LABELLING OF HUMAN RESTING LYMPHOCYTES BY CONTINUOUS INFUSION OF \(^{3}\text{H}\)THYMIDINE. I. CHARACTERIZATION OF CYTOPLASMIC LABEL

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SUMMARY

After continuous \(^{3}\text{H}\)-TdR infusion in vivo or incubation with \(^{3}\text{H}\)-TdR in vitro human blood lymphocytes were examined by light-microscopic and electron-microscopic autoradiography. Using relatively long autoradiographic exposure times (50–300 days) not only nuclear but also cytoplasmic labelling was visualized, the cytoplasmic label being present in up to 96% of the cells. The cytoplasmic label was predominantly associated with the mitochondria and was removed from the cells nearly completely by treatment with DNase but not with RNase or cold perchloric acid. It is concluded that this cytoplasmic label mainly represents \(^{3}\text{H}\)-TdR incorporated into mitochondrial DNA which is continuously renewed in an average turnover time of 14 days or less. This value is compatible with a turnover time of 11 days for mitochondrial DNA in mammalian cells reported in the literature.

INTRODUCTION

As part of a programme concerning lymphocyte kinetics in man, cell production and turnover rates were investigated in lymphoproliferative disorders and other malignant diseases using \(^{3}\text{H}\)thymidine (\(^{3}\text{H}\)-TdR) administered by continuous intravenous infusion. The evaluation of the autoradiographed blood smears of the patients revealed an unexpected finding: Grains overlying lymphocytes were not exclusively associated with the nuclear areas but were also localized over the cytoplasmic rims.

In the present paper the kinetics of cytoplasmic labelling with \(^{3}\text{H}\)-TdR in vitro is described, and the nature of the cytoplasmic label is outlined by additional in vitro experiments, electron-microscopic and cytochemical procedures. The problem of nuclear labelling with \(^{3}\text{H}\)-TdR is the subject of a following paper (Schick et al. 1978).

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**Patients and Methods**

**Patients**

One patient (He. A.) with undisturbed haemopoiesis suffering from glioblastoma multiforme and 5 patients (Hu. J., Ha. H., Hō. J., St. E., Ki. C.) with chronic lymphocytic leukaemia (CLL) were examined. Some of their clinical data are summarized in Table 1.

<table>
<thead>
<tr>
<th>Patients ...</th>
<th>He. A.</th>
<th>Hu. J.</th>
<th>Ha. H.</th>
<th>Hō. J.</th>
<th>St. E.</th>
<th>Ki. C.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diagnosis</td>
<td>Gliobl.</td>
<td>CLL</td>
<td>CLL</td>
<td>CLL</td>
<td>CLL</td>
<td>CLL</td>
</tr>
<tr>
<td>White blood cell count</td>
<td>9100</td>
<td>7300</td>
<td>22000</td>
<td>35400</td>
<td>420000</td>
<td>1000000</td>
</tr>
<tr>
<td>% lymphocytes</td>
<td>26.8</td>
<td>58.6</td>
<td>85.4</td>
<td>97.6</td>
<td>97.3</td>
<td>&gt; 99</td>
</tr>
<tr>
<td>Pretreatment (free interval until (^3\text{H-TdR}))</td>
<td>Operation</td>
<td>Chemotherapy (2 months)</td>
<td>—</td>
<td>Chemotherapy (1 month)</td>
<td>Chemotherapy (2 years)</td>
<td>—</td>
</tr>
<tr>
<td>Treatment during the study (period between start of (^3\text{H-TdR}) and therapy)</td>
<td>—</td>
<td>Chemotherapy (3 weeks)</td>
<td>—</td>
<td>Splenic irradiation (3 weeks)</td>
<td>—</td>
<td>Splenic irradiation (3 weeks)</td>
</tr>
</tbody>
</table>

**In vivo labelling procedure and preparation of blood smears**

\([\text{Me-}^{3}\text{H}]\text{thymidine (sp. act. 5 Ci/mM; Radiochemical Centre, Amersham)}\) was administered by intravenous infusion at a constant rate for 3 days (in case Ha. H.), 4 days (St. E.), 7 days (He. A., Hō. J.), 9 days (Ki. C.) and 10 days (Hu. J.). Each patient received 0.1 mCi/kg body weight daily. Blood samples of 3 ml were taken at least twice daily within the first 2 weeks, then every 1-3 days up to the fourth week and thereafter about twice per week until the end of the study. From each sample leukocyte concentrates were prepared as described previously (Meuret & Hoffmann, 1973) and smeared on slides. All smears were fixed in absolute methanol for 30 min and then washed in running water for 2 h.

**Labelling of cultured lymphocytes**

From venous blood of 3 healthy donors mononuclear cells were isolated by the Ficoll Isopaque technique (Boyum, 1968). The resulting cell suspensions contained about 90% lymphocytes. The lymphocyte yield ranged between 50 and 60%. After 3 washes in MEM the cells were dispersed to a final concentration of 10⁶ cells/ml of a culture medium (MEM with 10% foetal calf serum and 1% antibiotic-antimycotic mixture: Gibco, Grand Island, New York) without any mitogen. Aliquots of 10 ml cell suspension placed into tissue culture flasks were incubated with 3 μCi \(^3\text{H-TdR}\) for 1-3 days at 37 °C in a 5% CO₂ in air atmosphere. During the incubation period the cell concentration decreased by about 8% per day. Part of the cultured cells was harvested every 24 h. After centrifugation of the culture fluid the cell-
free supernatant was pipetted off and stored at \(-36^\circ C\). All cells were washed 3 times with MEM. One half of the cells was resuspended in a minute amount of foetal calf serum and smeared on slides; from the other half dispersed in MEM containing 15\% foetal calf serum cytocentrifuge slides were prepared. All slides were fixed with 100\% methanol for 30 min.

Depression or cessation of \(^3\)H-TdR uptake of cultured cells may occur during the culture period, e.g. by enzymes released from viable or dead cells into the culture fluid (Marsh & Perry, 1964; Rubini, Keller & McCall, 1964). Therefore exponentially growing lymphoblasts of the lymphoblastoid cell line WI L 2 were incubated for 2 h at 37 \(^\circ\)C in 1- to 3-day-old media of lymphocyte cultures set up with an initial concentration of 0.3 \(\mu\)Ci/ml \(^3\)H-TdR. Thereafter cytocentrifuge slide experiments were performed.

**Enzymic digestion and acid extraction procedures for removal of DNA or RNA from smears**

Slide centrifuge preparations of lymphocytes cultured for 1–3 days in \(^3\)H-TdR containing medium were treated with deoxyribonuclease (DNase) or ribonuclease (RNase). In the in vivo experiments analogous preparations performed at the end of \(^3\)H-TdR infusion were incubated either with RNase or cold perchloric acid (PCA). Crystallized DNase and RNase (Serva, Heidelberg, W. Germany) were used each at 10 mg/100 ml of 20 mM Tris buffer adjusted to pH 7.3 and containing 45 mM MgCl\(_2\) and 5 mM CaCl\(_2\) (Love & Rabotti, 1963). The digestion periods were 12 h for RNase and 24 h for DNase, both at 37 \(^\circ\)C. Removal of RNA by acid extraction was performed with 10\% PCA for 36 h at 4 \(^\circ\)C (Baserga & Kisieleski, 1963). All slides were washed in tap water after the extraction procedures.

**Preparation of ultrathin sections**

Leukocytes obtained from patient Ha. H. at the end of a 3-day \(^3\)H-TdR infusion and lymphocytes labelled in vitro for 3 days were fixed in 0.1 M sodium cacodylate buffer (pH 7.4), containing 1.5\% glutaraldehyde and 1\% sucrose (370 mosmol), for 2 h at 4 \(^\circ\)C. In order to obtain pellets the thoroughly rinsed cells were centrifuged in polyethylene tubes (10,000 g, 5 min), the pellets were postfixed in veronal-buffered OsO\(_4\) (1\%, 2 h, 4 \(^\circ\)C), dehydrated in ethanol and embedded in Maraglas (Huhn & Stich, 1969; Spurlock, Kattine & Freeman, 1963).

**Autoradiography**

**Light microscopy.** Triplicate sets composed of normal and extracted smears were coated with Ilford K4 emulsion (in patients Hö. J. and Ki. C.) or Kodak AR 10 stripping film (in the other patients and in the in vitro experiments). The exposure times in the in vitro studies were 140 days (He. A.), 160 days (Hu. J., St. E.), 200 days (Hö. J.), 280 days (Ha. H.) and 300 days (Ki. C.) and 30 or 150 days in the in vitro experiments. After development the autoradiographs were stained with buffered Giemsa solution.

**Electron microscopy.** Ultrathin sections of light-gold interference colour were coated with Ilford L4 emulsion by means of a dipping machine (Vrensen, 1970). The thickness of the emulsion layer was 1–1.5 crystals (1 part emulsion to 1.4 parts of H\(_2\)O) and was controlled by the interference colour, which was found to be purple to yellow-green. Sections were exposed in a N\(_2\) atmosphere at 4 \(^\circ\)C for 200 days (cultured lymphocytes) and for 380 days (Ha. H.), developed with Microdol X for 4 min at 20 \(^\circ\)C, poststained with uranyl acetate and lead citrate by means of LKB grid boxes (Sjöström, Thornell & Hellström, 1973). Electron microscope: Zeiss EM 10.

**Evaluation of the autoradiographs**

**Light microscopy.** In each preparation to be evaluated 1000–5000 lymphocytes were scored in the case of the in vitro study and 100–200 in the case of the in vitro study. Using an ocular micrometer nuclear and cytoplasmic labelling was evaluated separately. Nuclear labelling was defined as the silver grains overlying the nucleus and the adjacent cytoplasmic area of
1 μm breadth. Cytoplasmic labelling was defined as the rest of silver grains overlying the cell and in addition the grains over a '1-μm zone' surrounding the cell border. These 1-μm zones were included because of the reach of ³H-irradiation in biological material (Feinendegen, 1967). The autoradiographic background was defined using activity-free control preparations processed along with the experimental ones. Then the mean background per nuclear and cytoplasmic area respectively was subtracted from the mean grain counts of lymphocytes evaluated in the experimental slides.

Electron microscopy. Grain densities were evaluated over single compartments (nucleus, cytoplasm, mitochondria, embedding medium) and junctional compartments (nucleus/cytoplasm, mitochondria/cytoplasm, cytoplasm/embedding medium) using the circle method (Williams, 1969). For background correction the grain density over the embedding medium was used. Frequency distribution of grains was tested by χ²-test.

RESULTS

Light-microscopic findings

In vivo (Fig. 1 A): In all patients the first lymphocytes with unequivocal cytoplasmic labelling appeared during the first day of ³H-TdR infusion. The labelling intensity of the cytoplasm as well as the frequency of cells showing cytoplasmic label increased until the end of ³H-TdR infusion (Fig. 2) and decreased thereafter slowly until the background level was reached after 4–7 weeks (Fig. 3). When the declining phase of the mean grain count curves is plotted semilogarithmically (Fig. 4) it can be interpreted as a simple exponential function with half lives (t₀.5) ranging from 5–7 to 14–5 days (mean 9.9 days).

In vitro (Fig. 1 B). In contrast to the in vivo situation the mean grain count of the cytoplasm in vitro increased particularly during the first day and thereafter only slightly (Fig. 5 A). In order to decide, whether this was due either to different metabolic processes in vivo and in vitro or only to a reduced ³H-uptake beyond the first day the additional experiment with the proliferating lymphoblasts was performed. The result is shown in Fig. 5B. The mean grain count of the lymphoblasts being in the S-phase of the cell cycle was highest after incubation in medium with freshly added ³H-TdR and became stepwise lower after incubation with 1, 2 or 3 day-old ³H-TdR containing medium. The decrease of labelling intensity of the lymphoblasts was exponential and corresponded to a t₀.5 of 23 h. This indicates a time-dependent exponential decrease of the availability of ³H-TdR for cellular synthesis in the lymphocyte cultures the precise biochemical reason being unknown. With the t₀.5 of the availability of ³H-TdR the mean grain count values of the cultured lymphocytes can be corrected to values to be expected in the case of constant ³H-TdR availability throughout the whole culture period using the following formula:

\[ MGC II = \frac{MGC I \cdot \lambda t}{1 - e^{-\lambda t}} \]

where MGC II = expected mean grain count, MGC I = measured mean grain count, \( \lambda = \ln 2 \): t₀.5 and t = duration of the lymphocyte culture. As shown in Fig. 5A′ the corrected labelling-intensity values of the cytoplasm and nucleus of resting lymphocytes incubated with ³H-TdR increase almost linearly with time.
Fig. 1. A. Leukocytes of a leukocyte concentrate smear from patient Hu. J. after a 10 days $^3$H-TdR infusion; 3 lymphocytes show distinct label over their cytoplasmic rims. × 1000. B. Lymphocytes of a healthy donor cultured 24 h in medium containing $^3$H-TdR; all cells show cytoplasmic and nuclear label. × 1000. C. EM autoradiographs of lymphocytes from a healthy donor cultured 72 h in medium containing $^3$H-TdR; the grains are predominantly localized over or close to the mitochondria. × 5000.
Cytochemical findings

After digestion with DNase the number of grains over the cell area was reduced by about 90% (90% over cytoplasm and 91% over nucleus) in cells cultured for 24 h, and by about 83% (79 and 86% respectively) after 72 h. By contrast no significant reductions of grains were found after RNase or PCA treatment in vitro and in vivo.

Electron-microscopic findings

According to the χ²-test, frequency distribution of grains (Table 2) does not correspond to a random distribution (P < 0.001). It is evident that there are no significant differences between the in vivo and in vitro data. The grain density over the cytoplasm was highest over the mitochondria: 10 grains/100 μm² in vivo as...
Labelling of human resting lymphocytes, I.

Fig. 3. Course of cytoplasmic mean grain count during and after $^3$H-TdR infusion in 4 patients. Each point represents the mean (± s.d.) of 4 single values evaluated in 2 analogous slides of 2 different samples both taken during 1 day. The mean background level and its standard deviation are given by the horizontal lines. The periods of $^3$H-TdR infusion are symbolized on the x-axis.

well as 26 grains/100 $\mu$m$^2$ in vitro and lowest over the mitochondrion-free cytoplasm: 0.7 grains/100 $\mu$m$^2$ (in vivo) and 2 grains/100 $\mu$m$^2$ (in vitro). The grain density over the junctional compartment of mitochondria/cytoplasm was lying between these extremes: 3 grains/100 $\mu$m$^2$ in vivo and 9 grains/100 $\mu$m$^2$ in vitro. Accordingly, it is very likely that the grains over this compartment originate from tritium incorporated within the mitochondria and not within the surrounding cytoplasm. Regarding the compartment
cytoplasm/embedding medium, however, it is assumed that the grains can be associated with the cytoplasm, because the background over the embedding medium has been subtracted from the grains over any compartment. For the sake of simplicity in the following the grains over the mitochondria and the mitochondrial cytoplasmic borderlines are combined and compared with the mitochondrion-free cytoplasmic area. The grain densities over the former and the latter are significantly different ($P < 0.001$, $\chi^2$-test). If the total activity of the cytoplasm is considered, it can be calculated that 57% (in vivo) and 67% (in vitro) of the cytoplasmic label is located in the mitochondrial region (Table 2).

**DISCUSSION**

The first question to arise is whether we are dealing with a true cytoplasmic labelling or only with (a) an absorption of tritium-labelled compounds on the cell surface or on intracellular structures; or (b) a scattering of autoradiographic grains over the cytoplasm resulting from $\beta$-emission from tritium molecules bound in the nucleus near the nuclear membrane.

The absorption hypothesis can be excluded for the following reasons: First, cytoplasmic labelling increased with time, while at least in the in vitro experiments the concentration of $^3$H-TdR in the culture medium decreased. Second, the label
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Fig. 5. A shows the average values of the mean grain counts observed in cultured lymphocytes of 3 normal donors; ●, mean grain count of nucleus; ○, mean grain count of cytoplasm. In B the mean-grain-count values of WI L2 lymphoblasts incubated for 2 h in cell-free medium of 1 to 3-day-old 3H-Tdr-containing lymphocyte cultures are plotted semilogarithmically. t½ = 23 h. A' shows the expected course of mean grain count when using the observed values of parts A and B for calculation. The nuclear labelling is discussed elsewhere (Schick et al. 1978).

Table 2. Percentage grain distribution compared with relative areas of various compartments of lymphocytes labelled in vivo and in vitro respectively

<table>
<thead>
<tr>
<th>Compartments</th>
<th>Lymphocytes labelled in vivo</th>
<th>Lymphocytes labelled in vitro</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Nucleus</td>
<td>Nucleus/cytoplasm</td>
</tr>
<tr>
<td>Relative areas, %‡</td>
<td>32.5</td>
<td>9.4</td>
</tr>
<tr>
<td>Grain distrib., %§, †</td>
<td>17.2</td>
<td>3.1</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Mitochondria + cytoplasm</th>
<th>Cytoplasm/ embedd. medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>Relative areas, %‡</td>
<td>33.4</td>
<td>9.7</td>
</tr>
<tr>
<td>Grain distrib., %§</td>
<td>20.5</td>
<td>4.5</td>
</tr>
</tbody>
</table>

* Without the compartments mitochondria + mitochondria/cytoplasm.
† Total areas (= 100%) of 5800 μm² and 13000 μm² were evaluated in vitro and in vivo respectively.
§ The grain distributions base on 110 grains in vivo and 179 grains in vitro.
‡ After elimination of newly formed lymphocytes (= 1.3% of all cells) characterized by high nuclear grain density (Schick et al. 1978).
was removed by DNase treatment. The other possibility, that cytoplasmic labelling might be simulated by scattering of nuclear label is ruled out by the appearance of grains over the cytoplasm in a distance of more than 1 μm from the nucleus even in ultrathin sections and by the light-microscopic observation that occasionally cells without nuclear labelling showed nevertheless cytoplasmic labelling.

Cytoplasmic labelling after application of $^{3}$H-TdR seems to be a general feature of human lymphocytes. We observed it in normal and leukaemic lymphocytes, both in vivo and in vitro. Although cytoplasmic labelling has been found in only 20–60% of the cells in vivo and 75 or 96% in vitro (autoradiographic exposure times of 50 or 150 days), it is assumed that 100% of the lymphocytes would disclose cytoplasmic labelling, if the $^{3}$H-TdR concentration applied or the autoradiographic exposure time used were high enough. Cytoplasmic labelling after $^{3}$H-TdR has already been reported for mouse spleen cells in vivo, but without quantitative autoradiographic data (Bryant, 1966) and for human blood lymphocytes in vitro (Prindull, Ron & Yoffey, 1976). In the last case less than 2% of the cells were found to be labelled after a flash of $^{3}$H-TdR (25 μCi/ml) and an autoradiographic exposure time of 45 days. Other investigators using relatively large total doses of $^{3}$H-TdR in the form of continuous or serial application (Clarkson et al. 1970; Denman, Denman & Embling, 1968; Haas, Bohne & Fliedner, 1969; Little, Brecher, Bradley & Rose, 1962; Robinson, Brecher, Lourie & Haley, 1965; Theml et al. 1973) or in form of flash injection (Harris, Pelc & Blackmore, 1973; Harris, 1975) in connexion with relatively long autoradiographic exposure times did not describe labelling of the cytoplasm of lymphocytes in humans (Clarkson et al. 1970; Theml et al. 1973), rats (Haas et al. 1969; Little et al. 1962; Robinson et al. 1965) and mice (Denman et al. 1968; Harris et al. 1973; Harris, 1975). Apart from species differences, this failure may be due to insufficient conditions for autoradiographic visualization of this kind of weak labelling mainly depends on rather high values of the dose of $^{3}$H-TdR, of the duration of $^{3}$H-TdR application, and of the duration of the autoradiographic exposure time. If one of these factors is decreased the other ones must be reciprocally increased. More than other authors we have compensated for the use of a relatively low dose of $^{3}$H-TdR with rather long application times and very long exposure times. Thus we easily succeeded in reproducible demonstration of cytoplasmic labelling.

The results reported give some information about the nature of cytoplasmic label after $^{3}$H-TdR. The cytoplasmic label is not water-soluble and is not identical with the acid-soluble pool of nucleotides and related compounds of low molecular weight, for it cannot be extracted by prolonged methanol and PCA treatment of the cells (Feinendegen, 1967). Moreover the label is resistant to extraction by RNase indicating that it does not belong to the RNA fractions. By contrast, DNase treatment removes 80–90% of the cytoplasmic label. Thus we assume that most of the label is $^{3}$H-TdR incorporated into cytoplasmic DNA. Since about 60% of the cytoplasmic label is localized to mitochondria the conclusion is drawn that most of the label corresponds to mitochondrial DNA. This is further substantiated by the kinetics of cytoplasmic labelling. Our results indicate that the metabolic process responsible for cytoplasmic labelling, presumably the metabolism of cytoplasmic DNA, has a
turnover time of about 14 days (or somewhat less if the possibility of the reutilization of \(^3\)H-TdR is taken into account: Baugnet-Mahieu & Gautier, 1972; Feinendegen, Heiniger, Friedrich & Cronkite, 1973). Accordingly the turnover time of mitochondrial DNA of mammalian cells (mostly rat liver cells) measured using biochemical methods has been reported to be 11.3 (7.5–12) days (Gross & Rabinowitz, 1969; Mitra & Bernstein, 1970; Neubert, Oberdisse & Bass, 1968; Parsons & Simpson, 1973).

It should be emphasized, however, that not all cytoplasmic label was found in the mitochondria and could be extracted by DNase. This rest of about 20% of label may be bound to protein (perhaps by incorporation of \(^3\)H into amino acids after catabolism of \(^3\)H-TdR) since it is not extractable by DNase, RNase and PCA.

Of special interest is the fact that not only the cytoplasm but also the nucleus of mitotically resting (Go) lymphocytes can be labelled after \(^3\)H-TdR. This phenomenon will be described and analysed particularly with regard to its influence on cell kinetic studies in a further paper (Schick et al. 1978).

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