INHIBITION OF MITOTIC ACTIVITY OF THE CORNEAL EPITHELIUM DURING CHRONIC PYROGENAL-INDUCED STRESS IN ALBINO RATS

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Injection of pyrogenal daily for 5 days caused no change in the diurnal index of labeled nuclei (ILN): it was 67.0% in the cornea of control rats and 69.1% in stressed rats. Cytophotometric analysis with a control for section thickness showed that chronic exposure to stress led to a significant increase in the number of tetraploid nuclei from 3.2 to 7.7%. There were no significant changes in the percentages of the other classes of nuclei. Neither polyploidization nor evidence of accumulation of cells in the G_2-phase was found. The absence of a progressive increase in the number of tetraploid nuclei or of more marked polyploidization against the background of prolonged depression of cell division and a stable ILN can be explained by the circadian nature of the response of mitotic activity to stress. Even during chronic exposure to stress the depression of cell division which was observed in the afternoon and evening was not found in the morning, at the peak of mitotic activity. This circadian shunt contributes to maintenance of the modal ploidy of the corneal nuclei.

KEY WORDS: moderate hypothermia; mitotic activity; cornea.

The writer showed previously that prolonged depression of mitotic activity of the corneal epithelium in chronic pyrogenal-induced stress is not accompanied by any change in the number of DNA-synthesizing nuclei [8]. This depression is determined by adrenal hormones [5]. A similar picture was observed during exposure to another stressor, namely moderate hypothermia.

The object of this investigation was to study the nature of inhibition of mitotic activity in the corneal epithelium during prolonged exposure to pyrogenal-induced stress.

EXPERIMENTAL METHOD

Male rats weighing 140-190 g were used. A state of chronic stress was induced by intravenous injection of pyrogenal (5 μg/100 g body weight) between 11 a.m. and noon for 5 days. Intact animals served as the control. The experiments were carried out 30 h after the final injection. It is at that time that depression of cell division is observed [7]. There were two series of experiments: in series I, to rule out any shift in the circadian rhythm between the level of DNA-synthesizing cells and mitotic activity, the diurnal index of DNA-synthesizing nuclei was determined autoradiographically. For this purpose, every 6 h during the 24-h period 2 μCi thymidine-^{3}H was instilled into the eye of a rat before sacrifice. The instillation was repeated 4 times in the course of 1 h (at intervals of 15 min), for thymidine, when applied to the cornea, is washed out much more rapidly than it is eliminated after parenteral administration. Autoradiographs were prepared from the corneas in the usual way. The index of labeled nuclei (ILN) of the stratum basale and stratum spinosum was determined after examination of 2000-3000 nuclei and expressed as a percentage. In this series of experiments 17 animals were used. In the experiments of series II, to assess the possible role of delay in the G_2 phase or of a higher degree of polyploidization as an alternative pathway of mitosis during stress, the DNA content in the epithelial nuclei was determined cytophotometrically. The corneas were fixed in a mixture of ethanol and acetic acid (3 : 1) and embedded in paraffin wax. Sections were cut to a thickness of 5 μ, which was measured accurately on the ORIM-1 microscope by the method described in [2].*

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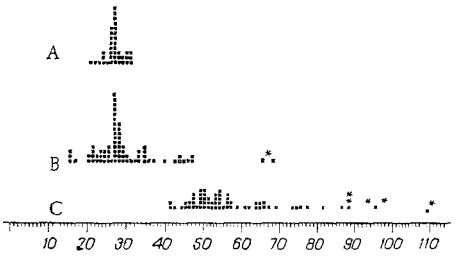

Fig. 1. Histogram of DNA content (in conventional units) in spermatozoa and corneal nuclei of albino rats depending on thickness of sections. A) Spermatozoa; B) section through cornea 3.48 μ thick; C) section through cornea 6.04 μ thick; *) metaphase figures.

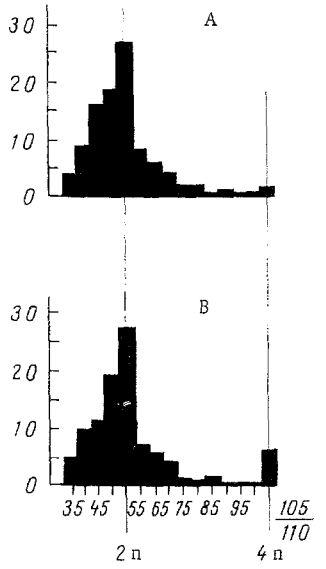

Fig. 2. Histograms of DNA content in nuclei of corneal epithelium from intact (A) and stressed (B) albino rats. Abscissa, DNA content (in conventional units); ordinate, frequency of discovery of cells with a given DNA content (in %).

After dewaxing of the sections hydrolysis in 5N HCl was carried out at room temperature, followed by staining with Schiff's reagent. Metaphase figures, as indices of the degree of ploidy [3], and spermatozoa taken from the epididymis and treated concurrently with the sections, were used as standards of ploidy for reference. The optical density of the nuclei and spermatozoa was determined in the Laboratory of Cytology, Institute of Human Morphology, Academy of Medical Sciences of the USSR, on a single-beam probe cytophotometer at a wavelength of 520 nm. Meanwhile, to determine the area of the nuclei, the sections were photographed and then drawn on chromatography paper, after which the drawings of the nuclei were cut out and weighed on an analytical balance. The DNA content was expressed in conventional units as the product of optical density and the weight of the nucleus. Altogether 50-80 nuclei from each animal were subjected to cytophotometry; altogether 18 animals – 10 intact and eight experimental – were used in this series.

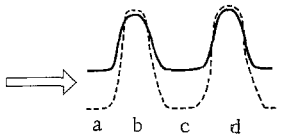

Fig. 3. Scheme showing changes in mitotic activity of corneal epithelium during chronic stress. Continuous line represents control animals, broken line experimental animals. a) Evening, 5th day; b) morning, 6th day; c) evening, 6th day; d) morning, 7th day. Arrow indicates stress for 5 days.

EXPERIMENTAL RESULTS

In the experiments of series I autoradiographic analysis showed that the diurnal ILN in the cornea of the control rats was 67.0 ± 2.7. No significant changes were observed in the stressed animals: their diurnal ILN was 69.1 ± 1.8. This is in agreement with the conclusions drawn from the writer's previous investigations, when chronic pyrogenal stress was shown not to change the number of DNA-synthesizing nuclei in the corneal epithelium.

In the experiments of series II determination of the thickness of sections revealed considerable variation, for even in the same strip of serial sections it could vary from 3.48 to 7.52 μ. The results of determination of DNA confirmed once again the need for verifying section thickness during cytophotometry. The distribution of nuclei by DNA content in sections from an intact animal, 3.48 and 6.04 μ thick, is shown in Fig. 1. Individual variations in a section 3.48 μ thick were less marked than those in a section 6.04 μ thick, in agreement with data in the literature [10]. The results of cytophotometry during analysis of the histogram were used with allowance for section thickness. The results were reduced to a standard thickness of 6.04 μ, for this height of section was found to correspond to partial agreement between 4 times the value of the DNA content of spermatozoa and the maximal value of the tetraploid standards, i.e., metaphase figures. The principal measurements were made on sections 3.71 and 4.06 μ thick; no sections thicker than 6.04 μ or thinner than 3.4 μ were used. Reconstruction of the nuclei places certain limitations on the identification of the class of ploidy to which they belong, but it is nevertheless possible to judge changes in the experimental animals compared with the intact. On the basis of examination of 423 corneal nuclei from stressed rats and 605 nuclei from control rats histograms were plotted (Fig. 2).

The results of these investigations indicate that the overwhelming majority (74.5%) of corneal nuclei in intact rats are diploid. Tetraploid nuclei, identified on the histogram from the zone of distribution of metaphases, accounted for 3.2% ($\sigma = 1.3$). Nuclei with an intermediate DNA content were found in 22.3% of cases. No nuclei with a DNA content of over 4n were found. Exposure to stress for 5 days led to a significant ($P < 0.001$) increase in the number of tetraploid nuclei, up to 8.7% in the corneas of the experimental rats ($\sigma = 2.25$). The number of diploid nuclei and also of nuclei with an intermediate DNA content showed no significant change (72.3 and 20.2% respectively). This twofold increase in the number of tetraploid nuclei was evidently the result of delay of the cells in the G_2-period, a characteristic feature of stress situations [1, 9]. The fact that depression of mitotic activity for several days was not accompanied by any progressive increase in the number of tetraploid nuclei or in the degree of polyploidization can probably be explained by the circadian character of the reaction of mitotic activity to stressors [4, 6, 7]. Depression of mitotic activity observed in the evening, 6 h after the final (5th) injection of pyrogenal [8], was not observed at 7 a.m. on the 6th day, but it developed again in the evening at 7 p.m. [7]. Cells delayed in the G_2-period during the afternoon and evening probably escape from the block in the morning, at the peak of mitotic activity, when the antimitotic effect of the stressors has disappeared [4, 6, 7]. This circadian shunt prevents accumulation of cells in the G_2-period in chronic stress. The scheme shown in Fig. 3 explains why the marked inhibition of cell division, lasting for 4 days after repeated action of the stressor, was not accompanied by any change in the number of DNA-synthesizing cells or in the degree of polyploidization of the nuclei.

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MECHANISMS OF POLYPLOIDIZATION OF MOUSE CARDIOMYOCYTES

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Most ventricular cardiomyocytes in mice aged 5-6 days are polyploid cells. By this time 60% of the cardiomyocytes have become binuclear and a further 10% have become mononuclear polyploid cells. Binuclear cardiomyocytes are formed as a result of acytokinetic mitosis, mononuclear tetraploid cells as a result of termination of mitosis in the initial phases.

KEY WORDS: cardiomyocytes; polyploidization.

Polyploidy is considered to be a feature which distinguishes the cardiomyocytes of primates from those of other mammals so far studied [14, 15], although it is now more than 10 years since Rumyantsev et al. [5, 7] found a certain number of polyploid cells in the rat myocardium. The study of polyploidy has been hindered by the fact that work has had to be done on tissue slices, when it is impossible to estimate the number of binuclear cells and determination of mononuclear polyploid cells (by DNA cytophotometry) gives rise to considerable distortion. The development of methods of dissociating the myocardium into single cells [2, 3, 10] has changed opinions on the composition of the cardiomyocyte population. Most cardiomyocytes in the myocardium of the adult mouse and rat have been found to be binuclear, i.e., definitely polyploid with respect to their combined genome. The present writers have shown [1] that with respect to DNA content some cardiomyocyte nuclei are tetraploid or octaploid. The object of this investigation was to study the origin of polyploid (mono- and binuclear) cardiomyocytes.

EXPERIMENTAL METHOD

Thymidine-^{14}C was injected subcutaneously in a dose of 1.5 μCi/g (specific activity 52 mCi/mmole) into 28 (CBA × C57BL/6)F_1 mice aged 3-4 days. The animals were killed after 1-36 h and the heart was placed whole in 10% formalin in Sorensen's buffer (pH 7.0). Next, in accordance with the method suggested in [2, 3], a cell suspension was prepared from the ventricles by dissociating them in KOH. One drop of suspension was placed on a slide. For DNA photometry, the Feulgen reaction was carried out on preparations of isolated cells (hydrolysis in 5N HCl for 10 min at 37°C, treatment with Schiff's reagent for 60 min at room temperature). The preparations were then coated with type M emulsion (Photographic Chemical Research Institute) and exposed for 14 days. The DNA content in the nuclei of the cardiomyocytes was measured on a Vickers M-86 microdensitometer. Before photometry, the preparation was drawn and the position of labeled cells marked on the drawing; the label was then removed. During photometry cells which had incorporated thymidine were located on this drawing. The mitotic index and the number of binuclear and labeled cells were determined by examination of 2000-3000 cardiomyocytes from each animal, in films stained by Giemsa's method. To determine the frequency of labeled mitoses at least 20 mitoses, usually 50-80, were found.

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