Induced Cervical Carcinoma of the Mouse
A Quantitative Cytologic Method for Evaluation of the Neoplastic Process*

I. KOPROWSKA,† J. BOGACZ,†‡ C. PENTIKAS, AND W. STYPULKOWSKI

(Department of Pathology, State University Medical Center, Brooklyn 3, N.Y.)

The success in producing cervical carcinoma by intracervical insertion of methylcholanthrene-soaked thread (4) and intravaginal paintings with 3,4-benzpyrene (9) opened a new approach to experimental work in this field. Von Haam and Scarpe (10) and Reagan et al. (7) first reported cytologic studies of this tumor. Following their publications it became apparent that a standardized method was necessary for recording the gradual appearance of the bizarre characteristics of exfoliated cells before and during the development of cervical carcinoma. Such a standardized method would have to be simple enough so that it could be used by workers with limited experience in cytology and, therefore, could not be based upon an arbitrary distinction between malignant and non-malignant cells—nor could it be based upon instrumental measurements of cells, not only because these are cumbersome, but also because, owing to the numerous characteristics of malignant cells, only a few are amenable to evaluation by precision instruments. The purpose of this report is to offer a practical method for recording the gradual appearance of bizarre cellular characteristics and for correlating their presence with the neoplastic process.

MATERIALS AND METHODS

Animals.—Approximately 4-6-week-old C57 virgina female mice from the Jackson Memorial Laboratory were used throughout this study. They were kept in separate cages in groups of five to ten mice, fed standard Purina Laboratory Chow, and given water ad libitum; although about 800 C57 mice were used during the 2 years of experimentation with induced cervical carcinoma, the actual quantitative cytologic assay of the neoplastic process is presented on the basis of a detailed study of 58 mice, of which 30 were subjected to carcinogenic treatment and 28 were entirely untreated controls. Thirty treated mice belong to the first group of animals painted with benzpyrene with the aid of an infant-size otic speculum. This group was chosen for a detailed cytologic study because the introduction of speculum in the painting procedure resulted in an almost simultaneous appearance of cytologic abnormalities in mice subjected to the same number of carcinogenic applications.

Carcinogen and methods of its application.—3,4-Benzpyrene obtained from Edcan Laboratories was used throughout this study. For the treatments a 1 per cent solution in acetone was prepared and applied 8 times weekly for 19 weeks, in the following manner: The mouse was kept flat on its back in the palm of the left hand of the operator, index and thumb holding the skin of the back of the neck and ring finger securing the tail. An infant size otic speculum (size 1 during the first few weeks and then size 2) was clamped at the desired level (about 6 inches above the working bench) on a metal stand. The mouse was moved forward until the speculum was inserted intravaginally. The source of light (in our case a microscope lamp) was placed behind the mouse. The operator, seated in front of the bench and holding the mouse with the left hand, directed the beam of light with a laryngoscopic headlight mirror, inspected the cervix, and, by passing through the speculum a cotton-tipped wire loop dipped in benzpyrene solution, painted the cervical portio and os. Reference to this technique of carcinogenic treatment was also made elsewhere (1, 3).

Preparation of vaginal smears.—From the treated and control mice, every other week for 10 weeks and then once a week throughout the duration of carcinogenic treatment, vaginal aspirates were obtained by use of a specially adapted eye-drop pipette immediately prior to painting with the benzpyrene solution. A drop of saline was injected into the vagina before aspiration to increase the amount of frequently scanty aspirates. Vaginal smears prepared from the aspirates were fixed and stained according to the standard Papainnamo technic for processing gynecologic sections (EA-50).

Histologic diagnosis.—Mice died spontaneously or were sacrificed in extremis between the 19th and 30th week. Five random animals, however, were sacrificed in the 19th week to evaluate histological lesions present at the time of completion of carcinogenic treatment. All uteri were fixed routinely in 10 per cent formalin and then cut lengthwise antero-posteriorly in order to retain the topographical relation to bladder and rectum and to show cervical and vaginal tissues in the same section. After the tissues were imbedded in paraffin, blocks were

Received for publication May 16, 1958.
RESULTS

Prior to the 11th week of treatment, cellular abnormality in the vaginal smears was noted only rarely, and in general there was no difference between the benzpyrene-treated mice and the controls. Smears were often scanty, but phases of the sexual cycle usually were recognizable. During or about the 11th week, cellular abnormality became more frequent in the benzpyrene-painted mice, but their intensity was still not striking (+). No changes were apparent in smears of the control mice. About the 17th week there was a sudden increase in the number of involvements and degree of abnormalities scored. Five or more plus symbols were consistently recorded for the treated mice. This type of smear pattern remained more or less constant for about 3 weeks and then was followed by further increases in the number and degree until practically all the cytologic criteria of malignancy were met. Smears of control mice remained unchanged. From the 19th week animals began to die or were sacrificed with large cervical tumors. These patterns appeared to be fairly constant for each of the treated animals. Because mice were dying during the experiment, average scores were calculated for compiling Chart 1. These average values were obtained by adding together the number of positive criteria of malignancy exhibited by mice of the same group in a given week and dividing the total by the number of surviving mice. The degree of intensity was disregarded, and ±, +, and ++ symbols were counted as equal.

Chart 1 shows clearly the progression as, from the 19th week, further criteria of malignancy became added to the score for the treated mice whereas, in contrast, the control mice showed only one or two criteria at most.

Chart 2 illustrates the progressive increase in intensity of the three nuclear factors, i.e., nuclear enlargement, hyperchromasia, and irregular nuclear borders in the fifteen treated mice which were still alive between the 12th and 23d weeks of observation. For the sake of clarity of presentation, no more than three cytologic criteria of malignancy are analyzed on this chart.

While in general no single cytologic criterion of malignancy alone was found pathognomonic for carcinoma, persistence of five or more of the factors graded one or two plus was consistently cor-


came related with histologically proved malignant les-

ions. An attempt was made to determine the order of appearance of the different cellular abnormalities, and it was noted that in some mice early abnormal cytologic patterns were characterized by nuclear enlargement, hyperchromasia, irregular nuclear borders, prominent nucleoli, bi- and multinucleation, and pronounced cytoplasmic vacuolization, thus resembling the cytologic pattern of dyskaryosis seen in cases of carcinoma in situ in humans (5). In other instances, the appearance of slightly elongated cells without pronounced nuclear abnormalities, as well as the presence of engulfment and keratinization, was reminiscent of human cytologic patterns of chronic inflammatory conditions. When the appearances of these various cell characteristics were calculated, the time of appearance of the various factors was about the same, except for the bizarre elongated cells with abnormal nuclei, which appeared later, as shown in Chart 3.

Chart 4 illustrates the simultaneous appearance
of prominent nucleoli, frequent binucleation, and cytoplasmic vacuolization.

While most of the cellular criteria of malignancy utilized in this work are commonly used for the cytologic diagnosis of human neoplasms, no reference in the literature was found regarding the diagnostic significance of localized areas of blue-staining cytoplasm (see Fig. 9). The chemical nature of such areas, which may be owing to cytoplasmic degeneration, has not been elucidated by the present study. However, “basophilic cytoplasmic inclusions” were scored consistently from the 17th week in smears of the treated mice.

In mice which died of cancer, it was sometimes observed that, during a period from 1 to 3 weeks prior to death, there was a decrease in their scores. This usually coincided both with the decreased exfoliation of epithelial cells in general and with the presence of numerous polymorphonuclear leukocytes and of red blood cells. In such cases usually fewer cells with scored factors were present than were seen in the smears of the same animals during the preceding weeks. The presence of blood and the finding of elongated bizarre cells and small keratinized cells with dark pyknotic nuclei would usually help to distinguish this type of smear from one seen prior to the 17th week of experiment.

A close correlation was noted between the cytologic findings and the clinical symptoms. Mice usually began to bleed on the 18th week of treatment, and speculum examination at that time revealed the presence of tumors.

Histologically early carcinoma was found only in three animals, and these were sacrificed after the last treatment in the 19th week. Of the 30 treated mice, eighteen died and twelve were sacrificed.

![Chart 2](image)

**CHART 2.**—Illustrates the progressive nuclear enlargement (Column A), hyperchromasia (Column B), and irregularity of nuclear borders (Column C) during the neoplastic process. Unshaded areas represent ± symbols, lightly shaded areas represent + symbols, and heavily shaded areas represent ++ symbols.

![Chart 3](image)

**CHART 3.**—Shows that bizarre, elongated cells appeared later than nuclear enlargement during the neoplastic process in surviving mice during 12-28 weeks of experiment.

![Chart 4](image)

**CHART 4.**—Illustrates the simultaneous appearance of prominent nucleoli, frequent binucleation and cytoplasmic vacuolization.
Histologic evidence of advanced carcinoma of the cervix and/or vagina (see Figs. 10-12) was found in 26 mice. One animal, with cytologic and clinical evidence of a malignant neoplasm, was found dead with tissues chewed up.

DISCUSSION

In the course of the experiments, observations were made which seemed to lead to the formulation of a practicable premise. Five cytologic patterns were distinguished with the following characteristics and order of appearance:

1. Absence or only sporadic presence of low grade scores (from the beginning of treatment to the 10th week).

2. Persistent presence of several factors with low grade scores (±) and occasionally a higher score (from 11th to 17th week).

3. Persistent presence of five or more factors with + scores usually including nuclear abnormalities, cytoplasmic vacuolization, and localized areas of cytoplasmic basophilia (apparent in the 17th or 18th week of treatment).

4. Persistent presence of most factors, some of which are graded ++, i.e., outstandingly pronounced. Red blood cells noted (19th to 26th week of experiment).

5. Diminution in scores. Poor exfoliation. Cells degenerated. Many polymorphonuclear leukocytes and red blood cells. This type of cytologic pattern, when observed, follows or displaces the preceding one.

One may conjecture that these cytologic patterns correlate with histologic diagnoses in the following manner:

1. No morphologically demonstrated lesion.

2. Nonspecific cervicitis or cervical dysplasia.

3. Early neoplastic lesion.

4. Advanced neoplastic lesion.

5. Necrotic, infected tumor, terminal stage.

The material utilized for this study, however, provides histologic correlation only for the later stages of the neoplastic process. Histologic correlation for earlier stages of the neoplastic process will be reported separately. Nevertheless, data are available to ascertain that the cytologic distinction between early and advanced stages of malignant disease is based upon realistic histologic findings. The problem of early neoplastic lesions still remains puzzling. Von Haam and Reagan (7, 10) admit their inability to distinguish, from smears, carcinoma in situ from invasive carcinoma. Von Haam's (10) statistical evaluation of exfoliated cells points to a quantitative rather than qualitative differentiation of these two conditions. However, he did observe that disturbance of the sexual cycle was present only in invasive carcinoma.

In the group of 30 treated mice, on which the present method of cytologic evaluation is based, all but three mice ultimately developed advanced cervical carcinoma, and these three had been sacrificed in the 19th week of treatment when “early carcinoma” with some invasion already was present. Thus, on the basis of tissue studies of animals included in this series, no correlation of cytologic and histologic findings in cases of indisputable intra-epithelial carcinoma is feasible.

In the course of study of the pathogenesis of early induced cervical carcinoma of mouse (to be reported separately) it was observed that early malignant lesions in mice offer a serious diagnostic challenge as to their intra-epithelial or invasive nature. The few indisputable carcinomas in situ which were seen were usually found at the edge of an infiltrating lesion or were co-existent with infiltrating carcinoma in another portion of the vaginal or cervical mucosa. Furthermore, a multicentric origin in those early carcinomas was common, and different portions of vaginal and cervical epithelium presented simultaneously different types of lesions. In addition, exophytic and infiltrating forms of tumor were often present in the same animal, and different portions of the same neoplasm frequently exhibited different degrees and types of histologic differentiation. Our histologic observations are similar to those recently published by Scarpelli and von Haam (8). That lesions produced with benzpyrene in the uterine cervix are also malignant by other than morphologic criteria was demonstrated in the case of a cervical carcinoma, originally induced chemically, which grew upon subcutaneous transplantation and ultimately became transformed into an ascites tumor (3). Because cells exfoliate from various sites of the cervix and vagina, it may be difficult to recognize, from the cytologic pattern of an induced cervical carcinoma, the presence of carcinoma in situ.

On the basis of our observations, however, certain significant conclusions about the stage of neoplastic process may be drawn from the cytologic patterns. The cytologic pattern of early carcinoma is characterized by persistent presence of at least five of the arbitrarily chosen cytologic criteria of malignancy, comprising more than one nuclear abnormality. Normal epithelial cells are usually seen. There is no complete cessation of sexual cycle, but it is often difficult to recognize the exact phase because of a persistence of polymorphonuclear leukocytes during estrus and because of the co-existence of different phases of the cycle in various portions of genital mucosa.
The cytologic pattern of advanced carcinoma prior to terminal necrosis is usually characterized by the presence of nearly all the cytologic criteria of malignancy including, as a rule, strikingly bizarre elongated cells. Few cells which don’t fulfill at least one criterion of malignancy are seen.

The cytologic pattern of advanced carcinoma in its terminal stage is characterized by scanty exfoliation of epithelial cells, with virtual disappearance of “normal” cells. Smears are loaded with polymorphonuclear leukocytes and red blood cells. There is no evidence of estrogenic activity since, in such cases, mucosa from which normal cells exfoliate is replaced by the malignant tumor.

Although daily vaginal smears probably would be required to determine the effect of the neoplastic process upon the estrus cycle, we were able to observe occasional cellular characteristics of estrus in smears exhibiting cytologic pattern of invasive carcinoma. It is of interest that Perry and others reported that there was no difference in the sexual cycle in animals which developed tumors and those which did not (6). The impression that the cytologic pattern of early carcinoma may have some definite meaning in the course of the development of a malignant neoplasm appears to be supported by the correlation with increased susceptibility of cervical cells to viral infections (2, 3).

For the present, the reported method of recording and scoring selected criteria of malignancy by several collaborators is sufficiently reliable to permit the diagnosis of the presence of a malignant neoplasm in individual mice. It also permits one to define cytologic patterns of early and advanced carcinoma even though it does not require making the distinction between malignant and nonmalignant cells. While cells, individual or in clusters, which exhibit simultaneous presence of several cellular criteria of malignancy are easily identified as malignant, there are numerous neoplastic cells in smears which defy such recognition by microscopic examination. This can be demonstrated by preparing a contact smear from the cut surface of a large induced carcinoma totally replacing the uterus. Such a smear undoubtedly contains malignant cells, but only some are sufficiently characteristic to be identifiable by careful scrutiny. However, the sum total of cellular abnormalities seen in such a smear is sufficiently indicative to determine the presence of a malignant neoplasm.

**SUMMARY**

Invasive cervical and/or vaginal carcinoma was induced in 100 per cent of C3H mice within a period of $4\frac{1}{2}$–5 months by applying 3,4-benzopyrene to the cervix through an otic speculum. The development of carcinoma may be evaluated accurately by a team of co-workers without taking measurements and without labeling individual exfoliated cells. By scoring cytologic criteria of malignancy in weekly vaginal smears, cytologic patterns are obtained which permit diagnosis of the presence of carcinoma, distinguish between early and advanced stages of malignant disease, but do not determine presence or absence of invasion. Smears from mice with early carcinoma were characterized by persistent presence of at least five of the factors, including usually more than one nuclear abnormality. As the neoplastic process extended, both the quantitative and qualitative scoring became higher. In terminal stages there was decreased exfoliation accompanied by marked infection and necrosis. This cytologic method of evaluation of a neoplastic process may also be used for determining the effect of substances being tested for interference with carcinogenesis.

**ACKNOWLEDGMENTS**

Authors are indebted to Doctors G. N. Papanicolaou, P. J. Fitzgerald, and B. McMahon for their reading and critical evaluation of the manuscript in preparation.

FIG. 1—9 show cells in smears stained by Papanicolaou's method and photographed under immersion oil with constant magnification ×1250.

FIGS. 10—12 show tissue sections stained with hematoxylin and eosin and photographed with magnifications indicated in corresponding descriptions.

FIG. 1.—Nucleated cells in vaginal smear of a control mouse with negative scores for criteria of malignancy after 20 consecutive weeks of observation.

FIG. 2.—Cells illustrating nuclear enlargement (+), hyperchromasia (+), and prominent nucleoli (+) found in a vaginal smear of a mouse 25 weeks after the beginning of benzpyrene treatment.

FIG. 3.—Bizarre, elongated (+), binucleated (+) cell with prominent nucleoli (+) is from vaginal smear of a mouse 21 weeks after the beginning of treatment.

FIG. 4.—A cluster (+) with cytoplasmic vacuolization (+) and a cell scored for engulfment (+) and keratinization (+) found in a vaginal smear of a mouse 22 weeks after the beginning of benzpyrene treatment.

FIG. 5.—Irregular nuclear borders (+) may be seen in a binucleated (+), bizarre, elongated (+) cell from a vaginal smear of a mouse 21 weeks after beginning of treatment.

FIG. 6.—Bizarre, cellular cluster (+) with prominent cytoplasmic vacuolization (+) and large (+), hyperchromatic (+) nuclei with irregular borders (+) found in a vaginal smear of a mouse 17 weeks after the beginning of benzpyrene treatment.

FIG. 7.—Binucleation (+) with nuclear enlargement (+) and prominent nucleoli (+) in cells from a vaginal smear of a mouse 22 weeks after the beginning of treatment.

FIG. 8.—Multinucleation (+), prominent (+) nucleoli and slight (±) elongation are illustrated in a cell from a vaginal smear of a mouse 21 weeks after the beginning of treatment.

FIG. 9.—A localized basophilic (+) area in the cytoplasm of a cell in vaginal smear of a mouse 23 weeks after the beginning of treatment.

FIG. 10.—Section of epidermoid carcinoma involving cervix and vagina of a treated mouse sacrificed 30 weeks after beginning of treatment. Figures 8 and 9 illustrate cells found in vaginal smear of the same mouse. Magnified ×50.

FIG. 11.—Different field of the same section of epidermoid carcinoma as shown in Figure 10 illustrates an epithelial pearl. Magnified ×500.

FIG. 12.—An area adjacent to the epithelial pearl illustrated in Figure 11 was chosen for illustration of cellular details in tissue. Magnified ×1250.
Induced Cervical Carcinoma of the Mouse A Quantitative Cytologic Method for Evaluation of the Neoplastic Process
