DIFFERENTIAL EFFECTS OF METABOLIC INHIBITORS ON CYTOPLASMIC MEMBRANE ASSOCIATED AND NUCLEAR DNA

M. R. Hall†, W. Meinke, D. A. Goldstein, and R. A. Lerner*

Department of Microbiology and Department of Experimental Pathology*, Scripps Clinic and Research Foundation, La Jolla, California 92037

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SUMMARY: The cellular functions necessary for transport of cytoplasmic membrane associated DNA from nucleus to cytoplasm have been investigated utilizing inhibitors of macromolecular synthesis. Hydroxyurea, fluorodeoxyuridine, cytosine arabinoside, and ethidium bromide did not prevent transport of cytoplasmic membrane associated DNA to the cytoplasm. In contrast, rifampicin and N-demethyl rifampicin totally inhibited the appearance of newly synthesized DNA on cytoplasmic membranes, while dimethyl-benzyl-demethyl rifampicin was partially inhibitory.

We have described a species of DNA (cm-DNA) associated with cytoplasmic membranes of human lymphocyte cell line, Wil_2 (1,2,3,4). cm-DNA represents about 2% of the total cellular DNA and is distinguished from the bulk of chromosomal and mitochondrial DNA, by its size, configuration, labeling properties with radioactive precursors, and genetic complexity (3,4). Denatured cm-DNA reassociates as two fractions whose rates of reassociation differ by about 4 decades (3,4). Approximately 70% of cm-DNA reassociates rapidly ($C_0t_{1/2}=2.3 \times 10^{-2}$) and is comprised of repeated sequences. However, these repeated sequences are homologous to only about 4% of the repeated sequences of nuclear DNA. About 30% of cm-DNA is comprised of sequences which reassociate about ten times faster than non-reiterated (unique) sequences of nuclear DNA ($C_0t_{1/2}=223$). This fraction has homology with about 11% of the unique sequences of nuclear DNA (4).

The finding that cm-DNA is apparently synthesized in the nucleus and subsequently exits to the cytoplasm (2) and that only a small amount of the total cellular DNA ever appears on cytoplasmic membranes, led to an investigation of the "processing" of cm-DNA. For these studies, Wil_2 cells were grown in suspension culture in the presence of certain metabolic inhibitors and their effect on transport of cm-DNA was measured by the ability of each inhibitor to block the appearance of cm-DNA in the cytoplasm.

These studies show that de novo DNA synthesis is not required for the transfer of pre-formed cm-DNA from the nucleus to the cytoplasm. However,

†present address: Department of Cell and Molecular Biology, Medical College of Georgia, Augusta, Ga. 30902

evidence is presented for involvement of a metabolic process which is inhibited by rifampicin and some of its derivatives.

MATERIALS AND METHODS

Cell culture: Detailed description of the methods and media used for growth, synchronization and 5-bromodeoxyuridine (BdUrd) labeling of the human lymphocyte line Wil_2 have been described (1,2,3).

Equilibrium centrifugation: Analysis of BdUrd labeled nuclear DNA and cm-DNA by equilibrium density centrifugation in CsCl gradients was as previously reported (2).

DNA synthesis: The effect of various drug treatments on DNA synthesis was determined by removing 2.0 ml aliquots from cell cultures and pulse-labeling for 30 min at 37 C with [^{14}C]-thymidine, 1.0 μCi/ml (54.1 mCi/mM). The pulse was terminated by addition of 10.0 ml of cold (4 C) Earles salts to the mixture and cells collected by centrifugation at 800 g for 5 min. The cell pellet was resuspended in 5.0 ml of cold (4 C) trichloroacetic acid (TCA), incubated 30 min in an ice-water bath, and acid precipitable material collected by filtering through Whatman GFC filters. Filters were rinsed with 95% ethanol, dried, and radioactivity determined by standard scintillation techniques (2).

RESULTS

In previous experiments, evidence for exit of DNA from nucleus to cytoplasm was obtained from pulse-chase experiments employing BdUrd in synchronized cell cultures (2). The procedure was to synthesize nuclear DNA substituted with BdUrd until the middle of S phase (approximately 20 hrs) and then to grow the cells in a "chase" medium without BdUrd for an additional 12 hrs. The appearance of "new" BdUrd substituted DNA on cytoplasmic membranes following the chase was interpreted as evidence of transfer of DNA from nucleus to cytoplasm.

To determine whether de novo DNA synthesis was required for exit of cm-DNA from the nucleus and to rule out the possibility that a small "private" pool of BdUrd was responsible for the appearance of additional cm-DNA substituted with BdUrd, cells were exposed to several inhibitors of DNA synthesis. Fig. 1 shows the effect of hydroxyurea (H.U.), fluorodeoxyuridine (FdUrd) and cytosine arabinoside (Ara-C) (open symbols) on total cellular DNA synthesis when added at 20 hrs after synchronization (i.e., middle of S phase). The solid circles show the pattern of total DNA synthesis during a normal cell cycle. It is apparent that all three inhibitors were effective in reducing uptake of [^{14}C]-thymidine to 2 to 3% of control values within 1/2 hr after addition.

The effect of inhibitors on the appearance of cm-DNA in the cytoplasm was studied using synchronized cells in which DNA had been uniformly labeled with [^{14}C]- thymidine. Cells were grown for 20 hrs in medium containing 20 μg/ml BdUrd, the culture divided into three equal portions and cells harvested by centrifugation. One third was lysed with NP-40, one third was resuspended in a thymidine-deoxycytidine chase medium and incubated an additional 12 hrs, and

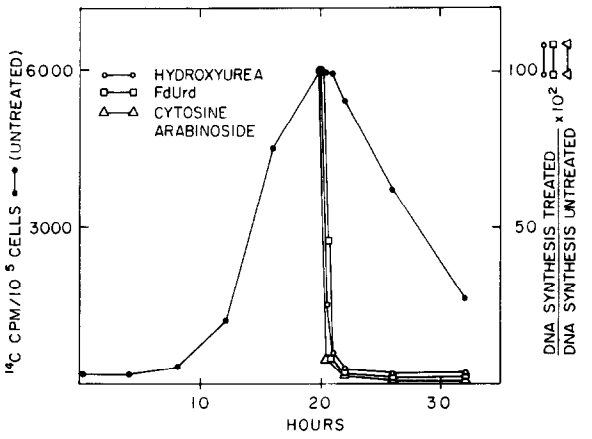

FIGURE 1. Effect of hydroxyurea (75 µg/ml), fluorodeoxyuridine (25 µg/ml) and cytosine arabinoside (50 µg/ml) on DNA synthesis during the cell cycle of synchronized lymphocytes. Lymphocytes, synchronized in the G1 phase of the cell cycle (5) were collected by centrifugation at 800 g for 5 min, washed in prewarmed (37° C) complete medium, and resuspended at a cell density of 5 x 10^5/ml in fresh complete medium, (t=0). Twenty hrs after synchronization, the culture was divided into four parts and the inhibitors were added to three of them. At selected times, throughout a 32 hr incubation period, 2.0 ml aliquots were removed and DNA synthesis determined as described in Materials and Methods.

the final third was incubated for 12 hrs in medium containing either H.U., Ara-C or FdUrd. Nuclear and cytoplasmic DNA was prepared from NP-40 lysed cells as previously described (2). CsCl equilibrium centrifugation profiles of nuclear and cm-DNA from control and H.U. treated cells are shown in Fig. 2. Under the control conditions the amount of BdUrd substituted light-heavy (LH) cm-DNA increased several fold during the 12 hr chase period (Fig. 2, bottom center). A similar amount of additional LH DNA appeared in the cytoplasm of cells treated with H.U. during the 12 hr chase period (Fig. 2, bottom right). Comparable results were obtained when Ara-C, FdUrd, or ethidium bromide were added during the chase period. From these experiments it is clear that in the absence of BdUrd and under conditions where cellular DNA synthesis is blocked, additional LH DNA continues to associate with cytoplasmic membranes.

Another group of inhibitors tested were rifampicin and two of its derivatives because of their varied inhibitory properties on eukaryotic and prokaryotic cells (6-11). Initially, rifampicin, N-demethyl rifampicin (AF/AP) and dimethyl-benzyl-demethyl rifampicin (AF/ABDP) were utilized at 50 µg/ml, 100 µg/ml and 250 µg/ml to determine their effects on growth of Wil_2 cells. At 100 µg/ml, rifampicin and AF/AP allowed normal cell growth and DNA synthesis, while AF/ABDP was toxic. At 250 µg/ml, all of the derivatives were at least partially inhibitory to cell

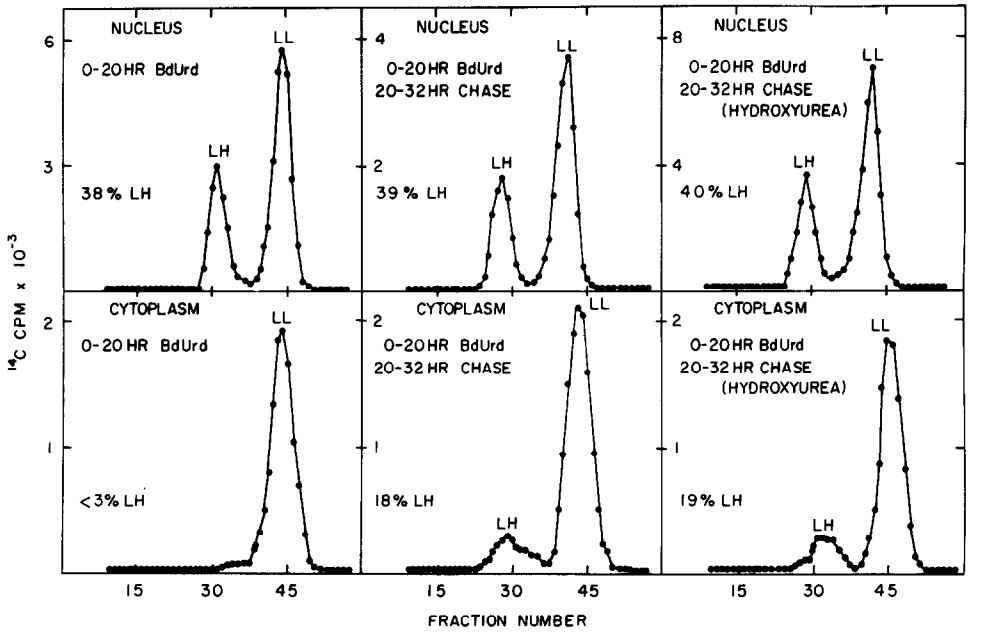

FIGURE 2. Equilibrium density gradient analysis of nuclear and cm-DNA prepared from control and hydroxyurea treated synchronized lymphocytes. Lymphocyte DNA was uniformly labeled with [^{14}C]-thymidine and the cells synchronized in the G1 phase of the cell cycle. Cells were "released" from G1 "arrest" and suspended in fresh complete medium containing BdUrd at a concentration of 20.0 µg/ml. After 20 hrs incubation, one third of the culture was collected and nuclear and cm-DNA prepared (2). Of the remaining two thirds, one third was resuspended in fresh "chase" medium containing 50.0 µg/ml thymidine and 5.0 µg/ml deoxycytidine, and the remaining one third was resuspended in fresh complete medium containing hydroxyurea at a concentration of 75.0 µg/ml. Both cultures were incubated for an additional 12 hrs, after which the cells were harvested and nuclear and cm-DNA extracted. DNA was centrifuged in solutions containing CsCl (initial density 1.72 g/ml) for 42 hrs at 35,000 rpm (25° C), fractions collected and analyzed as previously described (2).

growth and viability within 24 hrs. Therefore, rifampicin and AF/AP were utilized at 100 µg/ml, and AF/ABDP at 50 µg/ml.

To investigate the effects of derivatives of rifampicin on cm-DNA, pulse-chase experiments utilizing DNA substituted with BdUrd were performed as described above. Under control conditions the LH peak in the cytoplasm approximately doubled during the chase period. By contrast, in rifampicin treated cells, no "new" LH DNA appeared in the cytoplasm during the 12 hr chase. A similar result was observed when AF/AP was utilized. AF/ABDP did not completely inhibit the appearance of new LH DNA on cytoplasmic membranes (Table 1). Under similar conditions, when cultures were incubated for 32 hrs in the presence of BdUrd, treatment with rifampicin from 20 through 32 hrs did not effect the net incorporation of BdUrd into nuclear DNA.

TABLE 1

EFFECT OF INHIBITORS ON CYTOPLASMIC MEMBRANE ASSOCIATED DNA

Inhibitor	Concentration μg/ml	% Inhibition*
Hydroxyurea (H.U.)	75	3
Cytosine Arabinoside (ARA-C)	50	5
5-Fluorodeoxyuridine (FdUrd)	25	0
Ethidium Bromide	1	0
Rifampicin	100	96
N-Demethyl-rifampicin (AF/AP)	100	100
2,6-Dimethyl-4-benzyl-4-demethyl-rifampicin (AF/ABDP)	50	12

*Average of at least two experiments with each inhibitor. Values based on the amount of L.H. cm-DNA extracted from an inhibitor treated culture relative to a control culture.

The effect of rifampicin on cm-DNA does not appear to be due to alteration of overall RNA or protein synthesis. The polyribosome sedimentation patterns and ability to incorporate [^{14}C]-leucine into nascent polypeptides were approximately equivalent in samples from control or rifampicin treated synchronized cells at 20 and 32 hrs after release of cells from "G1 arrest". This does not, however, eliminate the possibility that rifampicin is effecting synthesis of a specific RNA or protein.

Table 1 summarizes the effects of the various inhibitors on the appearance of cm-DNA. Rifampicin and AF/AP almost totally inhibit this phenomenon, while H.U., FdUrd, Ara-C and ethidium bromide have little or no effect. AF/ABDP, which appeared to be the most toxic of the rifampicin derivatives, had only a minor inhibitory effect on the appearance of cm-DNA at the concentration employed in our experiments.

DISCUSSION

These studies employing inhibitors of macromolecular synthesis support the conclusion of our previous experiments that cm-DNA is a specific cytoplasmic DNA distinguishable physically and metabolically from the bulk of nuclear DNA. The fact that pre-formed DNA which has been substituted with BdUrd continues to appear in the cytoplasm when DNA synthesis is prevented by inhibitors with different mechanisms of action, indicates that cm-DNA results from exit of DNA from nucleus to cytoplasm and not from de novo synthesis employing precursors from a sequestered cytoplasmic pool.

It is clear that rifampicin and AF/AP prevent appearance of "new" cm-DNA; however, the mechanism by which rifampicin acts on cm-DNA is not apparent from our data. Rifampicin and its derivatives are known to produce several types of inhibitory effects on prokaryotic and eukaryotic cells and their viruses (6-11). In general, these effects on both eukaryotes and prokaryotes appear to concern the expression of a certain class of genes. As Sager points out, one indication of the different modes of transcriptional control of chromosomal and cytoplasmic genes is the fact that organelle RNA polymerases are inhibited by rifampicin, whereas this drug is without effect on nuclear transcription (12).

Finally, it is possible that the effect of rifampicin derivatives on cm-DNA results from one of several possible nonspecific effects. These include (1) inhibition of synthesis of proteins necessary for transport of cm-DNA, (2) change of a nucleus-cytoplasm equilibrium of cm-DNA, (3) binds to and saturates cytoplasmic membrane combining sites for cm-DNA, (4) prevents synthesis of linkages between cm-DNA and cytoplasmic membranes.

The ability to specifically inhibit the appearance of cm-DNA on cytoplasmic membranes may provide a means of determining its true function in the cell.

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