ARCHIVES OF BIOCHEMISTRY AND BIOPHYSICS
Vol. 189, No. 1, July, pp. 37-43, 1978

Human Placental *N*-Acetyl-β-D-Hexosaminidase Isozymes

Activity toward Native Hyaluronic Acid

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Received December 1, 1977; revised February 2, 1978

The activity of purified human hexosaminidases A and B toward hyaluronic acid (HA) isolated from cultured human skin fibroblasts was investigated. The cleavage of *N*-acetylglucosaminyl residues to monosaccharide *N*-acetylglucosamines by hexosaminidase isozymes was determined in the presence and absence of purified human β-glucuronidase. The pH optima of this reaction, with and without β-glucuronidase, were 4.5 for hexosaminidase A and 4.0 for hexosaminidase B. The hydrolysis of HA by both hexosaminidase isozymes proceeds linearily for at least 18 h in the presence of β-glucuronidase. Concentrations of 0.5–5 units of either isozyme showed a linear relationship with rate of hydrolysis. Without β-glucuronidase, hexosaminidase only cleaved the terminal *N*-acetylglucosamine residue. However, under optimal conditions, with β-glucuronidase, the hydrolytic activity of hexosaminidase B was about 30% as efficient as that of hexosaminidase A. Approximately 70% of the HA could be degraded by 5 units of hexosaminidase A in the presence of 0.5 unit of β-glucuronidase, as opposed to 25% degraded by hexosaminidase B. These results probably reflect intrinsic differences in the activities of the two isozymes. Since the substrate (HA) did not inhibit the hydrolysis of a synthetic substrate (4-methylumbelliferyl-β-glucosaminide) by hexosaminidase B, the linear kinetics of HA hydrolysis implies no product inhibition. These data indicate that native HA can be hydrolyzed by the combined activities of β-glucuronidase with hexosaminidase A or hexoaminidase B.

Mammalian β-*N*-acetylhexosaminidase (hexosaminidase) (EC 3.2.1.52) is an exoenzyme capable of hydrolyzing *N*-acetylglucosaminyl and *N*-acetylgalactosaminyl residues from the nonreducing end of various substrates. Robinson and Stirling (1) first separated hexosaminidase into two major isozymes, namely, hexosaminidase A and hexosaminidase B, which demonstrate identical kinetics on synthetic substrates regarding pH optima, K_m values, and specificity for different substrates and inhibitors (1–3, 31). Hexosaminidase activity toward various natural substrates has not been fully characterized. Information regarding this activity was obtained partially from studies of various disorders involving deficiency of one or both isozymes. In Sandhoff–Jatzkewitz disease (SJD),[1] both hexosaminidase A and hexosaminidase B are missing (4), leading to the accumulation of substances containing a terminal *N*-acetylhexosamine, e.g., lipids (GM_2 ganglioside, globoside, etc.) (5), glycopeptides (6), and mucopolysaccharides (MPS) (7). In classic Tay–Sachs disease (TSD), in which only hexosaminidase A is deficient (8), the major substance accumulated is GM_2 ganglioside (9). This observation suggested that hexosaminidase A is specific for the catabolism of GM_2, whereas other *N*-acetylhexosamine-containing substances can be cleaved by either hexosaminidase A or hexosaminidase B. *In vitro* studies confirmed that GM_2 is hydrolyzed efficiently

[1] Abreviations used: HA, hyaluronic acid; hexosaminidase, β-*N*-acetyl hexosaminidase; SJD, Sandhoff–Jatzkewitz disease; TSD, Tay–Sachs disease; MPS, mucopolysaccharides; CNAG, ε-aminocaproyl-*N*-acetylglucosylamine; SDS, sodium dodecyl sulfate; DEAE, diethylaminoethyl; CM, carboxymethyl.

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only by hexosaminidase A and not by hexosaminidase B. The latter isozyme, however, acts on the asialo derivative of GM_2 and on globoside (3, 10–12).

The activity of hexosaminidase on glycoproteins was studied by Aronson and De Duve (13). The possible involvement of the hexosaminidase isozymes in the catabolism of MPS such as hyaluronic acid (HA) has not yet been thoroughly investigated. Principally, the catabolism of the various MPS such as HA may proceed by two alternative pathways. In hyaluronidase-containing tissues, such as liver, HA may be broken into small fragments by the endohydrolytic activity of this enzyme (14) and further cleaved by hexosaminidase and β-glucuronidase to monosaccharides. However, in cells lacking hyaluronidase activity, such as cultured skin fibroblasts (15), the catabolism of HA may proceed only via the second pathway, namely, the combined activity of hexosaminidase and glucuronidase. Although some studies suggest that HA cannot be hydrolyzed without hyaluronidase (16), this mechanism was proposed as the only possible pathway in these cells (7, 15). The direct activity of hexosaminidase on small MPS substrate fragments, such as heptasaccharides (15) or trisaccharides (17), was studied using crude or partially purified enzyme preparations (18), and indicated that, while hexosaminidase A is capable of hydrolyzing these substrates, hexosaminidase B demonstrated considerably reduced, if any, activity. However, significant *in vivo* degradation of MPS by hexosaminidase B has been suggested indirectly (7).

The present study indicates that purified hexosaminidases A and B were both active in catabolyzing native HA, but with different efficiencies. Hexosaminidase alone hydrolyzed only terminal *N*-acetylglucosamine, but upon the addition of excess β-glucuronidase, a synergistic effect was observed, and under optimal conditions up to 70% of the substrate could be hydrolyzed.

MATERIALS AND METHODS

Cell culture. Skin fibroblasts from normal healthy individuals were propagated by standard techniques as previously described (19). The cells were grown in nutrient medium F-10 (HAM) (GIBCO Inc., Grand Island, New York) supplemented with 10% fetal calf serum (GIBCO) in 75-cm^2 Falcon tissue culture flasks in an atmosphere of 95% air and 5% CO_2.

Preparation of tritiated HA. Confluent monolayers of human skin fibroblasts (approximately 5×10^6 cells per culture flask, containing 1 mg of protein) were labeled with 5 μCi/ml of [^{3}H]glucosamine (13 Ci/mmol; The Radiochemical Centre, England) in glucose-free medium supplemented with 0.2 mg/ml of glucose for 48 h at 37°C. The medium was collected and the cells were harvested by trypsinization, washed twice with 0.9% NaCl, and disrupted by five cycles of rapid freezing and thawing. The insoluble residue was precipitated by centrifugation. The supernatant was collected (supernatant A) and the pellet incubated overnight with 300 μg of Pronase (Calbiochem, La Jolla, California) at 37°C in total volume of 2 ml of 0.1 M sodium phosphate buffer (pH 6.8). The undigested material was removed by centrifugation and the supernatant was combined with supernatant A. Four volumes of ethanol were added and the solution was allowed to stand at room temperature for 1 h. The resulting precipitate was collected by centrifugation (12,000*g* for 30 min) and dissolved in a minimal volume of triple-distilled water.

MPS from the incubation medium were collected in a similar manner. To this medium, 4 vol of ethanol were added and the precipitate was collected as described above. After Pronase digestion, the undigested residue was removed by centrifugation. The supernatant was combined with the intracellular material and dialyzed against five changes (3 liters each) of 5 mM Na-phosphate buffer at pH 7.0. The solution was applied to a DEAE-Sephadex A-50 column (1 × 10 cm) (Pharmacia, Sweden) preequilibrated with the same buffer. Stepwise elution with 50-ml fractions of increasing concentrations of NaCl was performed as described by Bach and Berman (20). Fractions eluted with 0.6–2.0 M NaCl, containing radioactivity associated with MPS (21), were pooled and dialyzed against five changes (3 liters each) of triple-distilled water. This material was lyophilized, redissolved in 50 μl of water, and applied to a cellulose acetate strip (2.5 × 16.75 cm; Sepharose III, Gelman Co., Ann Arbor, Michigan) for electrophoresis in 0.1 M Na-barbital buffer (pH 8.6) at 200 V and 4°C for 40 min. Standards of HA, dermatan sulfate, and heparan sulfate were run in parallel. By this technique, HA was easily separated from the sulfated MPS and from glycoproteins. The HA was located and extracted from the strip as described by Endo and Yosizawa (22).

Characterization of HA. The molecular weight of the tritiated HA (8×10^7 cpm/mg of uronic acid) was determined by gel filtration (Sepharose 4B, Sigma Chemicals, St. Louis, Missouri) and analytical ultracentrifugation (Beckman Model E ultracentrifuge equipped with schlieren optical system). Molecular weights by ultracentrifugation were calculated according to Svedberg and Pederson (23), and by gel filtra-

tion, the elution profile of HA was compared to that of standard MPS with known molecular weights. The HA was eluted from the column in one symmetrical peak. Uronic acid was determined according to Bitter and Muir (24). Total hexosamine was assayed by a modification (25) of the Elson and Morgan reaction (26) and sulfate by the method of Dodgson (27). The amino sugars were identified using an amino acid analyzer (Beckman Model 120 B) as previously described (20).

Enzyme preparations. Hexosaminidase isozymes A and B were purified from human placentas as previously described (28). The procedure included chromatography on a Sepharose-bound concanavalin A column followed by affinity chromatography with Sepharose-bound ϵ-aminocaproyl-*N*-acetylglucosylamine (CNAG) and DEAE-cellulose. Final purification of the A and B isozymes was achieved by ion-exchange chromatography on CM-cellulose and by gel filtration on Sephadex G-150, respectively. Purity of the isozymes was established by polyacrylamide gel electrophoresis in the presence or absence of SDS and by analytical ultracentrifugation. β-Glucuronidase, free of hexosaminidase and hyaluronidase, was partially purified from human placentas as follows: Supernatants of placental homogenates (about 35 liters) (28) were chromatographed on Sepharose-concanavalin A and Sepharose-CNAG columns as above, but from the latter column the nonabsorbed effluent fraction was collected and rerun on Sepharose-CNAG. The effluent was again collected and concentrated to 15 ml by ultrafiltration on an XM 50 filter (Amicon, the Netherlands). This solution was chromatographed on a Sephadex G-200 column (5 × 100 cm; flow rate, 14 ml/h) and fractions of 5.5 ml were collected (Fig. 1a). The fractions under the peak of glucuronidase activity were pooled, concentrated on an XM 50 filter, and rechromatographed on the same column. The glucuronidase peak was collected (Fig. 1b), pooled, concentrated to 20 ml, and frozen in small aliquots. The final specific activity of the glucuronidase preparations was 18–24 units/mg of protein.

Enzyme assays. Hexosaminidase and glucuronidase were assayed using 4-methylumbelliferyl-2-acetamido-2-deoxy-β-D-glucopyranoside and 4-methylumbelliferyl-β-D-glucuronide, respectively, as substrates (Koch Light, England), as described elsewhere (29). One unit of enzyme activity is defined as the amount of enzyme which hydrolyzes 1 nmol of substrate per hour, under the conditions specified.

The hydrolysis of hyaluronic acid (HA) was assayed as follows: [^{3}H]HA (15,000 cpm) was incubated in a total volume of 200 μl of 0.1 M Na-acetate buffer (pH specified for each experiment) containing 300 μg of bovine serum albumin (Grade A, Calbiochem) with hexosaminidase A or B (3 units), if not otherwise stated. Purified glucuronidase, when tested, was added in an amount of 0.5 unit per reaction. When incubations were longer than 3 h, two drops of toluene were added. Inactivated (by boiling) hexosaminidase A or B controls were incubated in parallel. The reaction was stopped by immersing the tubes in a boiling-water bath for 5 min. Then 100 μl of 1 M $NaHCO_3$ was added and undegraded MPS were precipitated with 1.4 ml of ethanol. The tubes were again immersed in boiling water for 1 min and centrifuged at 12,000*g* for 30 min. The supernatant was transferred to a scintillation vial and evaporated to dryness under a nitrogen stream. This dried material was dissolved in 0.5 ml of water and radioactivity was determined in 10 ml of Insta Gel (Packard, Downers Grove, Illinois). The efficiency of radioactivity counting was 25%.

Identification of product. The reaction product was identified by paper chromatography as previously described (12).

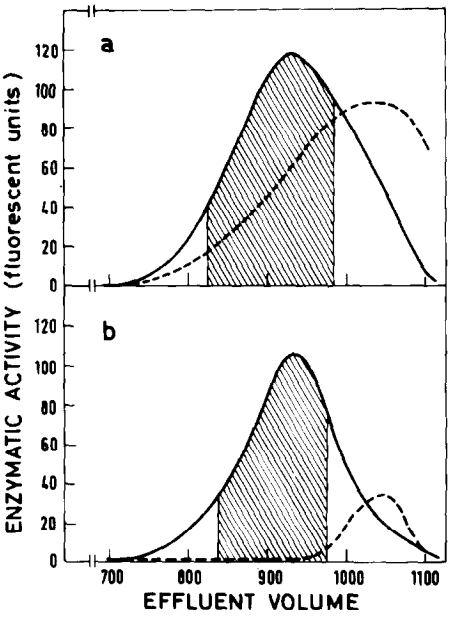

FIG. 1. Separation of β-glucuronidase (——) from hexosaminidase (-----) on Sephadex G-200. The sample (15 ml) was applied to the column (5 × 100 cm) and chromatographed at 4°C at a rate of 15 ml/h. The shaded areas represent the glucuronidase peaks pooled for further purification or for assay use. (a) First chromatography; (b) rechromatography on the same column.

RESULTS

The molecular weight of HA from cultured skin fibroblasts was found to be 2–2.5 × 10^5, by both ultracentrifugation and gel filtration. More than 80% of this material was degradable to small dialyzable fragments by fungal or testicular hyaluronidases. The molar ratio of uronic acid to hexosamine after acid hydrolysis was 1.2:1.

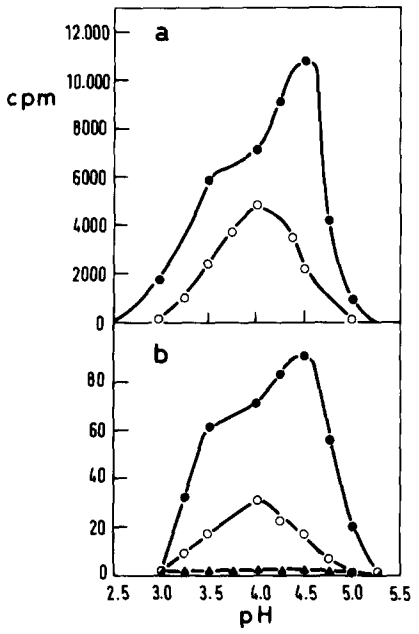

FIG. 2. The pH dependence of hexosaminidase activity toward tritiated HA. Three units of hexosaminidase isozyme were incubated with 15,000 cpm of [^{3}H]HA per incubation, as described in the text in the presence (a) or absence (b) of 0.5 unit of β-glucuronidase. Incubations were carried out for 20 (a) or 4 h (b). (●——●) Hexosaminidase A; (○——○) hexosaminidase B; (▲——▲), β-glucuronidase alone. Note the differences in scale between hydrolysis in the presence and absence of glucuronidase.

No sulfate was detected in the purified preparation. The major amino sugar detected after hydrolysis in 2 N HCl (20) was glucosamine (>90%). The electrophoretic mobility of the fibroblast HA on cellulose acetate was identical to that of the HA derived from human umbilical cord.

Figure 2 demonstrates the pH dependence of HA degradation by hexosaminidase A or B in the presence and absence of β-glucuronidase. The latter enzyme alone had no detectable activity on this substrate. Under optimal conditions, more than 90% of the radioactive product cleaved from HA by either hexosaminidase A or hexosaminidase B had an R_f value identical to that of *N*-acetylglucosamine on paper chromatography. No radioactivity was associated with glucuronic acid, free glucosamine, or di- or tetrasaccharides. Figure 2 indicates an optimal pH of 4.5 for hexosaminidase A and of 4.0 for hexosaminidase B. At the optimal pH for both isozymes, the addition of excess β-glucuronidase resulted in a considerably higher degradation of HA (Fig. 2a). However, the activity of hexosaminidase A differed significantly from that of hexosaminidase B, either alone or in association with β-glucuronidase. Under all conditions, hexosaminidase A was almost three times more active than hexosaminidase B in cleaving the *N*-acetylglucosaminyl residues. Following 18 h of incubation at pH 4.5, hexosaminidase A together with β-glucuronidase led to an almost 70% degradation of HA, in contrast to only 25% by hexosaminidase B.

Figure 3a describes the kinetics of HA hydrolysis by the hexosaminidase isozymes in the presence of β-glucuronidase. The reaction was almost linear for at least 18 h, with some increase in the rate of hydrolysis between 12 and 24 h. In the absence of β-glucuronidase, however (Fig. 3b), the maximal value of hydrolysis (about 0.4%) was obtained in 4–5 h of incubation.

Figure 4 shows a linear relationship between the rate of hydrolysis and the enzyme concentration. Titrations of β-glucuronidase activity indicate that the cleavage of glucuronic acid is not the rate-limiting step in the sequential degradation of HA; thus 0.2–0.5 units of β-glucuronidase resulted in identical kinetics, as shown in Fig. 4a.

Since a maximum of 0.2% of the *N*-acetylglucosamine residues is expected at the nonreducing end of the HA molecule (see Discussion), incubation with 1 unit of hexosaminidase alone brings about complete cleavage of all the terminal *N*-acetylglucosaminyl residues, thus reaching a plateau (Fig. 4b). On the other hand, β-glucuronidase causes the exposure of additional glucosamine residues, allowing the reaction to continue linearily, with a reaction rate proportional to hexosaminidase concentrations up to at least 5 units (Fig. 4a).

That hexosaminidase B is only 30% as active as hexosaminidase A may result from either intrinsic differences between the isozymes or inhibition of hexosaminidase B activity by the substrate. The latter possibility was ruled out since neither hexosaminidase A nor hexosaminidase B activity on the synthetic substrate was inhibited in

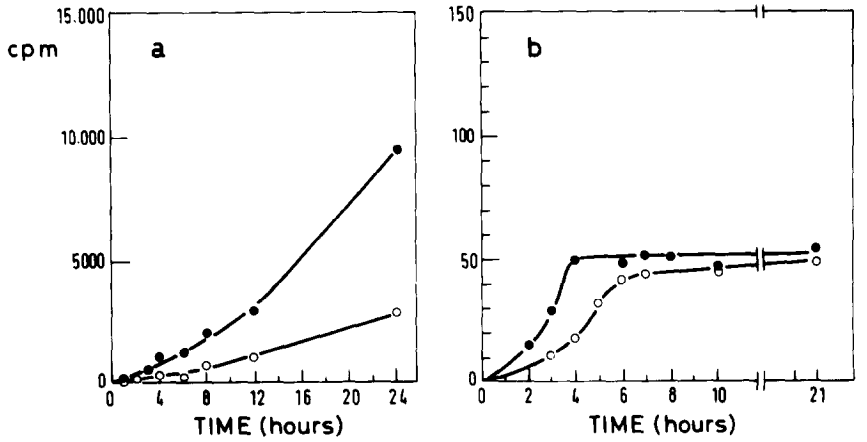

FIG. 3. Kinetics of hexosaminidase activity toward HA in the presence (a) and absence (b) of 0.5 unit of β-glucuronidase. Three units of enzyme activity were used in each experiment. Incubation was carried out at pH 4.5 for hexosaminidase A and at pH 4.0 for hexosaminidase B. (●——●) Hexosaminidase A; (○——○) hexosaminidase B.

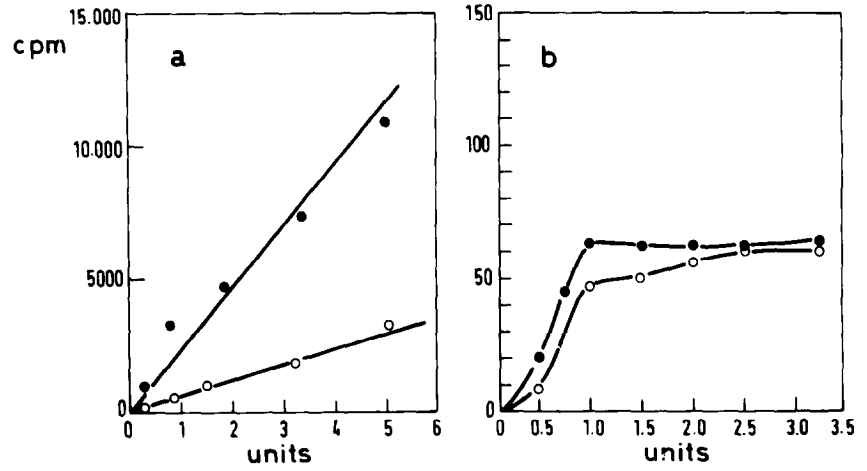

FIG. 4. Hexosaminidase activity as a function of enzyme concentration. Incubations were carried out for 18 h, at pH 4.5 for hexosaminidase A and at pH 4.0 for hexosaminidase B, in the presence (a) and absence (b) of 0.5 unit of β-glucuronidase. (●——●) Hexosaminidase A; (○——○) hexosaminidase B.

the presence of 15,000 cpm of [^{3}H]HA, which is the same concentration used in the incubation mixture.

DISCUSSION

The data presented above clearly indicate that human hexosaminidases A and B are both capable of hydrolyzing HA, although at different rates. This contradicts previous reports (15, 17) performed with small substrate fragments (tri- and heptasaccharides), using nonpurified enzymes from nonhuman sources, which suggest diminished, if any, activity of hexosaminidase B. Indirect evidence for the *in vivo* degradation of MPS by hexosaminidase B was previously suggested by Cantz and Kresse (7). Hexosaminidase B was about 20% as efficient in degrading accumulated MPS in cultured SJD fibroblasts as hexosaminidase A. This fact is further reinforced by the finding that hexosaminidase A-deficient TSD patients do not accumulate MPS in their cells (7, 15), unlike patients with SJD who lack both isozymes. The reduced hexosaminidase B activity is probably not due

to substrate inhibition, since HA was not inhibitory on the synthetic substrates. The linear rate of hydrolysis for 18 h (Fig. 4) rules out inhibition by the products and suggests that the differences in the activities of the two isozymes stem from intrinsic differences in their specificity toward HA. In fact, the time study indicates a slight increase in the rate of hydrolysis with time, which was particularly noted between 12 and 24 h. This might suggest that the enzymes become somewhat more active following partial degradation of the polymer. It should be pointed out here that highly purified preparations of hexosaminidase A and hexosaminidase B demonstrated identical specific activities on the synthetic substrates (31).

Since the purpose of this study was to compare the rates of hydrolysis by the two isozymes, no attempts were made to determine the total breakdown of HA by the enzymes using further additions of enzymes or incubations for longer periods, although most of the HA (about 70%) could be degraded by hexosaminidase A together with β-glucuronidase in our *in vitro* system.

According to its calculated molecular weight ($2\text{-}2.5 \times 10^5$), one HA polysaccharide chain contains approximately 500 *N*-acetylglucosaminyl residues, one of which (about 0.2%) is in the terminal position of the nonreducing end of the chain. The maximum radioactivity released under optimal conditions by either hexosaminidase isozyme (90–100 net cpm of a total 15,000) is somehow higher than the expected values (0.2%), but still indicates that the terminal sugar of the HA is *N*-acetylglucosamine which is hydrolyzable by hexosaminidase. Additional radioactivity was not released by increasing either the incubation time or the hexosaminidase concentration, unless β-glucuronidase was added. In that case, however, the combined activity of both enzymes led to the further degradation of most of the HA polysaccharide chain. The possibility that our preparations contained even small amounts of hyaluronidase activity was ruled out by the facts that degradation was not achieved by either enzyme alone and that the only product of the reaction was identified as *N*-acetylglucosamine. Interestingly, the pH optimum for each of the isozymes on HA is somewhat different, unlike the results obtained with synthetic substrates. Similarly, the combined activity of hexosaminidase A or B with glucuronidase was also optimal at different pH values.

The advantage of hyaluronic acid as a substrate over low molecular weight fragments is that its cleavage may better represent the natural degradation process in cells such as skin fibroblasts. Moreover, HA may be the immediate substrate for these enzymes, unlike sulfated MPS (dermatan and chondroitin sulfate), which require desulfatation before cleavage of the individual sugars (30). These results suggest that the lysosomal breakdown of HA in cells normally lacking hyaluronidase may proceed by the sequential activity of both hexosaminidase isozymes and β-glucuronidase on the two sugars in the HA molecule. *In vivo* degradation of HA in cultured human skin fibroblasts was reported recently (24); therefore, a total deficiency of hexosaminidase or β-glucuronidase is anticipated to result in the intracellular accumulation of mucopolysaccharides.

ACKNOWLEDGMENTS

The authors are greatly indebted to Mrs. Irit Kapeler for her skillful technical work. This work was partially supported by Stiftung Volkswagen Grant 34048 and Israeli Ministry of Health Grant 371.

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