HISTOPATHOGENESIS OF SQUAMOUS-CELL CARCINOMA INDUCED IN THE FORESTOMACH OF MICE BY INTRAMURAL INJECTION OF 20-METHYLCHELANTHRENE

HARLAN I. FIRMINGER and HAROLD L. STEWART, Pathology Section, National Cancer Institute, Bethesda, Md.

Squamous-cell carcinoma of the forestomach has been induced in mice by the oral administration and by the intramural injection of certain of the polycyclic carcinogenic hydrocarbons (1, 6). The morbid anatomy, histopathology, and histopathogenesis of this lesion have been described in mice ingesting 20-methylcholanthrene and 1,2,5,6-dibenzanthracene in aqueous oil emulsions (6). The present report deals with a similar study of squamous-cell carcinoma of the forestomach induced in mice by a single intramural injection of 20-methylcholanthrene with emphasis on the features of histopathogenesis (7).

MATERIALS AND METHODS

Mice of two genetic types were employed, consisting of 110 strain C57 brown and 110 A backcross hybrid animals, each type equally divided as to sex. They were from stocks maintained in this Institute in which previous experiments had shown animals of these genetic types to be susceptible to the induction of squamous-cell carcinoma of the forestomach. The A backcross hybrid mice were obtained by mating strain A females to strain C57 black males and backcrossing the females of the F1 generation to male strain A mice. The animals were 3 to 10 months of age when selected for the experiment and they were maintained on a diet of Purina laboratory chow and tap water.

Using intraperitoneal nembutal anesthesia supplemented by ether inhalation as required, the stomach was delivered through a left paramedian incision. A short-bevel, 27-gauge needle attached to a tuberculin syringe was inserted tangential to the serosal surface of the anterior wall of the forestomach at a point 2 to 3 mm. proximal to the limiting ridge. The needle was continued obliquely and superiorly to the left for a distance of 2 to 4 mm., at which point approximately 0.02 ml. of an aqueous suspension containing 0.6 mg. of 20-methylcholanthrene (table 1) was deposited in the submucosa. This produced a small bleb 3 mm. in diameter, which remained after withdrawing the needle. Following injection, the stomach was returned to the peritoneal cavity and the abdominal wound was closed.

1 Received for publication July 23, 1951.
2 Present address: Department of Oncology, University of Kansas Medical Center, Kansas City 3, Kans.
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Table 1.—Composition of aqueous 20-Methylcholanthrene suspension

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<th>Grams, percent</th>
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<tr>
<td>20-methylcholanthrene</td>
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<td>Aerosol O T</td>
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<td>Methocel (4,000)</td>
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<td>Ethyl alcohol (distilled water—q. s. 100)</td>
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pH adjusted to 7.4 with 0.1 N NaOH prior to making up to volume with water.

1 Prepared by Dr. Egon Lorenz, Chief, Biophysics Section, this Institute.

One mouse of each genetic type was killed by cervical dislocation approximately every 3½ days for 2½ months, and thereafter every 7 days for 12 months. Occasionally mice that died or that appeared moribund were substituted for those scheduled to be killed. The remaining mice were killed at the 77th week, when the experiment was terminated, unless during the interval they had developed large gastric tumors or appeared moribund, whereupon they were killed in order to obtain tissue free from post-mortem autolysis. The effective number of experimental mice was reduced from 220 to 165 by subtracting the animals that died post-operatively and those the tissues of which showed advanced post-mortem autolysis.

Twenty control mice (10 C57 brown and 10 A backcross animals) were given an injection of a control suspension identical with the experimental suspension except that it contained no 20-methylcholanthrene. Four control mice died within the first 3 months, providing control material for that period, and 11 died or were killed during the next 3½ to 17 months. Five were lost to the experiment because of post-mortem autolysis.

At autopsy the stomach was rapidly removed from the body, opened along the lesser curvature, spread out and pinned to a flat piece of cork, and fixed. A careful examination was made for metastases, implants, and other secondary neoplastic deposits. The tissues were fixed in Zenker-acetic-acid solution and occasionally in 10 percent formalin. They were embedded in paraffin, cut, and stained with hematoxylin and eosin. The sections of forestomach were routinely stained with, or by, hematoxylin and eosin (H and E), van Gieson's (VG), periodic-acid-Schiff's (PAS) (with and without previous diastase), phosphotungstic acid hematoxylin (PTAH), toluidine blue (TB) and crystal violet (CV), and were impregnated with silver by Wilder's or Laidlaw's methods for reticulin. Multiple sections were usually cut from the blocks of forestomach and serial sections were cut from selected blocks. Frozen sections were cut from a few formalin-fixed specimens of forestomach and examined under the polarizing microscope.

RESULTS

The mice, 165 in number, are classified into 2 major groups (table 2) according to the nature of the lesion of the forestomach. The pre-cancerous lesions, 66 in number (group 1) are further subdivided into those with (22 cases) and those without (44 cases) a diverticulum. There
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<td>Mice with precancerous lesions:</td>
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<tr>
<td>Without diverticulum</td>
<td>14</td>
<td>9</td>
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<td>15</td>
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<td>0</td>
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<tr>
<td>Both carcinoma and sarcoma</td>
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<td>0</td>
<td>0</td>
<td>1</td>
<td>4</td>
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<td>Mice autopsied</td>
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<td>13</td>
<td>13</td>
<td>22</td>
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<td>15</td>
<td>165</td>
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1 These specimens also showed precancerous changes.
are 4 additional specimens showing precancerous changes listed (table 2, footnote 1) under sarcoma of Group 2.

The malignant tumors, 99 in number (group 2), consist of 83 squamous-cell carcinomas, 4 sarcomas, and 12 mixed tumors (both squamous-cell carcinoma and sarcoma). A malignant gastric tumor is defined as a neoplastic lesion that infiltrates all coats of the viscus and forms a tumor mass on the serosa (6). Neoplastic lesions that did not achieve the magnitude consistent with carcinoma as defined are classified in the precancerous group. The first squamous-cell carcinoma of the forestomach was found in a mouse killed 60 days following injection. Of 132 animals autopsied 60 days or more following the injection, 95 or 73 percent showed squamous-cell carcinoma of the forestomach either alone or in association with sarcoma.

Statistical analysis of the data collected under the experimental conditions as outlined, for evaluation of the incidence of squamous-cell carcinoma, sarcoma, and mixed carcinoma and sarcoma by sex, by genetic type, and by sex within each genetic type revealed no significant differences, with the exception of a slightly lower incidence of squamous-cell carcinoma in strain C57 brown female mice.

**SARCOMA**

The forestomachs with sarcoma often showed ulceration and much of the wall of the viscus replaced by the neoplasm, which was usually large and bulky and which occasionally extended to adjacent structures and organs, including liver, spleen, pancreas, peripancreatic lymph nodes, and lateral abdominal wall. In one case there were nodular implants of sarcoma on the pelvic peritoneum. Microscopically the sarcomas were generally composed of spindle-shaped cells arranged in interlacing bundles (figs. 7 and 8) containing collagen and reticulum in intimate relationship with the tumor cells. Fibroblasts was demonstrated in only a few tumors and myofibroblasts not at all, although a diagnosis of leiomyosarcoma was occasionally entertained. Huge, bizarre, single and multinucleated tumor giant cells were sometimes observed (figs. 7 and 9).

In the muscularis and submucosa of a few specimens of forestomach there were focal areas of atypical cells, which did not appear to be epithelial cells and which were suggestive of early sarcomatous transformation (fig. 10). These areas were composed of interlacing, irregularly oriented, small bundles and groups of enlarged spindle-shaped cells showing more than the usual variation in nuclear size, shape, and staining. Two of these foci occurred in forestomachs showing squamous carcinoma, and conceivably could have represented areas of an unusually active stroma supporting the epithelial tumor. Another focus bordered upon a peritoneal abscess, the result of perforating carcinoma.

In 12 of the 16 animals with sarcoma there was also an associated squamous-cell carcinoma either partially or entirely separated from the sarcoma or intimately blended with it as carcinosarcoma (fig. 7).

**SQUAMOUS-CELL CARCINOMA**

The carcinomas presented the same gross characteristics as those previously described (6) and arose in relation to the site of injection of the methylcholanthrene (fig. 1). Their histologic characteristics varied moderately with respect to cell variation and amount of keratin in different tumors and different portions of the same tumor, but all were classifiable.
as squamous-cell carcinoma. A gland-like pattern occurred in one tumor
but, since the acinous-like spaces appeared to be lined by squamous cells,
and there was no associated mucus (PAS), the lesion was classified as
squamous-cell carcinoma.

All the carcinomas completely invaded the wall of the stomach, extended
laterally within the wall in all directions, and at times secondarily infil-
trated the mucous membrane of both chambers. After penetrating the
serosa, the tumors in 53 cases invaded adjacent organs and structures,
including spleen, liver, pancreas, omentum, lymph nodes, diaphragm, and
abdominal wall. Metastatic deposits were observed on the peritoneum in
3 animals (fig. 2), in the abdominal and mediastinal lymph nodes in 2
(fig. 3), in the lungs in 2 (fig. 6), and in the liver in 4 (fig. 5). In 13 addi-
tional mice, the liver contained multiple foci of atypical cells which,
although resembling neoplastic epithelial cells (fig. 4), showed no associated
keratin. Foci of atypical cells, indistinguishable from these, were ob-
served associated with unquestionable metastases. Such foci did not
occur in the liver of control mice or of the experimental mice without
carcinoma.

TRANSPANTATION

Out of 8 attempts at transplantation, 6 squamous-cell carcinomas and
2 sarcomas were successfully transplanted subcutaneously to other mice.
No attempt was made to transplant lesions developing earlier than 13
weeks following injection.

PRECANCEROUS AND ASSOCIATED LESIONS

Changes in stroma, blood vessels, and muscle: The fluid component of
the aqueous suspension was rapidly absorbed from the injection site,
leaving behind a deposit of crystalline methylcholanthrene in the sub-
mucosa. An acute inflammatory reaction developed in and about this
focus during the first 3½ days (fig. 12). This was characterized by the
presence of mononuclear and polymorphonuclear leukocytes and a few
fibroblasts. The cells, the walls of the blood vessels, and the connective-
tissue fibers in the center of the focus became necrotic early. There were
also degeneration and necrosis of the neighboring muscularis mucosae and
of the collagenic fibers of the lamina propria above the injection site, while
below it the muscularis propria appeared thickened and the adjacent
muscle fibers degenerated. Immediately around the necrotic focus, the
collagenic fibers of the submucosa were swollen, fragmented, angular,
granular, and strongly PAS-positive. These alterations gradually became
less pronounced as the distance from the inflammatory zone increased.
However, for some little distance beyond the immediate area of the in-
fiammatory focus, the collagenic fibers stained poorly with PAS and PTAH.
Here also, the submucosa presented a pale eosinophilic, homogeneous ap-
pearance, suggesting the presence of proteinaceous material filling the
intercollagenous spaces (figs. 15 and 26). The walls of the blood vessels
stained deep pink with PAS.
After 7 to 10 days, much of the necrotic material in the center of the focus had undergone liquefaction (fig. 13), and there was phagocytosis of methylcholanthrene crystals by macrophages, which had increased in number. Surrounding this central focus were macrophages and scattered fibroblasts embedded in a hyaline matrix, which stained deep red with eosin and dull pink with PAS. The small blood vessels in this region were often thickened and sometimes showed acute thromboangiitis. The lamina propria was edematous, particularly immediately below the hyperplastic mucosa, and there was beginning lymphangiectasia and telangiectasia, which increased with time. The area of damage in the muscularis propria increased in extent during the first week and showed patchy hyaline changes and fuchsinophilia (PAS). The adjacent muscle bundles became thickened and somewhat disarranged. Local peritoneal fibrosis ensued upon the extension of the inflammatory process to involve the serosa.

At 3 weeks, polymorphonuclear leukocytes were often prominent numerically in the exudate around the central focus containing the methylcholanthrene (fig. 14). The walls of many blood vessels at this time and subsequently showed irregular thickening, degeneration, and hyalinization (? fibrinoid degeneration). The involved portions of the vessel wall stained bright pink with PAS with and without prior treatment with diastase. Many vessels showed thromboangiitis. The injected crystalline material (fig. 14) became less evident after the first month (fig. 15), although in at least one specimen crystals were present 70 days postinjection. In animals autopsied later, multinucleated foreign-body giant cells were generally present, and macrophages persisted at the injection site for 3 months or more.

The area of the submucosa described at 7–10 days as showing a homogeneous eosinophilic appearance changed steadily with time. The tissue composing this area became more dense in appearance and stained rather diffusely, showing increasing eosinophilia (H and E) and fuchsinophilia (PAS). With PTAH the area stained a pale tan early, and later acquired an increasingly darker brown color. The staining reaction with VG changed from a pale orange through pink to red. No amyloid could be demonstrated. With time, a chronic inflammatory reaction characterized by focal collections of lymphocytes and plasma cells gradually made its appearance in the submucosa, well beyond the injection site.

In a few cases an ulcer extended into the injection site followed by evacuation of the crystalline and necrotic contents. In a few other cases a squamous-lined cystlike structure formed at the injection site, and persisted for months (fig. 27).

Changes in the mucosa: Acanthosis and hyperkeratosis of slight degree developed in the mucosa during the first 3½ days (fig. 12) following injection of methylcholanthrene. The cells of the basal layer became altered in their orientation and staining characteristics, and many of them appeared edematous and swollen. Individual, distorted cells containing elongated, hyperchromatic (pyknotic?) nuclei were interspersed between adjacent swollen basal cells. Immediately above the basal layer of cells
was a layer of compressed cells with deeply basophilic nuclei (fig. 12). Next above were the cells of the spinous layer, showing both an increase in the amount of cytoplasm and enlargement of the nuclei in which the chromatin was coarser and the nucleoli larger than normal. A few of the prickle cells contained multiple small eosinophilic granules (prekerato-hyaline granules?) in their cytoplasm. There was a prominent granular layer with an occasional cell containing a rather large nucleus and with keratohyaline granules that varied in size. The number of mitotic figures did not appear to be increased.

At the end of the first week the acanthosis and hyperkeratosis were sufficiently marked to produce a plaque 4 mm. in diameter on the mucosal surface of the forestomach. The disturbance of differentiation and keratinization of the cells was more pronounced. Some of the keratinized cells retained distorted pyknotic or ghost nuclei. A few large, round eosinophilic bodies resembling corps ronds were enmeshed in the layers of keratin. The normal wavy outline marking the junction between the mucosa and the lamina propria became accentuated, so that it resembled more the junction between the rete pegs and the papillary layer of the epidermis (fig. 20). At the tip of a few of these retia-like projections the cells of the basal layer were absent (fig. 16). There was one abnormal mitotic figure. A single projection of epithelial cells extended into the muscularis mucosae, which was the first evidence of infiltration.

By the tenth day the mucosal plaque, slightly larger now, was composed of several layers of keratin attached to the acanthotic mucosa, which was seven to eight cells in thickness compared to a normal mucosa only three or four cells thick. There were focal areas of atypical parakeratosis and small, loosely laminated spherules of keratin, both in the acanthotic mucosa and in the overlying keratin. Mitotic figures in the cells of the basal layer were frequent. As in the 7-day specimens, the tip of a few of the retia showed focal loss of basal cells.

At 2 weeks the mucosal plaque contained a central stellate cleft (fig. 11) and, as previously, showed atypicality of cells and alteration of differentiation and keratinization. In one of the two specimens studied, the reticular basement membrane was disrupted around two separate strands of epithelial cells that had infiltrated the submucosa (fig. 24).

Two 17-day specimens showed infiltration of epithelial cells into the submucosa (figs. 25, 28, and 31). One of these specimens (fig. 28) exhibited a diverticulum-like lesion that did not quite reach the serosa. In the mucosa lining the tip of this diverticulum was a focus of leukocytes, above which the keratin was interrupted. Around the focus of leukocytes the squamous cells were loosely arranged and dyskeratotic, and below it the basal cells were absent (fig. 31).

The other 17-day specimen showed a significant lesion (noted previously at 7 days and subsequently in a number of the forestomachs) characterized by inversion of keratinization at the blunt tip of one or more retia (figs. 17, 18, and 19). Normally the stratified squamous epithelium of the
mucosa keratinizes only in the direction of the lumen of the forestomach; whereas its deep aspect, which is in contact with the lamina propria, is covered by basal cells. In the lesion characterized by inversion of keratinization, the deep aspect of the individual rete was covered, not by basal cells, but by keratin. There was, then, bilateral keratinization both toward the lumen of the forestomach and in the direction of the underlying lamina propria. Depending upon the plane of the section, these two areas of keratin were either continuous or apparently separated from each other by a zone of atypical epithelial cells, most of which appeared to be spinous in character. The sides of the involved rete were covered by basal cells down to the level of the plug of keratin, at which point they curled inward to become continuous with the spinous cells, which were differentiating into keratin in both directions. The connective tissue stroma in contact with the keratin showed no inflammatory or foreign-body reaction.

Dyskeratosis: From the 3d to the 17th day as described, and indeed throughout the subsequent weeks of the experiment, changes developed in the acanthotic and hyperkeratotic mucosa which, for want of a better term, have been designated dyskeratosis. This dyskeratosis was characterized by prematurely keratinized single cells, corps ronds, and atypical parakeratotic cells. The viable cells around the dyskeratotic foci frequently exhibited alteration in size, shape, and staining characteristics.

A hitherto unobserved feature of the disorder of keratinization was the occasional presence of stainable cytoplasmic granules in the epithelial cells of the different layers of the mucosa. These altered cells were seen at different stages of the experiment but were most conspicuous in a specimen of forestomach examined 35 days following injection of the methylcholanthrene (figs. 20 and 21). The granules in the altered cells in the lower spinous and basal layers were acidophilic in the H and E and PAS preparations, reddish brown with PTAH, greenish yellow with VG, and black following silver impregnation. In the silver preparations, these granules were not only black but otherwise were morphologically identical to the keratohyaline granules in the cells of the granular layer. In the stained preparations, a gradual transition in staining characteristics could be traced between the granules within the epithelial cells of the different layers of the mucosa and those of the granular layer. The conclusion seemed inescapable that some of the keratohyaline granules in the granular layer were transformations of these newly observed granules in the cells deep in the mucosa.

Proliferation of basal cells: Following initial edema and distortion of the basal layer at 3 days, and the degeneration and focal loss of the basal layer observed at 1 week and 10 days, focal proliferation of the basal cells of the mucosa developed. This basal-cell proliferation was apparent first in a specimen examined 17 days following injection, and was frequently observed thereafter. The basal cells became irregular in size, shape, staining characteristics, and orientation, and formed budlike processes which imparted a scalloped appearance to the sides and base of the in-
olved retia (fig. 23). These budlike processes impinged upon, distorted, and often penetrated the muscularis mucosae following disruption of the basement membrane (figs. 22 and 23).

The most extreme example of basal-cell proliferation manifested itself as a papillary growth in a forestomach specimen 162 days after injection (fig. 32). The growth was composed almost entirely of sheets and cords of elongated, sometimes spindle-shaped basal-like cells with hyperchromatic, ovoid, or elongated nuclei. Several spherical masses of keratin were present in the depth of this growth. The transition from tumor cells to keratin was abrupt. The lesion bore a superficial resemblance to the seborrhoeic keratosis of man, although the infiltration at the base of the experimental lesion suggested its malignant potentiality.

**Infiltration:** When the altered cells of the mucosa showed evidence of aggressively infiltrating the stroma, or when an inflammatory process spread from the site of injection to involve the undersurface of the mucosa, the reticular basement membrane coincidentally became disrupted (figs. 22 and 23). However, after the infiltrating neoplastic cells had extended more deeply into the gastric tissues, the collagen and reticulum became condensed about them and apparently formed a new stroma.

The first infiltrating lesion in which the neoplastic epithelial cells penetrated all coats of the forestomach and formed a tumor nodule on the serosa, was observed in an animal killed 60 days following injection of methylcholanthrene. By definition, therefore, this represents the earliest example of carcinoma. Atypical epithelial cells were, however, observed infiltrating the muscularis mucosae at 7 days and the submucosa in one specimen at 13 days, and in two specimens at 17 days. Moreover, neoplastic foci in several specimens observed at biweekly intervals between 17 and 60 days, and thereafter exhibited more aggressive infiltrating characteristics although falling short of fulfilling the criteria of carcinoma as defined. Along with penetration of the wall of the forestomach in depth, the neoplastic tissue extended laterally from its points of origin, infiltrating the wall of both chambers of the stomach and of the esophagus; following penetration of the serosa many of the surrounding organs and structures became infiltrated by neoplastic tissue.

**DIVERTICULUM**

In table 2, 22 lesions were classified as a diverticulum (group 1). Although the wall of the forestomach regularly showed a defect and the deep aspect of the diverticulum was sometimes adherent to the spleen, liver, and other extragastric structures, still the stratified squamous epithelium lining some of these lesions appeared so innocent that a diagnosis of carcinoma could not be entertained (fig. 29). By contrast, other examples of the diverticulum exhibited neoplastic proliferation of the lining mucosa, with infiltration of the adjacent stroma and muscularis to a degree that made it difficult to distinguish these lesions from carcinoma. Still other diverticular lesions were associated with genuine carcinoma (fig. 30), and these specimens are included in group 2 of table 2. Some diverticular
lesions, when small and shallow, were distinguished with difficulty from the umbilicate lesion that develops in the forestomach of mice ingesting methylcholanthrene (6), and that so frequently eventuates in carcinoma. At the growing tip of many of the diverticula there were alterations of dyskeratosis in the epithelial cells as described at 17 days (fig. 31).

CONTROL MICE

No lesion of any type was found in the forestomach of the 15 control animals. In most of the specimens the exact site of injection could not be identified with certainty.

DISCUSSION

A single dose of 0.6 mg. of methylcholanthrene injected into the submucosa of the forestomach of mice, killed at varying intervals thereafter, induced squamous-cell carcinoma, sarcoma, mixtures of these growths, and precancerous lesions; the latter are believed to be of importance in the evolution of the malignant neoplastic process.

The term "precancer" has been used in clinical and experimental medicine to designate a group of lesions or conditions that are early cancers or cancers with or without limited infiltration or that are followed more or less consistently by cancer. Some of these are almost always followed by cancer sooner or later; others frequently give rise to cancer, while in still others cancer occurs only occasionally within the limits of the life span of the animal. In the present experiment, the lesions listed as precancerous in table 2 include all the forestomachs not showing carcinoma by definition. Many of the obviously neoplastic lesions showed infiltration of varying extent, sometimes just short of penetration of the serosa which, in this experiment, is the dividing line for distinguishing between precancerous lesions and carcinoma. Other lesions classified as precancerous were those occurring early in the experiment. The basis for considering these latter lesions as precancerous is validated by the high incidence of overt carcinoma developing under the conditions of this experiment.

The question as to when an induced neoplastic lesion of the forestomach warrants a diagnosis of carcinoma, constantly disturbs the investigator working in the field of experimental gastrointestinal cancer. In this as in previous reports from this laboratory carcinoma has been defined as a neoplastic lesion that infiltrates all coats of the viscus and forms a tumor mass on the serosa. For several reasons these criteria are not entirely satisfactory. On this basis some of the precancerous lesions in the present experiment fell just short of being classified as carcinoma. Some of the diverticula, although they extended through the serosa and also frequently exhibited neoplastic infiltration of the wall of the forestomach laterally, did not show neoplastic infiltration through the serosa. An attempt was made to diagnose carcinoma of the forestomach by the same general criteria employed in diagnosing gastric carcinoma in man, where the welfare of the patient is of paramount importance. Several pathologists familiar with the lesions of gastric carcinoma in man and in the experimental animal, and others less familiar with the experimental lesion,
were asked their opinion of a number of debatable lesions. The opinions were divergent; the tendency of the latter group was to classify more of the experimental lesions as carcinoma rather than as precancerous. Furthermore, a given pathologist's opinion varied from time to time. For example, one pathologist after examining the lesions listed under the heading of diverticulum, classified several of these as carcinoma. A few months later the same pathologist reviewing the same material classified them as diverticula. This points up a real dilemma. From the standpoint of interpretation of experimental results, the criteria employed are rigid and perhaps ultraconservative; however, they provide a margin of safety in arriving at conclusions from experimental work (8). From the standpoint of diagnosing human gastric cancer, adherence to such rigid criteria would be thoroughly unsound and misleading, for in this realm knowledge is more extensive and the purpose of the examination is different. It might be argued that infiltration of atypical cells, even in minor degree, is an adequate criterion of experimental cancer. If, however, this criterion is accepted, then it would be necessary to classify as carcinoma one lesion of the forestomach at 7 days, another at 13, two at 17 and several others prior to 60 days, postinjection, for all these specimens showed atypical epithelial cells infiltrating not only the lamina propria but beyond it. Infiltration of this degree may be observed in the forestomach of mice or rats subjected to a variety of experimental conditions such as starvation and vitamin deficiency, which do not evoke cancer. It might be argued further that no good purpose is served by making a distinction between precancerous lesions and carcinoma. Certainly in view of the high incidence of lesions of infiltrating and metastasizing carcinoma in the present experiment, the majority of the associated changes described as precancerous are in all probability carcinoma in miniature.

Seventy-one percent of the precancerous lesions in the present groups of mice were observed during the first 16 weeks of the experiment, and 29 percent in the succeeding weeks. In both periods similar lesions were observed. This lends support to the belief that many of the lesions observed in the latter period were also potentially malignant although the tempo of the neoplastic process was less rapid. Many of the changes in the forestomach interpreted as precancerous in the present experiment were also observed in a previous experiment in which methylcholanthrene was administered orally to mice (6). The present experiment is complicated by the puncture wound of the forestomach at operation and by the deposit of methylcholanthrene in the submucosa, with the attendant severe local inflammatory and degenerative changes. The gastric wall was weakened following destruction of the supporting tissues and this, it is believed, led to the development of the diverticular lesion, which was not observed in the feeding experiments. Although some or all coats of the wall of the forestomach showed a defect, and the deepest aspect of the diverticulum was sometimes adherent to spleen, liver, and other extragastric structures, the stratified squamous epithelium lining some of
the lesions appeared so innocent that a diagnosis of carcinoma could not be entertained. There seemed to be as much justification for separating this diverticulum from experimental carcinoma as for distinguishing between carcinoma and chronic diverticulitis in man. In some instances, the epithelial lining at the deepest extension of the diverticulum showed dyskeratosis and loss of basal cells. What role, if any, these dyskeratotic cells played in accelerating the deep extension of the diverticular lesion is problematical, but there was abundant evidence that precancerous changes and carcinoma developed. The diverticulum may have been preceded in some instances by the umbilicate lesion that occurred so frequently in the feeding experiments but was only occasionally observed in the injection experiment. In the feeding experiments localized damage to the supporting tissues of the forestomach was less severe, and this probably accounts for the failure of the umbilicate lesions to form diverticula.

The role of the degenerative changes in the stroma, muscularis, and walls of the blood vessels in the histogenesis of carcinoma in the injection experiments is probably similar to that of the analogous but less severe changes in the feeding experiments. The importance of these in the pathogenesis of carcinoma developing in the epidermis of the skin has been the subject of a great deal of discussion and speculation since the early studies of cutaneous carcinogenesis by tar and later by the hydrocarbons. In the present experiment an unusual lesion of undetermined nature was observed in the connective tissue of the submucosa adjacent to, and for some little distance beyond, the focus of methylcholanthrene. The involved tissue appeared edematous, but if this change was due to edema, then the edema fluid must have had a high content of a stainable material. With time, the areas of involvement became more dense in appearance and showed increasing eosinophilia and moderate fuchsinophilia. The connective-tissue stains suggested an increase in collagen. The nature of this change has not been fully elucidated.

Lesions characterized by acanthosis, hyperkeratosis, basal cell proliferation, and malignant dyskeratosis of the epithelium were observed in many specimens throughout the course of the present experiment and in many particulars were like these respective changes in the feeding experiments. A distinctive difference, however, was observed in the injection experiment. This was characterized by the appearance of prekeratohyaline granules in the cells of the basal and lower spinous layers. These granular objects were blackened in the preparations impregnated with silver, just as were the keratohyaline granules in the granular layer. With the staining procedures employed, although the granules at the two extremes of the width of the epithelium stained differently, those in cells in between showed transitions in staining characteristics. This, coupled with the fact that morphologically the granules in the cells of the basal layer and the keratohyaline granules in the granular layer were indistinguishable, lends credence to the belief that at least some of the latter were derived from the former.
The lesion that occurred at the blunt tip of one or more of the retia and that has been designated inversion of keratinization was not observed in the feeding experiments. This lesion occurred in specimens obtained at 7 and 17 days, and in a number of others subsequently. It was associated with loss of the basal layer of cells covering the tip of the rete and loss of the reticular basement membrane. This lesion showed keratinization taking place in two directions—toward the lumen of the forestomach, as normally, and toward the underlying stroma. The keratin was formed by spinous cells remaining after loss of the cells of the basal layer. The pathogenesis of this lesion, although imperfectly understood, would appear to be of significance as a possible early stage in the mechanism of infiltration.

**SUMMARY AND CONCLUSIONS**

A single dose of 0.6 mg. of methylcholanthrene in a concentrated aqueous methocel suspension was injected intramurally into the submucosa of the forestomach of strain C57 brown and A backcross hybrid mice. The experiment extended over a period of 77 weeks, during which time 99 of 165 treated animals developed a malignant tumor. Many animals were killed at intervals, according to a prearranged schedule, to determine the latent period of carcinoma and to afford material for a study of the histopathogenesis of forestomach carcinoma.

Of 132 animals autopsied following the latent period (after 60 days), 99 had a malignant tumor of the forestomach; of these 83 were squamous-cell carcinoma, 4 sarcoma, and 12 mixtures of squamous-cell carcinoma and sarcoma.

The complicated and often interrelated morphologic changes occurring in the squamous mucosa, stroma, blood vessels, and muscularis of the forestomach during the evolution of the malignant neoplastic process are described and illustrated and are compared and contrasted with those induced by oral administration of the carcinogenic hydrocarbons. The observations described emphasize the complexity of the process of experimental carcinoma and controvert the frequently expressed opinion that the mechanism of carcinogenesis resides in a single cell.

**REFERENCES**


Plate 43

The illustrations of the forestomach are all taken from the region of the injection site. The histologic sections illustrated are stained with hematoxylin and eosin unless otherwise stated. The period following injection is given in days. All the carcinomas are of the squamous-cell type, and all tumors are primary in the forestomach.

Figure 1.—Forestomach—carcinoma. 147 days. × 16.

Figure 2.—Abdominal peritoneum—implant of carcinoma. 196 days. × 120.

Figure 3.—Regional lymph node—metastatic carcinoma. 118 days. × 150.
PLATE 44

FIGURE 4.—Liver—focus of atypical cells, probably metastatic carcinoma. 91 days. × 1,120.

FIGURE 5.—Liver—metastatic carcinoma. 118 days. × 360.
PLATE 45

FIGURE 6.—Lung—metastatic carcinoma. 196 days. $\times 360$.
FIGURE 7.—Forestomach—mixed carcinoma and sarcoma. 151 days. $\times 95$.
FIGURE 8.—Forestomach—sarcoma. 161 days. $\times 85$. 
Figure 9. Liver—infiltrating sarcoma with bizarre tumor giant cells. 133 days. \( \times 340 \).

Figure 10.—Wall of forestomach—bundles of atypical spindle cells, possibly presarcoma. 56 days. \( \times 285 \).

Figure 11.—Esophagus, stomach, and duodenum—hyperkeratotic nodule of forestomach just proximal to limiting ridge overlying site of injection. 13 days. Photograph of gross specimen. Circa \( \times 4 \).
PLATE 47

FIGURE 12.—Forestomach degeneration and necrosis at injection site. Above this there is an infiltration of polymorphonuclear leukocytes in the submucosa, muscularis mucosae, and lamina propria. There is retia-like accentuation of the mucosal folds, which show cellular crowding of basal layer with variation in size and staining of nuclei and edema of cytoplasm. 3 days. × 180.

FIGURE 13.—Forestomach—Injection site with liquefaction centrally and degeneration of collagen at the periphery. 17 days. PTAH. × 95.
PLATE 48

Figure 14.—Forestomach—the injection site contains necrotic exudate and crystal clefts, some within macrophages. There are dilatation of capillaries and a smudged appearance of the submucosa. 21 days. X 95.

Figure 15.—Forestomach—to the left are vacuolated macrophages and other inflammatory cells bordering injection site. To the right the connective tissue is pale and smudged. There is degeneration of the muscularis propria. 31 days. X 140.
PLATE 49

FIGURE 16.—Forestomach—there are cellular variation in the mucosa and focal loss of basal cells. 10 days. $\times$ 690.

FIGURE 17.—Forestomach—a mucosal rete shows inversion of keratinization; plane of section is off-center (compare with that of two of the retia in figure 18) accounting for the bridge of cells interposed between the apparently separated masses of keratin above and below. 7 days. $\times$ 690.
Plate 50

Figures 18 and 19.—Forestomach—several mucosal retia showing loss of the basal layer with keratin and spinous cells in direct contact with the lamina propria. This appears to be a beginning stage of invasion of lamina propria. 17 days. \( \times 300 \).
Plate 51

Figure 20.—Forestomach—argyrophilic cytoplasmic granules in basal and spinous cells considered to be prekeratohyaline granules. 35 days. Wilder's silver impregnation for reticulum. $\times 415$.

Figure 21.—Higher power of area adjacent to that in figure 20, showing argyrophilic granules. Wilder's silver impregnation for reticulum. 35 days. $\times 1,400$. 

PLATE 52

Figure 22.—Forestomach—interruption and fragmentation of reticular basement membrane. Argyrophilic fibrils are interspersed in the keratin. 17 days. Wilder's silver impregnation for reticulum. × 300.

Figure 23.—Forestomach—penetration of reticular basement membrane by tongues of basal cells. 40 days. Wilder's silver impregnation for reticulum. × 300.
Plate 53

Figure 24.—Forestomach—budlike process of the mucosa has penetrated the muscularis mucosae and extends into submucosa. 13 days. × 1,340.

Figure 25.—Forestomach—the muscularis is necrotic and smudged. It is infiltrated by epithelial cells, which extend into the submucosa. 17 days. × 300.
Plate 54

Figure 26.—Forestomach showing acanthosis and hyperkeratosis of the mucosa with neoplastic invasion of the submucosa, which has a hyaline appearance. 56 days. × 38.

Figure 27.—Forestomach showing broad umbilicate lesion, which has extended through the muscularis propria but is confined by the thickened, chronically inflamed serosa. There are squamous-lined cystic structures on each side. 163 days. × 38.

Figure 28.—Forestomach—a diverticulum extends into the subserosa, which is thickened by fibrous inflammatory tissue. Much of the muscularis propria is destroyed in this area. Tip of diverticulum is shown in figure 31. 17 days. × 39.
PLATE 55

FIGURE 29.—Forestomach—large diverticular lesion with bulbous distention on the serosa. In all probability this lesion is in part the result of herniation of the mucosa due to damage to the supporting structures of the wall of the forestomach. 26 days. \( \times 42 \).

FIGURE 30.—Stomach, pancreas, and spleen—a diverticular lesion extends beyond the stomach and is attached to the spleen. There is a large carcinoma involving the neck and wall of the diverticulum and the wall of both chambers of the viscus. 141 days. \( \times 15 \).
PLATE 56

Figure 31.—Forestomach—tip of diverticulum illustrated in figure 28 showing loss of basal layer and irregularity of epithelial cells. \( \times 690 \).

Figure 32.—Forestomach showing papilloma composed of atypical basal cells and somewhat resembling a seborrheic keratosis. There are invasion of the submucosa and impingement on the muscularis propria. 162 days. \( \times 85 \).