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The Demonstration of Separate DNA Polymerase Activities In Intact Isolated Rat Liver Nuclei by Means of Response to Bleomycin and Arabinosyl Cytosine 5-Triphosphate (39113)

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(Introduced by D. R. Boggs)

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Rat hepatic nuclei, isolated and studied *in vitro*, tend to reflect changes occurring in DNA synthesis in the intact rat (1). Nuclei from normal rat liver synthesize minimal amounts of DNA, while nuclei from a partially hepatectomized rat show a marked increase in thymidine tri-phosphate incorporation. This increase probably represents continuation of elongation of replicating DNA strands begun *in vivo* (2). Studies of extracts of rat liver nuclei suggest that there are at least two separate DNA polymerase activities: a tightly-bound activity which rises in quantity along with DNA replication following hepatectomy and is inhibited by cytosine arabinoside 5′-triphosphate (ara-CTP), and a loosely-bound activity which rises little or not at all after partial hepatectomy and is insensitive to ara-CTP (3).

We have previously reported that Bleomycin causes dose-related and time-related increases of ^{3}H-thymidine triphosphate incorporation into DNA of isolated normal rat liver nuclei (4). This incorporation is presumably carried out by a DNA polymerase reacting to structural DNA damage produced by the Bleomycin.

The studies reported herein provide evidence that the increased ^{3}H-thymidine triphosphate incorporation which follows addition of Bleomycin to isolated rat liver nuclei is mediated by a DNA polymerase activity that is different from the polymerase activity that increases following partial hepatectomy.

Materials and methods. Male Sprague–Dawley strain rats (100 gm) were used as the source of liver; they were given food and water *ad libitum*. Nuclei were prepared from normal rats or from rats partially hepatectomized 24 hr earlier by ligation and excision of the left lateral and median hepatic lobes (1). The method of preparation, storage, and assaying of liver nuclei used in these experiments is modified after the method of Lynch *et al.* (2, 3, 4). Approximately 1 g of liver was rinsed in 0.3 *M* sucrose and suspended in 15–20 volumes of 0.2 *M* sucrose–4×10^{-3} *M* $CaCl_2$, with one stroke in a loose Dounce Homogenizer. The suspension was then homogenized with a loose-fitting rubber pestle, filtered through a monofilament nylon screen (100 mesh), and centrifuged at 750*g* for 4 min. The resulting pellet was resuspended in 18 ml of 2 *M* sucrose–10^{-3} *M* $CaCl_2$, layered over 13 ml of 2.2 *M* sucrose, and sedimented by centrifugation for 20 min in a Spinco SW 25.1 rotor at 20,000 rpm. Finally, the nuclei were either suspended in 2.5 ml of 0.3 *M* sucrose for immediate use, or frozen for later use by submersion of the butt of the tube containing the concentrated nuclei in liquid nitrogen. Each experiment was carried out on nuclei from one preparation, $1.2–2.0 \times 10^8$ nuclei, allowing 12–20 determinations per preparation. Since the experiments required up to 20 simultaneous determinations, duplicate determinations were not carried out routinely. However, duplicate and triplicate determinations, using the identical technique in other experiments, show a standard error of less than 7.0% of the mean value. Background counts were less than 25 cpm for all experiments; the reported data are not corrected for background.

Suspended nuclei were counted on a Coulter model B electronic cell counter and the concentration was adjusted to 10^7 nuclei/0.1 ml using 0.3 *M* sucrose. Thymidine triphosphate incorporation into 10^7 nuclei was meas-

ured at 37°C in 0.5 ml mixtures containing 3 m*M* $MgCl_2$, 2 m*M* ATP, 0.08 m*M* dGTP, dCTP, and dATP, 2% Ficoll, 1 m*M* EGTA, 2.5 m*M* cadaverine, and 0.016 m*M* ^{3}H-TTP (0.5 Ci/mmole), in 0.18 *M* Tris-HCl buffer (pH 8.2). Reactions were stopped with 1 ml of 1 *M* NaOH, and DNA was precipitated with 5 ml of a suspension 20 mg/ml of Celite (Johns–Manville) in ice-cold trichloracetic acid (10%). The DNA was sequentially redissolved (0.3 ml of 1 *M* NaOH), reprecipitated (8 ml of 5% trichloracetic acid), and centrifuged three times, the supernatant being discarded each time. The precipitate was then dissolved in 1 ml of 1 *M* NaOH and heated at 80°C for 15 min. The DNA was again precipitated with trichloracetic acid on a pad of Celite and was washed with acid, ethanol, and ether. The resultant pad of Celite was dissolved in hydroxide of hyamine, and counted in a toluene base liquid scintillation counting mixture (Liquiflour).

Ara-CTP was provided by Dr. Neil's laboratory or purchased from Terra–Marine Laboratories, La Jolla, California. It was dissolved in water, and used at a final concentration of 200 μM. (This concentration inhibits 98.8% of "proper" DNA synthesis in intact nuclei) (3).

Thymidine triphosphate was obtained from the New England Nuclear Corporation. Bleomycin was generously supplied by Dr. Karl Agre, Director of Pharmacological Research, Bristol Laboratories, Syracuse, New York. Bleomycin was dissolved in sterile distilled water immediately prior to use, and was used in a final concentration of 1500 μg/ml (approximately 1 m*M*).

Results. Normal rat liver nuclei incubated under control conditions incorporated ^{3}H-TTP for 20 min, but no additional incorporation was observed between 20 and 60 min (Fig. 1, a). When ara-CTP (200 μM) was included, the rate of incorporation was decreased during the first 30 min, but equaled the control by 1 hr (Fig. 1, b). Bleomycin (1.5 mg/ml) produced an increase in ^{3}H-TTP incorporation (Fig. 1, c) in comparison to control values throughout the incubation period. Ara-CTP partially inhibited the total ^{3}H-TTP incorporation occurring in the presence of Bleomycin at all points except 1 hr (Fig. 1, d). The 1 hr point exceeded that of Bleomycin alone.

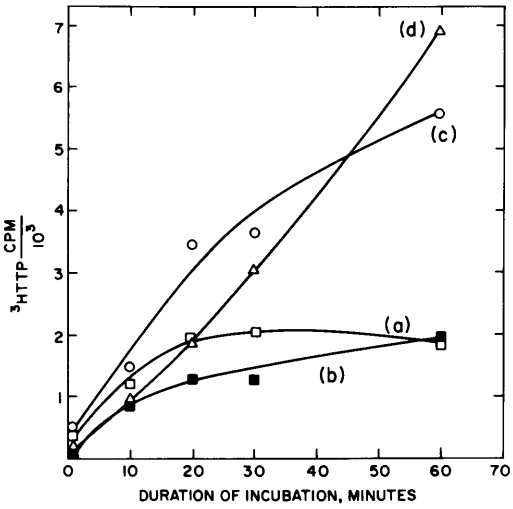

FIG. 1. ^{3}H-TTP incorporation into normal isolated liver nuclei over 1 hr of incubation. Each point represents one determination of ^{3}H-TTP incorporation into DNA of isolated nuclei during incubation at 37°C for the duration indicated on the abscissa. The content of the standard mixture used in each tube is as described in the text under Materials and Methods. All determinations for the experiments illustrated in Fig. 1 were carried out simultaneously on nuclei from a single liver preparation from a normal, nonhepatectomized rat.

Curve (a). Normal nuclei prepared from a normal rat and incubated in the standard 0.5 ml mixture. Curve (b). Normal nuclei incubated in the standard 0.5 ml mixture to which ara-CTP has been added to a final concentration of 200 μM. Curve (c). Normal nuclei incubated in the standard 0.5 ml mixture to which Bleomycin has been added to a final concentration of 1500 μg/ml (approximately 1 m*M*). Curve (d). Normal nuclei incubated in the standard 0.5 ml mixture to which Bleomycin (1 m*M*) and ara-CTP (200 μM) have been added.

Nuclei from partially hepatectomized rats ("regenerating" nuclei), demonstrated increased ^{3}H-TTP incorporation compared to normal rat liver nuclei (Fig. 2, a, vs Fig. 1, a). Addition of ara-CTP reduced ^{3}H-TTP uptake at all points to less than 35% of control (Fig. 2, b), principally due to inhibition occurring during the first 20 min of incubation.

When Bleomycin alone was added to nuclei from partially hepatectomized rats, ^{3}H-TTP incorporation during the first 30

min was reduced relative to controls (Fig. 3, a, vs. Fig. 2, a), but thereafter, incorporation increased. Addition of ara-CTP, as well as Bleomycin, resulted in further inhibition of the rate of incorporation of ^{3}H-TTP during the first 10 min, but thereafter, incorporation increased in a linear fashion up to 1 hr (Fig. 3, b).

Discussion. The mode of action of the antitumor antibiotic, Bleomycin, has been the subject of a number of reports (4–9). It has been demonstrated that Bleomycin inhibits DNA synthesis, as measured by thymidine incorporation, in HeLa S-3 cells (8, 9, 10), Ehrlich ascites cells (9), and *E. coli* B (8). Tobey demonsrated that in Chinese hamster cells exposed to Bleomycin at 100 μg/ml, initiation and termination of DNA synthesis occur at normal rates, but cells accumulate in G-2 (9). Nagatsu *et al.* (10) demonstrated that a large fraction of transplanted VX^{-2} cells treated with Bleomycin ended up with a G-2 content of DNA.

We have previously reported that PHA-stimulated lymphocytes also show dose-related inhibition of DNA synthesis when exposed to Bleomycin, and we were able to demonstrate that this is not related to the initial stimulatory events following addition of PHA. However, there was a suggestion that Bleomycin in low dosage caused increased incorporation of ^{3}H-thymidine into PHA-stimulated lymphocytes, and markedly increased thymidine triphosphate incorporation was observed in hepatic nuclei exposed to Bleomycin (4). These data suggest that Bleomycin produces DNA damage that is repaired by intranuclear repair systems. Similar findings have been noted in cultured mouse fibroblasts (11), and in an *in vitro* system utilizing partially purified DNA polymerase from *E. coli* B (12). In the former case, repair of DNA was suggested, while in the latter, it is suggested that degradation of the primer and product DNA by Bleomycin led to stimulation of DNA polymerase early in the reaction, followed by inhibition later.

The present studies show that in nuclei obtained from normal rat liver, virtually all of the DNA synthesis measured as ^{3}H-TTP incorporation occurs during the first 20 min of incubation. This incorporation is partially inhibited by ara-CTP. The portion

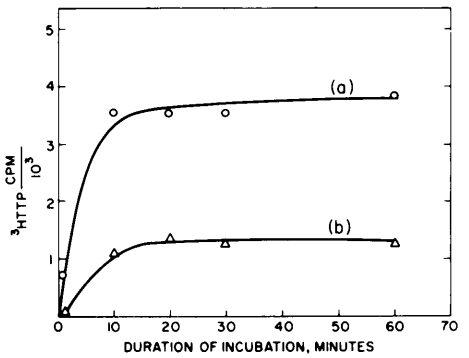

FIG. 2. ^{3}H-TTP incorporation into DNA of nuclei from liver of a rat that had undergone partial hepatectomy 24 hr earlier. Nuclei were incubated in the same mixture as normal nuclei. (All data from Figs. 2 and 3 were obtained from simultaneous experiments carried out using nuclei from a single liver preparation.)

Curve (a). Nuclei from liver of rat which had previously undergone partial hepatectomy, incubated in standard 0.5 ml mixture. Curve (b). As in (a), except that ara-CTP (200 μM) was added prior to incubation.

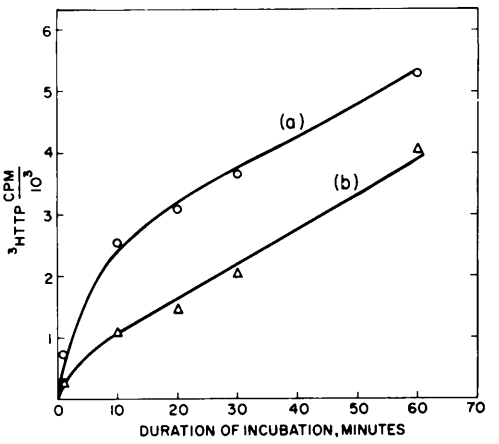

FIG. 3. ^{3}H-TTP incorporation into DNA of nuclei from liver of a rat that had undergone partial hepatectomy 24 hr earlier. Nuclei were incubated in the same mixture as normal nuclei. (All data from Figs. 2 and 3 were obtained from simultaneous experiments carried out using nuclei from a single liver preparation.)

Curve (a). Nuclei to which Bleomycin (1 mM) was added prior to incubation. Curve (b). Nuclei to which both Bleomycin (1 mM) and ara-CTP (200 μM) were added prior to incubation.

that is inhibited by ara-CTP represents *de novo* DNA synthesis in progress in the liver nuclei at the time of their isolation (3). The portion that is insensitive to ara-CTP probably represents "unscheduled" DNA polymerase activity, and may be a "repair" response to the trauma of preparation (13, 14). The apparent loss of the inhibitory effect of ara-CTP observed by 1 hr of incubation suggests an increase in ara-CTP-insensitive polymerase activity between 30 min and 60 min. This is attributed to the ability of ara-CTP itself to act as substrate for the ara-CTP-insensitive polymerase, particularly late in the incubation period, when there may be substrate depletion.

The addition of Bleomycin to normal nuclei produces an increase in ^{3}H-TTP incorporation, which is sustained over the entire hour of incubation. The addition of both ara-CTP and Bleomycin produces a sustained increase that is slightly smaller at all points except 1 hr. This small initial inhibitory effect of ara-CTP is again attributed to inhibition of *de novo* DNA synthesis in progress in the liver nuclei at the time of their isolation (3). The large increase in uptake produced by the addition of Bleomycin is attributed to activity of an ara-CTP-insensitive DNA polymerase reacting to DNA breaks produced by Bleomycin. The apparent reversal of the inhibitory effect of ara-CTP observed by 1 hr of incubation is explained as previously described.

Nuclei isolated from partially hepatectomized rats showed a two- to threefold increase in ^{3}H-TTP incorporation in comparison to normal nuclei. This increment is most notable in the first 10 min of incubation. It is markedly inhibited by ara-CTP. The addition of Bleomycin to nuclei from a partially hepatectomized rat apparently resulted in partial inhibition of ^{3}H-TTP incorporation relative to controls during the first 30 min, but this was followed by an increase over the remaining 30 min. Addition of ara-CTP as well as Bleomycin had a marked effect on total ^{3}H-TTP uptake, but this reflected inhibition during the first 10 min only. Thereafter, uptake increased, was a linear function of time, and was not influenced by the presence of ara-CTP. The DNA polymerase primarily responsible for the early increase in uptake is markedly inhibited by ara-CTP (Fig. 2, a). Bleomycin also appears to inhibit incorporation due to this polymerase, but the possibility that Bleomycin is merely degrading the newly synthesized DNA and liberating the incorporated ^{3}H-TTP, rather than blocking the polymerase directly, cannot be excluded. The parallel components of these curves, between 20 and 60 min, represent linear and prolonged ^{3}H-TTP uptake that is insensitive to ara-CTP. This incorporation is apparently due to a different polymerase that is activated in response to DNA damage caused by Bleomycin, and is not inhibited by ara-CTP.

These data are consistent with the previously reported work of Lynch *et al.*, in which liver nuclei were shown to contain at least two DNA polymerases that can be separated by 5% Brij 58 extraction (3). One of these enzymes, a tightly-bound activity that increases following partial hepatectomy, is inhibited by ara-CTP and is thought to represent normal replicase activity. The other, a loosely-bound activity that is present in both normal and regenerating nuclei, does not increase following partial hepatectomy, is insensitive to ara-CTP, and may represent a "repair" polymerase.

Grisham (14) and Kaufman (13) have studied this putative repair polymerase activity, showing that it can occur in all nuclei, regardless of their position in the cell cycle, and that the *in vitro* incorporation of ^{3}H-TTP into liver nuclei by this activity is not semiconservative. They have further shown that the activity of this type of polymerase is initiated by various manipulations that affect the confirmation and integrity of DNA (e.g., high shear preparation of nuclei, creation of single strand breaks, and 3′-hydroxyl termini with deoxyribonuclease I).

Our data are consistent with the hypothesis that single strand DNA breaks produced by Bleomycin (7) can also result in the initiation of this activity, and that this activity is distinguished from the activity that rises following partial hepatectomy (putative replicase) by its lack of inhibition by ara-CTP. Similar results have recently been reported by Friedman *et al.*, who have observed stimula-

tion of ^{3}H-TTP incorporation into DNA of two other types of isolated animal cell nuclei by the related antibiotic, Phleomycin (15).

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