EFFECT OF CAFFEINE ON POSTREPLICATION REPAIR IN HUMAN CELLS

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ABSTRACT DNA synthesized shortly after ultraviolet (UV) irradiation of human cells is made in segments that are smaller than normal, but at long times after irradiation the segments made are normal in size. Upon incubation, both the shorter and the normal segments are elongated and joined by the insertion of exogenous nucleotides to form high molecular weight DNA as in nonirradiated cells. These processes occur in normal human cells, where UV-induced pyrimidine dimers are excised, as well as in xeroderma pigmentosum (XP) cells, where dimers are not excised. The effect of caffeine on these processes was determined for both normal human and XP cells. Caffeine, which binds to denatured regions of DNA, inhibited DNA chain elongation and joining in irradiated XP cells but not in irradiated normal human or nonirradiated cells. Caffeine also caused an alteration in the ability to recover synthesis of DNA of normal size at long times after irradiation in XP cells but not in normal cells.

INTRODUCTION

Caffeine has a radiosensitizing effect on mammalian cells (Rauth, 1967; Domon and Rauth, 1969) but not on human cells (Wilkinson et al., 1970). This sensitizing effect is most demonstrable during the "S" phase of the cell cycle (Rauth, 1967; Domon and Rauth, 1969; Kuhlman et al., 1968).

DNA synthesized immediately after UV irradiation of mammalian cells is made in segments that are smaller than normal (Cleaver and Thomas, 1969; Rupp et al., 1969; Lehmann, 1972; Buhl et al., 1972 *a*). In mouse L5178Y (Lehmann, 1972) and human (Buhl et al., 1972 *a*) cells, the size of these small regions is approximately equal to the distance between the dimers in the parental DNA.

However, at long times after irradiation mammalian cells synthesize DNA in segments as long as those synthesized by nonirradiated cells (Meyn and Humphrey, 1971; Lehmann and Kirk-Bell, 1972; Buhl et al., 1973), whether the dimers have been removed or not (Buhl et al., 1973). This phenomenon probably represents a repair system which alters the dimer to allow for normal DNA replication to occur past it. Upon incubation of irradiated cells containing small segments of DNA, the segments are enlarged to form high molecular weight DNA as in nonirradiated cells (Cleaver and Thomas, 1969; Rupp et al., 1969; Lehmann, 1972; Buhl et al., 1972 *a*). Examina-

tion of the elongation and joining process indicates that exogenous nucleotides are inserted between the labeled smaller segments and that there are two distinct size regions of insertion, small ($\sim 10^3$ nucleotides) and large ($>10^5$ nucleotides) (Lehmann, 1972; Buhl et al., 1972 *b*).

These data suggested the following model: DNA replication proceeds until it reaches a dimer, where the polymerase stops and skips over the dimer leaving a gap of $\sim 10^3$ nucleotides in one of the strands. The gap is then filled by an unknown repair system. Caffeine inhibits the enlargement process in mammalian cells (Cleaver and Thomas, 1969) but not in normal human cells (Fugiwara and Kondo, 1972); and human cells are capable of excising large numbers of the pyrimidine dimers, whereas murine rodent cells are not (Regan et al., 1968; Setlow et al., 1972). From these observations it can be surmised that binding of caffeine to the dimer inhibits elongation and joining of the short segments of DNA synthesized after irradiation, resulting in the radiosensitizing effect.

We have examined the effect of caffeine on DNA replication in irradiated and nonirradiated normal human cells and in cells derived from an excision-defective human mutant with the clinical disorder xeroderma pigmentosum (Cleaver, 1968). Our findings suggest that caffeine binds more strongly to DNA containing dimers than to nonirradiated DNA, and that caffeine does not appreciably affect DNA replication in irradiated normal human cells but retards DNA replication in irradiated XP cells.

MATERIALS AND METHODS

Cell Lines and Media

The cell lines used were HS-WP, derived from a normal Caucasian foreskin, and SG-XP4, derived from an individual with uncomplicated XP. Cells were normally grown in minimum essential medium (Eagle, 1959) supplemented with 10% fetal calf serum and the nonessential amino acids at 37°C in a water-saturated atmosphere of 2% CO_2 in air. 1 or 2 days before each experiment the cells were trypsinized (0.25% trypsin) and plated in minimum essential medium supplemented with 10% calf serum and the nonessential amino acids on plastic Petri dishes (Permanox, Lux Scientific Corp., Thousand Oaks, Calif.) at approximately 20,000 cells per dish.

Pretreatment, UV Irradiation, Pulse Labeling, and Incubation

Each dish of cells was washed with warmed phosphate-buffered saline (Dulbecco and Vogt, 1954) and exposed to UV radiation from a germicidal lamp at a rate of 10 ergs/mm^2 · s. The cells were then washed and incubated in a warmed medium for 0.5 h. Caffeine (1 mM) was added 0.5 h before, simultaneously with, or immediately after pulse labeling. The DNA was pulse labeled by addition of 12–50 μCi/ml of [methyl-^{3}H]thymidine (sp act, 14.1–18.1 Ci/mmol) for a prescribed time (usually 30 min).

Sedimentation of DNA in Alkaline Sucrose

The cells were washed with and suspended in an ice-cold isotonic ethylenediaminetetraacetate (EDTA) solution (Setlow et al., 1969), sedimented in a refrigerated centrifuge (Sorvall RC-2B, Ivan Sorvall, Inc., Norwalk, Conn.), and resuspended in cold EDTA solution. They were then irradiated with 2-krad gamma rays from a ^{60}Co source at a rate of 3.65 krad/min. The gamma irradiation causes a few single strand breaks in the DNA to permit sedimentation without excess

entanglement of the DNA strands (Lehmann and Ormerod, 1970). The cells were then immediately lysed in a 0.2% sodium dodecyl sulfate solution on top of a 3.6 ml gradient of 5–20% sucrose, 0.1 M NaCl, and 0.1 M NaOH in a 4 ml polyallomer tube.

The gradients were centrifuged at 40,000 rpm for 75–100 min at 20°C in the SW56 rotor of a Spinco model L3-50 Ultracentrifuge (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.). Aliquots (0.125 ml) were collected through a hole punched in the bottom of the tube onto Whatman No. 17 paper strips (Carrier and Setlow, 1971). The strips were washed once in 5% trichloroacetic acid, twice in ethanol, and once in acetone (Bollum, 1966), then were dried, cut, and placed into vials with a toluene–2,5-bis-[2-(5-*tert*-butylbenzoxazolyl)]thiopene (BBOT) scintillator; and the radioactivity was counted in a liquid scintillation spectrometer. The molecular weights were calculated by computer, using the relationship observed by Abelson and Thomas (1966) and confirmed by R. B. Setlow (unpublished results), with single strands of T4, λ, and ϕ X174 DNAs as standards (Freifelder, 1970). The relationship found was that single-strand molecular weights are proportional to the 2.62 power of the distance sedimented.

RESULTS

Fig. 1 shows the effect of caffeine on DNA replication and on the repair system recovery of the ability to synthesize DNA in segments of normal size at long times after UV irradiation in normal human cells. Caffeine caused a reduction in the size of DNA segments synthesized by nonirradiated cells and also reduced the already smaller DNA segments synthesized after irradiation. When caffeine was added and the irradiated cells were pulse labeled at long times after irradiation, the newly synthesized DNA segments were equal to the segments synthesized by nonirradiated cells treated with caffeine during the pulse labeling period. Fig. 2 shows the effect of caffeine on elongation and joining of the shorter DNA segments synthesized after UV irradiation. In

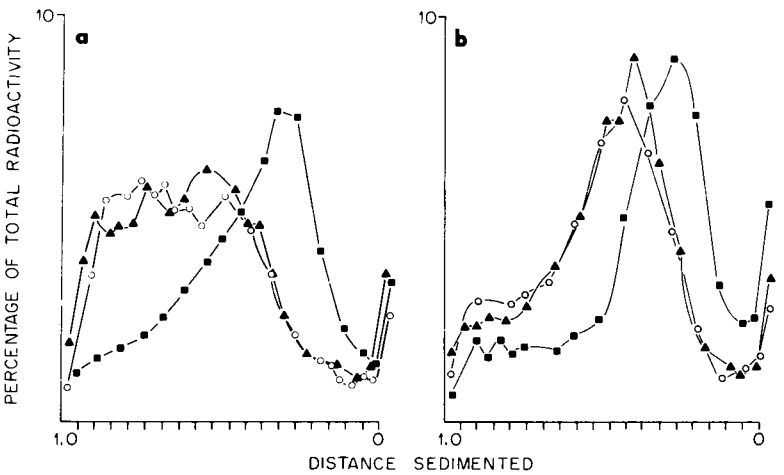

FIGURE 1 Alkaline sucrose gradient profiles of DNA from normal human cells, line HS-WP, which were either (*a*) not treated or (*b*) treated with 10^{-3} M caffeine added 0.5 h before pulse labeling. The cells were either nonirradiated (○) or irradiated with 100 ergs/mm^2 UV at 0.5 h (■) or 18 h (▲) before being pulse labeled for 0.5 h and sedimented for 100 min.

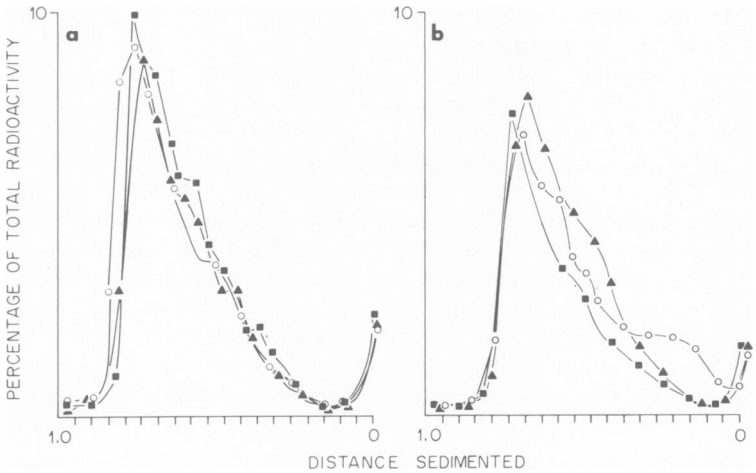

FIGURE 2 Alkaline sucrose gradient profiles of DNA from normal human cells, line HS-WP, which were either (*a*) not treated or (*b*) treated with 10^{-3} M caffeine added 0.5 h before pulse labeling. The cells were either nonirradiated (○) or irradiated with 100 ergs/mm^2 UV at 0.5 h (■) or 18 h (▲) before being pulse labeled for 0.5 h. The cells were further incubated in minimum essential medium supplemented with 10% fetal calf serum and the nonessential amino acids (*a*) with or (*b*) without 10^{-3} M caffeine for 18 h before being sedimented for 75 min.

all cases the smaller DNA segments were enlarged to form very high molecular weight DNA. This is in agreement with preliminary observations reported by Fugiwara and Kondo (1972) for UV-irradiated HeLa cells.

Fig. 3 shows the effect of caffeine on DNA replication of irradiated and nonirradiated XP cells. Again caffeine caused a reduction in size both in the DNA segments

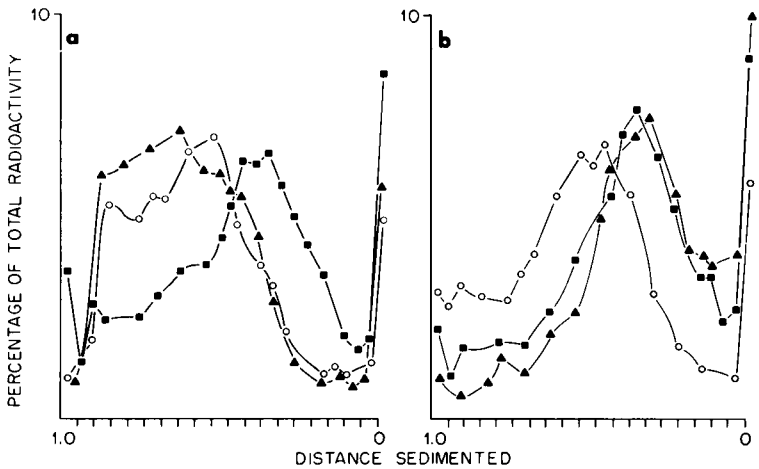

FIGURE 3 Alkaline sucrose gradient profiles of DNA from XP cells, line SG-XP4, which were either (*a*) not treated or (*b*) treated with 10^{-3} M caffeine added 0.5 h before pulse labeling. The cells were either nonirradiated (○) or irradiated with 100 ergs/mm^2 UV at 0.5 h (■) or 18 h (▲) before being pulse labeled for 0.5 h and sedimented for 100 min.

synthesized by nonirradiated cells and also in the smaller segments synthesized after UV treatment. At long times after irradiation the pulse-labeled DNA was synthesized in segments equal to those synthesized immediately after irradiation and pulse labeling; i.e., the apparent repair observed as recovery of the ability to synthesize DNA in segments of normal size at long times after irradiation was nullified by caffeine. This contrasts with the results for normal human cells (Fig. 1).

Fig. 4 shows the effect of caffeine on elongation and joining of the shorter segments synthesized after irradiation. Although caffeine did not retard the elongation of the shorter segments synthesized after caffeine treatment, it inhibited the complete elonga-

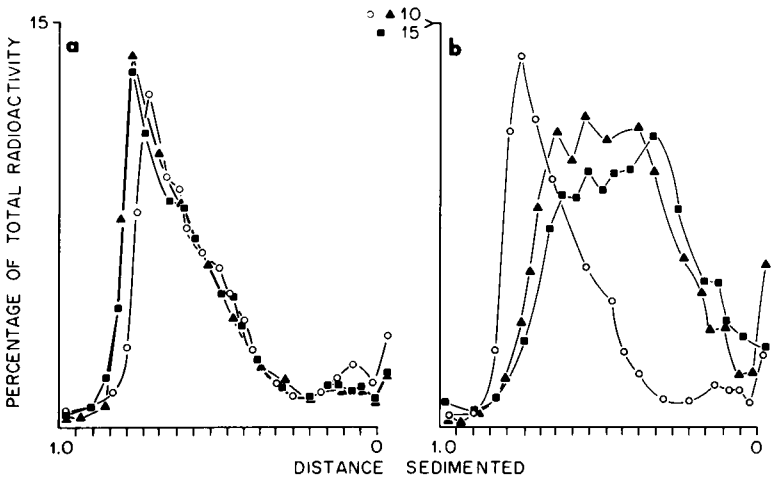

FIGURE 4 Alkaline sucrose gradient profiles of DNA from XP cells, line SG-XP4, which were either (*a*) not treated or (*b*) treated with 10^{-3} M caffeine added 0.5 h before pulse labeling. The cells were either nonirradiated (○) or irradiated with 100 ergs/mm^2 UV at 0.5 h (■) or 18 h (▲) before being pulse labeled for 0.5 h. The cells were further incubated in minimum essential medium supplemented with 10% fetal calf serum and the nonessential amino acids (*a*) with or (*b*) without 10^{-3} M caffeine for 18 h before being sedimented for 75 min.

tion and joining of the short segments synthesized after UV irradiation. Qualitatively similar results were obtained whether the caffeine was added 0.5 h before, simultaneously with, or immediately after pulse labeling and therefore the data are not shown.

DISCUSSION

Consideration of the present results and of previously reported data indicates that either the current model of postreplication repair (Fig. 5) or the conceived notion of how caffeine reacts with DNA is inconsistent with the findings. Caffeine is believed to bind to denatured regions of DNA, such as at a replication point or the site of a pyrimidine dimer, and to single-stranded regions of DNA, such as would be left around a pyrimidine dimer during the next round of DNA replication (Fig. 5). The reduction

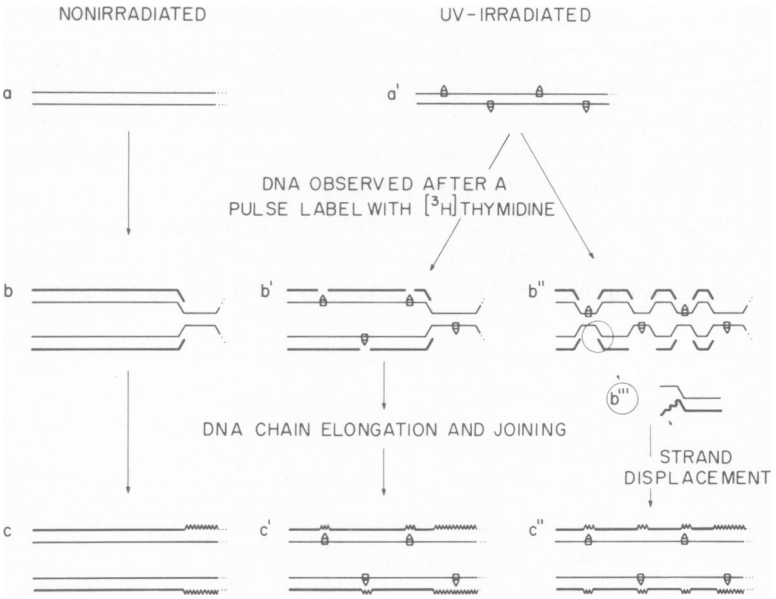

FIGURE 5 DNA replication in nonirradiated and UV-irradiated human cells. Nonirradiated parental DNA (———), pyrimidine dimers (△), [^{3}H]thymidine-containing newly synthesized DNA (———), newly synthesized DNA (———). DNA synthesis in nonirradiated cells is continuous (*b*), but in UV-irradiated cells the newly synthesized DNA contains either one gap for each dimer in the strand directly opposite the dimer (*b'*) or two gaps for each dimer, with gaps in both strands of the new DNA (*b''*). The insert (*b'''*) describes the detail of the interruption in DNA replication and the existence of a strand displacement, which would occur when the parental DNA strands reform the original double helix (B. Strauss, personal communication). Completion of the DNA replication in nonirradiated cells (*c*) and in UV-irradiated cells (*c'*, *c''*) is also shown. Examination of the elongation and joining process indicates that there are two different-sized regions completed by insertion of exogenous nucleotides in irradiated cells: large regions, which represent completion of the replication units present in nonirradiated cells (*c*), and small regions, which represent completion of the discontinuities caused by UV-induced pyrimidine dimers (*c'*, *c''*) (Lehmann, 1972; Buhl et al., 1972 *b*).

in size of newly synthesized DNA of nonirradiated cells treated with caffeine supports the notion that caffeine binds to the denatured regions of the replication fork. The inhibition of DNA chain elongation and joining of the short segments synthesized after irradiation of XP cells but not normal cells, and also the apparent suppression in the repair system which results in synthesis of DNA in segments of normal size at long times after irradiation in cells which do not remove pyrimidine dimers, indicate that caffeine binds to the denatured regions of DNA containing dimers. Caffeine apparently binds more strongly to the denatured regions caused by the dimers than to the denatured regions at the replication points, because DNA chain elongation is not inhibited in nonirradiated cells but is in UV-irradiated XP cells. It should be noted that the inhibition is not complete in the XP cells.

The caffeine-dependent suppression of the repair system, which is exemplified by

synthesis of DNA in segments of normal size at long times after irradiation, has not been observed when theophylline, which is similar to caffeine structurally, has been used with other cell lines (Lehmann and Kirk-Bell, 1972). Caffeine affects normal DNA replication to a much larger extent than does theophylline (Lehmann and Kirk-Bell, 1972); perhaps the apparent regression in the repair results from the larger effect of caffeine on DNA replication rather than from a true suppression in the repair. However, it should be noted that caffeine inhibits the complete elongation and joining of DNA segments synthesized at long times after irradiation to the same extent that it does small segments synthesized shortly after irradiation. If the caffeine binds to the dimer and this complex (dimer-caffeine) inhibits DNA replication in XP cells but not in normal human cells, it can be construed that the dimer-caffeine complex must be removed before DNA replication can proceed past that point. It has previously been shown that the repair endonuclease requires double-stranded DNA as template (Carrier and Setlow, 1970); therefore, if one is postulating gaps opposite the dimer (*b'* in Fig. 5), one should expect DNA chain elongation and joining to be inhibited in excision-proficient as well as excision-deficient cells. The experimental results indicate that DNA chain elongation is not inhibited by caffeine in normal human (excision-proficient) cells, so the gaps must not be formed opposite the pyrimidine dimer during DNA replication after UV irradiation.

The following modifications to the previously presented model of postreplication repair are suggested (*b''* in Fig. 5). DNA replication proceeds until it reaches a dimer, where it stops in both strands or perhaps proceeds for a small distance in the strand not containing the dimer. A strand displacement can then result when the parental DNA anneals to form the original helix in the dimer region (B. Strauss, personal communication). Before DNA replication can proceed, one of several things must happen. The dimer must be excised (which occurs to a great extent only in normal human cells); the dimer must be altered, as indicated by the recovery of the ability to synthesize DNA of normal size at long times after irradiation in cells which do not excise dimers; or a special polymerase must synthesize past the dimer before it is altered or excised. Caffeine does not prevent excision repair in human cells (Regal et al., 1968; Cleaver, 1969), but it does cause a suppression of the repair system, which results in synthesis of DNA in segments of normal size at long times after irradiation in XP cells. Neither the existence of an additional polymerase which specifically synthesizes past dimers nor its sensitivity to caffeine has been established; however, because Chinese hamster B14 cells, which have a greatly reduced excision ability (Setlow et al., 1972) and lack recovery of the ability to synthesize DNA in segments of normal size at long times after irradiation, are able to elongate and join the short DNA segments synthesized after irradiation (Meyn and Humphrey, 1971), a special polymerase must be invoked to synthesize past the dimer. Completion of DNA replication would result in regions of two distinct sizes (*c'* and *c''* in Fig. 5).

The modifications in Fig. 5 indicate that two discontinuities should occur for each dimer. However, as previously indicated, the size of the newly synthesized DNA

segments after irradiation of human and mouse L5178Y cells approximately equals the distance between dimers. The discrepancy between the observed data and the suggested model cannot be explained with existing data, but several points should be considered. The size of the short, newly synthesized DNA in irradiated cells was measured with a relatively long pulse label (usually 30 min). During this time many of the dimers could be altered or gaps could be filled (repaired), and normal DNA replication would proceed past them and no discontinuity would occur. Hence the small segments would not represent the true result. This suggestion is supported by the observation of shoulders or skewing of the DNA profiles toward high molecular weight DNA in irradiated cells. Shorter pulse times may result in smaller DNA and should answer the question. Additional evidence that smaller DNA could result is that caffeine causes a reduction in the size of the already small DNA segments synthesized after irradiation. The smaller segments synthesized after UV and caffeine treatment were approximately 0.66 the size of those synthesized after UV irradiation alone (Fig. 1 in Buhl et al., 1972 *a*). (The ratio was determined by comparing the number average molecular weights after corrections were made for pulse labeling of nonirradiated cells and represents a change from $\sim 4.4 \times 10^6$ daltons to $\sim 3.0 \times 10^6$ daltons.) This indicates that DNA is made in segments smaller than the distance between dimers. However, at this time we have not been able to demonstrate whether the reduction results from a general effect of caffeine which causes DNA to be made in smaller segments in nonirradiated cells or a specific effect which results from binding to the region of UV-induced dimers, causing an interruption of DNA replication.

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