A TEST FOR MUTATION THEORY OF CANCER: CARCINOGENESIS BY MISREPAIR OF DNA DAMAGED BY 4-NITROQUINOLINE 1-OXIDE

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Summary.—Evidence for a mutation theory of cancer is presented by reviewing the experimental work on 4-nitroquinoline 1-oxide (4NQO) carcinogenesis. 4NQO almost completely mimics u.v. light and produces 4NQO-purine adducts on DNA. When 4NQO-treated cells are held in liquid medium under appropriate conditions, the 4NQO adducts disappear from DNA, in parallel to decrease of premutational damage in Escherichia coli, or pretransformational damage in cultured mouse cells. Post-treatment with caffeine greatly diminishes the yields by 4NQO of mutants in E. coli, malignant transformants in cultured mouse cells and tumour nodules in the lung of mice. Potentially tumourigenized stem cells in the lung remain sensitive to selective killing by caffeine for at least 5 days after 4NQO treatment, in spite of their DNA being apparently replicated, an indication that carcinogen-damaged DNA in the stem cell can be transmitted to its successive daughter stem cells for many generations. This peculiar characteristic is discussed as a possible lead to the crux of the mutation theory of cancer in vivo, and a model for carcinogenesis is proposed.

That cancer arises from mutations in somatic cells has long been a popular theory (Bauer, 1928; Boveri, 1929; Burnet, 1957) but it has seldom been subjected to rigorous experimental tests. On the other hand, the view that cancer is caused by viruses or abnormal cellular differentiation has been more rigorously tested experimentally (Dulbecco, 1969; Cold Spring Harbor Symp., 1974; Pitot and Heidelberger, 1963; Heidelberger, 1975). This difference may simply reflect the fact that our knowledge of animal viruses and cellular differentiation in mammals is more advanced than that on mutation in mammals. In spite of the pioneering work by Russell, Russell and Kelly (1958) in mutagenesis in mice, the complexity has hindered the progress of mutation research in higher forms. Therefore, to test the mutation theory of cancer, knowledge of the molecular mechanisms of mutagenesis in microorganisms must be applied (Witkin, 1969; Kondo et al., 1970; Drake, 1970, Doudney, 1976). This paper gives a brief review of experimental work (Kondo, 1976), a discussion on the mutation theory of cancer, and presents a model for carcinogenesis.

Molecular nature of 4NQO damage to DNA

4-Nitroquinoline 1-oxide (4NQO) mimics u.v. radiation in its mutagenic and lethal effects. Two u.v.-sensitive strains, Hs30R (uvrA-) and Hs30(uvrB-), which we isolated from B/r-type u.v.-resistant strains of E. coli B, H/r30R, and H/r30, (arginine-requiring (arg-) with an amber-type nonsense mutation at the argF locus) obtained from E. M. Witkin, showed about 30-fold higher sensitivity than the parental strains to both 4NQO and u.v. with respect to loss of colony-forming ability and mutation to arg+.
prototrophy (Kondo and Kato, 1968; Kondo et al., 1970).

Like some other chemical carcinogens (Miller, 1971), 4NQO reacts with DNA only after activation by cellular metabolism. 4NQO is first reduced to 4-hydroxyaminoquinoline 1-oxide (4-HAQO), then converted to aminoacyl-4HAQO by reaction with an appropriate amino acid, a reaction catalysed by aminoacyl-tRNA synthetase in the presence of ATP. Finally, aminoacyl-4HAQO produced 4NQO guanine or adenine adducts (Tada and Tada, 1976; Nagao and Sugimura, 1976), e.g. 3-(N^6. or N^1-adenyl)-4-aminopyridinium 1-oxide (Kawazoe et al., 1975; Nagao and Sugimura, 1976). Both 4NQO-guanine and 4NQO adenine adducts are excised from DNA of E. coli and cultured cells (mouse and human) if they possess excision repair ability for u.v.-induced DNA damage (i.e., pyrimidine dimers). Adducts are not excised from DNA of a wvrA strain of E. coli or from cells obtained from a xeroderma pigmentosum patient, which lack excision repair ability for pyrimidine dimers (Ikenaga, Ichikawa-Ryo and Kondo, 1975a; Ikenaga et al., 1975b). However, a small fraction of 4NQO-guanine adducts are labile, and disappear slowly from DNA in the absence of excision repair. This could be the cause of the X-ray-type DNA repair synthesis which occurs in 4NQO-treated xeroderma pigmentosum cells (Regan and Setlow, 1973). Thus, the majority of 4NQO damage can be regarded as u.v.-like. The number of 4NQO-purine adducts per lethal hit is almost equal to that of pyrimidine dimers in E. coli and cultured cells (Table).

![Graph](image)

**Fig. 1.**—(A) Decrease in 4NQO-induced premutational damage (○: for arg^+ mutations) (Kondo and Kato, 1968) or 4NQO-purine adducts (△, □) (Ikenaga et al., 1975b) during holding 4NQO-treated cells of E. coli strain H/r30R in nutrient broth at 37°C. (B) Decrease in 4NQO-induced pre-transformational damage (○) (Kakunaga, 1974) and disappearance of 4NQO-purine adducts from DNA (△) (Ikenaga et al., 1975b) in 4NQO-treated mouse cells during post-incubation in a non-DNA-replicating state.

**Excision repair of 4NQO-induced pretransformational damage**

4NQO-induced premutational damage and 4NQO-purine adducts decrease in parallel for wild-type cells of E. coli during post-4NQO incubation in nutrient broth at 37°C (Fig. 1). In a subclone, A31, of BALB/3T3 (Kakunaga, 1974) the yield of transformed cells also diminished with time of holding the 4NQO-treated cells under non-DNA-replicating conditions (contact inhibition), in reasonable agreement with the disappearance rate of 4NQO-purine adducts from DNA observed by Ikenaga et al. (1975b) (Fig. 1). From these results and others (Kakunaga, 1973; Ikenaga et al., 1975a, b; Kondo, 1976) we conclude that excision of 4NQO-purine adducts from DNA occurs at a rate similar to the rate of decrease of 4NQO-induced biological damage, i.e., mutation in E. coli or transformation in mouse cells. In addition, we conclude that excision repair in E. coli and mouse cells is error-free for 4NQO-purine adducts.
Evidence for misrepair carcinogenesis

In *E. coli*, a second type of repair exists (Setlow and Setlow, 1972) which is completely lost in *recA*− strains. This strain is immutable by u.v., 4NQO, and various other mutagens (Witkin, 1969; Kondo et al., 1970). *E. coli* strains defective at the gene *lexA* (exrA) are barely mutable by u.v. though they are not as sensitive to killing by u.v. as *recA*− strains (Witkin, 1969, 1976). Post-replication repair is a branched process, with an error-prone branch dependent on the *lexA* gene product, and an error-free branch, which deals with defects in daughter strands produced by inhibition of replication opposite the pyrimidine dimers (or 4NQO-purine adducts) in the template strands (Witkin, 1969, 1976; Setlow and Setlow, 1972; Kondo, 1976). However, no *lexA*−-equivalent clone is available for mouse. Witkin and Fair-Quharson (1969), however, demonstrated that caffeine post-treatment of u.v.-irradiated *E. coli* partially mimics the phenotype of the *lexA*− strain.

As observed for u.v. mutagenesis, post-4NQO treatment with caffeine (which was added to agar plates) enhanced up to an intermediate dose, but diminished at a higher dose, the 4NQO-induced mutation in *E. coli* wild type, whereas 4NQO mutagenesis in an excisionless strain was suppressed with increasing caffeine doses (Fig. 2). The frequency of transformation produced by 4NQO in mouse cells decreased with increase in caffeine dose administered for 48 h after 4NQO treatment (Kakunaga, 1975) (Fig. 2).

However, caffeine post-treatment enhanced up to intermediate doses, but diminished at a higher dose, the N-acetoxy-2-fluorenylacetamide - induced transformation in Syrian hamster cells (Donovan and DiPaolo, 1974) (Fig. 2). Nomura (1976) found that the average number of tumour nodules per lung in female mice treated with a single injection of 4NQO (12.5 μg/g) was decreased by about 70% by caffeine (5 injections of 100 μg/g at 6- or 12-h intervals) ad-

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**Fig. 2.**—Comparison of the caffeine-post-treatment effects on transformation in rodent cells, mutations in *E. coli* and lung tumourigenesis in mice. (A) Transformed cells induced in mouse cells (Kakunaga, 1975) and Syrian hamster cells (Donovan and DiPaolo, 1974). 4NQO-treated mouse cells and N-acetoxy-2-fluorenylacetamide-treated hamster cells were post-treated with caffeine at indicated doses for appropriate time before seeding on fresh plates to score transformed foci. (B) Mutations to prototrophy (arg+) induced by 4NQO (Ishikawa-Ryo, H. and Kondo, S., unpublished). 4NQO-treated cells of *E. coli* strains H/r30R (wild type) and Hs30 (turB; excisionless) were plated on semi-enriched agar plates containing caffeine at indicated concentrations. (C) Lung tumours induced in mice by a single dose of 4NQO (12.5 μg/g) (Nomura, 1976). Five doses of caffeine (100 μg/g each) at 6-12-h intervals were given before 4NQO (▽: the last dose 12 h beforehand) or after 4NQO (○: in the 0-36 h; ●: in the 120-156 h).
ministered immediately or 5 days after 4NQO (Fig. 2).

From a knowledge of postreplication repair of u.v. damage in cultured mammalian cells (Hanawalt and Setlow, 1975) and of the effects of caffeine (Fujiwara and Kondo, 1972), and by analogy with u.v. mutagenesis in E. coli (Witkin, 1969; Doudney, 1976), we interpret the above results by postulating that 4NQO-induced damage to template DNA produces defects in daughter DNA strands after replication, which are usually sealed by normal DNA strands, but occasionally by anomalous strands with mutated regions. Further, the presumed error-prone postreplication repair is inhibited by caffeine, which results in selective killing of cells with 4NQO-damaged DNA, including potentially tumourigenized stem cells. Nomura (1976) also observed that the mortality of 4NQO-treated mice was increased from virtually zero to about 30% by caffeine post-treatment which was nontoxic when administered alone. Similarly, caffeine at 50 to 200 μg/ml greatly enhanced lethality of 4NQO-treated cultured mouse cells (Kakunaga, 1975). In contrast, caffeine concentrations (1000–2000 μg/ml) which were effective in suppressing mutations, only marginally enhanced lethality in 4NQO-treated (Ichikawa-Ryo and Kondo, unpublished) and u.v.-irradiated (Witkin and Farquharson, 1969) cells of excisionless E. coli strains. These results indicate that caffeine-sensitive, error-prone repair is a minor fraction of postreplication repair in E. coli, but a major fraction in cultured mouse cells. Caffeine does not suppress excision repair in mouse (Kakunaga, 1973) and human cells (Regan, Trosko and Carrier, 1968), in contrast to E. coli.

**Time of fixation and expression of mutations and neoplasia**

Mutations are not immediately expressed after u.v. mutagenesis in E. coli (Witkin, 1969; Doudney, 1976). Similarly, 4NQO-induced premutational damage in E. coli was “fixed” (being made insensitive to suppression by caffeine treatment) only during the first post-4NQO DNA replication (Kondo, 1976). 4NQO-induced pretransformational damage in cultured mouse cells also becomes insensitive to suppression by caffeine during the first DNA replication after exposure (Kakunaga, 1975).

In E. coli, mutations to prototrophy are dominant and their mutant character fully appears after the first post-u.v. (Witkin, 1969) or post-4NQO (Kondo, 1976) division, whereas recessive mutations to ColB+ (colicine-B-resistant mutations due to change in cell membrane character) are fully expressed only 3 to 4 cell generations after the mutation is fixed (Ishii and Kondo, 1972). Similarly, 4NQO-induced malignant transformation in cultured mouse cells was fully expressed only 3 to 4 cell generations after its fixation (Kakunaga, 1974). Harris et al. (1969) demonstrated that malignancy can be suppressed when malignantly transformed cells are fused with nornmalignant cells. Thus, we conclude that neoplastic transformation of cultured mouse cells by 4NQO is caused by recessive mutations.

**Discussion and concluding remarks**

Carcinogenic hydrocarbons (now known as mutagens) induce latent skin tumours (initiation), which then develop to gross tumours under stimulation by croton oil or other non-carcinogenic agents (promotion) (Berenblum, 1969). The time between initiation and promotion has little effect on the latent period and the tumour yield. If initiation means production of stem cells with an irreversibly altered biological character, then, because of presumed geometric proliferation, “initiated” stem cells will, sooner or later, be either lost from or predominate in the local, compartmented stem cell population to which they belong, as is the case of mutants produced in a finite population (Crow and Kimura, 1970). Thus, the time interval between initiation
and promotion would be expected to affect the latent period and the tumour yield. This contradiction suggests that carcinogen-"initiated" stem cells may not always proliferate geometrically. Cairns (1975) has proposed that a stem cell divides into a mortal, differentiating daughter cell and an immortal daughter stem cell, and that the immortal daughter cell always receives the DNA molecules which have the older of the two parental strands, whilst the mortal daughter collects the molecules with the younger parental strand. This idea fits the data reported by Nomura (1976) as argued below.

Caffeine treatment (5 injections at 6-12-h intervals over a 36-h period) produced mortality in about 30% of mice within 2 to 5 days (probably due to intestinal death) when administered either immediately, or 5 days after 4NQO. Caffeine alone, 4NQO alone, or 4NQO treatment 12 h after the end of caffeine treatment gave negligible lethality. The number of tumour nodules per lung in female mice inducible by 4NQO was reduced by about 70% by caffeine given either immediately or 5 days after 4NQO, and by about 30% by caffeine pretreatment ending 12 h beforehand (Fig. 2). Caffeine alone was non-tumourigenic. Thus we conclude that the effect of caffeine persists no more than one day after injection and that 4NQO-treated stem cells of intestine and lung retain almost the same sensitivity to killing by caffeine for at least 5 days after 4NQO. Caffeine is effective only when it is present during the first post-u.v. or post-4NQO DNA replication in potentiating u.v.-induced lethality (Rauth, 1967; Fujiwara and Kondo, 1972) or 4NQO-induced lethality (Kakunaga, 1975) in cultured mouse cells. This may also be the case for in vivo stem cells. Therefore, selective killing by caffeine of potentially tumourigenized cells (about 70%) either immediately or 5 days later could imply that about 70% of the total stem cells in the lung, which are in the proliferative state and have 4NQO-damaged DNA, enter S phase (DNA synthesis) during the 2 days after 4NQO, and yet 3 days later (i.e., 5 days after 4NQO) about the same number of stem cells with similarly damaged DNA enter S phase at about the same rate. Similarly, skin papillomas "initiated" in mice by 7,12-dimethylbenz(a)-anthracene (DMBA) remain sensitive to suppression by actinomycin D (which greatly inhibits DNA synthesis (Bates et al., 1968) and postreplication repair after u.v. (Fujiwara, 1975) as caffeine does) for many days after DMBA, provided that promotion by croton oil is given after actinomycin D (gelboin, Klein and Bates, 1965; Hennings and Boutwell, 1967). Actinomycin D destroys the cells of skin treated by DMBA 34 days previously, but not those subjected to croton oil post-treatment 2 days before actinomycin D. This indicates selective killing of cells with DMBA damage (Hennings and Boutwell, 1967). Thus, we may conclude that stem cells potentially tumourigenized by an appropriate carcinogen remain sensitive to selective killing by caffeine because of the persistence of carcinogen-damaged DNA and in spite of their DNA being replicated. This will occur if the carcinogen damage in the older of the parental DNA strands of treated stem cells is made resistant or inaccessible to excision repair and then transmitted to offspring stem cells but not to mortal, daughter cells (Fig. 3). This kind of model may partly explain why human peripheral lymphocytes with "unstable" chromosomal aberrations persist for some years after exposure to ionizing radiations (Bender, 1969).

If the above modification of Cairns' model holds true, "promotion" could correspond to production of invasive and proliferative mutant stem cells from stem cells with DNA damage. This could occur when the orderliness of an unchanging population of stem cells in mature animal organs is partly broken down by abnormal differentiation, by ageing, or by carcinogen exposure (Fig. 3).
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