Cytidine 5'-Diphosphate Reductase and Thymidine Kinase Activities in Phytohemagglutinin-stimulated Lymphocytes of Normal Subjects of Various Ages and Patients with Immunodeficiency

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Summary

The activities of CDP reductase and thymidine kinase in 10^6 to 5×10^6 phytohemagglutinin (PHA)-stimulated lymphocytes isolated from 2 to 5 ml of peripheral blood of individual subjects were measured. The activities of CDP reductase (pmol/h/10^7 cells) and thymidine kinase (nmol/h/10^7 cells) were high in infants, 698 ± 307 and 64.2 ± 20.2, constant in subjects of 1–40 years old, 401 ± 181 and 38.1 ± 15.3, and low in persons of more than 80 years old, 121 ± 113 and 22.3 ± 17.8, respectively. The ratio of thymidine kinase to CDP reductase activity increased with age, indicating that dependency on the salvage pathway of DNA synthesis in lymphocytes increased with age. The activities of CDP reductase and thymidine kinase were reduced in patients with the hyperimmunoglobulin E syndrome, congenital cytomegalovirus infection, anhidrotic ectodermal dysplasia with hyperimmunoglobulin A, Bloom's syndrome, immunodeficiency with hyperimmunoglobulinemia, and Down's syndrome. The clinical symptoms of these diseases seem to be due to impaired DNA synthesis of PHA-stimulated lymphocytes, but the degrees of reduction of enzyme activities were generally greater than that of thymidine incorporation in these patients.

Abbreviations

PHA, phytohemagglutinin
HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

Ribonucleotide reductase and thymidine kinase are thought to be rate-limiting enzymes in the *de novo* and salvage pathways, respectively, of DNA biosynthesis. Since the activities of these enzymes have been shown to be increased in rapidly proliferating tissues, such as tumors (8, 24, 28), developing organs (8, 12, 16), and regenerating liver (15, 28), it has been thought that these activities closely reflect the rate of DNA synthesis (25, 28).

Deficiencies of adenosine deaminase and purine nucleoside phosphorylase are observed in severe combined immunodeficiency. Immunodeficiency is thought to be due to inhibition of ribonucleotide reductase by the accumulation of deoxyadenosine triphosphate in adenosine deaminase deficiency (6) and deoxyguanosine triphosphate in purine nucleoside phosphorylase deficiency (5), because both deoxynucleotides are strong inhibitors of mammalian ribonucleotide reductase (9). From these consid-

erations, ribonucleotide reductase activity in lymphocytes may closely reflect the manifestation of immunity.

On stimulation by PHA, lymphocytes are transformed into cells showing enhanced DNA synthesis and consequently increased thymidine incorporation into DNA. Thymidine kinase catalyzes the first and rate-limiting step from thymidine to DNA in this pathway. Since measurement of [^{3}H]thymidine incorporation is reported not to be a precise assay of cell proliferation (2), measurements of ribonucleotide reductase and thymidine kinase activities should indicate better whether immunodeficiency and various other diseases are attributable to impaired DNA synthesis. These enzymes are difficult to assay in normal tissues because their activities are so low (25), but their activities are considerably increased in PHA-stimulated lymphocytes (18, 27). This paper reports studies on the ontogenic patterns of change in activities of these enzymes, involved in the *de novo* and salvage pathway, respectively, in PHA-stimulated lymphocytes from individual subjects and the relationship between the enzyme activities and thymidine incorporation, as a measure of the overall capacity of the salvage pathway, in children with immunodeficient diseases.

MATERIALS AND METHODS

Chemicals. CDP, ATP, thymidine, dithioerythritol, HEPES, snake venom (*Crotalus adamanteus*) and Dowex-1 (chloride form) were purchased from Sigma Chemical Co. (St. Louis, MO). ^{14}C-labeled CDP and thymidine, and PCS II were obtained from the Radiochemical Centre (Amersham, England). Ficoll-Paque was from Pharmacia Fine Chemicals (Uppsala, Sweden), PHA from Wellcome, RPMI 1640 medium from M. A. Bioproducts (Walkersville, MD) and DEAE-cellulose paper (Whatman DE81) from Whatman Co. (Clifton, NJ). All other reagents, which were of the highest grade available, were purchased from Wako Pure Chemical Co. (Tokyo, Japan).

Assay Methods. The following standard methods were used, unless otherwise stated. Lymphocytes from 2 to 5 ml of peripheral blood were isolated by centrifugation on a Ficoll-Paque gradient. They were then washed twice and cultured at a density of 1×10^6 cells/ml in RPMI 1640 medium with 10% fetal calf serum containing 1 μg/ml of PHA for 48 h at 37°C in a humidified atmosphere of 5% CO_2 in air. They were divided into three groups. Two groups were collected by centrifugation at 1500 rpm

for 10 min and suspended, respectively, in 50 μl of 50 mM HEPES buffer, pH 7.5, for CDP reductase assay and in 50 mM Tris-HCl buffer, pH 7.5, with 5 mM KCl for thymidine kinase assay. They were stored at −70°C until use. CDP reductase (EC 1·17·4·1) was assayed by the method of Steeper and Steuart (23), as modified by Takeda and Weber (24), and thymidine kinase (EC 2·7·1·21) was assayed by the method of Weber *et al.* (28), with modification for the conditions required for PHA-stimulated lymphocytes. The medium for assay of CDP reduction consisted of 33 mM HEPES buffer, pH 7.5, 10 mM dithio-erythritol, 8 mM Mg-acetate, 4mM ATP, 0.025 mM FeCl$_3$, 0.1 mM ^{14}C-labeled CDP (6 μCi/μmol) and 1 × 10^6 to 5 × 10^6 cells. The incubation mixture for thymidine kinase assay consisted of 50 mM Tris-HCl buffer, pH 7.5, 5 mM ATP, 2.5 mM MgCl$_2$, 1 mM [2-^{14}C]thymidine (1 μCi/μmol), and cells. For study of thymidine incorporation, one group of viable lymphocytes that had been cultured for 48 h was pulsed with 0.05 μCi of [^{14}C] thymidine/10^6 cells. The cells were collected after 2 to 24 h by centrifugation at 1500 rpm for 10 min and the medium was discarded. The cells were washed three times with saline, suspended in 1 ml of distilled water, and stored at −70°C. Radio-activity was determined in a liquid scintillation counter after addition of 5 ml of PCS II.

Statistical Analysis. Student's *t* test was employed to calculate the significance of differences.

RESULTS

Development of Assays for Ribonucleotide Reductase and Thymidine Kinase Activities in Small Amounts of PHA-stimulated Lymphocytes. The optimum assay conditions were found to be as described in "Materials and Methods." CDP reductase in PHA-stimulated lymphocytes was similar to that in other mammalian cells in that ATP and dithioerythritol were absolute requirements. The CDP reductase and thymidine kinase activities in unstimulated lymphocytes were 7.5 ± 0.1 pmol/h/10^7 cells and 1.8 ± 0.6 nmol/h/10^7 cells, respectively, although in practice, more than 10^8 lymphocytes were required to obtain a reliable value for CDP reductase activity. Maximal increases in activity were observed when the culture medium contained 0.3–3 μg/ml of PHA. As shown in Figure 1, the CDP reductase and thymidine kinase activities in PHA-stimulated lymphocytes increased 4-fold and 2-fold, respectively, in 24 h and reached maxima in 48 h, which were maintained until 72 h. Both activities decreased to 50–60% of the maximum by day 6 of
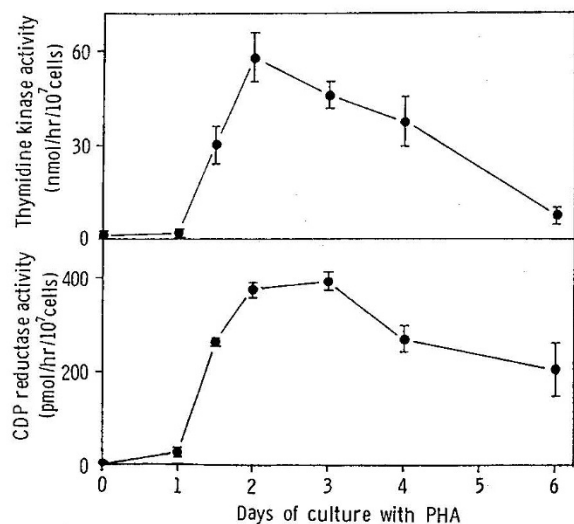

Fig. 1. CDP reductase and thymidine kinase activities during transformation of human lymphocytes. Lymphocytes from the same individual were cultured for the indicated times with phytohemagglutinin. Enzyme activities were determined as described in "Materials and Methods." *Points* and *bars* are mean ± SD from triplicate determinations.

culture. Under the present conditions, the reactions were linear for 60 min with 1 × 10^6 to 5 × 10^6 lymphocytes.

CDP Reductase Activity, Thymidine Kinase Activity, and the Ratio of the Two Activities in Subjects of Different Ages. As shown in Figure 2, CDP reductase activity in lymphocytes from infants was significantly higher than that in cells from older persons, was similar in cells of subjects of 1 to 40 years old, and was markedly reduced in persons of over 80 years old. Relatively wide variations were found in the CDP reductase activities of different normal persons, but the standard deviations of CDP reductase and thymidine kinase activities in the same subjects were 7.9 and 14.5%, respectively. Thus, the values were reproducible in repeat experiments and the lowest activities in different age groups of up to 40 years old were almost the same.

Thymidine kinase activity in different age groups showed a pattern of change similar to that of CDP reductase activity, but its maximum activity in each group was 2- to 3.5-fold the minimum activity, whereas that of CDP reductase was 4- to 8-fold the minimum activity. The minimum activity of thymidine kinase was highest in infants, constant in subjects of 1 to 40 years, and greatly reduced in persons over 80 years old.

The ratio of thymidine kinase to CDP reductase activity increased with age, and the ratios in groups over 7 years old were significantly higher than that of infants (Fig. 3). This age-related increase was very marked in aged persons.

CDP Reductase and Thymidine Kinase Activities in Various Diseases. The enzyme activities were measured in individual patients with growth and immunological disturbances to determine whether the symptoms were due to impaired DNA synthesis. The CDP reductase and thymidine kinase activities were both decreased in patients with the hyperimmunoglobulin E syndrome, congenital cytomegalovirus infection, anhidrotic ectodermal dysplasia with hyperimmunoglobulin A, Bloom's syndrome, and immunodeficiency with hyperimmunoglobulinemia (Table 1) and in 7 of 17 patients with Down's syndrome. No disease in which only one enzyme activity was reduced was observed. The ratio of thymidine kinase to CDP reductase activity tended to be high in patients with reduced enzyme activities, particularly in those with congenital cytomegalovirus infection and immunodeficiency with hyperimmunoglobulinemia (Table 1). Both activities were normal in milk allergy, xeroderma pigmentosum, and Cockayne's syndrome. The activities were also not decreased in endocrine diseases (pituitary dwarfism, adrenogenital syndrome, cretinism, and hyperthyroidism), congenital metabolic disorders (adrenoleukodystrophy, histidinemia, ornithine carbamoyltransferase deficiency, Wilson's disease, and Morquio's syndrome), congenital heart disease, progressive muscular dystrophy, or Turner's syndrome.

Rates of Thymidine Incorporation in Normal Subjects and Patients with Reduced Activities of CDP Reductase and Thymidine kinase. Thymidine incorporation into lymphocytes of normal subjects after 48-h incubation with PHA increased with time to a maximum of 6 h, as shown in Figure 4. Thymidine incorporation into lymphocytes from patients with low enzyme activities, such as those with congenital cytomegalovirus infection and immunodeficiency with hyperimmunoglobulinemia, was also linear for 6 h, but then continued to increase. This indicates that the thymidine incorporation rate should be determined in a period of within 6 h for identification of patients with impaired DNA synthesis. The thymidine incorporation rates in 2 h by cells from patients with reduced activities were lower than those by normal cells (Table 1). It is noteworthy that in these patients the reduction of enzyme activities, and particularly CDP reductase activity, were greater than that of thymidine incorporation.

DISCUSSION

It has been observed (22) that the pool size of deoxynucleotide in mammalian cells is small and supports DNA synthesis for only a short time. Assays of ribonucleotide reductase and thy-
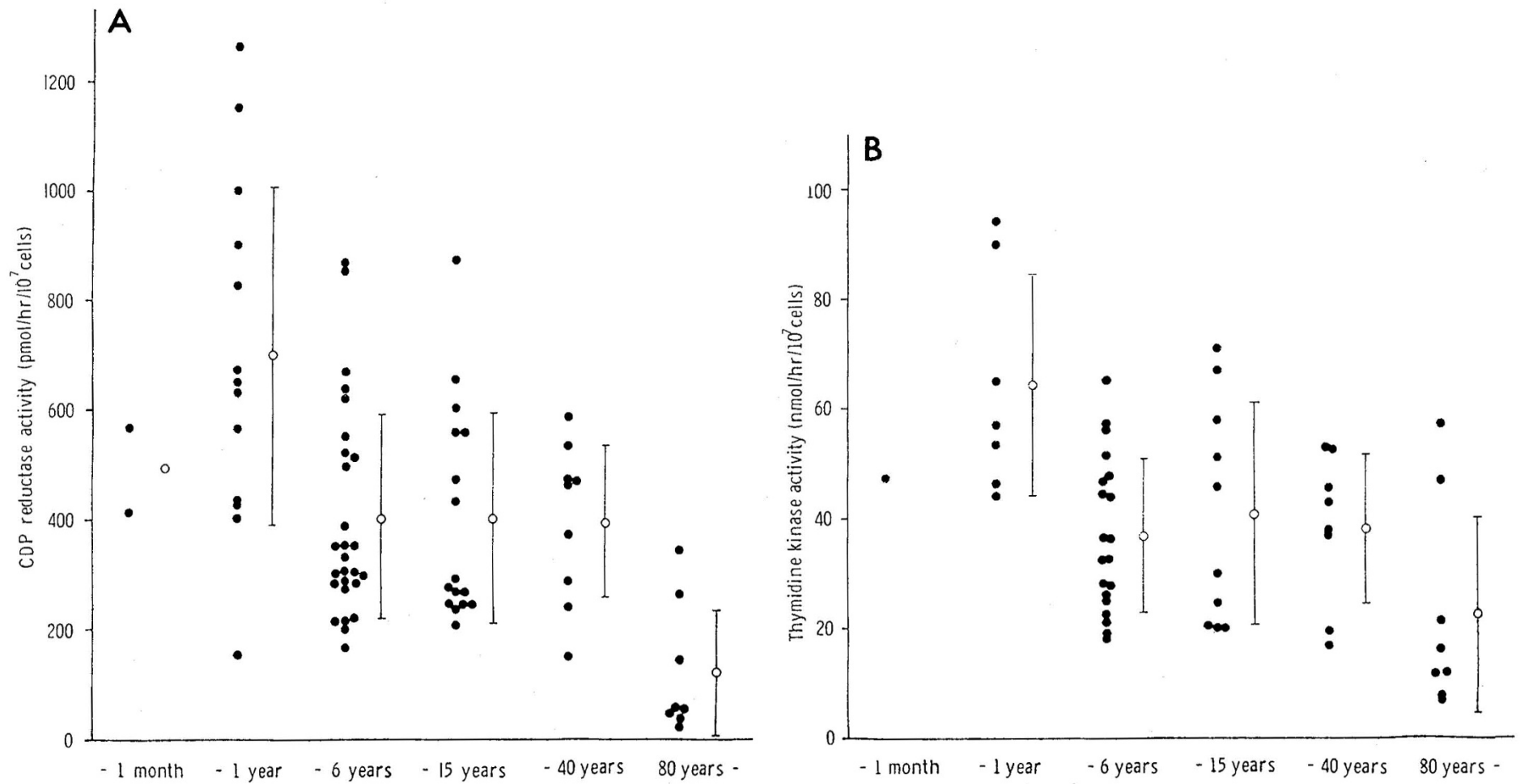
Fig. 2. *A*, CDP reductase activity (pmol/h/10^7 cells) in PHA-stimulated lymphocytes from normal subjects of different ages. The means ± SD (ranges) for the groups of <1 year, 1–6 years, 6–15 years, 15–40 years, and >80 years were: 698 ± 307 (155–1260), 402 ± 189 (165–869), 402 ± 191 (207–872), 395 ± 137 (145–584) and 121 ± 113 (20–344), respectively. The value for the group of <1 year was significantly different from those for the groups of 1–6 years ($P < 0.01$), 6–15 years ($P < 0.01$), 15–40 years ($P < 0.01$), and >80 years ($P < 0.001$). That of the group of >80 years was also significantly different from those for the groups of <1 year ($P < 0.001$), 1–6 years ($P < 0.001$), 6–15 years ($P < 0.01$), and 15–40 years ($P < 0.001$). *B*, thymidine kinase activity (nmol/h/10^7 cells) in PHA-stimulated lymphocytes from normal subjects of different ages. The means ± SD (ranges) for the groups of <1 year, 1–6 years, 6–15 years, 15–40 years, and >80 years were: 64.2 ± 20.2 (44.0–94.1), 36.9 ± 13.9 (18.2–65.1), 40.7 ± 20.1 (19.9–70.6), 37.9 ± 13.5 (16.9–52.6), and 22.3 ± 17.8 (6.5–57.1), respectively. The value for the group of <1 year was significantly different from those for the groups of 1–6 years ($P < 0.001$), 6–15 years ($P < 0.05$), 15–40 years ($P < 0.02$), and >80 years ($P < 0.01$). That of the group of >80 years was also significantly different from those for the groups of <1 year ($P < 0.01$) and 1–6 years ($P < 0.05$).
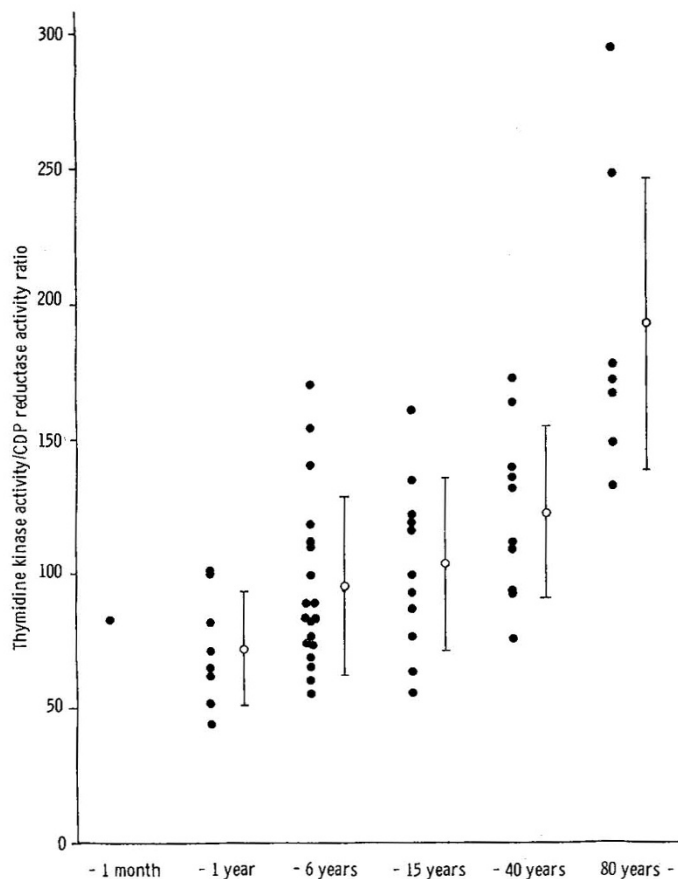
Fig. 3. Ratios of thymidine kinase to CDP reductase activity in different age groups. The mean ± SD (ranges) for the groups of <1 year, 1–6 years, 6–15 years, 15–40 years, and >80 years were: 72 ± 21 (44–101), 95 ± 33 (55–170), 103 ± 32 (55–160), 122 ± 32 (75–172), and 191 ± 54 (132–590), respectively. The value for the group of <1 year was significantly different from those for the groups of 6–15 years ($P < 0.05$), 15–40 years ($P < 0.01$), and >80 years ($P < 0.001$). That of the group of >80 years was also significantly different from those for the groups of <1 year ($P < 0.001$), 1–6 years ($P < 0.001$), 6–15 years ($P < 0.001$) and 15–40 years ($P < 0.01$).
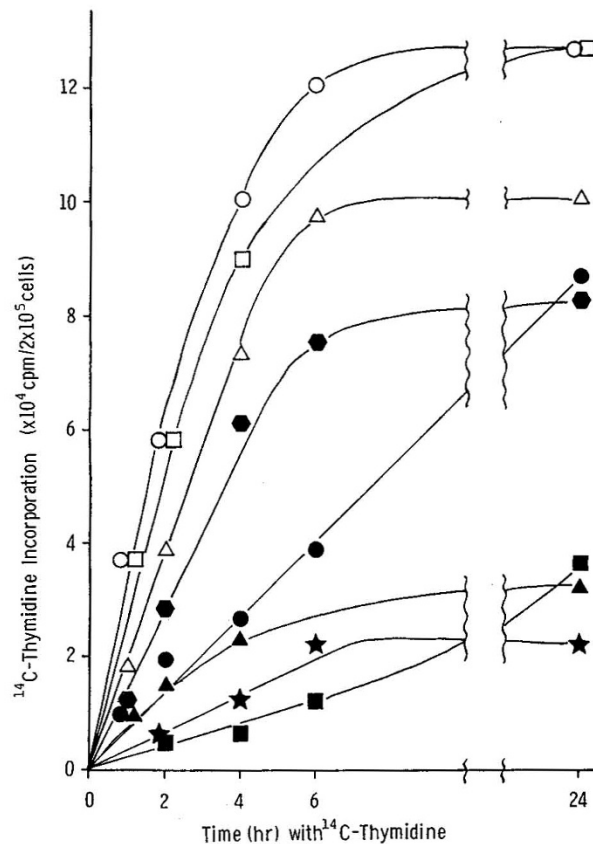

Fig. 4. Relationship between thymidine incorporation and time of incubation with [^{14}C]thymidine. Cells were incubated for 48 h with PHA before experiments as described in "Materials and Methods." The means ± SD of the activities of CDP reductase (pmol/h/10^7 cells) and thymidine kinase (nmol/h/10^7 cells) and thymidine incorporation in 2 h (cpm/h/2 × 10^5 cells) in normal subjects were 401 ± 181 ($n = 52$), 38.1 ± 15.3 ($n = 38$), and 23,898 ± 5,021 ($n = 21$), respectively. Normal (O, □, △), congenital cytomegalovirus infection (●), anhidrotic ectodermal dysplasia with hyperimmunoglobulin A (◖), immunodeficiency with hyperimmunoglobulinemia (■), Bloom's syndrome (▲), and hyperimmunoglobulin E syndrome (★).

Table 1. *Activities of CDP reductase and thymidine kinase, the ratio of thymidine kinase to CDP reductase activity, and rates of [^{14}C]thymidine incorporation in various immunodeficient diseases**

	CDP reductase activity (pmol/h/10^7 cells)	% of mean in normal subjects	Thymidine kinase activity (nmol/h/10^7 cells)	% of mean in normal subjects	Ratio of thymidine kinase to CDP reductase activity	% of mean in normals	[^{14}C]Thymidine incorporation (cpm/h/2 × 10^5 cells)	% of mean in normal subjects
Hyperimmunoglobulin E syndrome	102 ± 13 (4)	25	14.3 ± 1.4 (4)	38	140 ± 11 (4)	147	9,076 ± 163 (2)	38
Congenital cytomegalovirus infection	71 ± 29 (2)	10	9.1 ± 0.7 (2)	14	144 ± 49 (2)	200	10,125 ± 584 (2)	42
Anhidrotic ectodermal dysplasia with hyperimmunoglobulin A	61 ± 25 (4)	15	7.6 ± 3.9 (4)	20	139 ± 20 (4)	146	13,707 ± 2,334 (3)	57
Bloom's syndrome	45 ± 35 (7)	11	6.1 ± 2.1 (5)	16	147 ± 48 (4)	120	6,440 ± 2,854 (6)	27
Immunodeficiency with hyperimmunoglobulinemia	20 (2)	5	6.9 ± 1.9 (2)	18	343 ± 68 (2)	333	2,032 ± 706 (3)	9

* The mean value ± SD (ranges) for thymidine incorporation in normal subjects was 23,898 ± 5,021 (17,037–35,669) cpm/h/2 × 10^5 cells. Numbers in parentheses are numbers of repeat determinations in the same patient.

midine kinase activities are required to obtain reliable biochemical parameters of cell proliferation, which may reflect the capacities of humans for growth and immunity.

Tyrsted *et al.* (27) reported that more than 1.8 × 10^7 PHA-stimulated lymphocytes were required to measure CDP reductase activity. However, by determining the optimal conditions, we developed a micromethod in which the activity could be determined using 10^6 to 5 × 10^6 lymphocytes obtained from 2–5 ml

of peripheral blood. On 48-h culture of the cells with 1 μg/ml of PHA, when the cells from both young and aged subjects were only first generation metaphase cells (26), the activities of CDP reductase and thymidine kinase both reached maxima. Although peripheral lymphocytes of normal children showed a wide range of activities after 48 h of PHA stimulation, these activities were fairly reproducible and repeated tests on individuals gave similar results.

The CDP reductase and thymidine kinase activities of human lymphocytes are both higher in infants than in older subjects (Figure 2). Thus, the rate of DNA synthesis in T-lymphocytes is higher in infancy. Lymphocyte transformation in response to respiratory syncytial virus is significant in patients of 6 months of age or younger (21). Lymphocytes from newborn babies react more than those from adults to nonspecific stimulations with mitogens, such as PHA and concanavalin A (4). Our results on enzyme activities are consistent with this finding of a high response of lymphocytes of infants. Cell-mediated immunity is known to show age-related decrease. It has generally been thought that the number of PHA-responsive cells in cultures in peripheral lymphocytes decreases as a function of the age of the donors (10, 11, 19, 29). The proliferation kinetics of PHA-stimulated lymphocytes from young individuals appeared to be relatively homogeneous when compared with that of an aged group (26). This might be explained by a difference in the mean cell cycle duration in cultures from young and aged subjects. We found that the CDP reductase and thymidine kinase activities of lymphocytes from subjects of 1–40 years old were constant, but that the activities were decreased in cells from persons of over 80 years old. Moreover, the ratio of thymidine kinase to CDP reductase activity in lymphocytes showed an age-related increase, especially in persons of over 80 years old. This indicates that the predominant pathway of DNA synthesis in transformed lymphocytes gradually alters from the *de novo* to the salvage pathway with age. These results suggest that the *de novo* pathway is more important than the salvage pathway for lymphocyte transformation, a possibility that was also supported by the findings of Bernheim *et al.* (2). These enzyme activities seem to be good parameters of the rate of DNA synthesis, because their correlations with thymidine incorporation were 0.8788 (CDP reductase) and 0.8535 (thymidine kinase) in normal controls. However, the kinetic patterns of thymidine incorporation in patients with reduced enzyme activities were not consistent after pulse labeling with thymidine for 6 h. The thymidine incorporation rate in 2 h by cells from these patients was lower than normal (Table 1). Therefore, the results indicated that diseases with low enzyme activities might be due to impairment of DNA synthesis in lymphocytes. It has been reported that the diseases listed in Table 1 involve impaired cell-mediated immunity (3, 7, 13, 14, 17, 20). In addition, the degrees of reduction of enzyme activities were greater than that of thymidine incorporation in these patients. This absence of a parallel between the enzyme activities and thymidine incorporation is very interesting and indicates that aberrant immune function is a manifestation of a defect in enzyme induction. Thus, a dissociation between thymidine incorporation and enzyme activities might be observed when DNA synthesis in lymphocytes is impaired to various degrees. A dissociation of thymidine incorporation and DNA polymerase activity has also been observed in human PHA-stimulated lymphocytes after x-ray irradiation (1).

An alternative explanation for the discrepancies between the enzyme activities and [^{3}H]thymidine incorporation is that the assay of cell proliferation by thymidine incorporation is not accurate. Consistent with this idea, it has been reported that the value of (cpm incorporated/number of S phase cells) varied when assayed in PHA-stimulated lymphocyte cultures after 2-h [^{3}H] thymidine pulses on successive days (2). This variation was attributed to change in the rate of thymidine transport, endogenous thymidine synthesis, or DNA replication.

The ratio of thymidine kinase to CDP reductase activity tended to be high in patients with reduced enzyme activities. The ratio was particularly high in cases of congenital cytomegalovirus infection and immunodeficiency with hyperimmunoglobulinemia that showed aberrant kinetics of thymidine incorporation after 6-h exposure to thymidine. These results suggest that the salvage pathway of DNA synthesis predominates in lymphocytes with reduced activities. These enzyme activities did not reflect ability for DNA repair, because they were normal in patients with xeroderma pigmentosum and Cockayne's syndrome. It is particularly interesting that results suggested the existence of a defect in the DNA biosynthetic system as well as the DNA repair system in Bloom's syndrome. No abnormalities in enzyme activities were found in patients with endocrine diseases or various diseases with growth disturbance. It is concluded from the present work that the assay of both enzyme activities is a very useful and sensitive method for diagnosis of lymphocyte dysfunction due to a defect in DNA synthesis and that it may have a different significance from assay of thymidine incorporation.

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Bilirubin Photooxidation Products in the Urine of Jaundiced Neonates Receiving Phototherapy

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Summary

Bilirubin-IXα photooxidation products were detected by high performance liquid chromatography in the urine of neonates undergoing phototherapy for hyperbilirubinemia. The *in vivo* photoproducts were identified by chromatographic comparison with authentic synthetic standards using two complementary methods. Bilirubin photooxidation products were not detected in urine from jaundiced infants not receiving phototherapy. The specific photoproducts identified in the urine include propentdyopents, hematinic acid imide and its hydrolysis product (3-carboxy-2-methyl-2-hexenedioic acid), and the hydrolysis product (2-vinyl-3-methyl-maleic acid) of methylvinylmaleimide. Their total urinary concentrations were low (0.2–0.9 mg/dl) during phototherapy. These observations show that photooxidation of bilirubin clearly does occur during phototherapy. They are consistent with the view that, although photooxidation is not the major photochemical event associated with phototherapy, it can and clearly does occur concurrently with photoisomerization.

Abbreviations

UCB, unconjugated (4Z, 15Z)-bilirubin-IXα
HPLC, high performance liquid chromatography

Phototherapy is used routinely in hospitals for treating neonatal jaundice (hyperbilirubinemia). Patients are irradiated with white or blue light to enhance elimination of the yellow cytotoxic metabolite (4Z, 15Z)-bilirubin-IXα, which is produced in copious quantities in mammals by degradation of heme (2). Under normal metabolic conditions, UCB is detoxified by conjugation in the liver and excreted in bile. When conjugation is impaired, UCB accumulates in plasma and partitions into extravascular tissues, including the brain (23). About 150,000 infants per year in the United States develop plasma UCB levels of 15 mg/dl (3), and a large number of these receive some form of treatment (phototherapy) to remove or accelerate the excretion of UCB

from the body, apparently reducing the risk of bilirubin encephalopathy (23).

The mechanism of UCB elimination in jaundice phototherapy has been investigated for many years. At first it was thought that the important photochemical event was accelerated catabolism of UCB to water-soluble derivatives that were excreted principally in bile and, to some extent, in urine (18). In this connection, a considerable effort was directed toward studies of bilirubin photooxidation (7, 21), wherein singlet oxygen, *i.e.* molecular oxygen in its first electronic excited state and known to be cytotoxic (25), was thought to be the oxidizing species (7). Recent data indicate that singlet oxygen may not be implicated and that bilirubin photooxidation may proceed via radical intermediates (6). Mechanistic questions aside, the products of *in vitro* photooxidation have been well characterized (7).

The widely held belief that bilirubin photodegradation is the responsible mechanism for lowering serum UCB in neonatal phototherapy (5) began to erode with the discovery of an apparent augmented excretion of UCB in the bile of Gunn rats (19) and jaundiced babies (10) undergoing light irradiation. This important finding was extremely intriguing because UCB normally cannot be excreted by the liver unless it is converted to polar conjugates, principally glucuronides (2). To accommodate the confirmed (11, 21) biliary excretion, $Z \rightleftarrows E$ photochemical configurational isomerization at the *meso* carbon-carbon double bonds of (4Z, 15Z)-bilirubin-IXα was proposed (7, 8) to give more polar *E*-isomers, isomers that could more easily cross the liver. The hypothesis was subsequently tested (15) and confirmed in Gunn rats (12, 24). More recently, the structures of the *E*-isomers have been proved, and it has been shown that these isomers are formed in jaundiced rats exposed to blue light and in humans during phototherapy (14, 16). At present, current evidence favors the photochemical configurational isomerization of UCB (to *E*-isomers) as one of the most rapidly occurring photochemical events in phototherapy.

Of what importance is UCB photodegradation, however? In early studies with Gunn rats, Ostrow (20) suggested that UCB