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AUTORADIOGRAPHIC EVIDENCE OF INCREASED INCORPORATION OF ASPARTATE AND OF CARBAMOYL ASPARTATE IN FIBROBLASTS FROM A LESCH-NYHAN PATIENT

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SUMMARY Lesch-Nyhan patients are deficient in hypoxanthine-guanine phosphoribosyltransferase. Changes in the levels of enzymes which participate in "de novo" synthesis of pyrimidines have been detected in erythrocyte lysates from these patients. In this paper we present the first evidence, using autoradiography, for an increased rate of production of pyrimidines from aspartate and from carbamoyl aspartate in intact cultured fibroblasts from a Lesch-Nyhan patient. The significance of these findings is briefly discussed.

Patients with the Lesch-Nyhan syndrome (1) show a deficiency of hypoxanthine guanine phosphoribosyltransferase (HGPRTase) (E.C.2.4.2.8), and an increased rate of "de novo" synthesis of purines resulting in excessive production of uric acid. Other enzyme abnormalities have been identified in erythrocytes of Lesch-Nyhan patients. These involve increased levels of four of the enzymes which participate in the "de novo" synthesis of pyrimidines (2,3). However, it is not known: a) if the levels of these enzymes are also increased in cells containing the entire complement of enzymes for "de novo" synthesis of pyrimidines, and b) if there is an increase in the overall rate of pyrimidine formation in nucleated cells.

In this paper we present the first evidence for an increased rate of production of pyrimidines from aspartate and from carbamoyl aspartate in intact fibroblasts cultured from a Lesch-Nyhan patient.

MATERIALS AND METHODS (6-^{3}H) hypoxanthine (1.5 Ci/mmol) and L-(2,3-^{3}H) aspartic acid (12 Ci/mmol) were from The Radiochemical Center, Amersham. (^{3}H) carbamoyl aspartate (0.5 Ci/mmol) was synthesized from L-(2,3-^{3}H) aspartic acid and sodium cyanate (4). Fetal bovine serum and Eagle's minimum essential medium (MEM) were from Flow Laboratories (Irvine, Scotland);cycloheximide was from Sigma. Other chemicals were of the highest purity available.

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Cell cultures. Fibroblasts were cultured by the explant method from a skin biopsy of a 4-year-old patient with the Lesch-Nyhan syndrome (see results). The cells were grown in monolayer in 25 ml Falcon flasks at 37°C in Eagle's MEM supplemented with 20% fetal bovine serum, penicillin (100 UI/ml) and streptomycin (100 µg/ml). Experiments were done after 5-9 passages of the cells. Normal control fibroblasts were obtained from the skin of a healthy child;they were cultured under the same conditions.

Labelling of the cells. To cells, grown on coverslips for 48 h after the last passage (5th-9th) was added fresh medium containing (^{3}H) hypoxanthine, 30 µCi/ml (20 µM). After 5 hours incubation the medium was removed and the cells were washed with Dulbecco's solution, fixed and processed for autoradiography (see below). When (^{3}H) carbamoyl aspartate was used, 50 µCi/ml (0.1 mM) were added to the cells and the incubation time was 4 h. (^{3}H) aspartic acid was used at 5 µCi/ml (4 µM) for 1 h. When used, cycloheximide, at 200 µg/ml of culture medium, was added 10 min before the isotope, and was also present during the entire period of incubation with (^{3}H) aspartate.

Autoradiographs were done by the "stripping film" technique (5) with Kodak AR10 film, exposed for 8 days at 4°C. After development with D-19 (Kodak) and fixation with Agefix (Agfa) the cells were stained with Giemsa's solution.

RESULTS AND DISCUSSION The patient exhibited the characteristic neurological abnormalities of the syndrome (6), excreted increased amounts of uric acid and hypoxanthine, and the erythrocytes had 6% of the normal HGPRTase activity.

There was very extensive labelling of normal fibroblasts (Fig. 1a) when incubated with (^{3}H) hypoxanthine. Fibroblasts from the patient's skin incorporated practically no radioactivity (Fig. 1b) under the same conditions.

When (^{3}H) carbamoyl aspartate was incubated with normal fibroblasts there was some incorporation of radioactivity with preferential labelling of the nuclear and nucleolar structures (Fig. 2a). This is consistent with the role of carbamoyl aspartate in the synthesis of pyrimidines. The cells from the Lesch-

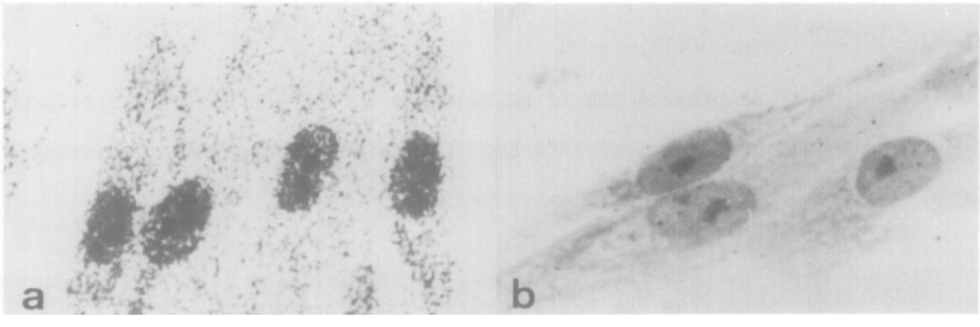

Fig. 1. Incorporation by normal and Lesch-Nyhan syndrome fibroblasts of (^{3}H) hypoxanthine. The experimental conditions were as described in the text. a) normal fibroblasts b) Lesch-Nyhan fibroblasts.

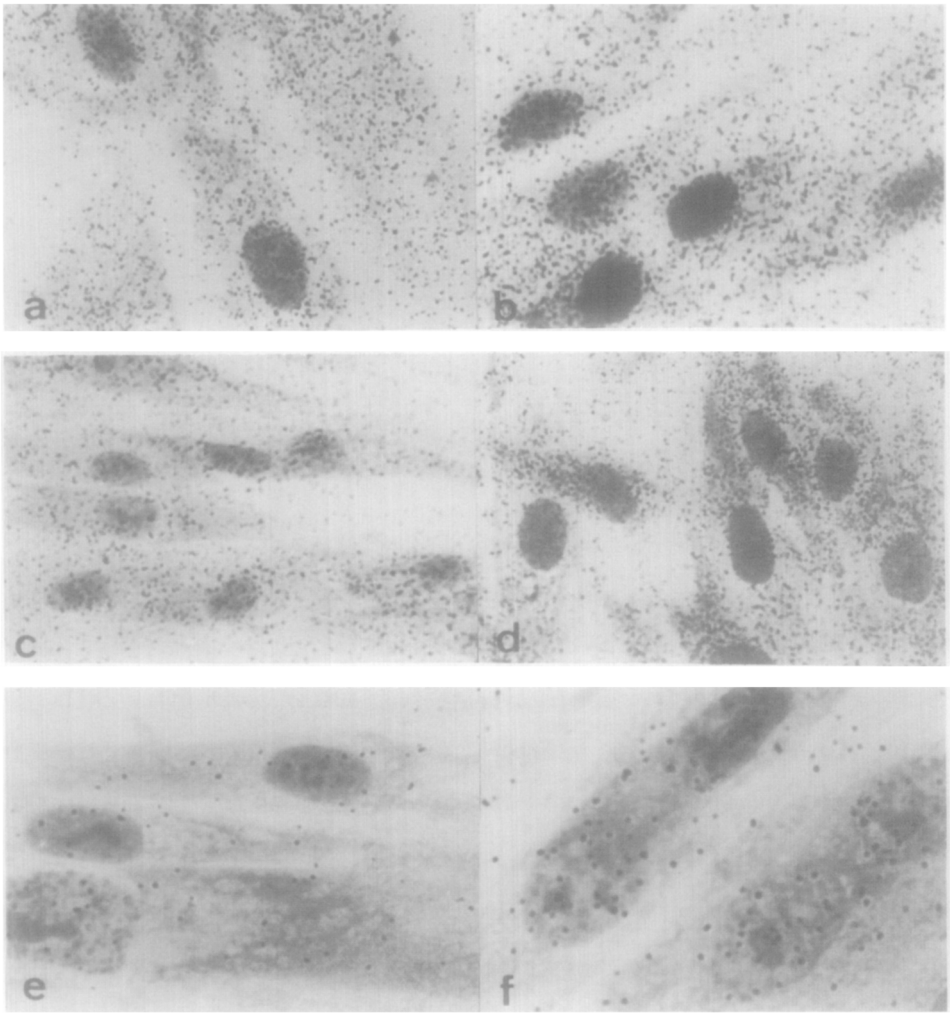

Fig. 2. Incorporation by normal and Lesch-Nyhan syndrome fibroblasts of [^{3}H]carbamoyl aspartate, [^{3}H]aspartic acid and [^{3}H]aspartic acid in the presence of cycloheximide. The experimental conditions were as described in the text. a) and b) normal and Lesch-Nyhan syndrome fibroblasts, respectively, in the presence of [^{3}H]carbamoyl aspartate; c) and d) normal and Lesch-Nyhan syndrome fibroblasts, respectively, in the presence of [^{3}H]aspartic acid; e) and f) normal and Lesch-Nyhan syndrome fibroblasts, respectively, in the presence of [^{3}H]aspartic acid and cycloheximide.

Nyhan patient when incubated with [^{3}H]carbamoyl aspartate (Fig. 2b) showed a similar distribution of grains over the different cellular structures. While the results are qualitative, comparison of the cells from the Lesch-Nyhan patient with the normal fibroblasts clearly shows an increase. Thus, in the presence of carbamoyl aspartate, fibroblasts from Lesch-Nyhan patients synthesize pyrimidines faster than control fibroblasts.

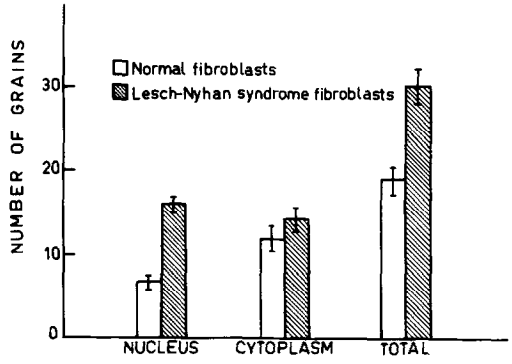

Fig. 3. Number of grains over the nucleus, cytoplasm and entire cell in 100 normal and Lesch-Nyhan fibroblasts after incorporation of (^{3}H)aspartic acid in the presence of cycloheximide. The experimental conditions were as described in the text.

When the cells were incubated with (^{3}H) aspartate, more grains were seen in the Lesch-Nyhan (Fig. 2c) than in normal fibroblasts (Fig. 2d). While in normal cells the label is evenly distributed over the entire cell body, there is a greater concentration of grains over the nucleus of Lesch-Nyhan cells. This suggests also a more rapid incorporation of aspartate into pyrimidines in Lesch-Nyhan than in normal cells;this interpretation, however, is complicated by the incorporation of aspartate into proteins. To clarify this point, the incorporation of (^{3}H)aspartate by both Lesch-Nyhan and normal fibroblasts was studied in the presence of cycloheximide (200 µg/ml). With blockage of protein synthesis there was a marked reduction in the number of grains. While the number of grains over the Lesch-Nyhan fibroblasts was also greatly reduced, they were more numerous than in the control cells and were more concentrated over the nuclear structures, as would be expected if they were due to labelling of pyrimidines (Figs. 2e,2f).

The smaller number of grains over the cells (as a consequence of the treatment with cycloheximide) made a quantitative estimation of their number feasible. As shown in Fig. 3, they are more numerous in the nuclei of Lesch-Nyhan cells (approximately three-fold) than in normal fibroblasts; the label of the cytoplasm is very similar for Lesch-Nyhan cells and normal fibroblasts. It appears that the aspartic transcarbamylase (E.C.2.1.3.2) is also increased in these

cells. Indeed, aspartate transcarbamylase in erythrocyte lysates from the patient was five times that of normal erythrocytes, as expected (3).

Thus, the increased levels of enzymes of the pyrimidine pathway reported in erythrocyte lysates from Lesch-Nyhan patients (2,3) are paralleled by the increased synthesis of pyrimidines from aspartate or carbamoyl aspartate in fibroblasts from these patients, and may have functional significance. It is not clear why the deficiency of a key enzyme of the purine salvage pathway should be associated with an increase in the pyrimidine biosynthetic pathway and should result in the bizarre neurological symptoms of the Lesch-Nyhan syndrome.

The fact that these patients have decreased HGPRTase activity and increased uric acid production should not obscure the increase in 5-phosphoribosyl-1-pyrophosphate (7) and in the enzymes of the pyrimidine pathway (2,3) which may be just as important in the pathogenesis. In this regard, it would be of much interest to determine whether other reactions involving 5-phosphoribosyl-1-pyrophosphate, such as the synthesis of histidine or of tryptophan, are also affected. Such studies are now under way.

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