Studies in Squamous Metaplasia in Rat Bladder

I. Effects of Hypovitaminosis A, Foreign Bodies, and Methylcholanthrene**

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SUMMARY

The effects of vitamin A deficiency, foreign bodies in situ, and methylcholanthrene in the production of squamous metaplasia in the epithelium and changes in the stroma of the urinary bladder of rats were studied as isolated stimuli and in combination. The results showed a variable contribution by the separate stimuli, with a summation of stimuli yielding earlier and more marked changes.

Vitamin A deficiency was a dominant factor in causing keratinization of the bladder epithelium in the rat. It is singularly effective with a foreign-body stimulus. Foreign bodies alone, even methylcholanthrene-paraffin pellets, in rats on stock diet, did not stimulate marked keratinization but did promote hyperplastic changes. The same foreign bodies in the avitaminotic animals often caused a marked epidermalization.

The degree of roughness of the surface of the foreign body appears to be a factor particularly in stimulating hyperplasia. Further, more atypical cytology was found when paraffin was the foreign body as compared with glass beads. The cytologic distortion as well as the metaplastic changes were more marked when methylcholanthrene was added to the paraffin.

Stromal changes included inflammatory infiltration and increase in PAS and Alcian Blue staining in animals with foreign bodies in the bladder irrespective of diet, and these changes were not present in the stroma of bladders of animals on vitamin A-deficient diet alone.

This investigation deals with a study of the metaplastic process in relation to vitamin A deficiency, and foreign-body and methylcholanthrene irritation.

Vitamin A deficiency causes squamous metaplasia of epithelia (20, 21). Mori (11, 12) found changes in the paraocular glands, salivary glands, and mucosa of the larynx and trachea in animals on low vitamin A diets. Wolbach and Howe (20, 21), Goldblatt and Moritz (8, 9), and Goldblatt and Benischek (7) described the relation of vitamin A deficiency to metaplasia in different organs. Changes described in the bladder included

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vitamin A in the liver, as determined with a fluorescence microscope. Curves of average body weight gain are shown in Chart 1.

The animals were then divided into the following 9 groups, with number of animals per group given in parentheses:

I. Stock diet (30 animals)
II. Stock diet+smooth glass bead in bladder (10)
III. Stock diet+rough glass bead (14)
IV. Stock diet+paraffin pellet (12)
V. Stock diet+paraffin pellet containing methylcholanthrene (12)
VI. Vitamin A-deficient diet (12)
VII. Vitamin A-deficient diet+rough glass bead (20)
VIII. Vitamin A-deficient diet+paraffin pellet (16)
IX. Vitamin A-deficient diet+paraffin pellet containing methylcholanthrene (16)

The glass beads and pellets measured 5–6 mm in diameter. The pellets were made with the paraffin used for embedding of tissues (melting point 56° C.). Pellets containing methylcholanthrene (Eastman Kodak Co.) were made as a 10 per cent suspension in paraffin.

The foreign bodies were inserted into the bladder surgically by the method of Jull (10), modified by closure of the bladder by external ligation of the full thickness of the fundus with catgut after approximation of the mucosal edges. In this way the contact of a continuous epithelial layer was preserved, with no suture material to act as an additional foreign body in the mucosa of the bladder.

The animals were sacrificed at different intervals and when they showed evidence of rapid decline. Some died of infection, mainly of the genito-urinary tract or of bronchopneumonia. The bladders were fixed in neutral calcium-formalin and stained by hematoxylin and eosin, periodic acid Schiff (PAS), with and without salivary digestion, and Alcian Blue (16). Special histochemical studies will be described elsewhere.

The amount of metaplastic change in the bladders was graded from 0 to 4+, according to the criteria given in Table 1.

RESULTS

EPITHELIAL CHANGES

The bladder mucosal changes in animals grouped by time intervals of up to 7, 8–20, and 21–40+ weeks are given in Table 2. Since no significant differences were noted between males and females, data are presented together.

Group I.—Stock diet; untreated. The bladders had a variable appearance, depending on the state of contraction or distention at time of death and fixation. The distended bladder (also studied by artificial distention with fixative during sacrifice) showed a thin epithelium, often reduced to a thickness of two cell layers (Fig. 1). The superficial layer consisted of larger cells containing PAS and Alcian Blue-positive material. The mucosa of the contracted bladder was four to five cell layers thick (Fig. 2).

Group II.—Stock diet; smooth glass beads inserted in the bladder. None of the bladders showed marked changes. One male animal of two examined after 18 months gave a suggestion of epithelial hyperplasia and pyknosis of the nuclei. Of the females, one of three animals killed after 15 weeks showed some epithelial hyperplasia and a 1 + metaplasia. The others showed a less marked change.

Group III.—Stock diet; rough glass beads inserted in the bladder. Of seven females and seven males, three females and five males showed a 1 + modification of the mucosa. One male showed a 2+ change on gross examination after 7 weeks, but the histological section apparently missed this area, so that it is charted as a 1+ alteration. No significant changes were found in the other bladders.

Group IV.—Stock diet; with paraffin pellets inserted in the bladder. Of six males and six females, three females and five males showed a 1+ modification of the mucosa. One male showed a 2+ change on gross examination after 7 weeks, but the histological section apparently missed this area, so that it is charted as a 1+ alteration. No significant changes were found in the other bladders.

Group V.—Stock diet; paraffin pellets containing methylcholanthrene inserted in the bladder.
Nine males and three females were included. The animals that died before the 3d week showed no changes. Thereafter 1+ changes, with mitosis, hyperchromatism, and some pleomorphism became evident. One male, 27 weeks after implantation, and one female, at 39 weeks, showed a maximal 4+ distortion of the mucosa.

Group VI.—Hypovitaminosis A regimen; no foreign body in bladder. Of seven males and five females, all showed some alteration of the bladder mucosa, though two killed at 5½ and 9 weeks, respectively, could not be classified as 1+ by the criteria listed. With the exception of a female at 24 weeks showing a 3+ change (Fig. 6), the others, killed between 5½ and 42 weeks, exhibited changes classifiable as 1− and 2+ only (Fig. 5). There was no close correlation between degree of response and duration of experimental treatment.

Group VII.—The rats were kept on avitaminotic A diet for 3 weeks, then had a rough glass bead inserted in the bladder and were maintained in hypovitaminosis with vitamin A supplement for periods varying from 1½ to 37 weeks. Ten males and ten females were available. The changes in the bladder mucosa were more marked and appeared earlier than in any group considered thus far. Of seventeen rats studied up to 20 weeks, three showed 3+ changes (Fig. 7) and nine a 4+ alteration. Three rats with 4+ changes showed papillomatous projections. The other rats showed less marked distortions of the bladder. Again no definite correlation with the duration of bead insertion was found.

Group VIII.—Hypovitaminotic regimen as in group VII; paraffin pellet inserted into the bladder. Ten males and eight females were studied. The changes were more marked than in Group VII. The few rats surviving for only short

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<td><strong>MUCOSAL CHANGES IN THE BLADDER, GROUPED BY TIME PERIODS</strong></td>
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*Classification of degree of change in Table 1.*
periods, 4 days, 1½ weeks, and 2 weeks, respectively, showed least change. Of thirteen animals studied between 8 and 20 weeks, twelve showed marked (4+) changes in the bladder, all within 12–15 weeks after the insertion of the paraffin pellet (16–19 weeks after being placed on the deficient diet). One male rat showed a 3+ change at this period. Two animals, vitamin-deficient for 23 and 24 weeks, respectively, yielded 3+ and 4+ changes. In six animals of Group VIII, calculous incrustation of the pellet was found.

**Group IX.**—Hypovitaminotic regimen as in group VII; paraffin pellet containing methylcholanthrene inserted in bladder. Eight males and eight females were studied, showing a 2+ change 3 weeks after operation; all of the animals that survived more than 3 weeks after the operation showed 4+ changes (Figs. 4, 8). One female showed this degree of alteration after 12 days. The most marked mucosal changes were found in this group, with pleomorphism of cells, variation in nuclei, mitosis, and deep infoldings of the epithelium (Fig. 8). All these cytological features were more marked than in any other group. In three instances, cytological features of carcinoma were found in limited areas of the mucosa, but the basement membrane was intact. Calculi were found in three rats.

**Stromal Changes**

The stroma of the bladder of the animals with simple hypovitaminosis A, including those with 3+ or 4+ changes, did not demonstrate an increase of staining with PAS and Alcian Blue. However, an increase of PAS or Alcian Blue-positive mucopolysaccharides was found in the animals with foreign-body and methylcholanthrene stimulation, whether they were fed the stock or the vitamin A-deficient diet. The increase of the PAS and Alcian Blue-positive material was also found surrounding the capillaries that extend into the epithelium and was seen whether or not other significant changes were present in the epithelium. Inflammatory changes were not remarkable in the vitamin A-depleted animals without foreign bodies (Fig. 6). Bladders with foreign bodies showed some inflammatory changes. This was minimal with the glass beads and more marked with the paraffin (Fig. 4) and the methylcholanthrene-paraffin pellets (Fig. 8). The animals that succumbed soon after the operation usually showed acute, diffuse inflammatory changes in the bladder. In animals that survived for longer periods, a chronic inflammatory process, characterized by mononuclear cells, mast cells, and larger fibroblasts, was found.

Calculi were found in several animals with an implanted foreign body, particularly with paraffin, whether on vitamin A-depleted or regular diet. No study was done of bacterial infection (19). Calculi were more common in males than in females. No high incidence of calculi was found in the group on a vitamin-deficient diet alone without a foreign body. Such calculi were described by Osborne and Mendel (14), Fujimaki (6), and van Leerum (17, 18).

**DISCUSSION**

Vitamin A deficiency is a dominant factor in causing keratinization of bladder epithelium. It is singularly effective with an accompanying foreign-body stimulus. Foreign bodies alone, even methylcholanthrene-paraffin pellets, in rats on stock diets did not stimulate marked keratinization, but hyperplasia of epithelium was present. The same foreign bodies in avitaminotic animals often caused marked epidermidalization. The degree of roughness of the surface of the foreign body appears to be a factor in stimulating hyperplasia. A chemical factor may also be operative in the paraffin used for pellets, for more atypical cytology was found when paraffin was the foreign body in the bladder than when a glass bead was used (Fig. 3). The paraffin pellets showed encrustation more often, and the roughness of the encrusted pellet may constitute a significant factor for the variations encountered. The cytologic

**Fig. 1.**—Normal distended bladder with mucosa reduced to two layers; a superficial (a) and a basal layer (b) of cells, and underlying stroma (s). X 1250.

**Fig. 2.**—Normal contracted bladder showing increased number of cells in epithelial layer (e) with a papillary folded projection of epithelium (f). The arrow is directed to a region where the superficial epithelium is cut tangentially. In this plane the cells are larger than when cut transversely (s) and can mimic squamous metaplastic epithelium. X 580.

**Fig. 3.**—Group IV. Regular diet with paraffin pellet in bladder for 37 weeks, showing 1+ modification, with epithelial hyperplasia, variation in size and staining properties of the cells and slight deepening of the epithelium (d). Subepithelial stroma shows inflammatory infiltrate (a). No keratinization present. X 350.

**Fig. 4.**—Group IX. Gross epidermidalization. Markedly keratinized mucosa of bladder of male rat maintained on vitamin A-deficient diet for 18 weeks, with methylcholanthrene paraffin pellet for 14½ weeks (4+ change). Pellet at lower left (p).
FIG. 3.—Group VI. Bladder mucosa of female rat on vitamin A-deficient diet for 9 weeks (1+ change). The cells are somewhat larger and the epithelial layer is irregularly thickened and folded; no keratinization is present. ×380.

FIG. 4.—Group VI. Bladder mucosa of rat on vitamin A-deficient diet for 24 weeks without foreign body in bladder. This animal showed the most marked modification in this group (3-4+). The epithelium is relatively thin but exhibits a thick layer of keratin. A thin granular layer (g) is present and some early papillation (p). No inflammatory cells are present in the stroma. ×380.

FIG. 5.—Group VII. Bladder mucosa of rat on vitamin A-deficient diet for 7 weeks, with rough glass bead for 4 weeks (3+ change). Marked keratosis without prominent keratin zone (g). ×380.

FIG. 6.—Group IX. Bladder mucosa of rat on vitamin A-deficient diet for 18 weeks, with methylcholanthrene paraffin pellet for 15 weeks, showing focal thickening and keratinization of the epithelium, a well developed granular layer (g), and deep penetration of epithelium (pe). Pleomorphism (ph) is noted at upper left. Inflammatory cells are seen in underlying stroma (st). ×140.
distortion as well as the metaplastic changes were much more marked when the carcinogen methylcholanthrene was added to the paraffin (Fig. 4).

No instances of invasive carcinoma were encountered. This may be owing to a relative resistance to cancer in the Sprague-Dawley strain of rat used, and it would be desirable to examine other strains of rat.

Jull (10) and Bonser et al. (2-4), using several carcinogenic agents, produced metaplasia, papillomas, and cancer in the bladders of dogs and mice. With higher concentration of methylcholanthrene (30 per cent) in paraffin pellets, Jull observed cancer. Rudali, Chalvet, and Winternitz (16) reported cancer in Wistar rats with methylcholanthrene. They did not report tumors in the rats in which only paraffin pellets were inserted in the bladder for over a period of 400 days. With the 10 per cent methylcholanthrene suspension used here, no definite invasive carcinoma was found. Experiments of longer duration with a higher concentration of methylcholanthrene are indicated.

Stromal changes were observed in animals with foreign-body stimulation, with and without methylcholanthrene, on both stock and deficient diet. These changes were not seen with simple hypovitaminosis, even where 3+ to 4+ changes had occurred. An alteration in the supporting stroma was stressed by Orr (13) as a factor in carcinogenesis, implying an effect of a metabolic interchange between the vascular system and the surface epithelium as a result of increased mucopolysaccharide content of the stroma.

Vitamin A may act directly on the oxidation-reduction mechanism of the epithelium (1) and affect the sulfhydryl and disulfide linkages implicated in the process of keratinization.

REFERENCES
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