survival of male rats given 7,12-dimethylbenzanthracene (DMBA). Procedures to which rats of each group were subjected are marked with an X. Adrenalectomy was performed 10 days prior to DMBA; methylcholanthrene, 10 mg administered 24 hours prior to a toxic dose of DMBA; hydrocortisone, 25 mg intramuscularly, twice daily until death of rat; DMBA, a single oral dose of 50 mg/100 gm body weight.

*Animal group number.

significant adrenal necrosis caused by DMBA once changes due to trauma (as judged by controls) had been discounted.

Of the 4 animals in which a thread impregnated with DMBA (100 mg/ml olive oil) was sewn through the left adrenal and one impregnated with olive oil was sewn through the right adrenal, one DMBA-treated animal died under anesthesia and three survived. Survivors were sacrificed on the 4th day.

In one survivor, microscopic examination revealed the DMBA thread in the cortex with no evidence of peripheral cortical necrosis or tissue reaction, while in the contralateral adrenal, there was focal necrosis with no thread visible in the section. In a second, there was focal necrosis in both adrenals at the site of entry of the needle, and threads failed to appear in the tissue sections; in the third, there was total necrosis of the gland in which the olive oil-impregnated thread was inserted, and about 70% necrosis of the adrenal in which DMBA-thread had been sewn.

Gross identification of the threads was difficult, and it seems possible that in some cases the thread may have worked its way out of the adrenal to be buried in the heavy accumulation of granulation tissue that surrounded these glands.

There is no evidence, however, that the direct application of DMBA to adrenocortical tissue causes necrosis, since in at least two of the three cases, trauma alone could have been responsible.

When DMBA in olive oil was injected into the left adrenal and olive oil alone to the right, none of the 4 rats showed more than minute foci of necrosis in the region of the injection site,
despite the fact that residual vacuoles indicated that at least some of the inoculated material remained in the cortex during the four-day term of the experiment.

In another experiment, DMBA or 3-MC powder was introduced, via the bevel of a 19-gauge needle, beneath the capsule of the left and right adrenals respectively. In all but two instances microscopy revealed the presence of a necrotic focus, and in 5 of 8 adrenals recovered, crystal spaces were seen in the cortex. However, except for focal necrosis in the region of the crystals, no evidence of total adrenal necrosis occurred, and the demarcation between healthy and damaged adrenal cortical tissue was distinct, with no suggestion of tissue damage beyond the limits of the lesion.

Thus, the direct instillation of DMBA into adrenal glands fails to provoke adrenal damage comparable to that noted after oral administration, despite the fact that local concentrations in the adrenal would appear to be much higher after injection, compared to oral administration. (Thus, 0.01 ml of a solution of 50 mg DMBA per ml of olive oil would deposit 0.5 mg of DMBA to the injection site. The concentration per mg of adrenal tissue, calculated on the basis of even distribution after an oral dose of 50 mg/100 gm body weight would approximate 0.0005 mg, so that a thousand-fold concentration of systemic DMBA would have to occur in the adrenal tissue for it to approximate that deposited at the injection site.)

The Effect of DMBA in Male Adrenalectomized Rats

Table 3 reveals the result of DMBA administered orally under different experimental conditions to adrenalectomized and intact rats. It demonstrates that adrenalectomized rats are as susceptible to the lethal effects of DMBA as intact rats, and that 3-MC given 24 hours prior to DMBA was almost as effective in prolonging the life of adrenalectomized as intact rats, so that preservation of the adrenals does not account for the protection that 3-MC offers against the lethal effects of DMBA.

Hydrocortisone offers no benefits to rats protected by 3-MC, but does seem to prolong life in adrenalectomized rats, compared to intact rats given DMBA but not 3-MC (Groups #2, #4, #5, and #8 in Table 3).

Thus DMBA causes severe, often lethal toxicity in the absence of the adrenals, and the protection of the adrenals by 3-MC is concomitant with, and possibly related to, the diminished severity of the generalized toxic reaction usually caused by DMBA.

DISCUSSION

Since the experiment in which DMBA or 3-MC powder was introduced directly into the adrenals revealed crystal deposits at the injection site, it would seem that these crystals are too slowly solubilized to influence the adjacent tissues under the acute terms of the experiment. The same conditions probably exist for the impregnated cotton thread. However, the DMBA dissolved in olive oil also failed to cause extensive adrenal damage; this certainly shows that DMBA is not active when applied directly to the adrenal. The advantage of direct application is that it eliminates generalized toxicity and bone marrow damage as possible factors in the genesis of adrenal necrosis. However, experimental evidence exists that not DMBA, but one of its metabolites, is the active factor, and that the chemical alteration of DMBA occurs in the liver (3, 15). Thus it will be important to test the effect of the active metabolite when instilled directly into the adrenal, an experiment that is yet to be done. Although it has been inferred continually in the literature that adrenal necrosis is caused by DMBA or its metabolites, the best that can be stated at present is that the administration of DMBA initiates a series of events that results in adrenal necrosis which occurs subsequent to the acute generalized toxicity and bone marrow destruction.

That the adrenal necrosis is an indirect effect of DMBA toxicity is also suggested by the fact that similar cortical lesions follow a wide variety of injuries, such as those caused by diphtheria toxin, acute infections, severe burns, etc. The adrenal changes reported in guinea pigs after diphtheria toxin or burns is pertinent, since after either insult, as in the case of DMBA, the adrenal necrosis can be prevented by hypophysectomy (13, 14), but animals die, nonetheless, from systemic toxicity (1). Gablik et al. (6) showed that diphtheria toxin, after incubation with tissue from susceptible animals, develops the ability to damage tissue from animals that are ordinarily resistant to it; this suggests that an intermediary substance is the toxic agent.

Thus, because of the delayed toxicity of DMBA, and because in the absence of the adrenals it still causes a severe generalized tissue toxicity, it may be that the adrenal effect is dependent upon widespread tissue damage, and that, as with diphtheria toxin, a tissue substance acting either alone or in combination with DMBA, or one of its degradation products, produces the adenotoxicity.

As seen in Table 3, adrenalectomized rats given 3-MC have less generalized toxic reaction and lower mortality than those not given 3-MC; this alone may be responsible for the adrenal protection effected by 3-MC, rather than direct interference of 3-MC with DMBA or its by-products at the adrenal receptors. Indeed, in none of the experiments reporting protection by various substances against adrenal necrosis by DMBA is there mention of the clinical course of the rat. Certainly if less generalized toxicity occurred after protective measures (as does happen when 3-MC is given), one would be forced to conclude that the protective measure diminished the general toxic effects of DMBA, including that on the adrenal, but not that the adrenal alone was protected. To date there has been no separation of these factors in the literature.

With respect to survival, if the first shock can be overcome, one of two events may occur: either the animal will die of complications of pancytopenia, or else small islands of residual marrow cells will survive and help to regenerate the marrow. In this case, antibiotics would be expected to increase survival. The cause of the initial shock is still undetermined, and experiments will have to be performed that rule out bacteremia, endotoxic shock, etc.

Three characteristics of adrenal apoplexy (Waterhouse-Friederichsen syndrome) as described in man are hemorrhage, necrosis, and thrombosis of either the adrenal veins or the venules and sinusoids of the adrenal medulla. At autopsy, these
changes may be found in varying degree and combination (11, 12).

However, separate studies by Plaut (9) and Bove (2) in routine autopsies failed, as we have failed, to develop a relationship between cortical necrosis and adrenal vein thrombosis, one frequently occurring in the absence of the other, so that no sequential pathogenesis has yet been developed to explain or define the picture of adrenal apoplexy. The vascular system of the adrenals is abundant, so that thrombosis of small sinusoids would not be expected to cause much additional damage. However, thrombosis of the entire sinusoidal system or larger vessels (not found in our experiments) would certainly be expected to increase the cortical damage.

Originally, DMBA toxicity was thought to exclude the adrenal medulla (7), but our experiments have demonstrated significant medullary lesions which may occur prior to evidence of cortical necrosis. The morphologic changes in the medulla (Fig. 3) are similar to those observed by Rich (10) in the adrenal cortex of individuals who died of "adrenal apoplexy," which he characterized as "tubulization" of cortical cords (10). He postulated that they were caused by initial focal necrosis of marginal cells of the cortical cords which permitted ingress of blood products from the adjacent sinusoids and has illustrations strikingly similar to our Fig. 3 which demonstrate similar pathologic features in the cords of the medulla.

We can conclude, therefore, that no pathogenetic relationships among adrenal necrosis, hemorrhage, and thrombosis can be adduced from the evidence, and that the part played by the adrenal medulla in cortical necrosis is still obscure. Since, however, in both experimental and human pathology the adrenal lesions that accompany severe systemic toxicity of various etiologies are strikingly similar, it would seem appropriate, until proved otherwise, to consider the damage to the adrenal caused by DMBA to be a manifestation of reaction to severe cellular injury, dependent on normal adrenal-pituitary function, rather than a direct toxic effect.

Perhaps this system could serve as a model for the study of the pathogenesis of this particular ill-defined category of adrenal disease.

REFERENCES

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