Use of phytohaemagglutinin stimulated lymphocytes to study effects of hypoxanthine-guanine phosphoribosyltransferase (HGPRT) deficiency on polynucleotide and protein synthesis in the Lesch-Nyhan syndrome

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Summary. The incorporation of [^{14}C]thymidine and [^{14}C]uridine into the nucleoprotein, and [^{14}C]phenylalanine into the protein by phytohaemagglutinin (PHA) stimulated lymphocytes from a patient with the Lesch-Nyhan syndrome [hypoxanthine-guanine phosphoribosyl transferase (EC 2.4.2.8. HGPRT) deficiency] and controls, was studied over 72 hours of incubation, with and without azaserine to block *de novo* purine biosynthesis. No difference was observed between the values obtained for Lesch-Nyhan and control lymphocytes, when PHA-stimulated without added azaserine. The percentage reduction in the incorporation of precursors into nucleoprotein and protein after PHA stimulation in the presence of azaserine was more obvious in the lymphocytes of the patient with the Lesch-Nyhan syndrome than in the controls after the shorter incubation periods at the lower rates of synthesis. Blocking the *de novo* purine biosynthetic pathway, in control PHA stimulated lymphocytes, inhibited transformation, whereas loss of the purine salvage enzyme HGPRT did not have this effect.

These results are compatible with the view that the brain and bone-marrow damage that occur in the Lesch-Nyhan syndrome are the result of lack of HGPRT in tissues with little *de novo* purine biosynthetic capability. Other tissues with both purine biosynthetic and salvage pathways are less vulnerable to the enzyme defect. Some possible mechanisms by which HGPRT deficiency could act are discussed. We suggest that inability to increase the supply of guanylic acid (GMP) in response to a mitotic stimulus may mediate the effect of HGPRT deficiency.

The Lesch-Nyhan syndrome is a rare sex-linked recessive neurological disorder associated with the virtually complete absence of the purine salvage enzyme hypoxanthine-guanine phosphoribosyltransferase (EC 2.4.2.8; HGPRT; Table I). Occasional cases of severe gout and/or uric acid urolithiasis, sometimes with minor neurological abnormalities, are associated with partial HGPRT deficiency (Kelley *et al*, 1967, 1969).

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Purine ribonucleotides are formed either by *de novo* synthesis from small molecular weight precursors or by direct reaction of a purine base with phosphoribosylpyrophosphate (PRPP*), catalysed by the purine phosphoribosyltransferase enzymes (Fig. 1). Some tissues (e.g. fibroblasts, Rosenbloom *et al*, 1968) have both pathways, others (e.g. brain and bone-marrow) lack, or have very little purine *de novo* biosynthetic activity (Howard,

* α -D-ribofuranose-1-pyrophosphate-5 phosphate.

TABLE I
MAIN CLINICAL AND BIOCHEMICAL FEATURES
OF THE LESCH-NYHAN SYNDROME

- 1: Delayed motor development, apparent by 3 to 4 months
- 2: Choreoathetosis by 1 year
- 3: Compulsive self-mutilation at 18 months to 2 years
- 4: Spasticity at about 1 year
- 5: Aggressive behaviour
- 6: Mental retardation
- 7: Small stature
- 8: Brain weight less than normal with neuronal loss, astrocytic hypoplasia, foci of necrosis, and focal perivascular demyelination, principally affecting the cerebral cortex, cerebellum, and mid-brain nuclei
- 9: Megaloblastic anaemia
- 10: Hyperuricaemia and hyperuricaciduria
- 11: Virtually complete deficiency of hypoxanthine-guanine phosphoribosyltransferase (EC 2.4.2.8 HGPRT).

Kerson, and Appel, 1970; Lajtha and Vane, 1958).

Fibroblasts derived from patients with the Lesch-Nyhan syndrome show no impairment of growth *in vitro*, whereas granulocyte/macrophage progenitor cells cultured from bone-marrow fail to grow normally and form fewer colonies, which are also smaller than colonies from control subjects (McKeran *et al*, 1974). This suggests that similar restricted growth in brain development, as a result of lack of HGPRT in a tissue with little purine *de novo* biosynthetic ability, might be a factor in the pathogenesis of brain damage in the Lesch-Nyhan syndrome (McKeran *et al*, 1974).

These propositions have been further studied in phytohaemagglutinin (PHA) stimulated lymphocytes by blocking purine *de novo* biosynthesis with azaserine. Blast transformation in lymphocytes involves a rapid burst of polynucleotide synthesis with an acute demand for purine mono-

nucleotides (GMP and AMP). This need is normally met by purine synthesis *de novo* and by the HGPRT catalysed purine salvage pathway. Thus, in the presence of azaserine the lymphocyte becomes a model of a brain cell or a bone-marrow stem cell from the point of view of the metabolic pathway which it has available for purine ribonucleotide synthesis.

Methods

Cell separation. Blood was withdrawn under sterile conditions after an overnight fast from a patient with complete absence of HGPRT and controls of comparable age and sex. Lymphocytes were separated from heparinized blood (0.1 ml, 1:1000 heparin, no preservative added) by the ficol-trisil method (Boyum, 1968).

Growth of phytohaemagglutinin stimulated lymphocytes from patient with Lesch-Nyhan syndrome and controls. The pellet of cells was resuspended to a final concentration of 1×10^6 lymphocytes/ml in Dulbecco's modification of Eagle's medium, containing benzylpenicillin (200 units/ml), streptomycin base (0.2 mg/ml), and pooled human serum gelatin (final concentration 10% v/v). The cell suspension (200 μ l, 200 000 lymphocytes) was added to the wells of microtitre plates. Aliquots (10 μ l) of [2- 14 C]thymidine (8.3 nCi, 0.134 pmol), [2- 14 C]uridine (50 nCi, 0.826 pmol), and L[U- 14 C]phenylalanine (30 nCi, 0.057 pmol) were added to separate wells at 0, 24, and 48 hours, with incubation for 24 hours at 37°C in 5% CO₂ and air. Experiments were performed in triplicate on three occasions with and without PHA stimulation (10 μ l added at zero time) [each vial contained reagent grade PHA suspended in 25 ml Dulbecco's modified Eagle's medium with benzylpenicillin (200 units/ml) and streptomycin base (0.2 mg/ml)].

FIG. 1. A simplified diagram to show the major features of the purine biosynthetic *de novo* and salvage pathways in the production of purine ribonucleotides for DNA, RNA and protein synthesis.

Plates were centrifuged for 10 minutes at $250 \times g_{av}$ and 4°C . The supernatant was discarded, the individual cell pellets washed once with NaCl (200 μl , 0.154 M), trichloroacetic acid (200 μl , 1 M) and finally with methanol (200 μl). Each cell pellet was dissolved in NaOH (200 μl , 1 M) and aliquots (100 μl) counted in 10 ml of a scintillator solution having the following composition: 2,5-diphenyloxazole (PPO) 6 g, p-bis (2-5-phenyloxazolyl) benzene (POPOP) 0.12 g, Triton ($\times 100$) 500 ml, methanol 100 ml, xylene 1 litre. The efficiency of counting was 71%.

Growth of phytohaemagglutinin stimulated lymphocytes from a Lesch-Nyhan patient and controls in presence of azaserine. The experiment described above was repeated on two occasions, with duplicate wells containing 0.3 mmol azaserine to inhibit the conversion of α -N-formylglycinamide ribonucleotide (FGAR), to α -N-formylglycinamide ribonucleotide and thus block the 4th reaction on the pathway of purine biosynthesis *de novo*.

Results

The incorporation of [^{14}C]thymidine, and

FIG. 2. The incorporation of (a) [^{14}C]thymidine, (b) [^{14}C]uridine and (c) [^{14}C]phenylalanine into the nucleoprotein and protein of Lesch-Nyhan and control PHA stimulated lymphocytes. ● Mean and extreme range of 6 observations on blood in the same patient with the Lesch-Nyhan syndrome. ○ Mean and extreme range of 6 observations on blood from 2 control subjects.

[^{14}C]uridine into the nucleoprotein, and [^{14}C]phenylalanine into the protein of PHA stimulated lymphocytes from a patient with the Lesch-Nyhan syndrome and control patients is shown in Fig. 2. No reduction was detected between the values obtained for the Lesch-Nyhan patient and controls with PHA stimulation.

The percentage reduction in the incorporation of [^{14}C]thymidine, and [^{14}C]uridine into the nucleoprotein, and [^{14}C]phenylalanine into the protein of lymphocytes from a patient with Lesch-Nyhan syndrome and controls with PHA in the presence of 0.3 mmol azaserine is shown in Fig. 3. Azaserine progressively inhibited the incorporation of precursors into lymphocyte nucleoprotein and protein. This was more obvious in the lymphocytes from the patient with Lesch-Nyhan syndrome during the

FIG. 3. The percentage reduction in the incorporation of (a) [^{14}C]thymidine, (b) [^{14}C]uridine, and (c) [^{14}C]phenylalanine into the nucleoprotein and protein of Lesch-Nyhan and control PHA stimulated lymphocytes in the presence of 0.3 mmol azaserine

● Mean and extreme range of 4 observations on blood in the same patient with the Lesch-Nyhan syndrome. ○ Mean and extreme range of 6 observations on blood from 2 control subjects.

first 24 hours of incubation than in the control subjects' cells. Maximum inhibition (80 to 100%) of the incorporation of the three precursors had been achieved by 72 hours of incubation in cells from both sources.

Discussion

The present experiments show that blocking purine synthesis *de novo* impairs the ability of lymphocytes to produce DNA, RNA, and protein in response to a strong mitotic stimulus (Fig. 3). HGPRT deficiency alone did not have this action (Fig. 2) though it increased the effect of inhibiting purine synthesis *de novo* (Fig. 3). These results show that purine synthesis *de novo* is more important than purine salvage during rapid cell replication. They are also compatible with the hypothesis that tissues such as brain and bone-marrow with inherently low capacity for purine biosynthesis *de novo* and lacking HGPRT activity in the Lesch-Nyhan syndrome would be unable to synthesize DNA, RNA, and protein adequately, especially during phases of rapid cell division, as in the perinatal period, in the case of the brain.

This specific vulnerability to HGPRT deficiency, which tissues with little capacity for purine biosynthesis *de novo* show, could be alleviated *in vivo* by derepression of this metabolic pathway in the tissues concerned. However, no purine biosynthesis *de novo* was demonstrable in granulocytes from patients with the Lesch-Nyhan syndrome, as measured by the incorporation of [¹⁴C]formate into formylglycinamide ribonucleotide (FGAR) in the presence of azaserine (R. O. McKeran, 1974, unpublished data), indicating that derepression had not occurred in these cells.

Chambers, Martin, and Weinstein (1974) showed that stimulating mouse spleen lymphocytes with the mitogen concanavalin A, increased purine biosynthesis *de novo* by 10 to 100-fold, and that this was associated with induction of phosphoribosylpyrophosphate synthetase (ATP:D-ribose-5-phosphate pyrophospho transferase, EC 2.7.6.1., PRPP synthetase) which was stimulated by cyclic GMP. Green and Martin (1974) reported that cyclic GMP is an allosteric activator of PRPP synthetase. The biochemical lesion in the Lesch-Nyhan syndrome prevents the normal conservation of guanine as GMP. However, enough of this nucleotide is presumably produced by purine biosynthesis *de novo* to maintain adequate levels for the induction of PRPP synthetase, because normal DNA synthesis occurs when Lesch-Nyhan lymphocytes are stimulated by PHA (Fig. 2). Shields (1974) has

also emphasized the role of cyclic GMP in cell growth.

The brain damage in the Lesch-Nyhan syndrome has been ascribed to the action of an unidentified toxin (Nyhan, 1973). We consider that the alternative GMP starvation theory (Table II) is more likely to be correct, because of the importance of GTP for macromolecule-synthesis and neurotubule function (Olmsted and Borisy, 1973) and cyclic GMP in neurotransmission (Ferrendelli *et al*, 1970; Lee, Kuo, and Greengard, 1972; Weight, Petzold, and Greengard, 1974; Ferrendelli, Kinscherf, and Kipnis, 1972; Ferrendelli, Chang, and Kinscherf, 1974).

TABLE II
THEORIES AS TO CAUSE OF BRAIN DAMAGE IN
LESCH-NYHAN SYNDROME

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- 1: Toxic metabolite theory
 - 2: GMP starvation theory leading to:
 - a) Impaired nucleic acid synthesis
 - b) Impaired GTP dependent protein synthesis
 - c) Impaired GTP dependent glycoprotein synthesis (GTP dependent mannosyl transferase)
 - d) Lack of cyclic GMP leading to impaired cell growth, and neurotransmission
 - e) Impaired GTP dependent neurotubule function
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It is of interest that 5-hydroxytryptamine acts at some sites by stimulating guanylate cyclase (EC 4.6.1.2) and thus the production of cyclic GMP (Sandler *et al*, 1975). Lack of GMP in the Lesch-Nyhan syndrome might be expected to inhibit this reaction and the function of 5-hydroxytryptamine dependent neurones might be particularly susceptible to damage. There has been a recent report that 5-hydroxytryptophan, which is the immediate metabolic precursor of 5-hydroxytryptamine decreased the compulsive self-mutilation in a small series of patients with the Lesch-Nyhan syndrome (Mizuno and Yugari, 1974). This claim is being examined further.

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