Desoxyribonucleic Acid in Epidermal Carcinogenesis Induced by Methylcholanthrene*

Christopher Carruthers, Ph.D., and V. Suntzeff, M.D.

(From The Barnard Free Skin and Cancer Hospital, St. Louis 3, and Department of Anatomy, Washington University Medical School, St. Louis 10, Missouri)

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The role in cellular activities of desoxyribonucleic acid combined with proteins is well known, and the importance of this compound for normal and cancerous cells has been reviewed recently by Stowell (7). Since we have recently completed analyses of minerals in epidermal carcinogenesis (1) as a part of our investigations, it seemed desirable to determine quantitatively the desoxyribonucleic acid content of the epidermis during this transformation. The integration of the chemical, physical, and histological studies in our series has been reviewed by Cowdry (2).

EXPERIMENTAL PROCEDURE

Desoxyribonucleic acid was determined by means of the Dische diphenylamine reaction (4), as used by Seibert (6). The blue color was measured with a Coleman spectrophotometer at wave length γ 600. The standard curve was made with a very pure sample of sodium desoxyribonucleate kindly given to us by Dr. Jesse Greenstein, of the National Cancer Institute.

In extracting the nucleic acid from the epidermis we followed essentially the procedure of Johnson and Harkins (5). A sample of the epidermis was ground in a mortar with a 5 cc. portion of ice-cold distilled water until the mixture was finely divided. It was then poured into a 50 cc. centrifuge tube with the aid of two 5 cc. portions of distilled water and three 5 cc. parts of 10 per cent ice-cold sodium hydroxide. The centrifuge tube was placed in an ice bath in a refrigerator for 3 to 4 days, and stirred occasionally with a small glass rod. At the end of this time the contents of the centrifuge tube were made just acid to litmus with glacial acetic acid and centrifuged. Both filtrate and precipitate, after washing with distilled water, gave a brownish color with the Dische reagent, and the filtrate was therefore unsuitable for measuring the desoxyribonucleic (hereafter denoted by DKN) acid content. Sufficient concentrated hydrochloric acid was accordingly added to the filtrate to make it just acid to Congo red paper; a flocculent white precipitate occurred immediately, which, when filtered off, gave a strong blue Dische reaction. To the hydrochloric acid filtrate was added an equal volume of 95 per cent alcohol, but no precipitation took place. Then a portion of this alcohol mixture was neutralized and heated on a steam bath to drive off the alcohol. The Dische reaction was negative on this mixture as well as on the neutralized and concentrated hydrochloric acid solution, both results showing that all the DKN had been precipitated.

The final procedure was as follows: After precipitation of proteins with acetic acid near pH 7.0 in the centrifuge tube, the tissue residue and proteins were centrifuged and the supernatant liquid was filtered into a 150 cc. beaker. Tissue residue and proteins were washed several times with a few cc. of distilled water, centrifuged, and the supernatant fluid added to the filter paper, which was finally washed with a few cc. of distilled water. The nucleic acid was then precipitated from the well-buffered solution in the beaker by the addition of concentrated hydrochloric acid until just acid to Congo red paper, but before this was done, 5 or 6 drops of a 10 per cent solution of aluminum sulphate were added to ensure complete coagulation of the acid. The DKN was next transferred to a 50 cc. centrifuge tube, centrifuged, and then allowed to drain. To the acid in the centrifuge tube was added 12 cc. of distilled water and 24 cc. of Dische reagent (ample for the tissue samples employed). Upon stirring the acid went into solution, after which it was transferred to an 8×1 inch Pyrex digestion tube. The latter was then placed in a boiling water bath for 10 minutes to develop the blue color, a solution containing 3 cc. of distilled water and 6 cc. of Dische reagent, heated at the same time with the unknown, serving as a blank. After the tubes had been cooled to room temperature by immersion in beakers of tap water, the intensity of the blue color was measured. The blank, with little color if any, was used as a reference fluid, which automatically compensated for the same amount of color developing in the unknown in the Coleman spectrophotometer. Occasionally the blue solutions were slightly

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turbid, and then were cleared by sucking them through a sintered glass filter funnel before measuring the color intensity.

The DKN content was determined on the transplantable squamous cell carcinoma that we have used in our previous studies (1). When samples of the tumor were finely ground in a mortar and treated with an equal volume of 10 per cent sodium hydroxide, as was the epidermis, a very viscous and jelly-like solution resulted, in contrast to the freely flowing suspension obtained from normal and hyperplastic epidermis. However, the viscosity of the alkaline solution gradually diminished and at the end of 4 or 5 days' refrigeration it flowed freely, and could be treated exactly as was the epidermal suspension for the precipitation of the DKN.

New Buffalo and Swiss mice of both sexes, painted with 0.6 per cent methylcholanthrene solution in benzene as in our previous investigations (1), were employed. The animals were sacrificed 5 days after the last application of the carcinogen.

RESULTS

The results, expressed as milligrams of desoxyribonucleic acid per 100 mgm. of tissue, appear in Table I. The epidermis of normal untreated mice contained an average of 0.935 mgm. DKN per 100 mgm., while the benzene-treated had an average of 0.967 mgm. per 100 mgm. Therefore benzene alone had no appreciable effect upon the DKN content. The epidermis of mice killed 5 days after 1, 2, and 3 applications of the carcinogen in benzene contained averages of 0.584, 0.578, and 0.624 mgm. of DKN respectively; these figures are 37, 37, and 33 per cent less than the normal. The epidermis of mice sacrificed 5 days after 6 and 12 paintings had averages of 0.741 mgm. and 0.681 mgm. of DKN per 100 mgm. respectively, or 21 and 24 per cent less than normal. Five samples of squamous cell carcinoma contained 0.582 mgm. of DKN per 100 mgm., which was about 38 per cent less than normal. This decrease can be largely explained from the cellular morphology of the tumor (Tumor II of Cooper, Firminger, and Reller [10]), which is well differentiated with enlarged vesicular nuclei, and with a tendency to keratinize.

Since the DKN values for hyperplastic epidermis were below normal the following experiment was carried out according to the method of Dische (4), to ascertain whether the DKN was more firmly bound to the cell proteins in hyperplastic epidermis than in normal, and also to determine whether any of the DKN was precipitated with the proteins at pH 7 when the cold sodium hydroxide suspension of epidermis was neutralized with glacial acetic acid. Three samples of hyperplastic epidermis from mice that had received 3 paintings of methylcholanthrene on alternate days and were sacrificed 5 days after the last application, were treated with cold sodium hydroxide and the proteins precipitated with glacial acetic acid as described in the experimental procedure. After the

TABLE I: DESOXYRIBONUCLEIC ACID IN EPIDERMAL CARCINOGENESIS

Number of mice	Number of paintings	Time after first treatment to killing of mice, days		Desoxyribonucleic acid per 100 mgm. tissue, mgm.
		NORMAL, UNTREATED MICE		
8				0.882
10				0.970
8				0.860
7				1.027
33 (total)			Average	0.935
		BENZENE-TREATED MICE		
8	3	10		0.924
8	3	10		0.978
8	3	10		1.001
24 (total)			Average	0.967
		METHYLCHOLANTHRENE-TREATED MICE		
3	1	5		0.545
3	1	5		0.623
6 (total)			Average	0.584
3	2	8		0.574
3	2	8		0.583
6 (total)			Average	0.578
3	3	10		0.618
3	3	10		0.639
4	3	10		0.615
10 (total)			Average	0.624
5	6	17		0.775
7	6	17		0.718
5	6	17		0.729
17 (total)			Average	0.741
4	12	31		0.680
5	12	31		0.708
5	12	31		0.769
4	12	31		0.656
18 (total)			Average	0.703
		TUMORS		
7				0.605
5				0.523
5				0.567
5				0.595
5				0.621
27 (total)			Average	0.582

proteins and alkali-insoluble cell constituents had been washed several times with distilled water by centrifugation, to remove the DKN extracted by the cold sodium hydroxide, the proteins and cell constituents were treated with 10 cc. of $\frac{N}{2}$ sodium hydroxide and then heated on a steam bath for 2 hours. The volumes were kept constant at 10 cc. by the occasional addition of distilled water. At the end of 2 hours the solutions were neutralized with glacial acetic acid, allowed to stand for 1 hour, and filtered. To 5 cc. of the filtrates were added 4 volumes of 95 per cent alcohol, and the mixtures were allowed to stand overnight to precipitate completely any DKN that might be present. The minute amounts of precipitate were centrifuged and dissolved in water, and the Dische reaction was found to be negative on all 3 samples. The remainder of the filtrates were made just acid to Congo red paper in 15 cc. centrifuge tubes and the small amounts of precipitate were centrifuged and dissolved in water; the Dische reaction was negative on one sample, and only a barely visible bluish tint was observed in the other two. Cold sodium hydroxide therefore extracted practically all the DKN from the epidermis, and there was no evidence of a firmer combination of DKN with cell proteins in hyperplastic epidermis than in normal.

DISCUSSION

The chemical evidence for disturbance of nucleic acids in tumors has been given by Stowell (7), and need not be recapitulated here. Since our studies were confined to normal, hyperplastic epidermis, and a squamous cell carcinoma they are comparable to those of Stowell, who used a special microphotometer to measure the relative absorption of monochromatic light in sections of tissue stained by the Feulgen reaction for thymonucleic acid (8). He found that normal mouse epidermis contained more thymonucleic acid per unit volume than did hyperplastic skin (8), and the latter contained less of the acid as the hyperplasia increased. Moreover, Stowell and Cooper found that the thymonucleic acid content of human hyperplastic epidermis decreased by 24 per cent per area, whereas the mean amount of acid per cell increased in some preparations and decreased in others (9).

The decrease in the desoxyribonucleic acid content of hyperplastic mouse epidermis as measured microchemically is in agreement with these observations (8), except that in our studies the mean amount of DKN was increased with increasing hyperplasia. With respect to carcinoma, some of Stowell's specimens contained more, some less DKN per unit volume (7), while all our samples of a transplantable carcinoma had less of the acid than did normal or benzene-treated epidermis. As stated by Stowell and Cooper (9), the decrease in the DKN content of hyperplastic cells is probably due to the increased size of the cells, earlier established by Cowdry and Paletta (3).

SUMMARY

Hyperplastic epidermis contained less desoxyribonucleic acid than did normal or benzene-treated mice, and a transplantable squamous cell carcinoma had less of the acid than did normal epidermis, and about the same amount as the early hyperplasias.

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