LOSS OF DNA REPAIR CAPACITY DURING SUCCESSIVE SUBCULTURES OF PRIMARY RAT FIBROBLASTS

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ABSTRACT

Cultures of fibroblasts from newborn rats and successive subcultures of these cells were treated with 4-nitroquinoline-1-oxide to induce DNA repair. DNA from the cultures was examined by velocity sedimentation in alkaline sucrose gradients immediately after drug treatment and after a post-treatment incubation period of 3 h. Early passage cells were able to repair the damage that appeared as single strand breaks, however, by the seventh subculture this activity was not apparent. Measurements of repair synthesis showed a partial loss of this capacity with successive subculture. The results fit a model in which 4NQO causes two kinds of DNA modification, one of which is alkali labile and appears as a single-strand break. Both modifications are subject to excision repair, but each is recognized initially by a specific endonuclease. In the late passage cells, the endonuclease specific for the alkali labile modification is absent.

It has been well documented that the capacity for excision repair of DNA of established rodent cell lines such as mouse (9, 13, 16), hamster (12), and rat (3) is much less than that of established human cell lines (10, 12, 16). Mouse L cells seem to lack completely the ability to excise thymine dimers from their DNA (9), although they can perform other kinds of repair (16, 17) which can be interpreted to mean that the L cell lacks the thymine dimer-specific endonuclease but possesses other endonucleases plus the rest of the array of repair enzymes necessary to repair DNA damage other than the thymine dimer type. The supposed absence of thymine dimer specific endonuclease in the L cell is not due to an absolute genetic deficiency in the mouse, however, because Ben Ishai and Peleg (2) showed that primary cultures of mouse cells possessed virtually the same capacity to excise thymine dimers as human HeLa cells. However, when the mouse cells were subcultured, this ability declined and, by the seventh subculture, was undetectable.

We were interested in the generality of the loss of DNA repair capacity of cultured rodent cells, and so we undertook a study of the phenomenon in primary and successive subcultures of rat cells. 4-nitroquinoline-1-oxide (4NQO), a powerful carcinogen which mimics ultraviolet light in several of its biological effects (7, 15), was used as the damaging agent, and two measures of DNA repair were examined. First, repair synthesis was measured by the amount of radioactive thymidine incorporated into unreplicated DNA. Second, the disappearance of single-strand breaks was followed by the technique of sedimentation in an alkaline sucrose gradient. Previous studies indicated that the single-strand breaks observed resulted from the action of alkali on a 4NQO-induced modification of DNA and that the disappearance of the single-strand breaks was due to the enzymatic removal of the modification (17). In the present study, both kinds of repair activity

were readily demonstrable in primary cultures but fell progressively with successive subculturing so that, by the seventh subculture, repair synthesis was about 15% of its starting value while removal of alkali-labile modifications was not detectable.

MATERIALS AND METHODS

Newborn rats were used for the preparation of fibroblasts. The inner abdominal walls of four to six rats were dissected, minced with scissors, and then stirred for 30 min in a solution containing 0.5% trypsin in 0.015 M sodium citrate and 0.135 M KCl (pH – 7.8). After passage through sterile surgical gauze, the preparation was centrifuged. The cell pellet thus obtained was dispersed in Minimal Essential Medium (Grand Island Biological Co., Grand Island, N. Y.) to a density of 1×10^5 cells per milliliter. The medium was supplemented with penicillin, streptomycin, tylocin, and 10% fetal-calf serum. 30-milliliter portions of this cell suspension were then placed in 400-ml medicine bottles and incubated at 37°C in 5% Co_2, 95% air. After 2-3 h to allow the cells to stick to the bottles, the medium containing loose cells was poured off and replaced with fresh medium. Under these conditions, the cells doubled in 20–24 h. Cells in confluent monolayers thus generated were retrieved by scraping with a rubber policeman, diluted in half with fresh medium, and transferred into new bottles. After such transfer had taken place 12 times, the generation time was lengthened to approx. 36 h.

The ability of primary and subcultured rat cells for repair synthesis was assayed by the incorporation of [^{3}H]thymidine into the unreplicated DNA isolated by isopycnic centrifugation in CsCl. Monolayer cultures at various stages of subculturing containing 5×10^6 cells were obtained as described above and were treated with 1×10^{-5} M 4NQO or saline (controls) for 1 h. A further incubation was carried out for 3 h in fresh medium containing 5 μCi/ml of [^{3}H]thymidine (20 Ci/mmol) and 10^{-2} M hydroxyurea. 5-Fluorodeoxyuridine (10^{-6} M) and 5-bromodeoxyuridine (0.017 mM) were added one-half hour before 4NQO treatment and were maintained in the cultures until the cells were collected after the post-treatment incubation. The isolation of DNA and centrifugation in CsCl were detailed previously (4).

To examine the rejoining of DNA strand breaks, 1×10^6 cells in plastic Petri dishes (60 mm diameter) were labeled for one generation time with 0.5 μCi/ml of [^{3}H]thymidine. 4NQO (10^{-5} M) treatment was carried out for 1 h. The cells were then analyzed immediately or after a 3 h recovery in fresh medium. Sedimentation in alkaline sucrose gradients was performed as previously described (4, 17). Briefly, the cells were lysed for 10–12 h on top of 5–20% alkaline sucrose gradients contained in tubes for the Beckman SW 50.1 rotor. (Beckman Instruments, Palo Alto, Calif.). Sedimentation was carried out for 4.5 h at 15,000 rpm. Fractions containing 0.2 ml were collected from the top of the centrifuge tubes, and fraction number 13 corresponds to a sedimentation coefficient of 165S and single-stranded mol wt of 5 $\times 10^8$ daltons.

RESULTS

Figure 1 shows the banding positions of the rat fibroblast DNA in CsCl. Since bromodeoxyuridine was used in the experiment, semiconservatively replicated DNA has an elevated buoyant density. The presence of hydroxyurea, however, has effectively inhibited the formation of these "hybrid" molecules. Little or no "heavy" DNA was seen. The unreplicated (parental) DNA detected by OD measurements at 260 nm has a buoyant density of 1.70–1.71 g/cm^3. A comparison of the two sets of panels in Fig. 1 shows that 4NQO treatment has induced the incorporation of [^{3}H]thymidine into parental DNA. In an earlier study with myogenic L_6 cells, we showed that this incorporation was a consequence of repair synthesis and was not due to either a terminal addition reaction or a nucleotide pool effect (4). The specific activities of these fractions were measured, and, as shown in Table I, the repair synthetic activity decreased as the cells were subcultured. This decrease was not due to dying cells because it preceded by a considerable time the deceleration of cell growth, and at the seventh subculture a small amount of repair incorporation was still evident. The small amount of repair synthesis seen in the unirradiated control cultures is probably a technical artifact, and no significance is attached to it.

Figure 2 shows the alkaline-sedimentation pro-

TABLE I

Effect of Subculturing on Repair Synthesis in Primary Rat Fibroblasts

Subculture Generation	Repair synthesis			
	Controls		4NQO-treated	
	(1)	(2)	(1)	(2)
	cpm/μg DNA			
Primary	1.9	7.9	89.1	101.6
1		10.3		100.8
2	3.5		91.0	
3		6.4		56.2
4	7.7		31.2	
5		12.3		49.1
7	5.9		14.2	

The specific activities of parental DNA (see Fig. 1) were measured after the DNA had been dialyzed.

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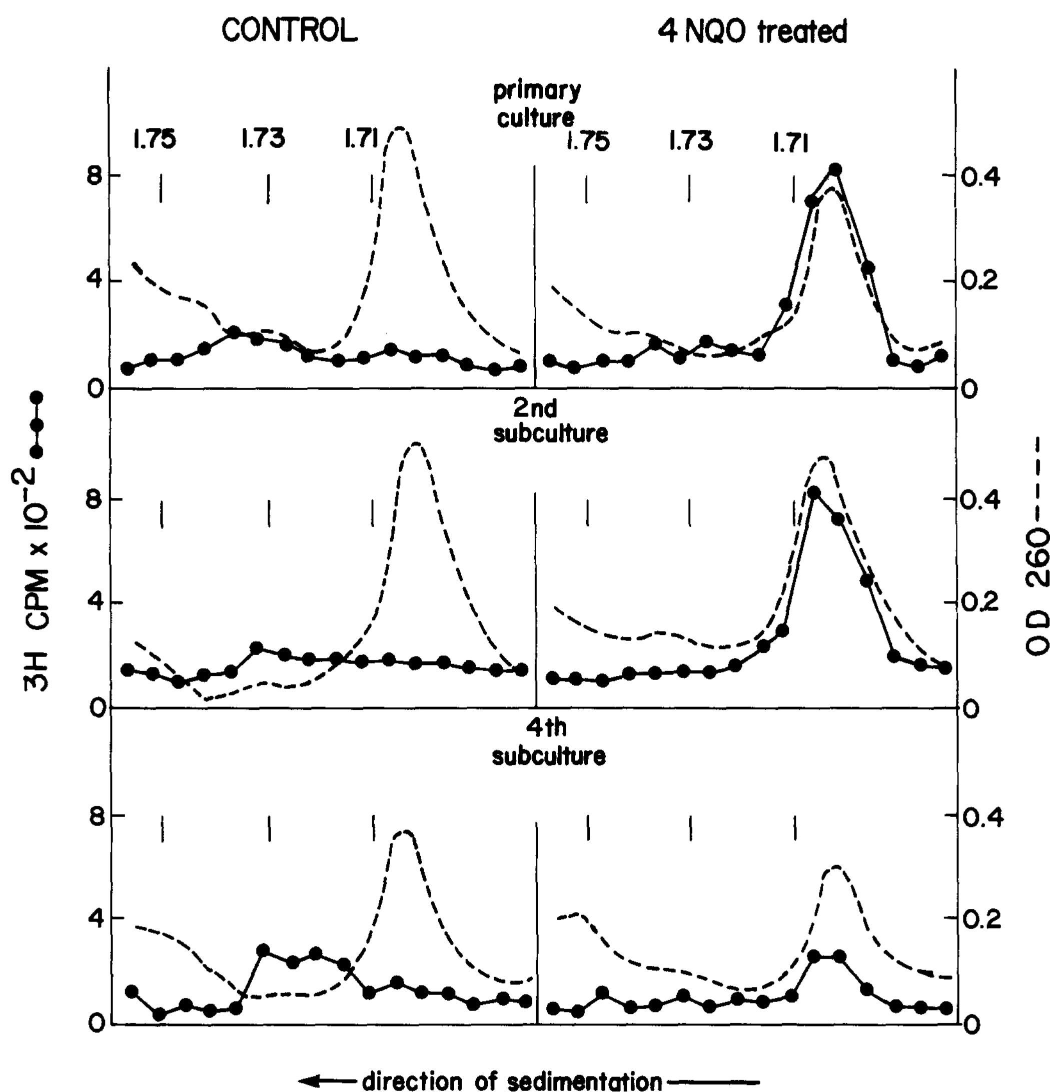

FIGURE 1 DNA repair synthesis profiles for rat fibroblasts at different subculture generations.

files of DNA from the cultured rat fibroblasts after treatment with 4NQO. The 4NQO treatment has caused the DNA to sediment more slowly, indicating the formation of single-strand breaks. This has been interpreted as the effect of alkali on DNA modified by 4NQO (4, 17). With a 3-h posttreatment incubation, DNA from the primary culture partially regained its size, indicating that some modifications on its DNA were removed. When cells from different subculture generations were examined in this way, a progressive decline in efficiency to remove the 4NQO modification was seen, and by the seventh subculture generation this type of repair was no longer apparent.

DISCUSSION

The decline in DNA repair capacity demonstrated in this study for successively subcultured rat fibroblasts parallels the decline in the ability of successively subcultured mouse cells to excise thymine dimers from their DNA (2) and thus provides another example from rodent cells of a culture-associated loss of repair capacity. This culture-associated loss of DNA repair capacity is also reflected in a number of established rodent cell lines (3, 9, 12, 13, 16). In contrast, human cells in culture have retained their capacity for DNA repair and only at the stage of senescence has a

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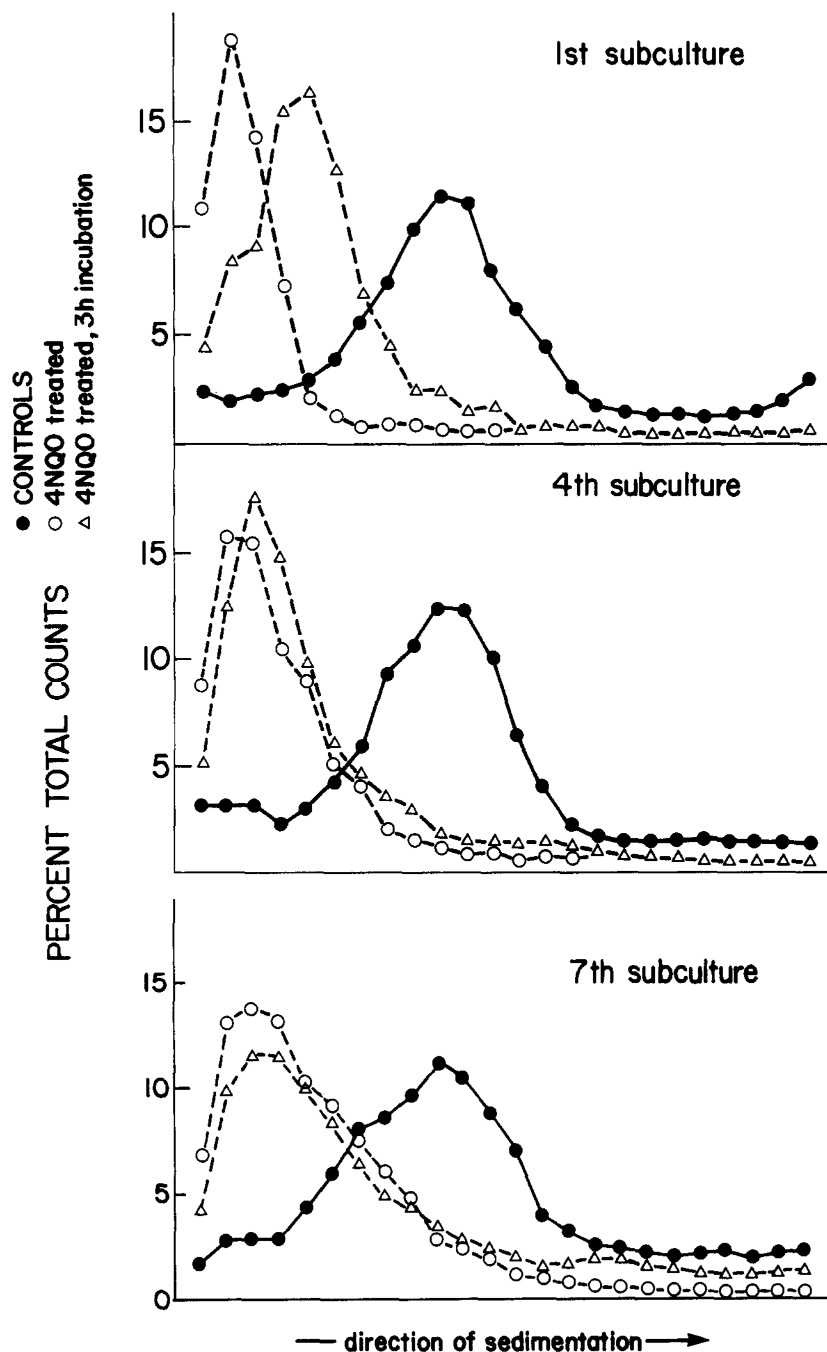

FIGURE 2 Sedimentation profiles of DNA from 4NQO-treated rat fibroblasts at different subculture generations.

significant decline in repair activity been noted (5). The loss of DNA repair capacity among cultured rodent cells is not a completely general phenomenon. Exceptions are seen in the mouse line BALB/c3T3, which exhibits high levels of DNA repair (11), and in the line A31-714, a derivative of BALB-3T3, which is as competent as human cells in removing covalently bound adducts of 4NQO (8). These exceptions may be due to the stringent subculturing protocol that defines the

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3T3 cell lines (1). Ben Ishai and Peleg (2) drew attention to the correlation between loss of repair capacity upon subculturing mouse cells and the acquirement of the malignant state by subcultured mouse cells (14). BALB/c cells also become malignant when the standard 3T3 subculture protocol is not adhered to (1). Another factor that may be at the base of the difference between human and rodent cells is the great difference in lifespan of the two species. The earlier mortality that characterizes rodent cells may be derived from an earlier loss of DNA repair capacity (6). Appropriate subculture conditions may well affect these processes.

Recently, we studied 4NQO-induced repair in the myogenic mouse cell line, L6 (4). This established line also has retained the capacity for DNA repair. However, it was found that after the myoblasts had fused and become myotubes, there was a partial loss in their capacity for repair synthesis and a complete loss of ability to remove DNA modifications that were observed as single-strand breaks in an alkaline sucrose gradient. A model to account for the observations was proposed. The similarity of the present results suggests that the same model applies. In essence, 4NQO induces two kinds of modification in DNA which can be removed by enzymatic repair processes. When the modifications are not removed, one of them appears as a single-strand break during alkaline sucrose sedimentation. The two modifications are recognized by separate endonucleases. Myoblasts or primary fibroblasts contain both activities, but myotubes or subcultured fibroblasts contain only one. In myotubes or subcultured fibroblasts, repair synthesis is reduced, and the unrepaired modification is observed as a persisting single-strand break in alkali. It should also be noted that 4NQO must produce a third modification in DNA which is observed as an irreparable strand break in alkali. That is, both myoblasts and primary fibroblasts can only partially restore the molecular weight of 4NQO-damaged DNA to its control size.

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