NUCLEAR ULTRASTRUCTURE OF THE TRANSFORMING LYMPHOCYTE DURING INHIBITION OF DEOXYRIBONUCLEIC ACID SYNTHESIS WITH HYDROXYUREA

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SUMMARY

The pattern of decondensation of heterochromatin in the transforming lymphocyte was found to be unaffected by hydroxyurea although DNA synthesis, which normally accompanies the latter part of this decondensation, was greatly inhibited. It is suggested that metabolic inhibition of DNA synthesis may result in atypical sites of DNA synthesis when the inhibitor is removed, since such sites are normally partly governed by an ordered sequence of decondensation of heterochromatin.

INTRODUCTION

Hydroxyurea has been shown to inhibit DNA synthesis in a variety of mammalian cells (Young & Hodas, 1964; Sinclair, 1965; Pfeiffer & Tolmach, 1967; Gale, 1968). The mode of action of hydroxyurea is not certain although interference with ribonucleotide reduction has been suggested (Young & Hodas, 1964). Hydroxyurea has been commended as an effective agent for producing a synchronous population of cells (Sinclair, 1965).

Evidence from several sources suggests that only chromatin in the decondensed form is active as a template for DNA synthesis (Littau, Allfrey, Frenster & Mirsky, 1964; Hay & Revel, 1963; Meek & Moses, 1963; Milner & Hayhoe, 1968). Many cell types contain part of their chromatin in the condensed form of heterochromatin during interphase and this must therefore decondense in order to replicate. It seems important to establish whether this decondensation of chromatin is impeded if DNA synthesis is inhibited.

It has been shown that transformation of the lymphocyte by phytohaemagglutinin (PHA) is characterized by a progressive and eventually complete decondensation of condensed chromatin. The latter part of this decondensation is accompanied by DNA synthesis in the first S-phase (Milner & Hayhoe, 1968). Transformation of lymphocytes induced by pokeweed mitogen occurs in the presence of hydroxyurea, although DNA synthesis is greatly inhibited (Topping, Kierski & Cooper, 1968).

In this study the transforming lymphocyte has been used to investigate the effect of inhibition of DNA synthesis on changes in nuclear ultrastructure.
MATERIALS AND METHODS

Lymphocyte cultures from two normal donors were set up as previously described except that the final leucocyte concentration was adjusted to $1.5 \times 10^6$ cells/ml (Milner, 1969). Three cultures from each donor were treated differently. To (a) was added PHA and $4 \times 10^{-4} \text{M}$ hydroxyurea, to (b) PHA only, and to (c) neither PHA nor hydroxyurea was added.

After incubation for 48 h $[^3\text{H}]$thymidine was added to each culture at a concentration of $0.5 \mu\text{c} / \text{ml}$. After 30 min further incubation the cultures were terminated. They were processed for light-microscope autoradiography and electron microscopy as previously described (Milner, 1969). Slides were stained with Leishman’s stain for morphology and periodic acid/Schiff (PAS) and haematoxylin for autoradiography.

The number of mitoses in 2000 cells from each culture was counted. The percentage of cells labelling with $[^3\text{H}]$thymidine was estimated from a count of 1000 cells for each culture. The percentage of lymphocyte nuclei showing a completely decondensed pattern was assessed by counting 500 cells in each culture with the electron microscope.

RESULTS

Preliminary experiments showed that a concentration of $4 \times 10^{-4} \text{M}$ hydroxyurea permitted transformation of lymphocytes to occur normally although there was minimal DNA synthesis. The morphology of cells at 48 h, where hydroxyurea was present from initiation of the culture, was directly comparable with a control culture without hydroxyurea.

Table 1. Effect of hydroxyurea on a 48-h culture of lymphocytes with PHA

<table>
<thead>
<tr>
<th>Culture</th>
<th>PHA</th>
<th>Hydroxyurea</th>
<th>Mitoses</th>
<th>$[^3\text{H}]$thymidine-labelled cells</th>
<th>Nuclei with totally decondensed pattern</th>
</tr>
</thead>
<tbody>
<tr>
<td>1a</td>
<td>+</td>
<td>+</td>
<td>o</td>
<td>1.0</td>
<td>7.0</td>
</tr>
<tr>
<td>1b</td>
<td>+</td>
<td>0</td>
<td>o.6</td>
<td>34</td>
<td>6.8</td>
</tr>
<tr>
<td>1c</td>
<td>0</td>
<td>0</td>
<td>0.02</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2a</td>
<td>+</td>
<td>+</td>
<td>o</td>
<td>0.6</td>
<td>7.2</td>
</tr>
<tr>
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<td>+</td>
<td>0</td>
<td>0.8</td>
<td>37</td>
<td>7.2</td>
</tr>
<tr>
<td>2c</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

At 48 h the percentages of mitoses, of cells labelling with $[^3\text{H}]$thymidine and of cells with a totally decondensed nuclear pattern were estimated. The results are shown in Table 1.

It can be seen that there was a complete absence of mitoses and minimal DNA synthesis in the absence of PHA or the presence of hydroxyurea. In the cultures containing hydroxyurea a very small percentage of cells was lightly labelled. Auto-
radiographs exposed for the same length of time are shown for PHA cultures with and without hydroxyurea in Figs. 1 and 2 respectively. It was not possible to quantitate the effect on DNA synthesis further by grain counting, since at a time of exposure of the autoradiographs which produced any significant labelling in the hydroxyurea cultures the cells in the control cultures were too heavily labelled for the grains to be counted.

The ultrastructural morphology of cells after 48 h in the three types of culture is shown in Figs. 3-5. In the culture without PHA there was no perceptible decondensation of nuclear heterochromatin (Fig. 3), but cultures with PHA showed a comparable morphology whether hydroxyurea was also present or not (Figs. 4, 5). The extent of decondensation was quantitated by estimating the percentage of cells which had a totally decondensed nuclear pattern. It will be seen, however, in Figs. 4 and 5 that other cells with relatively small amounts of heterochromatin were present, and these formed a considerable proportion of the total cells.

DISCUSSION

Transformation of the lymphocyte involves nuclear changes of gradual decondensation of condensed chromatin and the latter part of this decondensation is coincidental with the first S-phase of DNA synthesis (Milner & Hayhoe, 1968). The present results show that this decondensation is not affected if DNA synthesis is inhibited. Transformation of lymphocytes has also been shown to occur normally in the presence of another inhibitor of DNA synthesis, 5-fluorodeoxyuridine (Salzman, Pellegrino & Franceschini, 1966). It seems likely therefore that the present observations would also be found with other inhibitors of DNA synthesis.

When a metabolic inhibition of DNA synthesis is removed the ensuing S-phase is shorter than normal (Till, Whitmore & Gulyas, 1963; Rao & Engelberg, 1966; Pfeiffer & Tolmach, 1967). It has been proposed that some normally rate-limiting steps can carry on unimpeded in the presence of the metabolic inhibitor (Till et al. 1963). The present results suggest that decondensation of chromatin may be such a step.

Analysis of the extent of interference with the cell cycle in cells treated with hydroxyurea has suggested that only cells in the S-phase are impeded and those in the G1, G2 and M phases pass through these stages unaffected (Sinclair, 1965; Pfeiffer & Tolmach, 1967). There is therefore a pile-up of cells at the end of G1 and when the hydroxyurea is removed these cells pass synchronously into the S-phase.

During transformation, lymphocytes pass from the G0 through phase G1 and into the S-phase. The changes in nuclear chromatin previously shown normally to accompany the G0, G1 and S phases (Milner, 1969) are unaffected by hydroxyurea. Thus, it may be suggested, when this and similar agents are used for synchronizing cells, a population may be obtained which is functionally at the end of the G0-phase but morphologically with respect to nuclear chromatin at a different stage in the cell cycle.

Comings & Kakefuda (1968) have studied the initial sites of DNA synthesis in
human amnion cells released from a block to DNA synthesis with excess thymidine and amethopterin. They found that cells taking up [\(^{3}H\)thymidine in the first 10 min had no condensed chromatin and marked localization of grains at the nuclear membrane. They suggested that points of initiation of DNA synthesis for those replicons which replicate at the beginning of the S-phase are located at the nuclear membrane. In the transformed lymphocyte it seems that the nuclear membrane is an important locus of DNA replication in the majority of cells which have no readily discernable condensed chromatin (Milner, 1969). Such cells do not appear until later in the S-phase. If a peripheral band of heterochromatin is present, as it is in many cells, DNA replication is not at the nuclear membrane but at the borders of the heterochromatin adjacent to euchromatic areas (Milner & Hayhoe, 1968). It is possible that these different results may be due to study of different cell types. Nevertheless the present findings suggest that caution should be exercised in the interpretation of ultrastructural sites of DNA synthesis in cells recently released from a metabolic inhibition of such synthesis. The localization of DNA synthesis is partly dependent on changes in the pattern of condensed and decondensed chromatin, and these do not appear to be affected by inhibitors of DNA synthesis.

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REFERENCES


Nuclear ultrastructure and hydroxyurea


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Figs. 1, 2. Autoradiographs from 48-h cultures of PHA-stimulated lymphocytes labelled with [3H]thymidine. Stained with PAS and haematoxylin. \( \times 150 \).

Fig. 1. Cells from a culture containing \( 4 \times 10^{-4} \text{M} \) hydroxyurea. Only occasional cells are lightly labelled (arrow).

Fig. 2. Cells from a culture without hydroxyurea. A considerable proportion of cells is heavily labelled.

Fig. 3. Electron micrograph of cells from a culture of lymphocytes which contained neither PHA nor hydroxyurea. At 48 h there is no detectable change in heterochromatin from that of the original lymphocytes. \( \times 20000 \).
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Fig. 4. Electron micrograph of cells from a 48-h culture of transforming lymphocytes where PHA was present from initiation of the culture but not hydroxyurea. Cells with various amounts of heterochromatin are seen and one cell has none at all. × 7000.
Fig. 5. Electron micrograph of cells from a 48-h culture of transforming lymphocytes which had contained $4 \times 10^{-4} M$ hydroxyurea from its initiation as well as PHA. The nuclei include some with no heterochromatin at all and their morphology is comparable with that of a culture without hydroxyurea. $\times 7000$. 