Proc. Natl. Acad. Sci. USA
Vol. 83, pp. 4749–4753, July 1986
Cell Biology

Metabolic breakdown of [^{3}H]thymidine and the inability to measure human lymphocyte proliferation by incorporation of radioactivity

(thymidine phosphorylase/5-bromodeoxyuridine catabolism/isotopic fractionation/immunocompetence)

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Communicated by Alexander Hollaender, February 28, 1986

ABSTRACT **Quantitative measurement of the incorporation of tritiated thymidine into cultures of phytohemagglutinin-stimulated lymphocytes is routinely used as an indication of the immunocompetence of the cells and of their proliferation. The present experiments show that several components of human blood catabolize nucleosides, including thymidine, extensively. Most of the radioactivity from tritiated thymidine, for example, is quickly rendered unincorporable as the compound is metabolized to thymine and further breakdown products. Thus, cells continue to proliferate without incorporating radioactivity from the medium. Furthermore, variability in the degree of catabolism has been found from person to person, so that neither measurement of the depletion of radioactivity from the medium nor measurement of the amount of label incorporated into the cultures can be used as a quantitative indicator of cell proliferation or immunocompetence.**

When cells are allowed to proliferate in the presence of tritiated thymidine ([^{3}H]dThd), the radioactive nucleoside becomes incorporated into DNA and can be detected as labeled chromosomes in autoradiograms (1). Study of this phenomenon in proliferating cells gave the first indication that DNA replicates semiconservatively. That is, after one round of replication in the presence of the labeled precursor, both sister chromatids of the chromosome are radioactive, whereas after a subsequent round of replication in the absence of radioactivity, only one sister chromatid is labeled at any given region of the chromosome (2). As a result of the semiconservative nature of DNA replication, it is possible to follow the segregation of labeled DNA polynucleotide strands into chromatids through several replication cycles and to determine how many times proliferating cells have divided. This can be accomplished whether chromosomes are labeled with [^{3}H]dThd only during the first replication cycle or are labeled continuously with analogs of dThd such as 5-bromodeoxyuridine (BrdUrd) (3, 4). Thus, cell proliferation can be observed directly from the labeling patterns observed in chromosomes.

Because the incorporation of [^{3}H]dThd into chromosomes of proliferating cells sequesters the radioactive nucleoside from the medium, the loss of radioactivity from the medium is often used as an indication of cell proliferation, without recourse to any measurement of an increase in cell number or of segregation of label into sister chromatids. More commonly, however, the degree to which radioactivity from [^{3}H]dThd is actually incorporated into the DNA of phytohemagglutinin (PHA)-stimulated human lymphocytes is taken as a measure of the proliferation, and thus of the immunocompetence, of the cells (5–7). The reason for this is that only those lymphocytes that are immunocompetent respond to PHA and become transformed from G_0 cells into cycling (proliferating) blast cells.

That the degree of incorporation of radioactivity in cultures of lymphocytes might not be quantitatively related to cell proliferation was noted in autoradiograms of chromosomes showing that proliferating lymphocytes incorporated [^{3}H]dThd into their DNA for only one cycle and then continued to proliferate without further incorporation (8). The experiments reported here show that if human blood is cultured in the presence of [^{3}H]dThd, the nucleoside is quickly catabolized to unincorporable radioactive compounds that remain in the medium even though autoradiograms show that the cells are indeed proliferating. Thus, neither measurement of the depletion of radioactivity from the medium nor measurement of the amount of label incorporated into cells can per se be used as an indicator of the degree of cell proliferation.

MATERIALS AND METHODS

To see if labeled nucleosides are broken down in cultures of blood, we incubated 0.5 ml of heparin-treated human blood at 37°C in 5% CO_2/95% air with 5 ml of RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, penicillin at 50 units/ml, streptomycin at 50 μg/ml, and labeled nucleoside at 0.1 μCi/ml (1 Ci = 37 GBq). Some cultures that were PHA stimulated received 2% PHA-M (Difco). At intervals the medium was checked for total radioactivity, availability of the ^{3}H for incorporation into cells, and the movement of the label into various metabolites of dThd.

To estimate the amount of ^{3}H that remained in a form available for incorporation, we added the radioactive medium from the blood cultures to Chinese hamster ovary (CHO) cells. The CHO cells were first plated in Petri dishes and incubated in medium containing [^{14}C]dThd (50 mCi/mmol) at 0.01 μCi/ml for 24 hr to prelabel the DNA uniformly. The ^{14}C-labeled medium was then removed, the cultures were rinsed, and a 2-ml sample of the radioactive medium in which human lymphocytes had been grown was added. After 2 hr, this medium was removed, and the cells were rinsed with cold Dulbecco's phosphate-buffered saline (PBS) and placed on ice. The cells were then scraped onto filters, washed with 4% perchloric acid, and dried. The incorporated radioactivity was determined by scintillation spectrometry in Omnifluor (New England Nuclear) dissolved in toluene, and the results were obtained as a ^{3}H/^{14}C ratio. In any given experiment, the ^{3}H/^{14}C ratio obtained for fresh medium with no previous exposure to blood was taken to represent 100% availability of the ^{3}H for incorporation. All of the values were normalized to this value.

In the experiments in which the catabolism of dThd was monitored by an HPLC analysis of the medium from the

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Abbreviations: CHO, Chinese hamster ovary; PHA, phytohemagglutinin; RBC, erythrocyte.

blood cultures, 2-ml samples of the radioactive medium were deproteinized by the addition of 0.5 ml of 50% perchloric acid. The samples were then incubated at 4°C for at least 2 hr. After centrifugation, the supernatant was adjusted to approximately pH 6 by addition of 10 M KOH and then recentrifuged. Because some of the possible metabolites were unstable under these conditions, some of the samples were deproteinized by the addition of methyl alcohol (3 parts methyl alcohol/1 part sample) followed by centrifugation and evaporation of the methyl alcohol. [^{14}C]dThd and [^{14}C]-thymine were added to the samples as internal standards. Optical standards were dissolved in deionized water and measured at 208 nm.

The HPLC analysis itself was carried out at 28°C on a Hewlett–Packard 1082 liquid chromatograph using a 25 × 4.6 cm reverse-phase column with 10-μm C_{18} packing (Brownlee, Santa Clara, CA). The mobile phase was 8 mM ammonium phosphate at pH 5.5 with 7.5% (vol/vol) methyl alcohol, and fractions were collected and assayed for radioactivity in Aquasol. The fraction size was adjusted so that, in the experiment in which the metabolic products were determined, the material was collected in 95 fractions, whereas in those experiments in which simple loss of dThd was measured, the material was collected in only 14 fractions.

To determine which components of blood catabolized the [^{3}H]dThd to unincorporable products, freshly drawn blood was diluted 1:1 with PBS, layered on top of a Ficoll-Paque (Pharmacia) gradient, and centrifuged at 400 × g for 30 min. The plasma layer was recovered to look for catabolic activity in this fraction. The interface material was removed and washed in PBS and centrifuged at 300 × g for 10 min to retrieve the maximum number of cells. This fraction contained both lymphocytes and platelets. Half of this material was removed for further processing in Percoll (Pharmacia) to remove the platelets. Percoll was diluted to a density of 1.064 g/ml with Hanks' balanced salt solution. The mixture of lymphocytes and platelets was then layered onto the Percoll and centrifuged at 800 × g for 40 min. The pellet contained mostly lymphocytes, with only a very few contaminating platelets. Erythrocytes (RBCs) were obtained from both the top and the bottom of the RBC pellet found after the initial Ficoll-Paque separation.

A rough estimate by HPLC showed that our current batch of serum contained approximately 13 μM dThd. Thus, under our standard culture conditions of 10% serum, approximately 1.3 μM dThd was present.

Each experiment was repeated one to three times. The results shown in Figs. 1–5 are from representative experiments as there is some variation in the absolute response of blood from different donors (see below).

RESULTS

When medium containing [^{3}H]dThd at 0.1 μCi/ml was removed from PHA-stimulated whole-blood cultures, it was found to have at least 95% of the original radioactivity remaining even after 68 hr of incubation. The cells from these cultures, however, showed clear second- and third-division labeling patterns when examined by autoradiography, indicating that the cells had incorporated ^{3}H only in the first cycle after PHA stimulation, despite continuous exposure to the isotope. If fresh medium containing [^{3}H]dThd was added, ^{3}H was taken up readily and the labeling pattern changed, indicating that the cells were still capable of taking up and incorporating [^{3}H]dThd. When the radioactive medium from the blood cultures was added to CHO cultures, the CHO cells took up none of the ^{3}H. The radioactivity became unavailable for incorporation into CHO cells, whether the original blood cultures were PHA stimulated or not. Controls consisting of [^{3}H]dThd incubated in complete medium without human blood showed no loss in the incorporability of the label into CHO cells.

If the original cultures contained PHA-stimulated lymphocytes from a Ficoll-Paque gradient, there was very little diminution of ^{3}H, but it was still unincorporable. Packed RBCs appeared to cause a lesser conversion of the labeled precursor to unincorporable compounds. These latter results, however, could be caused by the presence of platelets (see below).

To investigate whether loss of the labeled methyl group of dThd was responsible for the decrease in incorporability, we incubated blood with dThd labeled with ^{3}H in the methyl group, in the 6 position of the ring, or in the 5′ position of the sugar for various lengths of time. Label from all three compounds became incorporable in CHO cells (Fig. 1), showing that loss of incorporability was not caused by a simple cleaving of the methyl group from the nucleoside. When the samples were warmed to 37°C and the condensate was collected on a "cold finger probe," only dThd labeled in the sugar produced radioactivity in the condensate. Only in this case, therefore, was the label metabolized to water.

To follow this loss of incorporable radioactivity with time, and to see if a similar phenomenon occurred with [^{3}H]BrdUrd, an analog of [^{3}H]dThd, we added either [^{3}H]dThd or [^{3}H]BrdUrd (27 Ci/mmol) at 0.1 μCi/ml to unstimulated cultures containing 10% whole blood for various periods of time. Whether [^{3}H]dThd or [^{3}H]BrdUrd was used, less than 50% of the ^{3}H was available for incorporation by CHO cells after 5 hr of incubation, and less than 10% by 24 hr. However, these results should not be taken to represent the kinetics of degradation of 15 nM dThd and 3.7 nM BrdUrd, because the culture medium with 10% serum already contained approximately 1.3 μM dThd—i.e., 100 times more dThd than was added.

At first the catabolism of [^{3}H]BrdUrd into unincorporable compounds was somewhat surprising because it is well known from sister chromatid exchange experiments that BrdUrd can be incorporated into human lymphocytes throughout several cell cycles of culture. Since these experiments are usually carried out in the presence of at least 10 μM BrdUrd, the experiment was repeated with the addition of 20 μM nonradioactive BrdUrd or dThd. Even with this relatively high concentration of nucleosides, [^{3}H]dThd and [^{3}H]BrdUrd were largely catabolized so that less than 20% of the original nucleoside remained after 27 hr of incubation (Fig. 2*A*). There was some indication that BrdUrd was more rapidly degraded than dThd, which is consistent with the finding of Nakayama *et al.* (9) that, *in vitro*, thymidine phosphorylase is more active toward pyrimidines with larger and more polar groups at the 5 position. Therefore, when this experiment was repeated for analysis by HPLC, [^{3}H]deoxyuridine, which has only hydrogen on carbon 5, was incubated

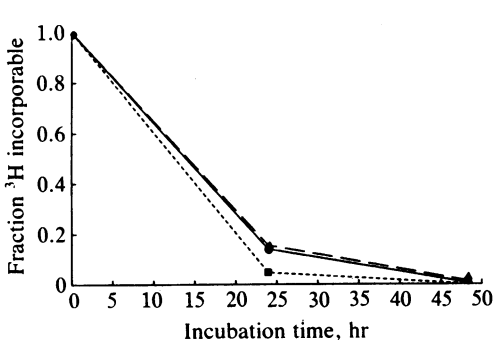

FIG. 1. Incorporation of ^{3}H by CHO cells from [^{3}H]dThd-labeled medium previously incubated with unstimulated whole blood. ●, Methyl-labeled [^{3}H]dThd at 0.1 μCi/ml; ■, ring (6)-labeled [^{3}H]dThd at 0.1 μCi/ml; ▲, sugar (5′)-labeled [^{3}H]dThd at 0.1 μCi/ml.

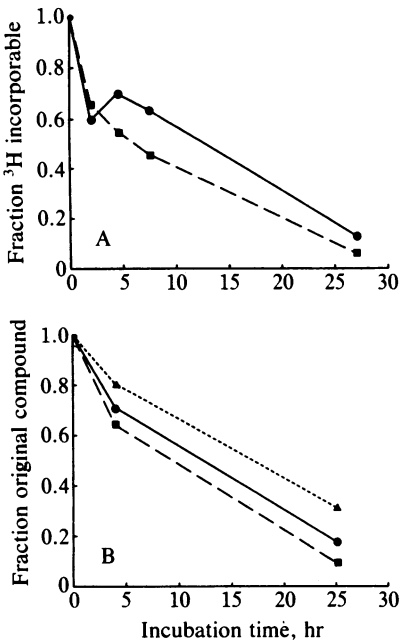

FIG. 2. Incorporation of ^{3}H by CHO cells (*A*) and ^{3}H remaining in the medium as the original nucleoside (HPLC analysis) (*B*) after incubation of unstimulated whole blood in medium containing the following: ●, 20 μM dThd plus [^{3}H]dThd at 0.1 μCi/ml; ■, 20 μM BrdUrd plus [^{3}H]BrdUrd at 0.1 μCi/ml; ▲, 20 μM deoxyuridine plus [^{3}H]deoxyuridine at 0.1 μCi/ml.

with whole blood. The results of the HPLC analysis (Fig. 2*B*) agree well with those of the CHO incorporation study—i.e., less than 20% of the nucleoside remained after 25 hr, and the deoxyuridine, as predicted, was degraded appreciably less than dThd or BrdUrd. The same pattern was observed when the experiment was repeated with blood from a different donor, although there was slightly more degradation of each compound (data not shown). When a still higher concentration of dThd (0.2 mM) was added to the cultures, the amount of degradation was greater, although at this concentration only 50% of the dThd had been broken down by 25 hr (Fig. 3).

All of these experiments were performed with unstimulated blood cultures, but it has been reported that actively cycling cells do not catabolize dThd (10). Therefore, to see if catabolism continues even after the lymphocytes have started to synthesize DNA, we added PHA to some cultures and found that there was no slowing of catabolism (Fig. 3).

When whole blood was fractionated by Ficoll-Paque and Percoll, then incubated with [^{3}H]dThd at 0.1 μCi/ml, some

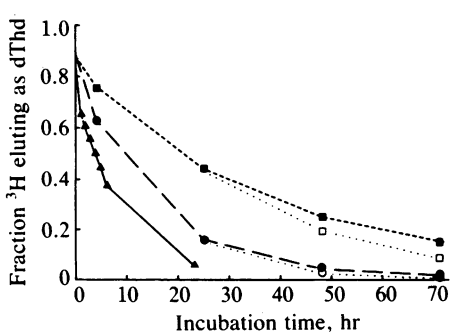

FIG. 3. HPLC analysis of ^{3}H remaining in the medium as [^{3}H]dThd after incubation with stimulated or unstimulated whole blood. ▲, 1.3 μM dThd, no PHA; ●, 20 μM dThd, no PHA; ○, 20 μM dThd, with PHA; ■, 200 μM dThd, no PHA; □, 200 μM dThd, with PHA.

degradation was observed in all fractions, as determined by the decreased incorporation of ^{3}H into CHO cells (Fig. 4*A*). In all cases, the cell content was adjusted to approximate the cell content of 10% whole blood, yet no fraction showed as much activity as the whole blood itself. The interface from the Ficoll-Paque gradient, which contained mostly lymphocytes, large numbers of platelets, and presumably small numbers of other cell types, showed considerable catabolic activity. When this fraction was reprocessed on a Percoll gradient of density 1.064 g/ml to remove platelets, most of the catabolic activity, along with the platelets, was lost. Washed RBCs, whether taken from the bottom or the top of the pellet from the Ficoll-Paque gradient, showed very little activity. The plasma fraction from the top of the Ficoll-Paque gradient showed moderate activity. These observations are consistent with the idea that platelets have a considerable amount of thymidine phosphorylase activity. Indeed, a thymidine phosphorylase has been purified from platelets (11) and observed by Pero *et al.* (12). We also noted that each time whole blood was washed some catabolic activity was lost, which was correlated with a concomitant loss of platelets.

When the blood fractionation experiment was repeated and the medium was analyzed by HPLC, the results agreed surprisingly well (Fig. 4*B*). This time a portion of the Ficoll interface was incubated after one wash in PBS and a portion was incubated after four washes. The former showed much more catabolic activity. The lymphocytes repurified on Percoll showed some activity, as might be expected since lymphocytes have been reported to have pyrimidine phosphorylases (13, 14). However, because even our purified lymphocyte preparations contained a few platelets, we cannot be certain that lymphocytes themselves do have catabolic activity. Samples were taken from the bottom as well as the top of the RBC pellet. The sample from the bottom contained no leukocytes or platelets (as determined microscopically)

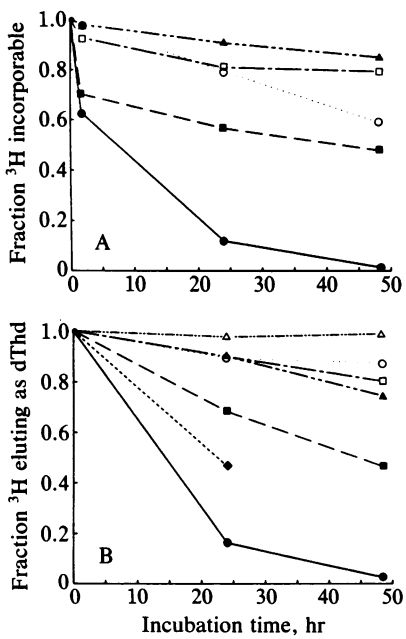

FIG. 4. Catabolic activity in separated blood fractions, as determined by CHO incorporation (*A*) or HPLC analysis (*B*). ●, Whole blood; ■, lymphocytes and platelets from a Ficoll-Paque separation, washed four times with PBS; ◆, lymphocytes and platelets, washed once with PBS; □, lymphocytes further purified on a 1.064-g/ml Percoll gradient; ○, plasma from a Ficoll-Paque separation; ▲, RBCs from the top of the pellet after a Ficoll-Paque separation, including some leukocytes and platelets; △, RBCs from the bottom of the Ficoll-Paque pellet, with no detectable contamination with other cell types.

and had no catabolic activity, whereas the fraction from the top was less pure and showed some breakdown of dThd.

Because platelets do not survive storage, a sample of blood was drawn and incubated with [^{3}H]dThd either immediately or after being stored at 4°C or 37°C for 10 days. Blood stored at 4°C for 10 days catabolized 96% of the [^{3}H]dThd in 25 hr, as did the unstored sample, but blood stored at 37°C catabolized only about 80% of the [^{3}H]dThd. The enzymes involved in degradation of dThd therefore seem quite stable and remain active in the plasma even after the platelets themselves have disappeared; indeed, thymidine phosphorylase activity has been reported in plasma (15–17). Because platelets may sustain various amounts of damage during centrifugation on a Ficoll-Paque gradient, this persistence of the enzymes could account for the activity in the plasma fraction observed in our blood fractionation experiments.

We have found some variation in the amount of dThd degradation from donor to donor. Blood from eight of our routine donors showed anywhere from 76% to 97% degradation by 24 hr. Although there are reports of dThd and BrdUrd catabolism in HeLa cells, mouse liver (18), and human fibroblasts (19), we found no breakdown products in the medium when we incubated confluent mycoplasma-free CHO, V79, DON, or BHK (hamster) cultures with [^{3}H]dThd for 24 hr.

To determine which compounds were formed as the original [^{3}H]dThd was progressively degraded, HPLC analyses were made of the medium at successive times after [^{3}H]dThd was added to the blood cultures (Fig. 5). ^{3}H was found to elute at new positions until no radioactivity remained as dThd. The first major product to appear coeluted with thymine; this is consistent with the catabolic scheme expected if a pyrimidine phosphorylase is responsible for the first degradative step, as found by Desgranges *et al.* (11). [When the starting compound was BrdUrd or dUrd, the first product to elute coincided with bromouracil or uracil, respectively (data not shown).] There was very little accumulation of dihydrothymine, as expected, since dihydrothymine dehydrogenase has been proposed to be the rate-limiting enzyme in the catabolism of dThd (20). Also, there was very little accumulation of radioactivity at the position of β-aminoisobutyric acid, which itself can enter many metabolic pathways, such as those for amino acid metabolism or the citric acid cycle, and thus be involved in the formation of many end products (21).

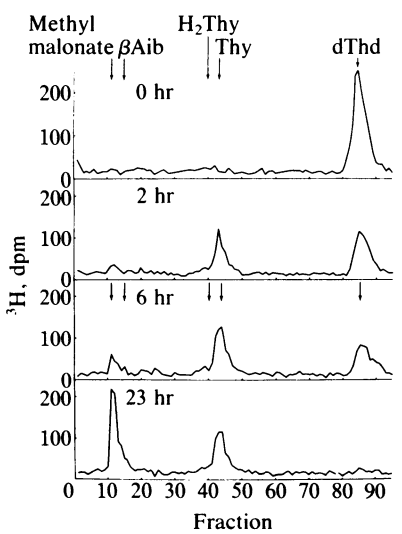

FIG. 5. HPLC analysis of medium after incubation of whole blood with [^{3}H]dThd for 0, 2, 6, or 23 hr. βAib, β-aminoisobutyric acid; H_2Thy, dihydrothymine.

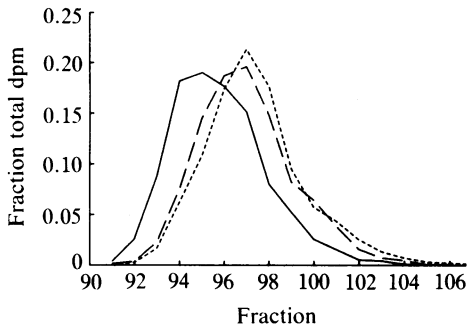

FIG. 6. Isotopic fractionation. —, Methyl-labeled [^{3}H]dThd; – –, ring (6)-labeled [^{3}H]dThd; ----, methyl-labeled [^{14}C]dThd.

Our HPLC analyses showed that dThd labeled with ^{3}H in the methyl group eluted 3–4% faster than dThd labeled with ^{14}C in the methyl group or with ^{3}H at the ring-6 position (Fig. 6). Unlabeled dThd appeared to elute with [^{14}C]dThd. This phenomenon, known as isotopic fractionation, has been described for several tritium- and deuterium-labeled compounds (22–25) but not for nucleosides, and it has been attributed to a change in the pKa of the nitrogen immediately adjacent to the deuterium or tritium. In dThd, however, two additional carbon atoms separate the ^{3}H on the methyl group from the closest nitrogen, so that it seems unlikely that the interaction between ^{3}H and nitrogen is responsible for this isotopic fractionation of dThd.

DISCUSSION

Human peripheral blood is widely used for both clinical and experimental investigations. Many protocols include the labeling of cells in culture either with a radioactive precursor ([^{3}H]dThd) or with BrdUrd. For instance, when cultures of blood are stimulated with PHA or with other lectins such as concanavalin A, lymphocytes (mainly T cells) become transformed into blast cells, enter S phase, and can incorporate [^{3}H]dThd into their DNA. Originally, morphological criteria such as the numbers of large blast cells present in the cultures, or the percentage of cells that incorporated [^{3}H]dThd, as observed in autoradiographs, were used to determine the immunocompetence of the cells and whether or not they responded to lectins or other antigens. Now, however, it is common practice simply to expose the cells to the lectin, incubate them with [^{3}H]dThd for several hours, and subsequently determine the incorporated radioactivity in the cultures as a whole by scintillation spectrometry.

This assay is claimed to be the most reproducible and quantitative technique for measuring the response of lymphocytes to a stimulus while avoiding a morphological analysis, and it is thought to be a sensitive indicator of lymphocyte transformation (6). The amount of ^{3}H incorporated is also taken as a quantitative measure of immunocompetence in various disease states, such as neoplasia (26, 27) and Alzheimer disease (28), as well as in aging (29, 30). This assay has also been used to monitor allergic response (31) and to detect previous exposure to pathogens (32).

Although it is true that those cells that respond immunologically to become transformed will enter S phase and thus incorporate [^{3}H]dThd, the results from our study indicate that the amount of ^{3}H incorporated in a culture is also a function of the amount of catabolism of dThd that occurs there. This catabolism, which is brought about by thymidine phosphorylase, occurs in leukocytes (this study; ref. 14), serum and plasma (this study; refs. 16 and 17), and platelets (this study; refs. 11 and 12). If the amount of this catabolism, which is quite variable from person to person (16), is not measured, then our ability to make quantitative measure-

ments of immunological competence by scintillation spectrometry is confounded (17, 33). As an extreme, one might find that in a given disease the same number of cells might be immunocompetent and thus respond to PHA, but that the amount of thymidine phosphorylase varies, leading to changes in the amount of ^3H incorporated, and thus to improper inferences about the degree of immunoregulatory impairment.

Although the amount of thymidine phosphorylase is found to be lower in homogenates of leukocytes from patients with chronic myelogenous leukemia than in leukocytes from normal people (14, 34), Pauly *et al.* (16, 17) found that cell-free plasma from patients with various neoplastic diseases has more thymidine phosphorylase than does plasma from healthy subjects. This metabolic activity led them to question the validity of defining cell proliferation by measuring [^{3}H]dThd incorporation in cells cultured in medium supplemented with plasma or serum. Thus, although it is true that patients with chronic myelogenous leukemia seem to be less immunocompetent than normal people, the variability in the amount of thymidine phosphorylase found in normal subjects, as well as the lower amount found in patients with the disease (16, 17), makes it difficult to reach any quantitative estimate of the effect of PHA stimulation simply by recording the amount of ^3H incorporated into cultures as a whole. A similar problem exists in making quantitative estimates of immunocompetence in aging people (35) or in animals (36, 37). That the amount of ^3H incorporated is not necessarily related to the number of dividing cells has already been pointed out by Carr *et al.* (7), who also noted that factors other than thymidine phosphorylase could affect the *in vitro* estimation of a blood culture's immunoresponsiveness to PHA.

Other types of experiments, too, can be affected by the presence of thymidine phosphorylase in platelets. For instance, if platelet lysates are added to cultures of other cells, such as glial cells, the lysates can decrease the amount of [^{3}H]dThd incorporated in 24 hr (33). Furthermore, in studies of sister chromatid exchanges (SCEs) found in human lymphocytes cultured in the presence of BrdUrd, it has been noted that the SCE frequency is dependent on the amount of BrdUrd available per replicating cell, and that this could be influenced by differences from one donor to another in the numbers of lymphocytes cultured (38). This has led to the use of different amounts of whole blood in cultures from different donors in attempts to ensure that each culture has the same number of lymphocytes (38, 39). This procedure, which changes the amount of whole blood, however, also changes the number of platelets, and thus the amount of thymidine phosphorylase in the cultures, and consequently emphasizes, rather than minimizes, the between-donor differences in the increase of SCE frequency as the amount of BrdUrd available per lymphocyte is increased (38).

From all of these experiments it can be concluded that (*i*) variation in thymidine phosphorylase levels, whether inherent or an artifact of the experimental protocol, can lead to erroneous interpretations of differences in the amount of incorporation of [^{3}H]dThd into DNA, and (*ii*) the amount of incorporation into lymphocytes after PHA stimulation may not be a reliable quantitative measure of cell proliferation or of the immunocompetence of the cells.

This work was supported by the Office of Health and Environmental Research of the U.S. Department of Energy (DE-AC03-76-SF01012).

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