SELECTIVE SEPARATION OF EPIDERMAL CELLS FOR CYTOFLUOROMETRIC ESTIMATION OF NUCLEAR DNA

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Received for publication January 26, 1978

A simple procedure used to isolate epidermal cells for the cytofluorometric estimation of their nuclear DNA content is described. The principal part of this method involves the microdissection of fixed tissue sections as well as ultrasonic irradiation. The loss of Feulgen-stainable nuclear DNA during treatment with ultrasonics, using lymphocytes and spermatozoa in control experiments, was estimated at approx. 2% of the original content. In using this method for determining the nuclear DNA content of human epidermis, it was found that the presence of individual cells was maximal in the stratum basale and minimal in the uppermost layer of stratum granulosum.

Ultraviolet microspectrophotometry of nucleic acids was first described by Caspersson (4). This quantitative histochemical method, based on the ultraviolet light absorption of nucleic acid purine bases at 257 nm wavelength, made possible the measurement of the nucleic acid content of individual cells. Cytophotometric methods based on this principle or the visible light absorption of specifically stained DNA have been used to show the nuclear DNA values in epidermal cells and their variations in various pathological status (13, 19, 26, 35, 39). However, there exist some difficulties in the cytophotometric Feulgen-DNA measurement using tissue sections; 1) the obvious difficulty of preventing the measured nuclei from including nuclei which are partially cut off and therefore contain only a small portion of the nuclear material, 2) in measuring a single nucleus, since the epidermal cells are in close contact with each other and their size and shape vary considerably, this measurement may come to include parts of the nuclei of the adjacent cells present in the measuring field, and 3) the nuclei of the epidermal cells in vertical sections vary in shape, often flattened and elongated. The disadvantages enumerated above could be eliminated by separating the epidermis into single cells, allowing Feulgen-DNA measurement in whole cells. The action of different enzymes has been applied to dissociate the epidermis into its constituents (1, 12, 21, 29, 32). Recently, Haag *et al.* (14) used diluted acetic acid to dissociate human epidermis. All chemical or enzymatical methods possess a common disadvantage in causing the nucleic acids

This study was supported partly by grant-in-aid for scientific research from the Ministry of Education, the Japanese Government (No. 248233).

to be measured to more or less decompose (7, 8, 37). Also, in using these techniques the topographical relationships are lost, entailing difficulties in the identification of various cell types. This study initially aimed to develop a simple method for the selective isolation of human epidermal cells, in which the Feulgen-stainable material would be affected as minimally as possible, and secondly, to estimate the nuclear DNA in each layer of the human epidermis.

MATERIALS AND METHODS

Biopsy skin specimens were removed from the thighs of five healthy males, age 22 to 25 years. They were fixed in 10% neutral-buffered formalin for 24 hr at 4°C, embedded in paraffin, and cut at 15 μ m thickness. They were dissected into small pieces under the dissecting microscope. After flotation in physiological saline, they were irradiated for 5 min with ultrasonics (300 W, 26 KC), and after being centrifuged at 400 g for 5 min, the sediments were smeared on glass slides. For controls, both normal lymphocytes and artificially-ejaculated spermatozoa were collected from five healthy males. They were floated in physiological saline, and then centrifuged at 400 g for 5 min. One portion of the cell sediment was smeared on a glass slide. Another portion was embedded in paraffin after being fixed in 10% neutral-buffered formalin, and cut at 15 μ m in thickness.

For the staining of DNA by the Feulgen method, the slides were rehydrated in distilled water for 10 min. Acid hydrolysis was performed in 5 N hydrochloric acid at 25°C for 40 min, followed by a 10 min rinse in running water. Pararosaniline Schiff's reagent was diluted to 0.05% in glycine buffer adjusted to pH 2.3. The diluted Schiff's reagent was chilled beforehand to 10°C in a cold water bath, and Schiff's reaction carried out at this temperature for 10 min (9). Then, the slides were transferred to a bisulfite rinse which had been continued at 10°C. After 3 changes of the bisulfite solution, the slides were washed thoroughly in running water for 1 hour, dehydrated with 100% ethanol and mounted with non-fluorescent embedding medium (Entellan, Merk). In order to stabilize the specific fluorescence, the slides were post-irradiated for 6 hr with green light containing the excitation peak for pararosaniline Feulgen staining (10, 11).

The microfluorometer Olympus MMSP-FR was used for cytofluometry (Olympus Optical Co. Ltd., Tokyo, Japan). For excitation, green light through an interference filter AL540 with the red-suppressing filter BG38 was used. The barrier filter used was R610.

RESULTS

1. *Selective isolation of epidermal cells*

Paraffin-embedded skin sections were dissected into small pieces containing various numbers of cells, then irradiated with ultrasonics. Fig. 1 shows the relationship between the number of cells in the small pieces to the number of isolated cells. When a small piece contained 5 or fewer cells, the number of isolated cells was about 75% of the original cell number in the small pieces (Fig. 2). The number of isolated cells decreased as the number of cells in the small pieces increased.

FIG. 1. The relationship of the cell number in small pieces to the number of isolated cells. Abscissa: Number of cells in small pieces. Ordinate: Isolation rate in %.

2. *Effect of hydrolysis conditions on the intensity of the Feulgen reaction*

Human lymphocytes and spermatozoa were smeared on glass slides. The air-dried smears were fixed in 96% ethanol for 20 min. Hydrolysis for 1 to 60 min was performed in the following four ways: 1) 1 N HCl at 60°C, 2) 1 N HCl at 25°C, 3) 5 N HCl at 60°C, and 4) 5 N HCl at 25°C.

Fig. 3 shows the curves of the mean fluorescence intensity for 1 N HCl hydrolysis at 60°C, 1 N HCl at 25°C, 5 N HCl at 60°C and 5 N HCl at 25°C. In 1 N HCl hydrolysis at 60°C, the curve changed rapidly and the period of optimum hydrolysis was very brief. In 5 N HCl hydrolysis at 60°C, the change in the curve of fluorescence intensity was tremendously rapid. The optimum hydrolysis time of 5 min was followed by a rapid decline in fluorescence intensity with increasing hydrolysis time. In 5 N HCl hydrolysis at 25°C, the change was much slower, with a higher fluorescence intensity. As the temperature and concentration of acid increased, the optimum hydrolysis occurred earlier. These results show that there is a considerable plateau of Feulgen reaction intensity by hydrolysis with 5 N HCl at 25°C.

3. *Effect of ultrasonics to the intensity of the Feulgen reaction*

Human lymphocytes and spermatozoa which had been floated in physiological saline were irradiated for 1 to 15 min with ultrasonics. As shown in Fig. 4,

FIG. 2(A). Small piece of stratum basale dissected from tissue sections. (B). Isolated single cell. Giemsa stain.

FIG. 3. Effect of acid concentration and temperature on the Feulgen intensity of spermatozoa (A) and lymphocytes (B). These specimens were fixed in 96% ethanol for 20 min and hydrolyzed in 1 N HCl at 60°C (—), in 1 N HCl at 25°C (---), in 5 N HCl at 60°C (---) or in 5 N HCl at 25°C (-.-). Abscissa: Hydrolysis time in min. Ordinate: Fluorescence intensity in arbitrary units.

FIG. 4. Effect of ultrasonic irradiation on the Feulgen intensity of spermatozoa and lymphocytes. Abscissa: Irradiation time in min. Ordinate: Fluorescence intensity in arbitrary units.

TABLE 1 *Effect of paraffin-embedding and ultrasonic irradiation procedure on the Feulgen intensity**

	Treated	Control
Spermatozoon	14.47 ± 0.6048	14.80 ± 0.6412
Lymphocyte	28.10 ± 1.7136	28.82 ± 1.8001

* Expressed as arbitrary units. Each figure represents the average of 200 determinants $\pm$ standard deviation of the mean. Experimental procedures as described in the text.

FIG. 5. Frequency distributions of the Feulgen intensity of individual cells in each epidermal layer. Abscissa: Relative fluorescence intensity of nuclear DNA to the diploid DNA ($2n$) of human lymphocytes. Ordinate: Number of cells measured.

FIG. 6. Frequency distributions of the Feulgen intensity of individual cells in each epidermal layer. Samples were irradiated with green light. Abscissa: Relative fluorescence intensity of nuclear DNA to the diploid DNA ($2n$) of human lymphocytes. Ordinate: Number of cells measured.

little change was found in fluorescence intensity after 5 minutes of irradiation. On the other hand, the effect of the paraffin-embedding and ultrasonic irradiation procedure on the fluorescence intensity of both lymphocytes and spermatozoa is shown in Table 1. The decrease of fluorescence intensity was about 2.2% in the case of spermatozoa, and 2.5% in lymphocytes after this procedure.

4. Cytofluorometric estimation of nuclear DNA in each layer of epidermis

The cytofluorometric data are shown in Figs. 5 and 6. The mean of the lymphocyte nuclei which were treated as skin samples was used as the reference value ($2n$). In the case of non-post-irradiated samples, the histogram of the basal cells indicated the greatest proportion of cells containing the $2n$ value of DNA, with some cells located around the $4n$ value. This tendency was more prominent in the samples irradiated with excitation light. Between the DNA values of $2n$ and $4n$ a relatively small number of cells were found. In the uppermost layer of stratum granulosum, however, the greatest proportion were found to be outside the DNA value of $2n$. No cells were found around the $4n$ value.

DISCUSSION

Epidermal cells are closely concentrated and their sizes and shapes vary considerably. Therefore, the measurement of a single nucleus may involve a portion of the nuclei of adjacent cells appearing in the measuring field. This possibility is furthered by the measurement of the maximal diameter, in order to ensure that the section contains a complete nuclei (1). Thus, it was necessary to separate and isolate intact nuclei of the epidermis. Various enzymes have been employed for this purpose; e.g., trypsin (5, 22), chymotrypsin (1, 33), collagenase (3), elastase (20), pancreatin (24), and hyaluronidase (15). Haag *et al.* (14) applied dilute acetic acid to dissociate epidermis into individual cells. However, these methods cause the nuclear DNA to decrease by about 30% as compared to purely mechanical

separation. Furthermore, in such preparations it is difficult to identify the cells of epidermal each layer. Iwasa (16) was successful in isolating the cells from the fixed tissues by ultrasonic irradiation following bromelain treatment. According to his work, the loss of nuclear DNA during preparation is about 6.3% in the case of lymphocytes, and 5.9% in Ehrlich ascites tumor cells. As shown in Table 1, the loss of nuclear DNA in the present method was about 2.2% in the case of spermatozoa, and 2.5% in lymphocytes. Thus, the present method seems to be useful for the isolation of epidermal cells by the cytofluorometric estimation of nuclear DNA.

Feulgen reaction is a common method for demonstrating DNA, and is also used widely as a quantitative modification in absorption cytophotometry. Kasten (18) investigated a great number of dyes with side chains similar to that of pararosaniline for their applicability in the Feulgen reaction. He found numerous substitutes for pararosaniline, some of which were known as fluorescent dyes from the classes of acridines, azines and xanthenes. Furthermore, Ploem (27) observed that pararosaniline Schiff stained nuclei also exhibit a red fluorescence when excited with green light. Using fluorescence, the excited molecules are light sources of their own and the total amount of light quanta emitted from the fluorescent structure is equally measured by a photocell, whether these structures are evenly or irregularly distributed within the measuring field. Thus, with cytofluorometry, the distributional errors inherent in absorption cytophotometry are avoided. However, there exist considerable problems such as fluorescence fading and partial non-linearity between fluorescence intensity and concentration of fluorescent substance. Böhm and Sprenger (2) and Prenna *et al.* (28) asserted that a linear relationship does exist between the content of Feulgen-DNA and the intensity of the fluorescent light, unless the concentration of the Feulgen dyes exceeds a certain limit. Fujita and his co-workers (9, 10, 11) noted that heavy staining with Feulgen dyes clearly destroys the proportionality between the Feulgen-DNA content and the total amount of fluorescence of the nucleus. Furthermore, they found that destruction of the primary fluorescence occurred in the background with stabilization of specific fluorescence of pararosaniline by irradiation of specimen with the strong excitation light. According to this study, proportionality between the amount of fluorescence and DNA content is perfectly maintained and the standard deviation in each class of ploidy came to be less than 5% of the mean DNA value. As shown in Figs. 5 and 6, the total fluorescence values of cell nuclei in each epidermal layer were widely distributed before irradiation with the excitation light. The number of basal cells between the fluorescence values of 2n and 4n ploidy-class decreased after the irradiation. Little change was found in the distribution pattern of fluorescence values, although the values of all the cell nuclei decreased to some degree. From these results, it seems likely that the amount of DNA decreases gradually during keratinization.

In general, the nuclei of epidermal cells disappear in the stratum corneum when keratinization proceeds normally. However, the mechanism of this disappearance is unknown. Although it has been suggested that a portion of DNA is contributed to cornified cells (25), direct chemical analysis of normal cornified cells showed no DNA (30). It is thought that deoxyribonuclease (DNase) is responsible for the first degradative step of DNA during the keratinization process,

although the role of DNase in the epidermis is still unclear. With histochemical and biochemical methods, some researchers (6, 17, 23, 31, 34, 36, 38) found a higher activity of DNase in the lowest level of the horny layer. These data suggest that the epidermal DNase plays a role in the disappearance of nuclei during physiological keratinization. The present results seem to correspond with this, although the actual mechanisms involved in the reduction of DNA in the final stage of cornified cell formation remain uncertain.

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