CELLS FROM PATIENTS WITH HOMOCYSTINURIA: A MODEL FOR STUDYING REPAIR MECHANISMS IN HUMAN CELLS

G. D. Zasukhina, Yu. I. Barashnev,
G. N. L'vova, T. A. Sinel'shchikova,
N. N. Shoniya, and A. N. Semyachkina

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Several models of molecular mechanisms of repair processes in human cells are now known: cells chronically infected with virus [3], mutant lines resistant to various mutagens. The most widely used model consists of cells from patients with hereditary diseases with a defect in their repair genes: xeroderma pigmentosum, ataxia-telangiectasia, Fanconi's anemia, etc. The present writers have described disturbances of repair activity in patients with Marfan's syndrome, which is found comparatively frequently in the population and is inherited as an autosomal-dominant condition [2]. All the systems studied are characterized by various defects of repair or replication. The discovery of new models by screening cells obtained from patients with hereditary diseases, using a test of repair activity to discover the defect, provides additional information on the mechanisms of repair of damaged DNA.

Homocystinuria is a genetically determined disturbance of metabolism of the sulfur-containing amino acid methionine, due to blocking of the enzyme cystathionine synthetase. As a result of secondary involvement of connective tissue in the pathological process in homocystinuria the phenotype of the disease is identical in some of its clinical parameters with Marfan's syndrome (involvement of the locomotor apparatus and eyes is similar in type).

The aim of this investigation was to study repair activity in cells of patients with homocystinuria.

EXPERIMENTAL METHOD

Two techniques were used: reactivation of damaged vaccinia virus, which enables the survival rate of the virus and induced mutagenesis to be assessed, and alkaline elution of DNA from membrane filters.

A culture of lymphocytes (48 h), obtained by the usual method from blood from healthy blood donors, was infected with vaccinia virus (Lister strain). Contact between virus and cells lasted 40 min at 37°C. The intracellular virus was treated with quanta of gamma-radiation (100 Gy) or with 10^{-5} M 4-nitroquinoline-1-oxide (4NQO) for 60 min. The samples were then washed to remove the mutagen, covered with fresh medium, and incubated for 24 h. To isolate intracellular virus the cells were frozen and thawed once or twice and titrated by the plaque method on a culture of chick embryonic fibroblasts. The survival rate of the vaccinia virus for each sample was calculated from the difference in titers of the virus in control and mutagen-treated cells. The mutability of the virus was estimated from the size of the plaques: plaques smaller than 2 mm in diameter were described as small, those measuring over 5-7 mm as large.

Alkaline elution was carried out by Kohn's method [4] with slight modification [5]. Peripheral blood lymphocytes, stimulated by phytohemagglutinin, were labeled 24 h after transplantation with ^{3}H-thymidine (1 mg/ml, 1 μCi/ml, specific activity 18.6 Ci/mmole) for 24 h, and then treated with 4NQO ($5 \cdot 10^{-6}$ M) for 30 min or with γ-rays (10 Gy). Repair after exposure to the chemical mutagen continued for 4 h, and after irradiation for 1 h.

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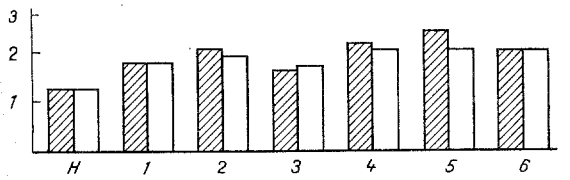

Fig. 1. Differences in survival rate of vaccinia virus treated with 4NQO (unshaded columns) or irradiated with gamma-quanta (shaded columns) in lymphocytes from healthy children (H) and children with homocystinuria (1-6). Ordinate, survival rate (log. PFU/ml).

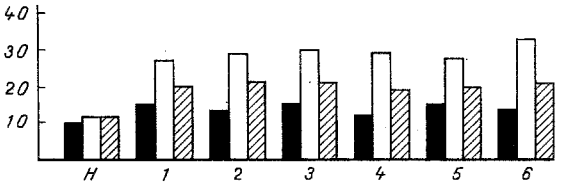

Fig. 2. Induced mutagenesis of vaccinia virus in lymphocytes from healthy children (S) and children with homocystinuria (1-5). Ordinate, number of small-plaque variants (in %). Black columns show spontaneous level of mutations, unshaded — after exposure to 4NQO, obliquely shaded — after gamma-irradiation.

The cells were harvested on millipore filters (1, 2μ, 24 mm) washed with cold Hanks' solution, and lysed at room temperature for 5 min in 5 ml of a solution of 0.2% laurylsarcosinate, 2M NaCl, 0.02M Na_2EDTA (pH 10.2). The filters were then washed with 3 ml of 0.02M Na_2EDTA (pH 10.2) and eluted with a solution of 0.1 N NaOH—Na_2EDTA (pH 12.6) for 2 h at a rate of elution of 0.3 ml/min, with collection of four 30-min fractions. Radioactivity was counted on Synpor filters (0.45 μ, 24 mm), the samples were precipitated after addition of bovine serum albumin (2 mg/ml) and TCA to 5% concentration. DNA remaining on the filter after elution was washed with 5% TCA and alcohol and the radioactivity of the acid-insoluble fraction was estimated by calculation of total radioactivity. The relative radioactivity of each fraction (K) was calculated by the equation:

$$K = (R_{tot} - \Sigma R_i)/R_{tot},$$

where R_{tot} denotes the total radioactivity of all eluted fractions plus radioactivity remaining on the filter after elution, and R_i denotes radioactivity of the i-th fraction.

4NQO and gamma-radiation were used as mutagens: they induced injuries which underwent "long" and "short" types of repair, respectively, and in turn, this allowed an integral investigation to be made of the functions of the two repair pathways in the cells.

EXPERIMENTAL RESULTS

The study of reactivation of vaccinia virus, treated with 4NQO or by γ-ray irradiation in lymphocytes obtained from children with clearly defined diagnostic features of homocystinuria revealed a marked decrease in survival rate of the virus (Fig. 1) in cells of all six patients studied. Incidentally, disturbances of both pathways of repair of damaged DNA after treatment both with 4NQO and with γ-rays were extremely rare in the patients' cells. For instance, only one patient, with xeroderma pigmentosum, was described in whom defects of repair of DNA, damaged by both ultraviolet and gamma rays, were found [1].

The level of induced mutations of the virus in the experiments with 4NQO and gamma-irradiation reached 12-15% in cells of healthy blood donors, whereas the formation of small-plaque variants in the patients' cells in experiments with 4NQO amounted to 30%, and in experiments with gamma-irradiation, to 20% (Fig. 2). Thus, the parameters of induced mutagenesis of vaccinia virus in cells of patients with homocystinuria were about twice as high as the spontaneous background.

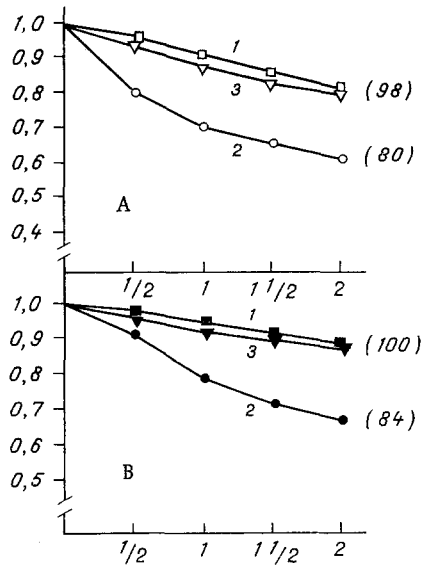

Fig. 3. Alkaline elution of DNA of lymphocytes from healthy blood donors, lysed on membrane filters. Abscissa, time of elution (in h); ordinate, relative radioactivity of eluted DNA fractions: A) Experiments with gamma-irradiation; B) experiments with 4NQO. 1) Control cells, 2) cells after treatment with mutagen, 3) cells after repair.

TABLE 1. Formation and Repair of DNA Breaks in Lymphocytes from Children with Homocystinuria

Experimental conditions	Healthy blood donors	Patients with homocystiria		
		№ 5	№ 6	№ 7
Gamma-irradiation (1 krad)	71±7	62	—	64
Gamma-irradiation + 60 min of repair	100±1	84	—	82
4NQO (5×10^{-6} M), 30 min	81±4	34	79	—
4NQO + 4 h of repair	102±2	83	79	—

Repair of DNA breaks in healthy donors' lymphocytes took place during 1 and 4 h after exposure to γ-rays and 4NQO, respectively (Fig. 3). The numbers on Fig. 3 are arithmetic mean values of relative radioactivity (in % of the control) of eluted DNA fractions. These parameters in lymphocytes of patients with homocystinuria after repair were 82.84% for gamma-irradiation and 79.83% for treatment with 4NQO, evidence of incomplete repair of the induced injuries (Table 1).

Thus, identical data were obtained for the cells of all patients with homocystinuria tested by the two methods, evidence of disturbance of repair of injuries to DNA induced both by γ-rays and by 4NQO. The method of reactivation of the virus in this case fixes the general disturbance of repair processes, whereas the method of alkaline elution reflects defects in the group of enzymes which, under normal conditions, perform resynthesis of RNA breaks. The results as a whole may serve as an additional laboratory diagnostic method for the detection of homocystinuria. Cells from patients with homocystinuria also provide a convenient model with which to study repair processes, for unlike most diseases described in the literature, they have disturbances of both the "short" and the "long" repair pathways.

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SURVIVAL OF XENOGRAFTS IN ANIMALS

A.N. Studitskii and N. N. Bosova

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Techniques of free autografting of muscles, developed in the writers' laboratory, have now become widely known and used both for research in experimental morphology and in surgical practice [8-12]. Various methods have been developed for preparing muscle tissue for transplantation of whole muscles and the conditions of its regeneration have been discovered [4, 6].

One favorable condition for free muscle grafting, as we know, is preliminary denervation, which induces a combination of changes in the muscle tissue that promote survival of autografted and homografted muscles [1-3, 5].

However, the most interesting subject from the theoretical point of view and the most promising for future use in surgical practice is interspecific grafting of whole muscles, to which this paper is devoted. We used the method of preparation of donor's tissue, suggested and developed by A. N. Studitskii, by wrapping it in cellophane film [7].

EXPERIMENTAL METHOD

There were three series of experiments. Wistar rats weighing 130-150 g were used as the donors. The recipients were C57BL mice, hamsters (*Mesocricetus auratus*), and guinea pigs.

Preparation of the donor's muscle: the right gastrocnemius muscle of the rats was wrapped in cellophane film, leaving the proximal and distal tendons free. Blood vessels and nerves supplying the muscle were left undisturbed. The muscle was kept under film for 9 to 12 months. Before grafting, the prepared muscle was removed from its bed, freed from the cellophane film, and cut into fragments corresponding in size to the excised muscles of the recipients. A fragment of donor's muscle was sutured by its end to the remnants of the proximal and distal tendons of the recipient's removed gastrocnemius muscle. The medial popliteal nerve was sutured to the graft.

In the experiments of series I rats were used as the donors and mice as the recipients. The material was fixed after 7, 14, 21, 30, 60, 270, and 330 days. In the experiments of series II the donors were rats and the recipients were hamsters. The material was fixed after 60 and 330 days. In the experiments of series III the donors were rats and the recipients were guinea pigs. The material was fixed after 3, 7, 14, and 60 days. Before fixation, contractile activity of the transplanted muscle was tested by stimulating the sutured nerves with an induction current. Histological preparations were stained with Heidenhain's azocarmine, with Romanovsky's azure-eosin, and Régaud's iron hematoxylin. Nerve endings were demonstrated by Bielschowsky's impregnation method.

EXPERIMENTAL RESULTS

A study of the state of the rat muscle after removal of the cellophane film at the end of 270-360 days showed that the muscles lost up to 40-60% of their weight. They were paler

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