COMPARATIVE AUTORADIOGRAPHIC ANALYSIS OF DNA SYNTHESIS BY SMALL INTESTINAL ENTEROCYTES IN VIVO AND IN VITRO

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When the method of autoradiography is used for diagnostic purposes in man, it is unacceptable to inject the radioactive substances both systemically and locally (for example, attempts have been made to inject them subcutaneously in order to reveal areas of growth of the epidermis [4]). The method of autoradiographic investigation of biopsy material has therefore begun to be used [2]. Pieces of tissue taken from the patient are incubated with a radioactive precursor, after which the rate of its incorporation into biomolecules of the nucleus and cytoplasm of the cells is determined and, on the basis of these data, conclusions are drawn regarding the intensity of the same processes in vivo, but this cannot be accepted as correct in the absence of appropriate verification. It is essential to establish to what extent the results of autoradiographic study of biopsy material agree with the results of the study of the same material in vivo. The subject of such an investigation may be synthesis of nuclear DNA in rapidly renewed cells. The rate of incorporation of the DNA precursor ^{3}H-thymidine is estimated by analysis of the labeling density of the autoradiographs. The traditional method of such an analysis, namely visual counting of grains of silver above test structures, is laborious, and is less accurate than photometric methods with automatic recording and processing of the data [3, 5, 6].

The aim of this investigation was to study DNA synthesis by epithelial cells of the intestine in experiments in vivo and in vitro and to establish whether it is possible to evaluate processes in vivo on the basis of the autoradiographic analysis of biopsy material.

EXPERIMENTAL METHOD

Epithelial cells from crypts of the mouse small intestine were used as the test object. DNA synthesis by enterocytes was compared after administration of the radioactive label in vivo and in vitro. In the experiments in vivo the animals were given a single intraperitoneal injection of ^{3}H-thymidine in a dose of 10 μCi/g. The animals were killed 1 h later and pieces of small intestine from 1 to 3 mm^3 in volume were taken, washed in medium 199, fixed successively in 1% solutions of glutaraldehyde and osmium tetroxide in suitable buffers, and the material was dehydrated, embedded in Epon-Araldite resin by the standard method, and semithin sections were cut from it [2]. For the experiments in vitro the mice were killed and pieces of small intestine were incubated with ^{3}H-thymidine (dose 10 μCi/ml, 1 h, 37°C), washed with fresh medium 199, and processes as described above. Semithin sections from material of both groups were covered simultaneously with type M emulsion, exposed for 4 days, developed with D19 developer, and stained with toluidine blue.

The morphological investigation was carried out with an Axiophot light microscope ("Opton," West Germany), using photometry in reflected light. The method of analysis chosen enabled grains of silver to be observed as luminescent points against a dark background [1]. Correlation between the intensity of reflected light and the number of grains counted visually was studied by the method of least squares. The signal from the amplifier of the photoelectric cell was led to an HP-85B computer. The data were analyzed by a program including measurement of the background from unlabeled nuclei and the intensity of reflected light, with deduction of the background, with the above-mentioned number of measurements, assessment of the standard error, Student's "t" and the significance of differences between the groups compared, for two groups of autoradiographs obtained in vivo and in vitro.

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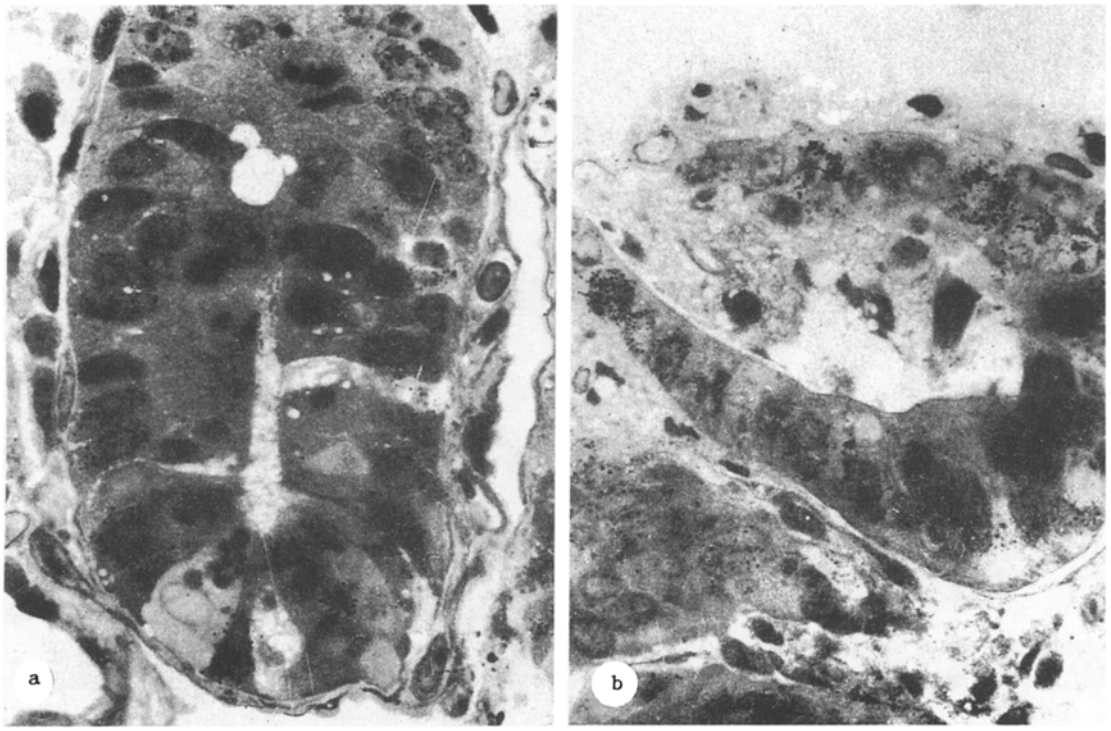

Fig. 1. Light-microscopic autoradiographs of epithelial tissue of mouse small intestine containing proliferating enterocytes labeled by injection of ^{3}H-thymidine in the crypts. a) In vivo, b) in vitro. 1000×.

EXPERIMENTAL RESULTS

Photographs of autoradiographs with intestinal crypt cells, reflecting DNA synthesis in vivo (Fig. 1a) and in vitro (Fig. 1b), are illustrated in Fig. 1.

No morphological differences in the structure of the crypts and of the cells in them were found in the two groups of autoradiographs, and differences in the intensity of labeling of cells identically distributed in the crypts, and also in the number and position of the labeled crypts in the sections, were observed. In longitudinal sections through the crypts, a density gradient of the label could be clearly distinguished in the direction from the base of the crypts to their middle, for the most intensively labeled cylindrical cells were located in the basal part of the crypts, where unlabeled Paneth's cells were seen. Correspondingly, in transverse sections through the crypts the intensity of labeling could vary depending on the height at which the section was cut through the crypt. However, on labeling in vivo the labeling density was found to be much higher than in vitro for cells correspondingly located in the crypts. Furthermore, in preparations obtained in vitro only crypts located at the edge of the fragment were labeled. It must be emphasized that in certain such crypts the labeling density was quite high and could reach 50% of the labeling density in vivo.

Before comparing quantitatively the labeling density in vivo and in vitro, we studied the possibility of automatic analysis of the preparations. The intensity of reflected light and the number of grains of silver, counted visually, were determined simultaneously for the same cell nuclei but from different preparations. The results for 140 arbitrarily chosen enterocytes are given in Fig. 2. The coefficient of correlation of the results obtained by the two methods reached 0.8, and the regression line plotted for them is described by an equation of the type:

$$I = 0.8N - 0.7.$$

Most of the points satisfy this relationship with an error of ≤ 20 %, which is admissible for such determinations [6] (the broken lines mark the boundaries of the corridors of errors within this same interval), but the error was greater in some measurements, for the size of the grain and possible fusion of the grains were not taken into account [5]. The labeling density

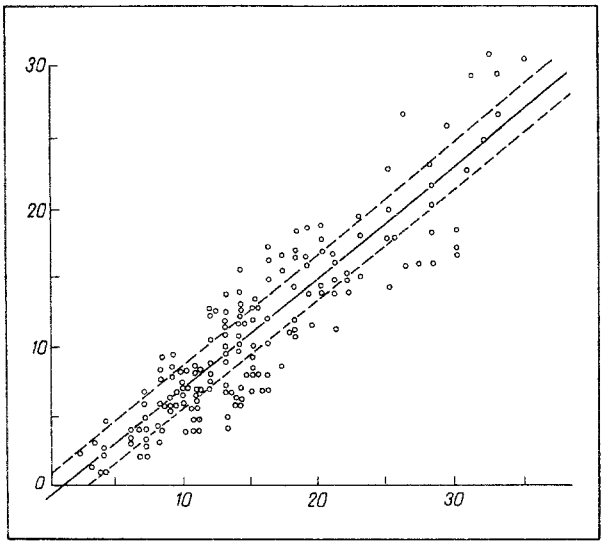

Fig. 2. Diagram of distribution of intensity of reflected light and of visual counting of grains of silver for labeled nuclei of small intestinal enterocytes and result of regression analysis of data. Abscissa, visual counting of grains of silver per nucleus (N); ordinate, intensity of reflected light from nuclei with label after deduction of background ($I - I_b$, arbitrary units).

TABLE 1. Results of Photometric Determination and Comparative Analysis of Labeling Density of Two Groups of Autoradiographs (10 preparations in each) Characterizing DNA Synthesis in Enterocytes Labeled in Vivo and in Vitro

Parameter		Autoradiographs	
		in vitro	in vivo
$I - I_b$		10,0	52,8
σ		0,9	4,7
j		50	54
Difference between means		42,8	
Student's "t'		8,8	
	p	$<0{,}001$	

was determined by a photoelectric method with an error of 5%. For grains of similar size, their number can be corrected by using the relationship thus found.

The results of photometric analysis of the two groups of autoradiographs are given in Table 1. The program which we developed greatly accelerated the quantitative analysis of labeling density and it can be recommended for the objective evaluation of autoradiographic data.

The significance of differences between the values compared was confirmed by a statistical method. This difference, in conjunction with the observed character of distribution of the label in the preparations in vivo and in vitro, indicates primarily that with labeling in vitro diffusion of ^{3}H-thymidine inside the pieces of material took place with great difficulty, and for that reason the number of labeled crypts was small. Consequently, biopsy specimens for autoradiographic investigation most be of minimal volume (1 mm^3). Inhibition of DNA synthesis did not take place in enterocytes of the biopsy material, and, as was pointed out above, with labeling in vitro some enterocytes with intensive labeling were found. The probability of reliable autoradiographic assessment is greater, the larger the number of biopsy specimens tested.

Thus epithelial cells of the crypts of the small intestine, which proliferate intensively in vivo, remain viable also in biopsy material. Under these conditions the principal characteristics of the proliferative process are maintained, but in labeled biopsy specimens the same labeling density is not achieved as during labeling in vivo. Because of slow diffusion of thymidine, cells located at the periphery of the incubated fragment are labeled. This must be taken into account during the autoradiographic

study of biopsy material. Meticulous standardization of the conditions of preparation of the autoradiographs also is important. Automated analysis of autoradiographs makes it easier to achieve the necessary accuracy and reliability of such investigations.

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