Desoxypyribose Nucleic Acid from Isolated Chromosome Threads in Experimental Epidermal Methylcholanthrene Carcinogenesis in Mice*

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(Received for publication August 15, 1946)

INTRODUCTION

In the major cancer research project conducted at this hospital, cytological, biochemical and biophysical changes that supervene in the epidermis of mice as a result of applications of methylcholanthrene have been followed. This approach to the problem of induction of malignancy has resulted in the discovery of distinct cellular and intercellular transformations leading to an early establishment of a new chemical equilibrium in the altered epidermis, which is maintained, substantially without further modifications, throughout the "latent period" of carcinogenesis. The steps by which these transformations are effected have been integrated and embodied in reports published by Cowdry (12-14).

The biological importance of nucleic acids in growth and development has recently been reviewed by Davidson and Waymouth (20) and by Davidson (19). There is increasing recognition of a causal connection between the processes connected with growth and the spatial distribution of nucleic acids in the cell. Thus disturbances in the cellular nucleic acids accompanying neoplasia have been noted by Caspersson and Santesson (7), Koller (24) and Stowell (33, 35). Stowell and Cooper (34) measured the relative amounts of Feulgen stained thymonucleic acid in normal epidermis, hyperplastic epidermis and epidermoid carcinoma with a microphotometric apparatus. They detected in general a fall in the thymonucleic acid content per unit volume of hyperplastic epidermis, and a rise in the carcinomas as compared with the normal epidermis. Carruthers and Suntzeff (6) have recently made quantitative determinations of desoxyribose nucleic acid in epidermal cells during carcinogenesis. They observed a progressive decrease in the amount of this substance from normal to malignant stages through the intermediate hyperplasia. The observations of these last investigators are in harmony with their earlier findings on the trend of biochemical changes in epidermal carcinogenesis. Our objectives have been (a) to isolate chromosomes from the epidermal and carcinomatosus cells and collect them as free as possible from other cellular constituents; (b) to determine quantitatively desoxyribose nucleic acid in these chromosomes during epidermal carcinogenesis.

MATERIAL AND METHODS

Swiss mice were treated with methylcholanthrene, as already described by Cowdry (12). Epidermis was separated from the underlying dermis at room temperature using the method devised by Suntzeff and Carruthers (36), which involves stretching the skin tightly over a board covered with transparent plastic and scraping the epidermis off the dermis. This technic is a distinct improvement over the one adopted earlier by Baumberger, Suntzeff and Cowdry (1) in that while the latter method employed a temperature of 50° C., the critical temperature for biological activities, for softening the collagenic fibers and thus to separate the epidermal layers from the corium, the new procedure is carried out at room temperature, thus eliminating the attendant dangers of water loss and deleterious effects on the cells.

The separated epidermis was immediately dropped
into vials containing physiological saline surrounded by freezing mixtures or dry ice. In the same manner solid pieces of transplanted squamous cell carcinomas, free from visibly necrotic areas, were dropped into similar containers. The lines of transplantable skin carcinomas of mice, induced by methylcholanthrene, employed by us were started nearly five years ago by Cooper, Firminger and Reller (11), and to date have been carried through 40 passages. Healthy and non-necrotic tumors, kindly donated by Drs. Cooper and Suntzeff, were cut up into small pieces, approximately 2 mm. in size, and were introduced subcutaneously into mice 6 weeks old. Some mice of the same age were also injected subcutaneously with fine fragments of the tumor suspended in physiological saline. The rate of growth of the transplanted tumor in the mice was at least double that of the injected population judged by the time lapse between introduction of the material and expression of optimum growth. Best results were obtained with tumors harvested 2 weeks after implantation. Although there were individual variations between tumors of this age, the majority of them showed little necrosis or cellular degeneration. The frozen tissues showed no visible structural alterations before isolation of chromosomes.

EXPERIMENTAL PROCEDURE

The technic of isolation of the chromosomes was that of Claude and Potter (9) and Mirsky and Pollister (25, 26) slightly modified. The basis of isolation was recognition of the fact that disruption by mechanical breakage of the nuclear and cell membranes releases the diverse components of the cellular system into the surrounding medium. As demonstrated by Bensley (2), Claude and Potter (9) and Mirsky and Pollister (25, 26) these components can be segregated, concentrated and purified by differential centrifugation. The final fraction readily lends itself to further study and chemical analysis. By using 1.5 per cent citric acid at about pH 3 as employed by Dounce (22, 23) and Ziegler (38), the nuclei were loosened but at this low pH nucleoproteins were undoubtedly denatured. Moreover it was thought that the cells were subjected to a milieu antagonistic to normal cellular activities. Hence this experimental procedure was abandoned.

Following the technic of Claude and Potter (9), who used leukemic cells of the C58 strain of mice and leukemic Wistar rats, the epidermal tissues were ground with sand in a mortar before centrifugation, but the results were unsatisfactory because it was extremely difficult to eliminate the fine particles of sand from the final fractions. The method that was finally developed consisted of grinding the separated epidermis, or the fragments of tumor, in a coarse mortar at a temperature of 1° to 2° C., until most of the cells and nuclei were crushed. The crushed mass was frozen, suspended in physiological saline at a temperature of about 1° to 2° C., and further disintegrated by subjecting to the action of a high speed Waring blender which had been cooled in dry ice before use.

Although it was comparatively easy to disintegrate cancer cells by this method, both normal and hyperplastic epidermal cells offered a great deal of resistance and longer treatment, both in the mortar and blender, was necessary to rupture them. Even so, some of the epidermal cells remained unbroken.

For a satisfactory separation, control of two variables was found to be of paramount importance, namely: the maintenance of a temperature between 0° and 5° C. and the regulation of pH from 7.2 to 7.4 throughout the course of the experiment. Under these conditions, the chromatin threads that are in suspension retain their distinctive fibrous appearance. The schedule of differential centrifugation was in all essential respects similar to that outlined by Claude. The instrument used was a model PR1 International Refrigerated Centrifuge adjusted to maintain a temperature of 0° C.

Two experimental errors are not completely eliminated: (a) Loss of some extremely fine chromatin strands near the limits of microscopical resolution because of their failure to sediment with the rest of the chromatin material, and (b) contamination of the chromatin threads by small amounts of cytoplasmic particles and mitochondria. Because these defects in the method are applicable more or less equally to all three types of tissues studied, they were not deemed serious enough to vitiate the results.

The chromosomes, thus isolated, are ready for microscopical examination and biochemical analysis. They are Feulgen-positive and give a distinct blue color when subjected to the diphenylamine reaction of Dische (21) as employed by Seibert (32), Dounce (23) and Car ruthers and Suntzeff (6). In quantitative evaluation of the deoxyribose nucleic acid the Dische reaction was applied to isolated chromosomes dried in vacuo, and the percentage transmission of the characteristic blue color at 600 m£ was measured with a Coleman spectrophotometer. The standard curve was made by Dr. Car ruthers from pure sodium deoxyribonucleate by plotting the concentration of this substance against the percentage transmission. The actual nucleic acid concentration was derived by multiplying the concentration of the sodium salt by 0.944.

OBSERVATIONS

Morphology.—Since most of the epidermal nuclei were in the resting stage and only a small proportion of them in various phases of mitoses, the bulk of the isolated chromosomes was evidently derived from the
resting nuclei. The position of the primary constric-
tion is clearly recognizable in many of the chromosomes,
especially in the stretched out condition. The centro-
meric regions appear as Feulgen-negative gaps between
the long and short arms of the chromosomes (Figs. 1 to
3). The final purified sample appeared white and had a
viscous consistency. Under dark field illumination the
chromatin appeared as distinctly fibrous, rod-shaped or
granular bodies of varying length but of more or less
uniform diameter, the latter being usually from 0.5 μ

Many of the thicker threads were presumably
derived by an elongation and partial despiralization of
the heteropycnotic or chromocentric regions so clearly
seen in intact resting nuclei. The thicker threads
were more heavily charged with thymonucleic acid, as
revealed by the Feulgen stain, than the finer strands.
This does not necessarily mean that there is inherently
a greater concentration of deoxyribonucleic acid per
unit length of these chromosomes. It is not at all
likely that a more tightly coiled state exists in them,
as shown by Coleman (10) in the case of the differ-
entially condensed X-chromosomes of the Acrididae and
by White (37) in the heteropycnotic X-chromosomes
of the Tettigoniidae and Acrididae.

On strictly morphological grounds it is difficult, if
not impossible, to distinguish between chromosomes
derived from normal, hyperplastic, or epidermal cells
(Figs. 1 to 3). This is hardly remarkable since micro-
scopic examination of the normal, hyperplastic and mal-
genous cells shows the same types of nuclear architecture—
chromatin strands and heteropycnotic masses on the so-called “net work” with the diameters of the intra-
nuclear strands corresponding with that of the isolated
fibrous chromatin. However, the chromosomes isolated
from squamous cell carcinoma appear to be more densely
stained with the Feulgen stain than those of the other
tissues. This agrees with the cytochemical findings
described below. Because of his failure to observe loose
ends in intact resting nuclei, Claude and Potter (9)
came to the conclusion that the chromatin threads were
oriented end to end to form a continuous filament. We
cannot agree; for chromosome threads are distributed in
random fashion throughout the nuclear space and allow-
ance has to be made for the fact that they represent structures spatially distributed in three dimensions. It
would, therefore, be extremely difficult to detect free
ends. Moreover, the weight of cytological evidence,
gained by a study of the mitotic cycle, militates strongly
against a continuous end to end alignment of chromo-
somes.

Cytchemistry.—The formed elements easily go into
solution in 1 molar sodium chloride and in distilled
water, leaving highly refractile granules. They may be
represents in the form of fibrous material by the
addition of water. Other physicochemical properties
detected in fibrous nucleoproteins by Minsky and Pollis-
ter (25, 26) and Minsky (27) have also been observed
but will not be described here.

Significant differences were found in the deoxyribose
nucleic acid content of the chromosomes of normal,
hyperplastic and carcinomatous cells (Table I). It
will be seen that the average content of deoxyribose
nucleic acid in normal epidermal chromosomes is 0.129
mgm. per mgm. of dry weight, or, roughly 13 per cent,
whereas in mice painted thrice with methylcholanthrene
it is 0.091 mgm. per mgm. of dry weight showing a
30 per cent drop in deoxyribose nucleic acid as com-
pared with the normal. Increasing the number of paint-
ings to 6 has no significant effect on the nucleic acid
content of the chromosomes.

TABLE I: DESOXYRIBOSE NUCLEIC ACID OF CHROMOSOMES
IN EXPERIMENTAL EPIDERMAL CARCINOGENESIS

<table>
<thead>
<tr>
<th>No. of mice</th>
<th>No. of paintings</th>
<th>Time after first treatment to killing of mice</th>
<th>DNA* per mgm. dry wt.</th>
</tr>
</thead>
<tbody>
<tr>
<td>13</td>
<td>1</td>
<td>0.108</td>
<td>NORMAL</td>
</tr>
<tr>
<td>11</td>
<td>2</td>
<td>0.136</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>2</td>
<td>0.154</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>2</td>
<td>0.118</td>
<td></td>
</tr>
<tr>
<td>46 (Total)</td>
<td></td>
<td>Average 0.129</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>3</td>
<td>0.098</td>
<td>METHYLCHOLANTHRENE-TREATED MICE—HYPERPLASTIC</td>
</tr>
<tr>
<td>10</td>
<td>3</td>
<td>0.083</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>3</td>
<td>0.090</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>3</td>
<td>0.092</td>
<td></td>
</tr>
<tr>
<td>41 (Total)</td>
<td></td>
<td>Average 0.091</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>6</td>
<td>0.091</td>
<td>SQUAMOUS CELL CARCINOMA</td>
</tr>
<tr>
<td>17</td>
<td>6</td>
<td>0.099</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>7</td>
<td>0.127</td>
<td></td>
</tr>
<tr>
<td>40 (Total)</td>
<td></td>
<td>Average 0.106</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>3</td>
<td>0.171</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>3</td>
<td>0.211</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>3</td>
<td>0.203</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>3</td>
<td>0.220</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>3</td>
<td>0.226</td>
<td></td>
</tr>
<tr>
<td>44 (Total)</td>
<td></td>
<td>Average 0.208</td>
<td></td>
</tr>
</tbody>
</table>

* DNA = Deoxyribose nucleic acid.

In malignancy, however, there is an increase which,
although it is variable in the different animals examined,
nevertheless reaches spectacular heights compared with
chromosomes of the normal and hyperplastic epidermises.
If, for instance, we exclude the average amount (0.171
mgm. per mgm. dry weight) of deoxyribose nucleic
acid in squamous cell carcinoma obtained from the first
8 mice examined (Table I), the remaining instances
are found to maintain a level which is more than 60 per cent above that in the chromosomes of normal epidermis and certainly more than double the nucleic acid content of chromosomes for hyperplastic epidermis. It must be pointed out, however, that in all such studies based on analyses of components of whole tissues, including averages of several samples, we are handicapped by not being able to recognize any differences that may be localized in the tissues. It is not inconceivable that a marked localized change in a few cells might be masked by including with them numerous others which are not altered in the same manner. Notwithstanding this, we believe that the general trend of changes in the desoxyribose nucleic acid content of normal, hyperplastic and carcinomatous epidermal tissues, as reflected in the newly established equilibrium, is sufficiently clear to warrant suggestive inferences.

DISCUSSION

The change that takes place in desoxyribose nucleic acid content of the chromosomes of hyperplastic epidermal cells and of squamous cell carcinoma from that of normal epidermis appears to be in conformity with the altered economy of the cell. It is not clear how methylcholanthrene operates to produce an altered yet unequivocal evidence that breakdown products of equilibrium at the precancerous level. Nor is there as content of chromosomes for hyperplastic epidermis. methylcholanthrene, under investigation by W. L. Simpson in this hospital, are the causal agents in differentiation into long and short arms, separated by Feulgen-negative gaps, which correspond to the position of the centromeres.

The photomicrographs were made from Feulgen stained preparations and are magnified approximately 700 diameters. In the ringed areas, the morphology of chromosomes is distinctly seen. Many of the chromosomes show a differentiation into long and short arms, separated by Feulgen-negative gaps, which correspond to the position of the centromeres.

1. Chromosomes isolated from normal epidermis.
2. Isolated chromosomes from hyperplastic epidermis.
3. Isolated chromosome concentrate from squamous cell carcinoma.
present, the vegetative nucleus undergoes a succession of polymitotic divisions giving rise to several generative nuclei. To all intents and purposes the pollen grain behaves like an "encapsulated tumor." This exceptional behavior of the vegetative nucleus is presumably brought about by action of the supernumerary heterochromatic B-chromosomes. The activity of so-called inert chromosomes would, therefore, seem to indicate that nucleic acid supply is a crucial variable in the causation of neoplastic growth. From this viewpoint Darlington (18) visualizes in normal growth a correct euchromatin-heterochromatin balance. Morbid mitoses, he thinks, are produced when this balance is upset.

From a detailed cytological analysis of chromosome behavior in 565 human tumors (carcinoma of the skin, esophagus, colon, rectum, larynx, lung, cervix, uterus and breast) Koller (24) has come to similar conclusions. He maintains that the quantitative change in nucleic acid metabolism revealed by chromosome behavior may be one of the deciding criteria of distinction between normal cells and tumor cells derived from them. According to him the normal functioning of a cell is conditioned by a specific heterochromatin-euchromatin balance. Since the amount of nucleic acid and the rate of its production determine the frequency of division, Koller argues that there must be a causal connection between abnormally high concentrations of desoxyribose nucleic acid and intensified division rate and malignancy.

That there is an increase in the amount of Feulgen-positive material in rat sarcoma as compared with normal rat fibroblasts was histochemically shown by Cowdry (15) from a study of tissue culture of these cells. Stowell (35) devised a photometric histochemical method for the determination of thymonucleic acid in normal, hyperplastic and neoplastic epidermis of mice and men, and established the order of decreasing mean amount of thymonucleic acid per unit volume of tissue and per cell. Hyperplastic epidermis in which the cells were large, contained less thymonucleic acid per unit volume of tissue than the normal epidermis and the carcinomas contained more desoxyribose nucleic acid per unit volume of tissue than the normal epidermis. Using homologous normal and tumor tissues (rat liver, rat hepatoma, mouse lung and mouse lung tumors) Schneider (30) discovered that the desoxyribose nucleic acid content of both tumors was much higher than that of their normal homologues.

In contrast to the findings of the previous workers Dounce (23) found no increase in the desoxyribose nucleic acid of nuclei isolated from Walker carcinosarcoma 256, normal liver and rat hepatoma 31, but actually observed a decrease in the hepatoma cells. It is difficult to explain this apparent discrepancy unless we admit that it is due to differences in technic, and the types of tumors used. Moreover, as Stowell (35) has shown, concentrations of desoxyribose nucleic acid vary greatly in different types of tumor and even in different portions of the same tumor. In addition, the rates of division and of growth have a pronounced effect on the quantitative relationships of the nucleic acids in the tumors. Thus, a high rate of division with its accompanying disturbances of euchromatin-heterochromatin equilibrium is more likely to cause a succession of chromosomal aberrations than one where the rates of division and growth are slow (cf. Koller). It is now known from the work of Brachet (5), Caspersen and Schultz (8), Schultz (31), Darlington (18), Painter (28, 29) and others that, since desoxyribose nucleic acid is lowest in resting nuclei, the presumption is justified that its synthesis is also very low. It is maximal at metaphase and in tumors with a higher mitotic index there would be a corresponding increase of desoxyribose nucleic acid. It should also be emphasized that any disturbances in the mitotic mechanism are bound to have their impact on the dividing cells. This is in conformity with the sorghum-phenomenon of Darlington and Thomas referred to earlier.

Carruthers and Suntzeff (6) found in hyperplastic epidermis of mice treated with methylcholanthrene and in squamous cell carcinomas less desoxyribose nucleic acid than in normal or benzene-treated epidermis. This does not really conflict with the present findings since their analyses were based on unit weights of tissue and not on unit weights of chromosomes. It should also be remembered in this connection that the decrease in the desoxyribose nucleic acid is probably due to the increase in the size of the cells as demonstrated by Cowdry and Paletta (16).

On the basis of present experiments, we are unable either to support or refute the concept of a definite causal connection between disturbed nucleic acid balance and neoplastic growth. It is clear, however, that the decrease in the desoxyribose nucleic acid values taking place in hyperplastic epidermis is not necessarily dependent on the nucleocytoplasmic relationships of the cells. It is actual, as revealed by cytochemical determinations of chromosomes on a dry weight basis. In squamous cell carcinomas instead of a projection of the altered equilibrium so manifestly seen in hyperplastic epidermis, we witness a notable modification. The desoxyribose nucleic acid is sharply increased. In normality, the nuclear system appears to be in a state of dynamic equilibrium in which there is a regular and periodic removal and replacement of desoxy and ribose nucleic acids, respectively. In malignancy this equilibrium is violently upset and there is a greater accumulation of desoxyribose nucleic acid in the chromosomes, resulting in a multitudinous series of mitotic aberrations of the
type described by Koller (24) and Darlington and Thomas (17). From the cytochemical evidence at hand the conclusion that there is a constant association of increased desoxyribose nucleic acid with squamous cell carcinoma is thus inescapable and because nucleic acids are so intimately bound up with the genetic structure of the cellular system, it would, therefore, seem that quantitative changes in their attachment or detachment from the chromosomes assume more than trivial interest. It is irrelevant for our argument whether this augmentation in carcinoma is the result of greater storage or greater synthesis of desoxyribose nucleic acid or lesser conversion of the latter into the ribose type.

We have remarked earlier that there are not sufficiently discernible features between chromosomes isolated from normal and abnormal tissues to warrant a distinction on a purely morphological basis. This does not necessarily mean that there are no enlarged chromosomes in neoplastic tissues, as observed by Biesele and Cowdry (4). Since only a few cells are in mitosis in the hyperplastic and carcinomatous cells and fewer still in metaphase, it would not be a simple matter to detect enlarged chromosomes among the enormous number of threads presented by chromosome concentrates. However, the earlier observations of Biesele on chromosomal changes in epidermal carcinogenesis and the assumption of polytene (multiple strand) structure of neoplastic chromosomes need to be revised. A valid criterion of distinction between chromosomes isolated from different states of epidermis would be the recognition of quantitative differences in their desoxyribose nucleic acid content.

SUMMARY

1. A mechanical method of separation and isolation of chromosomes from the tissues of mouse epidermis in normality, hyperplasia and squamous cell carcinoma is described.

2. Cytochemical determinations of desoxyribose nucleic acid from the three stages in carcinogenesis are given. Soon after the application of methylcholanthrene there is seen a sharp drop in the concentration of desoxyribose nucleic acid in the chromosomes, which is maintained at a new equilibrium level until the onset of carcinoma. In this state there occurs a striking increase in the desoxyribose nucleic acid content of the chromosomes, far above that of chromosomes in normal and hyperplastic epidermis.

3. The bearing of the disturbances of nucleic acid equilibrium on carcinogenesis is discussed.

ACKNOWLEDGMENT

We wish to express our grateful thanks to Dr. C. Car ruthers of this Hospital for his generous cooperation in the cytochemical aspect of this investigation. We are also appreciative of the many helpful suggestions offered by Dr. W. L. Simpson.

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