# Stimulation of protein accumulation in HeLa cells by inhibitors of DNA replication

## Ferritin

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Incubation of HeLa cells for 24 h with either hydroxyurea (HU), aphidicolin (APHI), thymidine (T) or butyrate (BU), substances used to inhibit replication and accumulate cells at the $G_1$/S interphase, followed by the elimination of the inhibitor and the addition of iron to the growth medium, results in an immediate (HU, APHI, T) or slightly delayed (BU) increased accumulation (18–24-fold higher than the basal level) of ferritin. Under the same experimental circumstances, 5-azacytidine is without effect. As a result of the action of these inhibitors on the structure of DNA, it is proposed that ferritin genes remain accessible to RNA polymerase allowing the accumulation in the cytoplasm of mature ferritin mRNA ready to be mobilized by iron for the production of ferritin molecules.

*Ferritin* *Cell synchronization*

## 1. INTRODUCTION

The increased accumulation of ferritin observed following the addition of iron to the growth medium of HeLa cells is thought to result from the mobilization of messenger RNA molecules preexisting in the cytoplasm [1,2].

Experiments conducted in this laboratory and aiming at accumulating HeLa cells at the $G_1$/S interphase with hydroxyurea (HU) before the addition of iron consistently gave higher outputs of ferritin.

Because HU inhibits ribonucleoside diphosphate reductase, an enzyme which is essential for DNA replication [3], we set out to analyze the action of various other inhibitors of DNA replication on the iron-mediated accumulation of ferritin.

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## 2. MATERIALS AND METHODS

HeLa cells were grown in suspension in minimal essential medium for spinner cultures (MEMS, Gibco F14) supplemented with yeast extract (Difco) and Oxoid nutrient broth no. 2, both at 2.5 g/l, 5% decomplemented (30 min at 56°C) newborn calf serum and 0.12% (w/v) methylcellulose. 140 ml exponentially growing HeLa cells were inoculated in each of 4 (A–D) identical 250-ml spinner flasks (Associated Biomedic System, USA), at cell densities of $7 \times 10^5$/ml (cultures A and B) or $10^6$/ml (cultures C and D).

Cultures C and D received the inhibitor which was either hydroxyurea (HU, 1 and 1.5 mM), aphidicolin (APHI, 5 $\mu$g/ml), thymidine (T, 2 mM), butyrate (BU, 5 mM) or 5-azacytidine (AZA, 5 and 10 $\mu$M), cultures A and B being used as reference were grown as such. The moment of addition of the inhibitors constituted $T_{24}$. 24 h later, following a low-speed centrifugation, the cells in each culture were resuspended in 120 ml

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fresh medium at a cell density of $9 \times 10^5$/ml, iron at a concentration of 108, 4 $\mu$g/ml, kept reduced by equimolar amounts of ascorbic acid, being added to cultures B-D. This moment was $T_0$.

The cultures grown at 37°C during the next 24 h were sampled (2 ml), 3 ($T_3$), 6 ($T_6$), 12 ($T_{12}$) and 24 ($T_{24}$) h later. The cells contained in the samples (taken in duplicate) corresponding to each time point were washed twice in phosphate-buffered saline (PBS) and finally resuspended in 2 ml PBS for protein determinations or PBS containing 3% (w/v) bovine serum albumin for ferritin determinations. Cells were then broken by 2 20-s sonications on ice applied in succession at power setting 4 of a Branson B12 sonifier, and protein determined [4]. The amounts of ferritin contained in the supernatant obtained by submitting the ultrasonic homogenate to a 10 min centrifugation at 10000 rpm and 4°C (Beckman J21 centrifuge, JA-14 rotor), were determined using an alkaline phosphatase enzyme-linked immunoassay (ELISA) developed in this laboratory.

## 3. RESULTS

While great care was exercised throughout these experiments to standardize the experimental procedure and obtain reproducible results, the levels of ferritin in control cultures fluctuated somewhat, increasing steadily as in fig.1 (curve A) or decreasing progressively from the moment the cells were centrifuged and resuspended in fresh medium (curves A in figs 2 and 4).

Treatment of such cultures with iron alone produced a transient increase in ferritin accumulation: following an initial lag of 3 h (curves B in figs 1–4), the relative proportions of intracellular ferritin rose some 6–8-fold above the control, reaching a peak 12 h later.

Incubation with HU (curves C and D in fig.1), APHI or T (curves C and D, respectively, in fig.2) and BU (fig.3, curves C and D), always failed to reveal any increase in ferritin accumulation during the first 24 h of the treatment with the respective inhibitors. Subsequent addition of iron however resulted in significant modifications.

With HU, APHI and T, ferritin accumulation started immediately at $T_0$ without the 3 h delay observed previously (butyrate-treated cells however showed a slight decrease during this period). It was

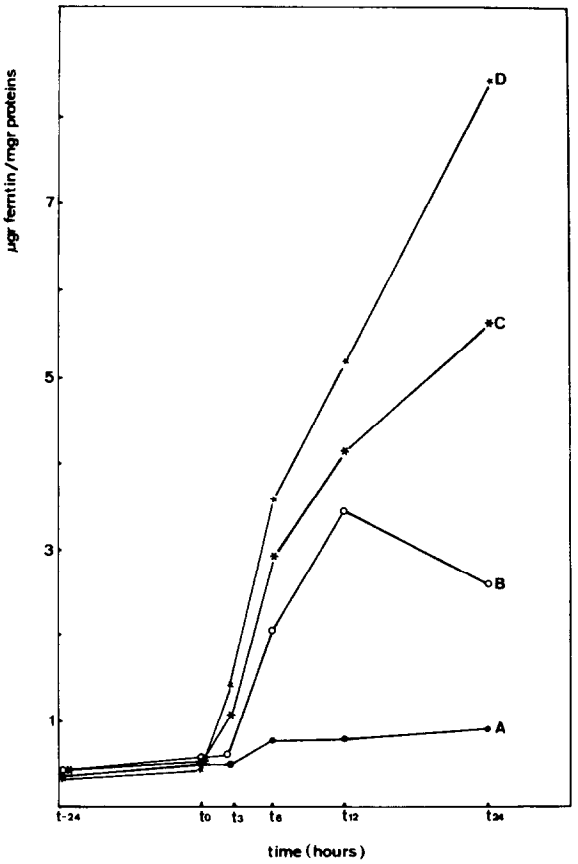


Fig.1. Influence of HU on the iron-stimulated synthesis of ferritin in HeLa cells. Four cultures (A–D) were initiated as described in section 2 and the proportions of intracellular ferritin evaluated at the times indicated. Cultures: A (control), cells grown 24 h in medium lacking any additive then centrifuged and resuspended in fresh medium; B, same treatment as for A with iron (108.4 $\mu$g/ml) being added after centrifugation C, cells grown 24 h in presence of 1 mM HU then centrifuged and resuspended in fresh growth medium ccontaining iron (108,4 $\mu$g/ml); D, same conditions as for C with the HU concentration being raised to 1.5 mM.

not until 6 h (curves C and D in fig.1) or 12 h (curves C and D in fig.2) after the addition of iron that the rates of ferritin accumulation began to level off. Cells treated with BU whether resuspended in fresh medium (fig.3 curve C) or in medium containing BU (fig.3 curve D) did not show this effect. Also, in all cases studied, the peak at $T_{12}$ disappeared, the final levels of intracellular ferritin attained being dependent on the concentration of

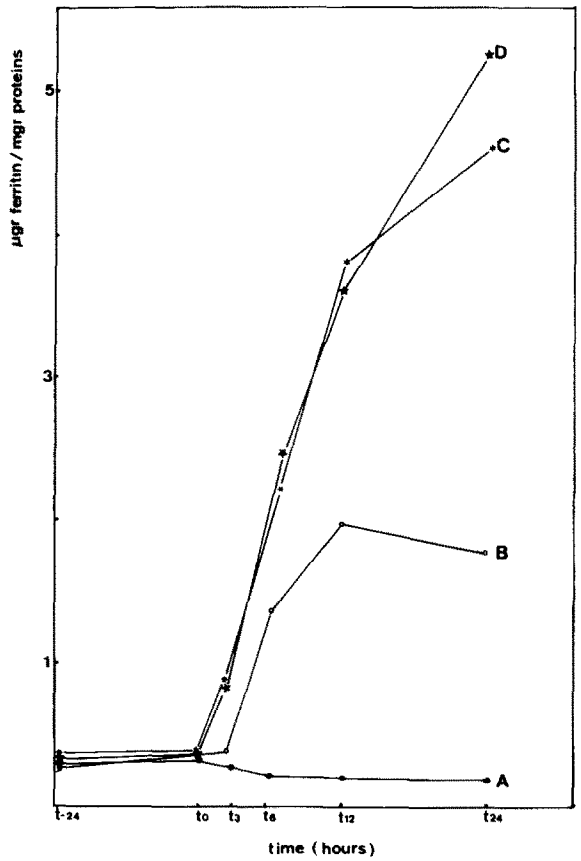


Fig.2. Influence of APHI and T on the iron-stimulated synthesis of ferritin in HeLa cells. Four cultures (A–D) were initiated as described in section 2. Cultures: A, control; B, same conditions as B in fig.1; C, same conditions as C in fig.1 with APHI (5 μg/ml) replacing HU; D, same conditions as C in fig.1 with T (2 mM) instead of APHI.

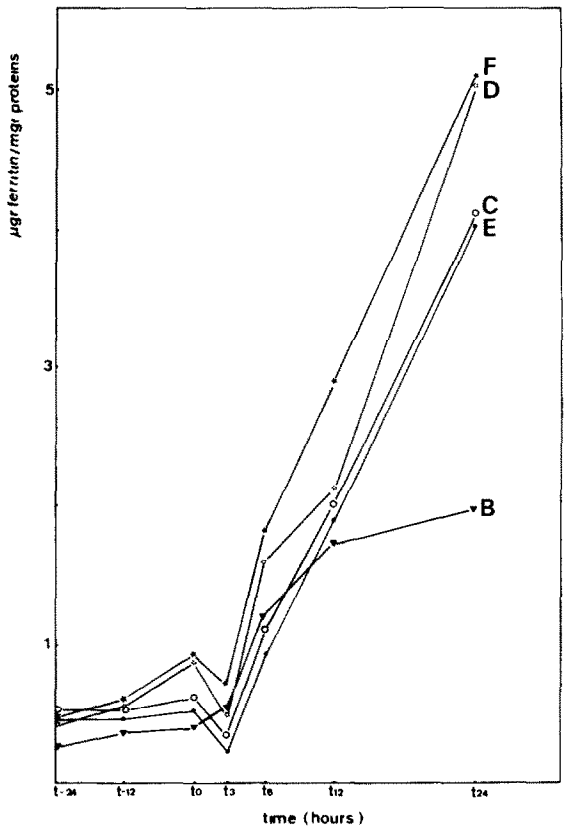


Fig.3. Influence of BU on the iron-stimulated synthesis of ferritin in HeLa cells. Five cultures (B–F) were initiated as described in section 2. Cultures: B, same conditions as B in fig.1; C, same conditions as C in fig.1 with BU (5 mM) replacing BU; D, same conditions as C with BU present throughout; E, cells first exposed to 1.5 mM HU and 5 mM BU for 24 h before being centrifuged and grown in the presence of iron alone; F, same conditions as E with AZA (5 μM) and BU (5 mM) replacing the mixture of HU and BU.

the inhibitor used (curves C and D in fig.1) as well as on their nature (fig.2, curves C and D), reaching values as high as 3-times the corresponding levels obtained with cells treated with iron alone (18–24-fold increase above the control). As compared to experiments where the inhibitors were used alone, neither the rate of accumulation nor the final amounts of ferritin observed increased when the inhibitors were combined (fig.3, curves E and F). AZA (fig.4) alone or in conjunction with iron did not increase the rate of ferritin accumulation.

## 4. CONCLUSIONS

HU, APHI, T and BU all have in common the ability somehow to interfere with DNA replication and accumulate cells at the $G_1$/S interphase [5–9]. Exposure of HeLa cells for 24 h to each of the 4 chemicals individually did not stimulate ferritin accumulation. When this treatment was followed by the addition of iron, ferritin accumulation took place immediately, reaching levels 3-times as high as those observed after treatment with iron alone (18–24-fold higher than the basal level).

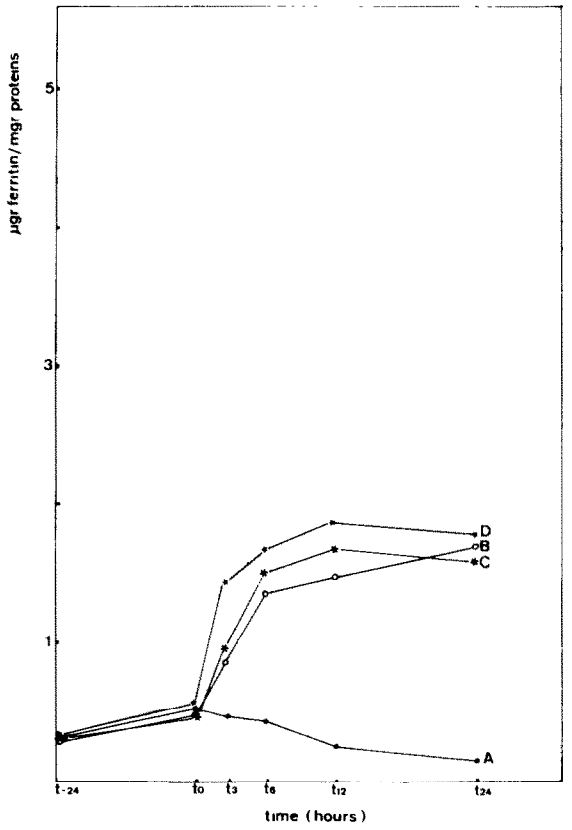


Fig.4. Influence of AZA on the iron-stimulated synthesis of ferritin in HeLa cells. Four cultures were initiated as described in section 2. Cultures: A, control; B, same conditions as B in fig.1; C, same conditions as C in fig.1 with AZA (5 $\mu$M) replacing HU; D, same conditions as C with the AZA concentration being raised to 10 $\mu$M.

This stimulatory action is reminiscent of the superinduction effect exerted by actinomycin D on ferritin accumulation [1]. More recently [10], the number of transferrin receptors present on human leukemic T cells was shown to increase as a result of treatment with the iron chelator desferrioxamine or HU. Thymidine and cytosine, arabinoside known for their inhibitory action on replication, also enhanced the phenotypic expression of transferrin receptor genes. One possible interpretation is prevention of the renewal of short-lived messengers by the inhibitors just mentioned above, so that at the translation level longer-surviving mRNAs such as ferritin mRNA have fewer mRNAs to compete with for ribosomes and initiation factors. Also, iron or HU inhibition of ferritin degradation cannot of course be ruled out by this type of experiment.

Another explanation is that, as a result of the action of these inhibitors on the structure of DNA, ferritin genes remained accessible to RNA polymerase allowing the accumulation in the cytoplasm of mature ferritin mRNA ready to be mobilized by iron for the production of ferritin molecules. This rests on the 2 following arguments. (i) The various inhibitors all act at different stages along the same metabolic pathway of DNA replication. Desferrioxamine and HU interfere with the supply of iron [10], thus on the activity of the non-heme iron-containing protein subunit M2 of ribonucleoside diphosphate reductase [11]. T is an inhibitor of the M1 subunit of the same enzyme [10] while APHI [6] and cytosine arabinoside inactivate $\alpha$DNA polymerase. In favour of this hypothesis, the second argument, which still remains to be firmly confirmed, stems from the fact that there are no particular reasons to believe that inhibitors of DNA replication act only on ferritin genes: if DNA structure remains accessible to RNA polymerase, then the amounts of other gene products coded by mRNA molecules of varying sizes should also increase. Preliminary evidence obtained in our laboratory indeed suggests that in HeLa cells, the amounts of $\beta$-chorionic gonadotrophin and fibronectin also immediately increase as a result of the addition of any of these inhibitors used alone, without iron.

More generally, the considerable enlargement observed with cells submitted for one generation to these inhibitors probably results from a boost in the accumulation of several specific proteins. Any new substances such as the bacterial siderophores parabactin and compound II [12] whose action is to accumulate cells at the $G_1$/S interphase are expected to belong to the same category of effectors and to produce the same stimulation of protein accumulation. Other modifications imparted on DNA structure such as by AZA, thought to act by inhibiting enzymes that methylate cytosine residues in eukaryotic DNA [13], remained without detectable effects on the accumulation of ferritin.

In view of the above considerations, the use of such inhibitors as synchronizing agents, because of their potential effects as initiators of unbalanced cell growth, must be considered with great caution [14].

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