Separation of three isoenzymes of N-acetyl- β -D-hexosaminidase from human tissues by cellulose acetate membrane electrophoresis

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SYNOPSIS The separation of N-acetyl- β -D-hexosaminidase isoenzymes from human tissues is used in the diagnosis and differential diagnosis of GM₂ gangliosidosis, since in type 1 the A isoenzyme is deficient and in type 2 both the A and B isoenzymes are deficient. Peripheral blood leucocytes are commonly used for these investigations, and the present study demonstrates that, in addition to these two isoenzymes, a third isoenzyme can be separated from leucocytes by cellulose acetate membrane electrophoresis. This isoenzyme is more anodic than the A and B isoenzymes and is similar to the hexosaminidase C isoenzyme recently reported in embryonic tissue extracts. The isoenzyme was also clearly demonstrable in human liver and kidney but was present in much lower concentrations in cultured cells. It could be demonstrated in leucocytes even after prolonged storage at -20° , and, like the A isoenzyme, is partially inactivated by a short exposure to heat.

Two isoenzymes of N-acetyl- β -D-hexosaminidase, called hexosaminidases A and B, have been separated from human tissues by a variety of techniques including starch (Okada and O'Brien, 1969) and polyacrylamide (Friedland, Schneck, Saifer, Pourfar, and Volk, 1970) gel electrophoresis, cellulose acetate membrane electrophoresis (Suzuki and Suzuki, 1970), isoelectric focusing (Sandhoff, 1969), and ion-exchange chromatography (Robinson and Stirling, 1968). The separation of these two isoenzymes is of important diagnostic value, since a deficiency of the A isoenzyme has been demonstrated in GM₂ gangliosidosis type 1, or Tay-Sach's disease (Okada and O'Brien, 1969), and of both the A and B isoenzymes in GM₂ gangliosidosis type 2, or Sandhoff's disease (Sandhoff, 1969).

Minor bands of hexosaminidase activity intermediate in migration between the A and B isoenzymes have been reported in some human tissues, initially by Young, Ellis, Lake, and Patrick (1970), and later by Price and Dance (1972) and Hayase and Kritchevsky (1973). The increase in serum hexosaminidase activity during pregnancy seems to be due to the appearance or amplification of such a band, which has been called hexosaminidase P (Stirling, 1972).

Another hexosaminidase isoenzyme has very recently been isolated from embryonic tissues (Poenaru and Dreyfus, 1973). This was more anodic than the A and B isoenzymes at pH 6.2 in cellulose acetate membrane electrophoresis and was called hexosaminidase C. It was also found in biopsies of adult liver and brain, but was reported to be very unstable and could not be demonstrated in tissues obtained at necropsy, from tissues stored at -20° for more than a few days, or from serum or urine.

In this laboratory the diagnosis and differential diagnosis of GM₂ gangliosidosis is made by cellulose acetate membrane electrophoresis of peripheral blood leucocyte extracts. We report here, for the first time, the separation of three hexosaminidase isoenzymes from these and other tissues useful in the investigation of these diseases.

Materials and Methods

Leucocytes were separated from fresh whole blood, anticoagulated with EDTA, by dextran sedimentation and differential lysis of erythrocytes (Nelken, Gilboa-Garber, and Gurevitch, 1960). Dextran T-110 was used at a final concentration of 20 g/l, and erythrocytes were lysed by exposure to hypotonic saline (2.5 g/l) for one minute. Lymphocytes were then separated by the density gradient method of

Harris and Ukaejiofo (1970), and a short-term culture was established by conventional techniques (Paul, 1970) in minimal Eagle's medium buffered with tricine and supplemented with fetal bovine serum (20%), phytohaemagglutinin (1 mitogenic unit/100 ml), and the antibiotics penicillin G (120 $\mu\text{g/ml}$), streptomycin (200 $\mu\text{g/ml}$), and amphotericin B (2.5 $\mu\text{g/ml}$). Mixed lymphocyte cultures were established in the same medium without phytohaemagglutinin.

Fibroblasts were grown from skin biopsies (Paul, 1970) in Ham's F10 medium buffered with bicarbonate and supplemented with calf serum (10%), fetal bovine serum (10%), tryptose phosphate broth (10%), and the antibiotics penicillin G (60 $\mu\text{g/ml}$) and streptomycin (100 $\mu\text{g/ml}$). The cells were detached and harvested by treatment with a solution containing trypsin and versene (Paul, 1970).

The lysosomal enzymes were extracted from these cell preparations, and also from histologically normal liver and kidney obtained at necropsy from an adult, by lysis in triton X-100 (Wattiaux and De Duve, 1956). Cell debris was removed by centrifugation, and the supernatant was kept at -20° until analysis. In some experiments lysates were prepared by freezing and thawing a cell suspension in deionized water.

Electrophoresis of the lysates was carried out at pH 6.2 in citrate-phosphate buffer (0.02 mol/l) on cellulose acetate membranes (Suzuki and Suzuki, 1970). A total hexosaminidase activity of approximately 1 mU (1 nmol/min) of each lysate was added to the membrane, and a run of 90 to 120 minutes

(at 0.4 mamps per cm width of membrane) gave good separation between the isoenzymes. These were visualized after the run by incubating the membrane at 37° for 15 to 60 minutes on filter paper impregnated with substrate solution (20 mg 4-methylumbelliferyl-2-acetamido-2-deoxy- β -D-glucopyranoside in 25 ml citrate buffer, 0.05 mol/l, pH 4.5) and illumination with ultraviolet light (365 nm). The bands of hexosaminidase activity then showed up as bright blue bands on a dark blue background, but they were enhanced when the membrane was placed on filter paper impregnated with glycine buffer (0.5 mol/l, pH 10.4). Photographs were taken as soon as possible since the 4-methylumbelliferone released by the hexosaminidase activity is very soluble in alkali and tends to diffuse through the membrane, causing spreading of the heavy bands and fading of the lighter bands.

Results and Discussion

In addition to the two major hexosaminidase isoenzymes (A and B) demonstrable in a number of human tissues (Robinson and Stirling, 1968) minor isoenzymes, intermediate between these two in mobility, have been reported (Young *et al*, 1970; Price and Dance, 1972; Hayase and Kritchevsky, 1973). In the present study we found that cellulose acetate membrane electrophoresis at pH 6.2 separates from some human tissues an isoenzyme more anodic than those previously described. This was first noticed as a faint band clearly separated from the A and B isoenzymes in leucocyte extracts (fig 1).

Fig 1 Separation of hexosaminidase isoenzymes from leucocytes (4, 6, 7), cultured fibroblasts (3, 5) and cultured lymphocytes (1, 2) by cellulose acetate membrane electrophoresis. Electrophoretic run — 2 h; substrate incubation — 15 min.

Fig 2 Electrophoresis of hexosaminidase isoenzymes in leucocyte lysates prepared from individuals varying in age from 6 days (9) to 28 years (6). Electrophoretic run — $1\frac{1}{2}$ h; substrate incubation — 1 h.

Fig 3 Electrophoresis of hexosaminidase isoenzymes in lysates prepared from leucocytes before (8) and after (7) heat inactivation, liver (3), kidney (5), cultured fibroblasts (2), mixed lymphocyte culture (4), phytohaemagglutinin-stimulated lymphocyte culture (6), and in a leucocyte lysate stored six weeks at -20° (1). Electrophoretic run $-1\frac{1}{2}$ h; substrate incubation -1 h.

On more prolonged incubation with the substrate the band became much more distinct and was invariably present in leucocytes prepared from individuals varying in age from 6 days to 28 years (fig 2). Although no bands of hexosaminidase activity intermediate in mobility between the A and B bands were noted, definite conclusions as to their absence could not be made since the bands are not so well resolved by electrophoresis as they are by ion-exchange chromatography or isoelectric focusing.

Poenaru and Dreyfus (1973) have isolated from embryonic tissues a hexosaminidase isoenzyme which also migrates to the anode faster than the more intense A and B bands. They were also able to demonstrate the same isoenzyme in adult liver, and since in the present study the leucocyte isoenzyme was found to be similar in mobility to the liver isoenzyme (fig 3), we have followed the nomenclature introduced by these authors in calling this isoenzyme hexosaminidase C.

All the three isoenzymes separated from leucocytes in the present study were stable during storage at -20° for at least six weeks (fig 3). The A and C isoenzymes were, however, less stable to heat treatment than the B isoenzyme. Both bands were markedly diminished in intensity, but were still visible, after electrophoresis of a lysate heated to 50° for one hour (fig 3). This is in contrast to the hexosaminidase C isolated from embryonic tissues since this was not visible in lysates stored more than a few days at -20° and was usually not demonstrable in tissues obtained at necropsy (Poenaru and Dreyfus, 1973).

Hexosaminidases A, B, and C were isolated from leucocyte lysates prepared either by treatment with triton X-100 to extract lysosomal enzymes (Wattiaux and De Duve, 1956) or by freezing and thawing a cell suspension in deionized water. They were also clearly visible after electrophoresis of extracts of human adult kidney and liver obtained at necropsy (fig 3). However, the fastest isoenzyme (C) was much less easily demonstrable in fibroblasts, cultured from skin, and lymphocytes, cultured either in the presence of phytohaemagglutinin or in mixed cell culture, even after prolonged incubation with the

substrate (figs 1 and 3). Hexosaminidase C was either not present in these cells or was present in very low activities.

Since Poenaru and Dreyfus (1973) found hexosaminidase C in embryonic liver, lung and brain, and later in adult liver and brain biopsies, and we have now found it in leucocytes and in some postmortem tissues, this isoenzyme, like the well known A and B isoenzymes, appears to be present in a wide variety of human tissues. Its physiological significance and its status in the GM₂ gangliosidoses is not known, however, and will be the subject of further study.

References

- Friedland, J., Schneck, L., Saifer, A., Pourfar, M., and Volk, B. W. (1970). Identification of Tay-Sachs disease carriers by acrylamide gel electrophoresis. *Clin. chim. Acta*, **28**, 397-402.
- Harris, R., and Ukaejiofo, E. O. (1970). Tissue typing using a routine one-step lymphocyte separation procedure. *Brit. J. Haemat.*, **18**, 229-235.
- Hayase, K., and Kritchevsky, D. (1973). Separation and comparison of isoenzymes of N-acetyl-β-D-hexosaminidase of pregnancy serum by polyacrylamide gel electrofocusing. *Clin. chim. Acta*, **46**, 455-464.
- Nelken, D., Gilboa-Garber, N., and Gurevitch, J. (1960). A method for simultaneous separation of human thrombocytes and leucocytes. *J. clin. Path.*, **13**, 266.
- Okada, S., and O'Brien, J. S. (1969). Tay-Sach's disease: generalized absence of a β-N-acetylhexosaminidase component. *Science*, **165**, 698-700.
- Paul, J. (1970). *Cell and Tissue Culture*, 4th ed. Livingstone, Edinburgh and London.
- Poenaru, L., and Dreyfus, J. C. (1973). Electrophoretic study of hexosaminidases. Hexosaminidase C. *Clin. chim. Acta*, **43**, 439-442.
- Price, R. G., and Dance, N. (1972). The demonstration of multiple heat stable forms of N-acetyl-β-glucosaminidase in normal human serum. *Biochem. biophys. Acta (Amst.)*, **271**, 145-153.
- Robinson, D., and Stirling, J. L. (1968). N-acetyl-β-glucosaminidases in human spleen. *Biochem. J.*, **107**, 321-327.
- Sandhoff, K. (1969). Variation of β-N-acetylhexosaminidase pattern in Tay-Sachs disease. *FEBS Letters*, **4**, 351-354.
- Stirling, J. L. (1972). Separation and characterization of N-acetyl-β-glucosaminidases A and P from maternal serum. *Biochim. biophys. Acta (Amst.)*, **271**, 154-162.
- Suzuki, Y., and Suzuki, K. (1970). Partial deficiency of hexosaminidase component A in juvenile GM₂-gangliosidosis. *Neurology (Minneapolis)*, **20**, 848-851.
- Wattiaux, R., and De Duve, C. (1956). Tissue fractionation studies. 7. Release of bound hydrolases by means of triton X-100. *Biochem. J.*, **63**, 606-608.
- Young, E. P., Ellis, R. B., Lake, B. D., and Patrick, A. D. (1970). Tay-Sachs disease and related disorders: fractionation of brain N-acetyl-β-hexosaminidase on DEAE-cellulose. *FEBS Letters*, **9**, 1-4.