Vol. 96, No. 3, 1980 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 16, 1980 Pages 1011-1016

INCREASED INCORPORATION OF ASPARTATE AND DECREASED INCORPORATION OF OROTATE IN FIBROBLASTS FROM LESCH-NYHAN PATIENTS AS REVEALED BY AUTORADIOGRAPHY

by Amelia Martínez-Ramón and Santiago Grisolía

Instituto de Investigaciones Citológicas

de la Caja de Ahorros de Valencia

Amadeo de Saboya 4, Valencia (Spain)

Received August 12,1980

SUMMARY While it has been known for many years that tissues from Lesch-Nyhan patients are deficient in hypoxanthine-guanine phosphoribosyl transferase, it has been demonstrated more recently that erythrocyte lysates show an increase in the levels of enzymes which participate in "de novo" synthesis of pyrimidines. Also, we were able to demonstrate, using autoradiography, an increased rate of incorporation of aspartate into the nucleus of intact cultured fibroblasts from a Lesch-Nyhan patient. We have now confirmed this finding. In this paper we show increased incorporation of aspartate into the nucleus of cells from three other patients. Interestingly, the incorporation of orotate is decreased in the fibroblasts from the four patients studied. The incorporation of leucine is the same in Lesch-Nyhan fibroblasts as in normal fibroblasts. The significance of these findings is briefly discussed.

The best molecular defect in the Lesch-Nyhan syndrome (1) is the deficiency (2) in hypoxanthine guanine phosphoribosyl transferase (HGPRT) (E.C.2.4.2.8) These patients also have an increase in the activity of ribose phosphate pyrophosphokinase (E.C.2.7.6.1); the increased level of phosphoribosyl pyrophospate should contribute to the increased "de novo" production of purines characteristic of these patients (3,4).

On the other hand, it has been found recently that there are a number of alterations in the metabolism of pyrimidines in erythrocyte lysates from Lesch-Nyhan patients (5,6), and more recently we have been able to show, using autoradiographic methods, an increased incorporation of the pyrimidine precursors carbamyl aspartic and aspartate in intact fibroblasts from a Lesch-Nyhan patient (7). The fibroblasts from this patient were almost entirely deficient in HGPRT. Although much attention has been given, and properly so, to the de-

0006-291X/80/191011-06$01.00/0

ficiency of the enzyme HGPRT and to the mental deficiency of these patients, the increase in the pyrimidine pathway demonstrated at the intact cell level is most interesting because it not only involves another major pathway but one which uses intermediates in common with the purine biosynthetic pathway.

Thanks to the kindness of Prof. Nyhan we have tested the fibroblasts from three other subjects showing the typical clinical manifestations, as well as the HGPRT deficiency of the Lesch-Nyhan syndrome. We show below, with the aid of autoradiographic methods, the high incorporation of aspartate in fibroblasts from these patients. Also, we have tested the incorporation of orotic acid in order to detect possible variations in orotate phosphoribosyl transferase (E.C. 2.4.2.10.) which is involved in one of the last steps of pyrimidine biosynthesis.

MATERIALS AND METHODS L-(2,3-^{3}H) aspartic acid (12 Ci/mmol), L-(4-^{3}H) leucine (Ci/mmol) and (5-^{3}H) orotic acid (15 Ci/mmol) were from the Radiochemical Center, Amersham. The bovine fetal serum and Eagle culture medium, minimum essential medium (MEM) from Flow Laboratories (Irvine, Scotland). Cycloheximide was from Sigma, and other chemicals used were of the highest purity available.
Cell cultures. The fibroblasts designated line V.G. were cultured by the explant method from a skin biopsy of a five-year old child with the Lesch-Nyhan syndrome. The lines D.G., J.R.R. and C.W.W. were fibroblasts from other patients with the same disease, 8,14 and 16 of age respectively, and were from the laboratory of Dr. Nyhan, (Department of Pedriatics, La Jolla) University of California in San Diego. The cells were grown in monolayer in 25 ml Falcon flasks at 37°C in Eagle's MEM supplemented with 20 % bovine fetal serum, penicillin (100 I.U./ml) and streptomycin (100 µg/ml). The control human fibroblasts were obtained from the skin of a normal child and were cultured under the same conditions.
Labeling of the cells. To cells grown on cover-slips for 48 h after the last passage (10,15), was added fresh medium containing (^{3}H) aspartic acid, 5 µ Ci/ml (4 µM) for 1 h. Cycloheximide (200 µg/ml culture-medium) was added 10 min before the isotope, and was also present in the medium throughout the incubation period with (^{3}H) aspartate. When (^{3}H) leucine was used, 2 µCi/ml (4 µM) were added to the culture medium for 1 h. In another experiment, cycloheximide was added with the same conditions as described for (^{3}H) aspartate. (^{3}H) orotic acid (10 µCi/ml) (8 µM) was added and the pulse was the same as for the other isotopes.

The autoradiograms were prepared using the "stripping film" (8) technique with Kodak AR10 film, exposed for 8 days at 4°C. After developing with D-19 (Kodak) and fixing with Agefix (Agfa), the cells were stained with a citrate-buffered Giemsa's solution.

RESULTS AND DISCUSSION The cells used in this study show a deficiency of HGPRT as evidenced by the lack of incorporation of (^{3}H) hypoxanthine and of (^{3}H) gua-

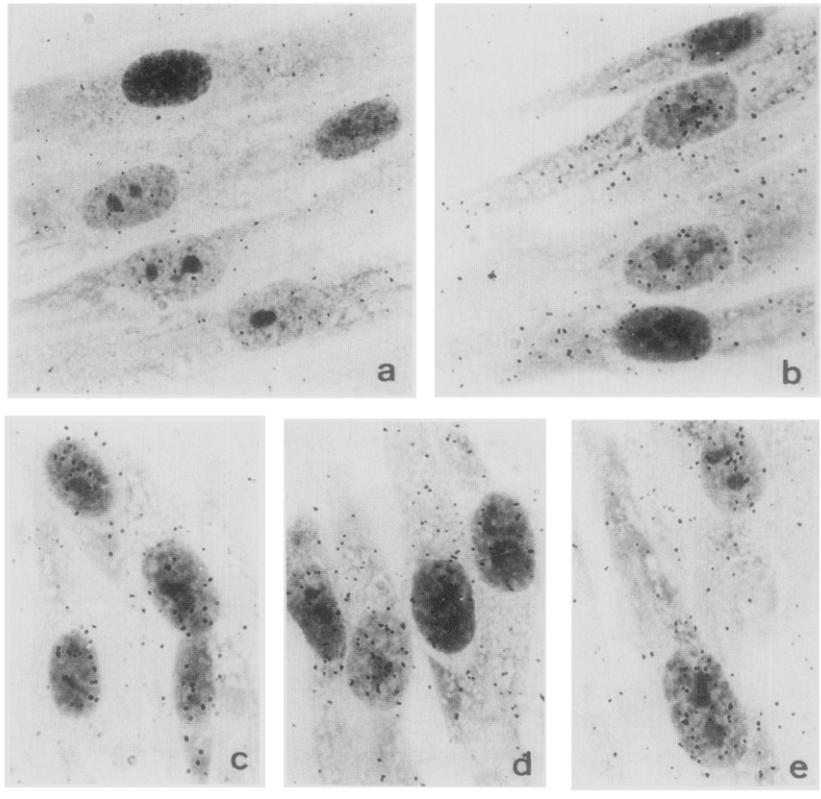

Fig. 1. Incorporation by normal and Lesch-Nyhan syndrome fibroblasts of (^{3}H) aspartic acid in the presence of cycloheximide. The experimental conditions were as described in the text. a) normal fibroblasts b) Lesch-Nyhan fibroblasts.

nine. We have shown previously that while it was possible to demonstrate a higher incorporation of (^{3}H) aspartate in Lesch-Nyhan fibroblasts than in normal fibroblasts, the large number of radioactive marks did not permit quantification. Therefore, the synthesis of protein was inhibited with cycloheximide, and under these conditions it was possible to quantitate the incorporation. Because of this, in the three cases tested now, we have used cycloheximide throughout to quantitate incorporation both in the nuclei and in the cytoplasm. The results are very similar to those obtained with the first case studied by us (7). Fig. 1 illustrates a larger incorporation of (^{3}H) aspartate in Lesch-Nyhan fibroblasts than in control fibroblasts . Also, as illustrated in Fig.3 and as expected, there is a larger number of marks in the nucleus than in the cytoplasm.

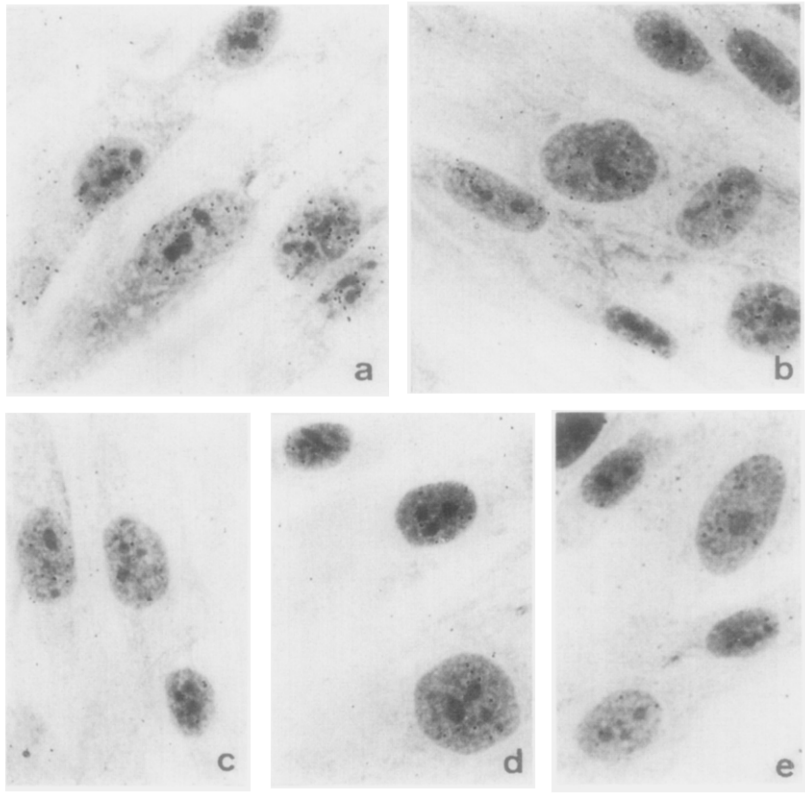

Fig.2. Incorporation by normal and Lesch-Nyhan syndrome fibroblasts of (^{3}H) orotic acid. The experimental conditions were as described in the text. a) normal fibroblasts b,c,d,e) Lesch-Nyhan syndrome fibroblasts.

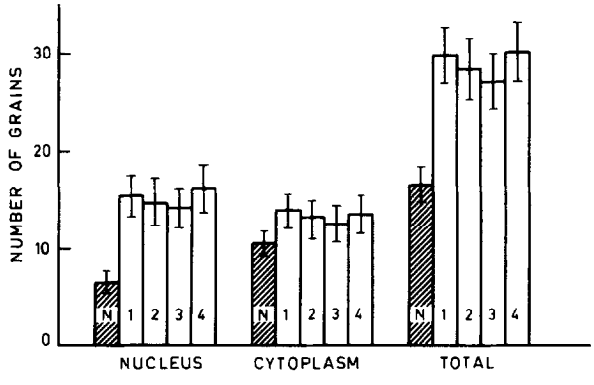

Fig. 3. Number of grains over the nucleus, cytoplasm and entire cell in 100 normal and Lesch-Nyhan fibroblasts after incorporation of (^{3}H) aspartic acid in the presence of cycloheximide. The experimental conditions were as described in the text. N) normal fibroblasts 1,2,3,4) Lesch-Nyhan syndrome fibroblasts.

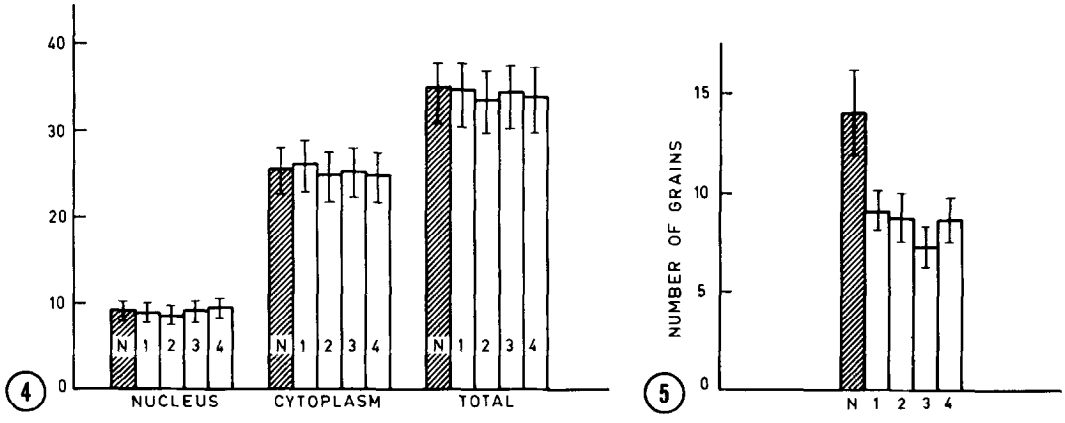

Fig. 4. Number of grains over the nucleus, cytoplasm and entire cell in 100 normal and Lesch-Nyhan fibroblasts after incorporation of (^{3}H) leucine. The experimental conditions were as described in the text. N) normal fibroblasts 1,2,3,4) Lesch-Nyhan fibroblasts.

Fig. 5. Number of grains over entire cell in 100 normal and Lesch-Nyhan fibroblasts after incorporation of (^{3}H) orotic acid. The experimental conditions were as described in the text. N) normal fibroblasts 1,2,3,4) Lesch-Nyhan fibroblasts.

Following the suggestion of Dr. Horecker, we checked the incorporation of (^{3}H) leucine to confirm that the incorporation of aspartate, under our conditions, was indeed reflecting mostly pyrimidine biosynthesis and was not due to incorporation into proteins. The incorporation of (^{3}H) leucine in the presence of cycloheximide resulted in almost complete lack of incorporation. Also, by using very low specific activities we have been able to quantitate the incorporation, and as is shown in Fig. 4, there are no differences in the extent of incorporation between the Lesch-Nyhan cells and those of controls. Also, as expected, with leucine there is a preferential labeling in the cytoplasm.

As illustrated in Figs. 2 and 5, when cells were incubated with (^{3}H)orotic acid, there was surprisingly less incorporation with Lesch-Nyhan fibroblasts. Again, in this case, the bulk of incorporation was in the nucleus. These results are difficult to interpret, particularly since an increase in orotate phosphoribosyl transferase has been shown in the erythrocytes of Lesch-Nyhan patients, while their leucocytes contain normal or slightly lower levels of the enzyme.

We do not know the mechanisms responsible for the increase in activity of enzymes for pyrimidine biosynthesis demonstrated by ourselves and others in Lesch-Nyhan patients. The new finding of decreased incorporation of orotate, demonstrated in this paper, indicates at first glance a decrease in orotate phosphoribosyl transferase activity. However, it is generally believed that the cells of these patients always show an increment in phosphoribosyl pyrophosphate, and in a thorough study of the enzymes of pyrimidine biosynthesis in Erlich ascites tumors (9) it has been shown that orotate phosphoribosyl transferase is activated by phosphoribosyl pyrophosphate.

Obviously, an extensive and thorough study of enzyme levels is necessary, particularly of orotate decarboxylase (E.C.4.1.1.23.) as well as of incorporation of precursors in intact cells of both the pathways of biosynthesis and of utilization of pyrimidines in cells of these patients. Such a study may not only shed light on the obviously complex metabolic alterations of these patients, but may also help in clarifying functional alterations, particularly those leading to behavioral manifestations.

ACKNOWLEDGMENTS We wish to thank W.L. Nyhan for the gift of three cell lines from Lesch-Nyhan patients, F. Thompson for help with the manuscript, and I. Salazar, F. Ripoll and M.J. Argulló for their expert technical assistance.

REFERENCES

1. Lesch, M. and Nyhan, W. (1964) Am. J. Med.,36, 561-570.

2. Seegmiller, J.E., Rosenbloom, F.M. and Kelley, W.N.(1967) Science, 155, 1682-1684.

3. Fox, I.H., and Kelley, W.N. (1971) Am. Int. Med., 74, 424-433.

4. Torrelio, B.M. and Paz, M. (1979) Biochem. Biophys. Res. Commun., 87, 380-387.

5. Beardmore, T.D., Meade, J.C. and Kelley, W.N. (1973) J. Lab. Clin. Med., 81, 43-52.

6. Kennedy, J. (1978) Biochem. Biophys. Res Commun., 80, 653-658.

7. Martínez- Ramón, A., Rubio, V. and Grisolía, S. (1979) Biochem. Biophys. Res. Commun., 90, 333-337.

8. Schmid, W. (1963) Cytogenetics, 2, 175-193.

9. Shoaf, W.T. and Jones, M.E. (1971) Biochem. Biophys. Res. Commun., 45, 796-802.