Catabolism of Exogenously Supplied Thymidine to Thymine and Dihydrothymine by Platelets in Human Peripheral Blood

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ABSTRACT

The interference of platelets with the estimation of unscheduled DNA synthesis in human peripheral mononuclear leukocytes following genotoxic exposure was studied. A 96% reduction in the unscheduled DNA synthesis value was achieved by incubating [3H]thymidine with platelet-rich plasma for 5 hr at 37°C. Using radioactive thymine-containing compounds, together with quantitative analyses based on thin-layer and ion-exchange chromatographies, we have shown that thymidine was converted to thymine which, in turn, was converted to dihydrothymine in platelet-rich plasma. The enzymes responsible were separated from platelet lysates by gel filtration and were identified as thymidine phosphorylase and dihydrothymine dehydrogenase. The phosphorylase reversibly catalyzed the formation of thymine from thymidine and converted bromodeoxyuridine to bromouracil. The dehydrogenase reversibly catalyzed the interconversion of thymine and dihydrothymine in a reaction dependent on NADP(H), and it was inhibited by diazauracil and by thymine. Nearly all the thymidine-catabolizing activity found in whole blood samples supplied exogenously with thymidine was accounted for by the platelets. Since most genetic toxicological tests that use blood samples do not involve removing platelets from the blood cell cultures, then it is concluded that precautions should be taken in the future to determine the influence of platelets on these test systems. This is particularly true for methods dependent on thymidine pulses such as unscheduled DNA synthesis, or those dependent on bromodeoxyuridine, such as sister chromatid exchanges, since this nucleoside is also a substrate for thymidine phosphorylase.

INTRODUCTION

The majority of laboratory tests used to monitor human populations for harmful effects of genotoxic exposures is carried out on peripheral blood leukocytes (3, 5, 10). No doubt, the use of blood is based on the ethical considerations that such sampling is relatively nontraumatic and expendable to the individual. The more common of these procedures such as estimations of UDS (12), sister chromatid exchanges (14), chromosome aberrations (11, 16), and mutational frequencies (1) have all been shown to be dependent on the amount of exogenously supplied dThd.

The determination of UDS is particularly affected by exogenously supplied dThd, because it is measured as the unscheduled incorporation of [3H]dThd into DNA at specific activities of [3H]dThd in the range of 10 to 30 Ci/mmol. Hence, the [3H]dThd concentration added to leukocyte cultures is quite low, which, in turn, would make this method unusually sensitive to any dThd catabolic activity present in the blood.

Earlier, we had found (13) that whole blood, or human mononuclear leukocytes cultured in the presence of PRP, gave lower UDS values than whole blood depleted of platelets or leukocytes cultured in the presence of PPP. Furthermore, it was shown that this was not due to inhibition of UDS by any of a number of factors associated with platelet metabolism; e.g., cyclic AMP, cyclic GMP, sodium arachidonate, or prostaglandin E2. On the other hand, platelets are known to possess dThd phosphorylase activity (6), and we have recently shown (12) dThd catabolism by platelet-contaminated leukocyte cultures under conditions which strongly reduced the calculated level of UDS. In our continuation of this work, reported below, we have more fully characterized the pathway and enzymes of dThd catabolism in platelets. We have also shown that it is the catabolism which interferes so seriously with UDS measurements, and by demonstrating the catabolism of BrdUrd, we point to the implications for protocols using that compound of contamination by platelets.

MATERIALS AND METHODS

Chemicals. NADPH was from Boehringer Mannheim, West Germany; 5-bromo-2'-deoxyuridine, 5-bromouracil, 5-deazauracil, 4,5(6,8)-dihydrothymine, thymine, and 5,6-dihydrothymidine were from the Sigma Chemical Co., St. Louis, MO; 5,6-dihydrothymine was from Koch-Light Laboratories Inc., Colnbrook, England. Thymidine and uridine were from Schwarz Laboratories Inc., New York, NY. Ribothymidine (5-methyluridine) was from P-L Biochemicals Inc., Milwaukee, WI. Deoxyguanosine was from the California Foundation for Biochemical Research, Los Angeles, CA; [5-3H]dThd (26.3 Ci/mmol) and [methyl-3H]thymine (53 Ci/mmol) were from Amersham International, Amersham, Bucks, England. Sephadex G-100, and protein standards for molecular weight determination (ribonuclease, chymotrypsinogen, ovalbumin, and bovine serum albumin) were from Pharmacia Fine Chemicals, Upssala, Sweden.

Preparation of Platelets from PRP. Fresh PRP or outdated PRP obtained from the blood bank at Lund University Hospital were prepared as described previously (13). Platelets were pelleted at 400 × g for 15 min and washed twice with 0.9% NaCl solution (saline). If isolated platelet concentrates were used, they were also cultured in physiological saline. If PRP was used, there were no additional cultural supplements present. In some cases, isolated platelets were stored as frozen pellets at −20°C for up to 2 months.

Preparation of Platelet Lysates. Washed platelets were suspended in physiological saline or in buffer solution (NaHPO4, 5 mM, pH 7.5; or ammonium carbonate, 15 mM, pH 7.2; or Tris/HCl, 50 mM, pH 7.4). A typical preparation was carried out as follows. Platelets from 2- to 3-liter blood were suspended in 12 ml of either physiological saline or buffer containing...
solution, were frozen and thawed, and were sonicated on ice for 10 periods of 5 sec. After another freezing and thawing, the suspension was centrifuged at 20,000 × g for 30 min. In some experiments, the supernatant obtained was further centrifuged at 100,000 × g for 1 hr in order to obtain a soluble cytosolic supernatant.

Gel Filtration. All manipulations were carried out at 4°. Sephadex G-100 was poured into a column (59 cm x 1.6 cm) and irrigated with 10 mM Na₂HPO₄ plus 5 mM mercaptoethanol at pH 7.5. Standard proteins or lystate in 0.5 ml were applied, and irrigation was continued as before. Fractions of 3 ml were collected. Protein elution was continuously monitored by A₂₆₀. The flow rate was adjusted to 0.4 ml/min. Fractions were concentrated 6- to 10-fold by freeze drying before they were assayed for dThd- and thymine-catabolism.

Elution volume (ml) 20 30 40
A₂₆₀ per fraction 0.5 1.0 1.5

Detection of Enzyme Activity. Enzyme activities were determined by incubating samples with radioactive substrate and then separating the products by ion-exchange chromatography or by TLC, or by a combination of both. TLC sheets were cut in 10-mm sections with the origin at the center of one such section, and the radioactivity in each was determined. When quantification of the dThd and thymine regions was desired, because of similar Rf values there was about a 5% overlap of these regions preventing quantitative resolution of mixtures of these compounds in this range. Further details of incubation conditions are given in "Results." The role of P₃ in the pathway of dThd breakdown was also shown (Chart 2). This was accomplished by incubating washed platelets from 2 ml of PRP at 37° for 5 hr in the presence of 2 μM [³H]thymine and then analyzing the incubation mixture for dihydrothymine by TLC. Under these conditions, there was nearly 100% conversion of thymine to dihydrothymine. These data, together with the timecourse studies on dThd breakdown, we have reported previously (i.e., the appearance first of thymine, followed by the appearance of dihydrothymine and the depletion of thymine) (12), and inhibition studies (see below), indicate that the pathway is dThd → thymine → dihydrothymine, as has been shown previously for other tissues (7). No further metabolism of

RESULTS

Pathway of dThd Catabolism. We have reported elsewhere (12, 13) that preincubation of [³H]dThd with PRP resulted in a time-dependent decrease in the [³H]thymine incorporation into DNA during UDS. For example, a 50% reduction in the UDS value was achieved by incubation of the precursor dThd for less than 2 hr. We have also shown (12), using ion-exchange chromatography, that during such preincubation, dThd was converted to thymine, as well as to another metabolite. While this metabolite eluted from the ion-exchange column in a region which overlapped considerably with the dThd region, it was not incorporated into DNA during UDS.

We have used TLC carried out as described in "Materials and Methods" to identify the dThd catabolite produced after incubation of 1 ml of PRP with 2 μM [³H]dThd for 5 hr at 37°. The most lipophilic standard, dihydrothymine, cochromatographed with the unknown metabolite both having identical Rf values of 0.71. We have confirmed (Chart 1A) that dihydrothymine also cochromatographed with the product on the ion-exchange column. It may be noted that (5,6)-dihydrothymine coeluted with the dThd catabolite, but the major component of 4,5(5,6)-dihydrothymine did not (Chart 1B).

The conversion of thymine to dihydrothymine by PRP was also shown (Chart 2). This was accomplished by incubating washed platelets from 2 ml of PRP at 37° for 5 hr in the presence of 2 μM [³H]thymine and then analyzing the incubation mixture for dihydrothymine by TLC. Under these conditions, there was nearly 100% conversion of thymine to dihydrothymine. These data, together with the timecourse studies on dThd breakdown, we have reported previously (i.e., the appearance first of thymine, followed by the appearance of dihydrothymine and the depletion of thymine) (12), and inhibition studies (see below), indicate that the pathway is dThd → thymine → dihydrothymine, as has been shown previously for other tissues (7). No further metabolism of

Chart 1. Cochromatography of the dThd catabolite and dihydrothymine on Dowex AG 50 ion-exchange resin. PRP was incubated with 2 μM [³H]dThd for 5 hr at 37° and then loaded onto the resin and chromatographed as described in "Materials and Methods." A, oo, radioactivity in the sample; o—o, A₀₀ of standard 5,6-dihydrothymine; oo, oo, radioactivity in the sample; oo—oo, A₀₀ of standard 4,5(5,6)-dihydrothymine.
dihydrothymine was observed by a more detailed analysis of other R2 regions on TLC. This pathway was also confirmed by TLC of [3H]dThd incubations with washed platelets from 3 ml of PRP suspended in physiological saline solution or with platelet lysates (4 to 16 mg protein/ml) prepared in the various buffers described above. Supernatant solutions obtained by centrifuging such lysates at 100,000 x g for 1 hr also carried out the aforementioned dThd catabolic pathway.

### Contribution of Platelets to dThd Catabolic Activity of Whole Blood

The higher values of UDS measurements obtained previously (13) when PPP was used in culture media instead of PRP suggested that most of the dThd catabolic activity was located in the platelets. We have therefore studied dThd catabolism in whole blood and in blood depleted of platelets.

Whole blood was cultured for 5 hr at 37° in the presence of either 4 μM [3H]dThd or 100 μM [3H]dThd. After incubation, the blood was centrifuged at 400 x g for 10 min and 2 to 3 μl of the PPP were subjected to TLC analysis as described in “Materials and Methods.” The blood sample containing 4 μM [3H]dThd was 100% converted to [3H]dihydrothymine, whereas at 100 μM, more than 95% of dThd was converted but to [3H]thymine only.

We have also compared blood depleted of platelets with whole blood for dThd catabolic activity. For this purpose, 3 ml of blood were centrifuged at 100 x g for 15 min, and the PRP was removed. The volume of the blood cell pellet was adjusted to 10 ml with physiological saline, and the sample was centrifuged again under the same conditions. This wash procedure was performed twice. The volume of the cell suspension was corrected to 3 ml with physiological saline. As a control, a sample of whole blood was centrifuged 3 times under the same conditions, but in this case no plasma was removed, and no washings were carried out. The platelet-depleted whole blood (200 μl) and whole blood control samples (200 μl) were incubated in the presence of 2 μM [3H]dThd for 1 hr at 37°. The dThd catabolic activity was monitored by TLC analysis as we have already described above. It can be seen in Table 1 that 200-μl samples of whole blood converted 37.8% of the [3H]dThd to [3H]thymine and [3H]dihydrothymine. Very little conversion (i.e., <5%) was observed if the whole blood was depleted of platelets by replacing the PRP with physiological saline.

### Enzymes of dThd Catabolism

The conversion of thymine to dihydrothymine has been shown in various tissues (7, 15). The enzyme responsible has been purified from rat liver (15). It is NADPH-dependent and is inhibited by diazauracil. Chart 2 shows that in human platelet lysates the conversion of thymine to dihydrothymine was also dependent on NADPH; we have also shown that this conversion was inhibited by diazauracil (data not shown). It may be concluded that a dihydroxopyrimidine (dihydrothymine) dehydrogenase is the enzyme responsible. In the presence of diazauracil, dThd was converted to thymine only, indicating that dihydrothymine arises from thymine as we have already demonstrated above.

The reversibility of the thymine to dihydrothymine reaction was carried out by suspending platelets from 2 ml of PRP in 1 ml of physiological saline solution fortified with 100 μM dThd. The platelets were incubated at 37° for 5 hr and then washed twice in physiological saline. The purpose of this step was to ensure that the platelets had a supply of NADPH. A parallel incubation of PRP with 2 μM [3H]thymine at 37° for 5 hr was used to provide a source of [3H]dihydrothymine. The supernatant from this incubation containing dihydrothymine was then used to resuspend the platelets already enriched for NADPH. Next, these platelets were incubated at 37° for 5 hr and analyzed for enzymatic activity by TLC as described in “Materials and Methods.” Chart 3 shows the composition of the supernatant before and after the final incubation of NADPH-enriched platelets. It is clear that the platelets were provided with dihydrothymine that was converted to thymine quantitatively.

Chart 4 shows the dependence on Pr of dThd conversion to thymine. This is consistent with the activity being dThd phosphorlyase rather than a hydrolyase. The reversibility of this step in whole platelets (Chart 5) was achieved by incubating 1.05 ml of PRP at 37° for 5 hr in the presence of [3H]thymine (10 μl), thymine (22 μl; final concentration, 2 μM), deoxyguanosine (80 μl; final concentration, 1 μM), and diazauracil (50 μl; final concentration, 5 μM). Deoxyguanosine was used to provide deoxyribose-1-phosphate for the transferase reaction (11) catalyzed by the phosphorlyase in the reverse direction. Diazauracil was used to protect the thymine from conversion by the dehydrogenase. The PRP reaction mixture was subjected to TLC and radiochemical analysis as described in “Materials and Methods.” Chart 5 shows that the major portion of thymine was converted to dThd.
Chart 3. Reversal of the dehydrogenase reaction in PRP (conversion of dihydrothymine to thymine). [3H]dThd (2 µM) was incubated with PRP for 5 hr at 37° to obtain a source of [3H]dihydrothymine. The product (••) was identified as [3H]-dihydrothymine by TLC, and this was then incubated for 5 hr at 37° with platelets isolated from PRP that had been incubated previously with 100 µM dThd (this had been done to ensure a pool of NADPH). The final incubation was also analyzed by TLC as described in "Materials and Methods" for the production of thymine.

Molecular Weights. Because of the differences in the molecular weights reported for dThd phosphorylase (6, 9) and dihydrothymine dehydrogenase (15), platelet lysate was subjected to chromatography on gel filtration media to separate these 2 enzymatic activities. The data in Chart 6 reveal that we have successfully separated the 2 enzymatic activities on a Sephadex G-100 column. The phosphorylase eluted from the column ranging from M, 65,000 to 90,000, while the dehydrogenase was excluded from the gel matrix and appeared in the void volume.
Catabolism of dThd by Human Platelets

**Table 2**

<table>
<thead>
<tr>
<th>Sample</th>
<th>dThd (%)</th>
<th>Thymine (%)</th>
<th>Dihydrothymine (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>11</td>
<td>12</td>
<td>73</td>
</tr>
<tr>
<td>B</td>
<td>&lt;5</td>
<td>18</td>
<td>76</td>
</tr>
<tr>
<td>C</td>
<td>&lt;5</td>
<td>94</td>
<td>&lt;5</td>
</tr>
</tbody>
</table>

* Control, no other additive.

**Table 3**

<table>
<thead>
<tr>
<th>Thymine added to PRP (nmol)</th>
<th>Dihydrothymine produced (nmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>41</td>
<td>6.2</td>
</tr>
<tr>
<td>1000</td>
<td>0</td>
</tr>
</tbody>
</table>

**Phosphorylase, and M, 235,000 (15) for dihydrothymine dehydrogenase.**

**Metabolic Inhibition.** The presence of dihydrothymine dehydrogenase in human platelets has not been reported by other investigators. We have sought an explanation for the divergence of our observations from those of other workers, particularly Desgranges et al. (6) who tested for, but could not detect, this activity. It can be seen (Chart 7) that dihydrothymine production varied with the concentration of dThd. At lower levels of dThd (up to 202 μM), all of the substrates were converted to thymine. Therefore, this was the level of thymine to which the dehydrogenase was exposed. In order to determine whether the enzyme is inhibited by thymine or by dihydrothymine, the following experiments were performed. One-mi aliquots of PRP were incubated with 2 μM [3H]thymine together with dihydrothymine at 0, 15, 25, 35, and 50 μM. The conversion of [3H]thymine to [3H]-dihydrothymine was determined by TLC analysis after an incubation period of 5 hr at 37°. There was a complete conversion (i.e., 100%) of [3H]thymine to [3H]-dihydrothymine, regardless of the amount of unlabeled dihydrothymine that was added to the various PRP incubations. Then, 1-ml aliquots of PRP were incubated with 2 μM [3H]dThd (Table 2, Samples A and B) and with 2 μM [3H]dThd plus 200 μM unlabeled thymine (Table 2, Sample C). Following 5-hr incubation of all samples at 37°, thymine (unlabeled) was added to Sample B (Table 2) to a 200 μM final concentration to provide a control for the possible effect of such levels of thymine on chromatography. After TLC analysis (Table 2), it was clear that no chromatographic distortion had occurred and that thymine had inhibited the production of dihydrothymine. The conversion of thymine to dihydrothymine was also checked by ion-exchange chromatography. As indicated previously (Chart 1A), this technique is unsuitable for samples containing both dThd and dihydrothymine, but for the separation of thymine and dihydrothymine in the absence of dThd it is perfectly adequate. Table 3 shows the inhibitory effects of thymine on dihydrothymine production. These data are consistent with the dehydrogenase being inhibited by thymine and not by dihydrothymine.

**Effects of Platelet Lysates on UDS.** It has been clearly shown that platelets influence genotoxic measurements in that they inhibit UDS induced by the mutagen NA-AAF (12, 13). Further
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Table 4

<table>
<thead>
<tr>
<th>Plasma added</th>
<th>Other additive</th>
<th>% of inhibition of UDS</th>
</tr>
</thead>
<tbody>
<tr>
<td>PRP</td>
<td></td>
<td>35</td>
</tr>
<tr>
<td>PRP, preincubated&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td>95</td>
</tr>
<tr>
<td>PRP, preincubated&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.48 µM [³H]dThd&lt;sup&gt;c&lt;/sup&gt;</td>
<td>30</td>
</tr>
<tr>
<td>PPP</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>PPP</td>
<td>Platelet lysate, 50 µl&lt;sup&gt;d&lt;/sup&gt;</td>
<td>47</td>
</tr>
<tr>
<td>PPP</td>
<td>Platelet lysate, 100 µl&lt;sup&gt;e&lt;/sup&gt;</td>
<td>67</td>
</tr>
<tr>
<td>PPP</td>
<td>Platelet lysate, 200 µl&lt;sup&gt;f&lt;/sup&gt;</td>
<td>67</td>
</tr>
<tr>
<td>PPP</td>
<td>200 µl G-100 fraction&lt;sup&gt;i&lt;/sup&gt;</td>
<td>31</td>
</tr>
</tbody>
</table>

<sup>a</sup> [³H]dThd (0.48 µM) added during 5-hr preincubation; no further [³H]dThd was added.

<sup>b</sup> Following preincubation of 0.48 µM [³H]dThd with PRP, further addition of [³H]-dThd was made for the 18-hr period used to estimate UDS.

<sup>c</sup> Standard lysate in physiological saline (see “Materials and Methods”).

<sup>d</sup> Lysate of platelets from 250 ml of blood. The 200 µl tested represented 50% of Fraction 5 from Sephadex G-100 chromatography as in Chart 6 and “Materials and Methods.”

The effects of various plasmas and other additives listed below were measured by their incorporation into the cultures during the 18-hr incubation for quantifying UDS. The effects of the various plasmas and other additives listed below were measured by their incorporation into the cultures during the 18-hr incubation for quantifying UDS.

The procedure for estimating platelet effects on UDS is published elsewhere (13). Briefly, after induction of DNA damage in platelet-depleted mononuclear leukocyte cultures by a standardized 10 µM dose of NA-AAF, UDS was quantified for 18 hr by pulse with 10 µCi/ml [³H]dThd in the presence of 10 µM hydroxyurea. The effects of the various plasmas and other additives listed below were measured by their incorporation into the cultures during the 18-hr incubation for quantifying UDS.

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DISCUSSION

We have reported previously that human blood platelets interfere with UDS measurements (12, 13). Preliminary observations suggested that this resulted from dThd catalysis, and initial studies on the catabolic pathway were reported (12). We have now studied dThd catabolism by PRP, by isolated platelets, and by platelet lysates.

It can be concluded that the dThd catabolic activity of whole human blood is located mainly in the platelets. It must also be noted that BrdUrd was substantially degraded by platelets and by whole blood and that this was after 18- to 24-hr incubation periods. In sister chromatid exchange analyses routinely carried out by some investigators, incubations of whole blood cultures are often for 48 to 72 hr (3, 5, 10). Therefore, we would like to emphasize that account should be taken of the potential effect platelets may have on genotoxicity tests that are dependent on exogenously supplied dThd or BrdUrd.

While our work has again indicated the presence of dThd phosphorylase in human blood platelets, we have also shown the presence of a thymine-converting enzyme. Dependence on NADPH and inhibition of diazouracil indicate that the enzyme is dihydrothymine (dihydropropyrimidine) dehydrogenase. We report that this enzyme is significantly inhibited by thymine concentrations in excess of 20 µM. This may be viewed in the light of a previous report on the rat liver enzyme. In that case (15), the Km for thymine was found to be 2.5 µM, much lower than the levels at which we noted significant inhibition. We conclude that this inhibition is the reason that other workers (6) did not find this activity in human platelets.

While we have carried out preliminary substrate/product inhibition studies, we have not made kinetic measurements with isolated enzymes. Thus, we cannot say how the kinetic behavior of whole platelets differs from that of platelet lysates. Perhaps dihydrothymine dehydrogenase inhibition is more readily detected in lysates; this would explain (Chart 4) why thymine was not fully converted to dihydrothymine. Alternatively, since the lysate used in that experiment had been thoroughly dialysed, the result may reflect instability of the dehydrogenase, or its requirement for an ion removed by dialysis.
In Table 1, the values for dThd catabolism are not in exact mathematical proportion to the platelet concentrations shown. This reflects the sensitivity of platelets to the manipulations to which the blood sample had been subjected. Release of, and thus loss of, a number of factors can be expected under these conditions. In this regard, aggregated platelets apparently do not release the dThd catabolic enzymes, since we have shown that human serum does not inhibit UDS, whereas human PRP from the same donor does (13). On the other hand, PPP prepared from PRP conditioned for 24 hr by incubation at 37° has more dThd phosphorylase activity than does PPP prepared from fresh PRP.4

REFERENCES

4 Unpublished results.

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