Early Development of Squamous-Cell Carcinoma in Methylcholanthrene-painted Chicken Skin*

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A high incidence of neoplasms in cyclically stimulated organs, such as the uterus and mammary glands, has been observed for many years. Worthington, Taber, and Wilson (9) attempted to test the cyclic aspect of stimulation, as opposed to continuous or no stimulation, as a contributing factor in susceptibility to chemically induced neoplasms. The comb of the capon was chosen for their experiments, because it could be easily observed and treated with carcinogens and could be continuously or intermittently stimulated by androgen administration. It was suspected that the comb was susceptible to carcinogenesis, since Hertz and Tullner (3) reported grossly abnormal combs in birds treated with methylcholanthrene.

In their experiments, Worthington, Taber, and Wilson painted one side of the capon comb with methylcholanthrene 6 times a week for a period of 4 months. Some of the birds received daily testosterone injections throughout the treatment period; some received testosterone every other week; and some were untreated. Although the epithelium of all combs showed marked hyperplasia, no carcinoma was observed either grossly or microscopically. The outstanding feature of the combs which were not under continuous androgen stimulation was the presence of numerous aggregations of lymphocytes in the dermal peripheral layer. Such aggregates appeared only occasionally in combs subjected to continuous androgen stimulation. It was apparent that some factor, or factors, in androgen-stimulated combs had inhibited the lymphocytic response to the carcinogen.

When the wing and adjacent breast skin of capons was painted with methylcholanthrene, the lymphocytic reaction occurred to the same degree in the dermis of both androgen-treated and untreated birds.1 The inhibition of the lymphocytic response in androgen-stimulated combs, then, was not duplicated in these other body areas by androgen administration.

The comb responds to androgen by the deposition of a mucoid material in the intermediate layer of the dermis (note B in Figs. 1, 2). This results in an increase in size and turgidity. There is no thickening of the epidermis. In fact, with the increasing size of the comb, sloughing of the horny layer takes place (2).

It seemed possible that the presence of this mucoid material was a factor in the inhibition of the lymphocytic response in androgen-stimulated combs. In this laboratory, Palmer2 extracted the mucoid from the comb and injected it subcutaneously in the wing, simultaneously painting the overlying skin with methylcholanthrene. No inhibition of the lymphocytic response occurred in this mucoid-treated skin. With this treatment, however, he observed two cases of early squamous-cell carcinoma in the eight birds receiving both mucoid and methylcholanthrene treatment for 42 days, although none was observed in eight birds treated with either methylcholanthrene or mucoid alone.

The appearance of carcinoma in Palmer's birds in so short a time suggested that the presence of mucoid in subcutaneous tissue might have an augmenting effect on carcinogenesis. Our present experiments were designed to test further the effects of methylcholanthrene in conjunction with comb-mucoid, either extracted and injected into the wing, or in situ in the androgen-stimulated comb. A preliminary report of our results has been published (4).

EXPERIMENTAL

Methylcholanthrene and mucoid treatment of wing skin.—The ventral surface of the left wing of twenty 16-day-old light-brown Leghorn females was painted with a 0.5 per cent solution of methylcholanthrene in benzene 4 times a week for 6 or 24 weeks. In addition, they received, on the same
days, subcutaneous injections of comb-mucoid extract. Fourteen birds were treated with methylcholanthrene alone (MC-controls), and four were treated with mucoid alone (Mu-controls). Feathers were plucked when necessary to clear the treated area.

a) Gross observations: Wings from the four Mu-controls showed some local reaction to the injections. The area frequently seemed tender, small sores appeared temporarily on some wings, and soft subcutaneous nodules were occasionally noted. On MC-control wings and MC + Mu-treated wings, a scablike crust appeared by the end of the 1st week. This usually disappeared by the 2d or 2d week and afterward reappeared and sloughed several times as treatment was continued. The birds that were not treated with methylcholanthrene in this experiment or in other birds in our flock. Duran-Reynals (1) reported pox lesions in the skin of Plymouth Rock chickens painted with methylcholanthrene and postulated an activation of the latent virus by the carcinogen.

b) Histological observations: The four Mu-control birds were killed after 6 weeks of treatment. Although some sections of each wing showed slight disruption of subcutaneous tissue, a diffuse inflammatory reaction, and occasional aggregations of lymphocytes in the dermis, most sections appeared normal. Since there was no indication of epidermal stimulation in these birds or in others similarly treated by Palmer, no Mu-controls were included in the group treated for 24 weeks.

TABLE 1

<table>
<thead>
<tr>
<th>No. of birds</th>
<th>Type of treatment</th>
<th>Age at first treatment (days)</th>
<th>Treatment period (weeks)</th>
<th>Site of treatment</th>
<th>Massive lymphocytic response</th>
<th>Carcinoma</th>
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<tbody>
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<td>6</td>
<td>wing</td>
<td>0</td>
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<tr>
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<td>6</td>
<td>wing</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>12 &quot; †</td>
<td>MC + Mu</td>
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<td>6</td>
<td>wing</td>
<td>12</td>
<td>3</td>
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<tr>
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<td>wing</td>
<td>9</td>
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<td>8 &quot;</td>
<td>MC + Mu</td>
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<td>wing</td>
<td>8</td>
<td>2</td>
</tr>
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<td>comb</td>
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<td>9 &quot;</td>
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<td>35</td>
<td>34</td>
<td>comb</td>
<td>2</td>
<td>3</td>
</tr>
</tbody>
</table>

* Mu = comb-mucoid extract; MC = methylcholanthrene; T = testosterone.
† Including one bird, with carcinoma, killed at 44 weeks.
‡ Lymphocyte aggregates were found in a small area only.

Five MC-controls and eleven MC + Mu-treated birds were killed after 6 weeks; nine MC-controls and eight MC + Mu-treated birds were killed after 24 weeks; one MC + Mu-treated bird was killed because of illness on the 81st day.

MC-control wings and those treated with MC + Mu were so similar that they may be grouped together for description. Epidermal hyperplasia and hyperkeratosis occurred in all and were more pronounced with longer treatment. The hard, pointed elevations which had developed on some wings were composed entirely of keratin. Ulceration was frequently observed. The dermis was thickened, and there seemed to be an increase in the number of fibroblasts and a decrease in collagenous fibers, this being more evident in birds treated for 24 weeks. Numerous aggregations of lymphocytes were present in the dermis, and there was no evidence that the presence of mucoid inhibited this reaction. In all wings there were areas of infiltration by many lymphocytes (not aggregated), large mononuclear cells which resembled monocytes, and fewer heterophils, eosinophils, and multi-nucleated giant cells. Necrotic areas were

Mucoid was extracted with 0.4 M NaOH for 72 hours. The extract was then washed with chloroform, precipitated with 95 per cent ethanol, filtered or centrifuged, and dried with acetone. For injections, this dry material was made up in a 2.5 per cent aqueous solution. Each injection consisted of 0.1 cc.
associated with this reaction in approximately half of the wings. The MC + Mu bird which was killed on the 31st day was found to have carcinoma of the treated wing. A study of sections of the wings revealed two more cases of carcinoma in MC + Mu-treated wings and two cases in MC-controls killed after 6 weeks. The incidence of carcinoma was not increased with continued treatment up to 24 weeks (Table 1). Autopsies at this time brought the total carcinoma yield to five (25 per cent) in MC + Mu-treated and five (36 per cent) in MC-control birds.

There were no histological differences in carcinomas of MC-control and MC + Mu-treated wings. Likewise, carcinomas observed after 24 weeks of treatment did not appear advanced over those observed after 6 weeks.

In all cases, the diagnosis of carcinoma was made on a histological and cytological basis. All the lesions observed fulfilled the following criteria: (a) infiltration of surrounding tissue; (b) numerous mitotic figures (sometimes bizarre); (c) large hyperchromic nuclei with prominent nucleoli; (d) varying degrees of anaplasia; (e) loss of polarity of cells; (f) pearl formation (Figs. 3, 4).

Methylcholanthrene treatment of unstimulated and androgen-stimulated combs.—Eighteen light-brown Leghorn males were castrated at 22 days of age. At this time, nine of them had a pellet of testosterone propionate, weighing approximately 38 mg., implanted subcutaneously. Beginning 13 days after castration (35 days of age), the left side of the comb of each bird was painted with methylcholanthrene 6 times a week for 34 weeks. Four of the androgen group and four of the group not treated with androgen were also painted on the left wattle. Additional testosterone pellets were implanted at 7—9 weeks and at 16—17 weeks, when a slight regression of the comb indicated a decrease in androgen stimulation.

a) Gross observations: Inhibition of androgen-induced comb growth occurred on the painted side, confirming the observations of Hertz and Tullner (8), Pachkis and Cantarow (6), and Worthington, Taber, and Wilson (9). No visible tumors and no fowl pox lesions were observed in any of the birds. By the end of the 1st week, a yellow crust appeared on the combs. This continually sloughed and reappeared throughout treatment. By the 28th week some of the androgen-stimulated combs began to have an unpleasant odor, evidently from dark necrotic material present on the painted surface.

b) Histological observations: In all androgen-stimulated combs, hyperplasia and hyperkeratosis were evident. The inhibition of growth on the painted side was correlated with a decreased thickness of the intermediate or mucoid layer on that side. Occasional aggregations of lymphocytes were present in the peripheral layer of the dermis (Fig. 2). Ulceration of the epidermis with dermal inflammation was frequently noted. In the peripheral layer there seemed to be some increase in fibroblasts and decrease in collagenous fibers, but this was not nearly so marked as in the methylcholanthrene-treated wings.

Epidermal hyperplasia was relatively more marked in combs not treated with androgen, and pegs of hyperplastic epidermis sometimes traversed the width of the combs. Ulceration was rare. Numerous aggregations of lymphocytes filled the dermal peripheral layer in places (Fig. 1). There was, in general, more disruption of the normal comb pattern in these than in androgen-stimulated combs.

The wattle, which is histologically similar to the comb, responds to androgen stimulation as does the comb, but to a lesser degree. As observed in sections of the comb, unstimulated and androgen-stimulated wattles differed in their response to methylcholanthrene. The wattles not stimulated by androgen showed more disruption of pattern, more marked hyperplasia of the epidermis, and a more massive lymphocytic response.

In diagnosing carcinoma of the comb, the same criteria were used as listed for the skin of the wing. In the androgen group, carcinoma occurred in three (33 per cent) of the combs (Table 1). One of these birds had been sacrificed at 24 weeks and was found to have another primary carcinoma in the treated wattle. This was the only wattle in which cancer was observed. Carcinoma was found in two (22 per cent) of the unstimulated combs (Fig. 5). There were questionable areas in the comb of one other bird in the androgen group and in two of the unstimulated group. Histologically, carcinoma did not differ in the two groups. Although the combs were treated longer, it appeared that carcinoma in the comb was less advanced than that in the wing.

Examination of the internal organs at autopsy revealed no evidence of metastases in any of the birds which developed carcinoma in these experiments.

DISCUSSION

Including the unpublished results of Palmer with the results of experiments reported here, the incidence of epidermoid carcinoma was found to be

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4 Grateful acknowledgment is made to Dr. Norman L. Heminway of the Schering Corporation for the pellets of testosterone propionate (Oreton-F) used in this experiment.
20 per cent (seven cases) in wings of birds painted with methylcholanthrene and simultaneously injected subcutaneously with a comb-mucoid extract, as compared with 28 per cent (five cases) in those treated with methylcholanthrene alone. The incidence of carcinoma was 33 per cent (three cases) and 22 per cent (two cases) in MC-treated, androgen-stimulated and unstimulated combs, respectively. These results indicate that the comb-mucoid, either extracted and injected subcutaneously in the wing or in situ in the comb, did not augment carcinogenesis as was first suspected, nor did it inhibit carcinogenesis.

The number of cases of carcinoma which were induced in these experiments and the short period of treatment necessary for the induction seem significant in view of the reported resistance of the fowl to the chemical induction of epidermoid carcinoma (7). We observed carcinoma in one bird killed at 31 days and in 25 per cent (four cases) of the birds killed 42 days after treatment had begun.

Although Duran-Reynals (1) reported the induction of grossly detectable epidermoid carcinoma in chickens after 6–8 months of treatment with methylcholanthrene, he did not state the time at which he could first diagnose carcinoma microscopically. Recently, Rigdon and Brashear (8) reported carcinomatous lesions appearing on chicken skin from 60 to 100 days after 30 daily treatments with methylcholanthrene. They reported no microscopic observations prior to the time the lesions were visible grossly.

The occurrence of fowl pox lesions in some of the chicks painted with methylcholanthrene and not in untreated chicks is in agreement with the results of Duran-Reynals (1). He gained the impression that there was a gradual transition from the early pox lesion to the squamous-cell carcinoma and speculated that the reason Peacock and Peacock (7) failed to induce cancers was that their chickens did not carry the latent pox virus.

In our experiments, the five pullets which developed carcinoma at 24 weeks had pox lesions. However, when examined microscopically the carcinoma was not found in close association with pox tissue. None of the five chicks which developed cancer at 4½ or 6 weeks and none of the five capons which developed carcinoma at 34 weeks had pox lesions, and no indication of pox could be detected microscopically. This indicates that the presence of a pox lesion was not a necessary prerequisite for the development of cancers in these experiments. It does not, however, rule out the possibility that the pox virus was present in the treated skin. In more recent investigations by Duran-Reynals and his associates,5 it was found that methylcholanthrene did induce cancers in a specific type chicken (cross between male white Rocks and female Rhode Island Reds) in the absence of pox lesions and detectable pox virus.

The dermal lymphocytic response observed in all MC-treated wing skin and in MC-treated combs and wattles of capons not treated with androgen was, apparently, similar to that reported by Duran-Reynals (1) and by Rigdon and Brashear (8). The relative absence of such a response in androgen-stimulated combs and wattles has been of interest to us. The fact that Worthington and Taber1 found that the lymphocytic reaction occurred to the same degree in the wings of chicks injected with androgen as in those with no hormone treatment suggested that androgen did not act directly to inhibit the reaction. Since the modified skin of the comb and wattle, but not the wing skin, responds to androgen by the deposition

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5 Summarized by the Committee on Growth in its Eighth Annual Report to the American Cancer Society, Inc., and quoted with the permission of Dr. Duran-Reynals.
of large amounts of mucoid, it seemed possible that this mucoid material might in some way inhibit the lymphocytic response.

Although we failed to inhibit the reaction by injecting an extract of comb-mucoid into the wing, the hypothesis stated above cannot be eliminated. Our extract was a crude one, and the amount injected did not approximate the amount present in androgen-stimulated combs.

Worthington, Taber, and Wilson (9) found that combs intermittently stimulated with androgen exhibited a lymphocytic response to methylcholanthrene comparable to that in nonstimulated combs. In two androgen-stimulated combs in our experiments we found small areas in which a massive lymphocytic reaction occurred. Each of these was in a place where treatment with the carcinogen had inhibited growth to such an extent that the edge of the comb was rolled over in a scroll-like fashion, and relatively little mucoid was present. These observations lead to the supposition that, if mucoid is capable of inhibiting the lymphocytic reaction, it must be present continuously, and in relatively large amounts.

Zweifach and Chambers (10) have shown that the gelatinous matrix which forms the supporting structure for capillaries can be softened by hyaluronidase, thereby increasing the permeability of the capillary wall in the cat and rat. Ludwig and Boas (5) have demonstrated that comb-mucoid is largely composed of hyaluronic acid. It seems possible that accumulation of hyaluronic acid around the capillaries in androgen-stimulated combs might decrease their permeability and prevent the massive infiltration of lymphocytes into the dermis. At the present time, experiments are being planned to test this hypothesis.

SUMMARY

Fourteen light-brown Leghorn females were painted with methylcholanthrene (MC) on the ventral surface of the wing 4 times a week for 6 or 24 weeks. Twenty others received, in addition to MC, subcutaneous injections of a comb-mucoid extract (Mu). After 6 weeks of treatment two of the five MC-controls and three of the twelve MC + Mu-treated birds had carcinoma of the wing skin. Of the birds treated for 24 weeks, three of the nine MC-controls and two of the eight MC + Mu-treated wings developed carcinoma. The dermis of all treated wings was infiltrated with numerous large aggregates of lymphocytes.

The combs of eighteen capons were painted with MC 6 times a week for 34 weeks. Nine of these were stimulated by androgen. Carcinoma was observed in two of the combs not stimulated by androgen and in three of the androgen-stimulated combs. Aggregates of lymphocytes were predominant in unstimulated combs, but almost absent in androgen-stimulated combs.

In these experiments, the presence of comb-mucoid appeared to have no augmenting or inhibitory effect on carcinogenesis. Likewise, the presence of fowl pox lesions did not appear to be a contributory factor.

Although androgen-stimulated combs failed to show the typical lymphocytic reaction to MC, the injection of a comb-mucoid extract did not modify this response in MC-painted wings.

REFERENCES

Early Development of Squamous-Cell Carcinoma in Methylcholanthrene-painted Chicken Skin

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