A Comparative Morphologic Analysis of Adult Onset Leukodystrophy With Neuroaxonal Spheroids and Pigmented Glia—A Role for Oxidative Damage

Zarina S. Ali, MS, J. Patrick Van Der Voorn, MD, and James M. Powers, MD

INTRODUCTION

Orthochromat leukodystrophy (OLD) is a heterogeneous group of noninflammatory progressive dysmyelinating disorders that are morphologically characterized by diffuse myelin loss and nonmetachromatic, sudanophilic scavenger cells (macrophages). Van Bogaert and Nyssen (1) are credited with the first report of the pigmented type of orthochromat leukodystrophy (POLD). The distinguishing morphologic feature of POLD is the presence of pigmented macrophages and other glia. The sudanophilic lipopigment autofluoresces and stains with diastase-peroxidase-Schiff (D-PAS), Ziehl-Nielsen, Masson-Fontana, and cresyl violet and variably for iron (2), all of which are characteristic of ceroid. Ultrastructurally, the pigment granules are reported to exhibit fingerprint, multilamellar, and granular profiles, also consistent with the lipopigment ceroid (2–9). The most frequent POLD phenotype, adult onset, presents clinically in the third to sixth decade. Initial symptoms include personality and behavioral changes along with abnormalities in orientation, speech, and memory. Spastic paresis, seizures, and dementia may develop in later stages. Neuroimaging findings are not diagnostic. An autosomal dominant inheritance pattern is found in some families (5, 7–9), whereas others may be autosomal recessive or dominant with incomplete penetrance (1, 4). Most, however, appear to be sporadic, and the basic biochemical or genetic defect(s) remains unknown. Recent biochemical analysis of myelin proteins suggests a defect in the myelin-associated glycoprotein (MAG) as a potential pathogenic cause in 1 family with an autosomal dominant adult-onset OLD (10). However, no pigmented cells were present, and only classical lipofuscin granules were seen ultrastructurally in oligodendrocytes.

Among the other types of OLD, Axelsson et al (11) first described an autosomal dominant “‘hereditary diffuse leukoencephalopathy with spheroids’” (HDLS) in a large Swedish family. Morphologically, HDLS displays widespread loss of myelin sheaths and axons, numerous neuroaxonal (axonal) spheroids, autofluorescent and sudanophilic lipid-laden macrophages and glialosis in cerebral white matter and, variably, in descending pyramidal tracts (11, 12). Two members of the family also displayed iron-positive macrophages (11). Clinically, patients present with cognitive, behavioral, and motor symptoms, similar to those seen in patients with POLD. Magnetic resonance imaging scans are nonspecific.
and do not allow for a definitive diagnosis (13). Since Axelsson’s original report, additional cases of autosomal dominant HDLS have been reported (8, 9, 12–17). Further ultrastructural (2, 12, 16) and immunohistochemical (8, 13, 15) characterization of these spheroids has demonstrated the accumulation of phosphorylated neurofilaments, mitochondria, ubiquitin, and amyloid precursor protein (APP). Recently, Marotti et al (8) and Itoh et al (9) described a 2-generation family and siblings, respectively, with overlapping clinical and pathologic features of HDLS-POLD. A review of the HDLS-POLD literature by Marotti et al (8) revealed that several cases of POLD, including the original report by Van Bogaert and Nyssen in retrospect, contain numerous axonal spheroids. This suggested a disease spectrum that includes HDLS and POLD or a common pathogenic link resulting in the same final morphologic pathway.

Oxidative damage may play an important pathogenic role in POLD, because glia and macrophages seem to contain an abundance of ceroid that represents an end product of oxidative damage to various biochemical moieties, including cellular lipids and proteins (reviewed in Reference 18). Although the mechanism of ceroid accumulation in POLD is unknown, Zeman and Siakotos (19) suggested several possibilities for lipopigment accumulation in the neuronal ceroid-lipofuscinoses (NCL): an abnormal turnover of total phospholipids, or abnormal composition of structural lipids, or increased peroxidation of native lipids. Mitochondria normally supply the major source of reactive oxygen species (ROS), which makes the phospholipids in the mitochondrial inner membrane (e.g. cardiolipin) particularly vulnerable to oxidative insult. Biochemical analysis of 1 patient with POLD (2) revealed a striking elevation in brain plasmalogens, which are enriched in myelin sheaths (20). Debate about the antioxidant function of plasmalogens exists, but several studies support a protective role of plasmalogens against ROS-mediated damage owing to the presence of a vinyl ether bond, which is reactive toward ROS (reviewed in References 20 and 21).

Further support for a pathogenic oxidative insult in HDLS-POLD is the presence of iron in macrophages. As a transition metal, iron is capable of reducing hydrogen peroxide into the highly toxic hydroxyl radical via the Fenton reaction (22). There is increasing evidence that iron-induced oxidative mechanisms underlie many neurodegenerative diseases (reviewed in Reference 23), including Alzheimer disease, Parkinson disease, and more recently, hereditary ferritinopathy (24). Mitochondrial respiratory enzymes are particularly vulnerable to iron toxicity (25). An oxidative pathogenic component also has been reported in a few primary diseases of myelin, such as adreno-leukodystrophy (ALD) (26). Brain tissue contains relatively few protective antioxidant enzymes or compounds, and its large amounts of polyunsaturated fatty acids represent specific targets for free radical attack, making the CNS especially sensitive to ROS-mediated damage (27). Oxidative damage, perhaps iron-induced, may represent a significant pathogenic mechanism in POLD, which results in ceroid accumulation, plasmalogen degradation (28), and a compensatory protective upregulation of plasmalogen synthesis in glia.

The clinical and pathologic similarities between HDLS and POLD have previously been described, primarily through a comparison of those cases reported in the literature (8). Most cases have not been extensively analyzed, and none have been directly compared in a single study. Here, we compare 5 cases diagnosed or reported as HDLS (8, 13) and 10 cases diagnosed as POLD, 6 of the latter having a family history of neurologic illness. We find that the current clinical, histologic, histochemical, immunohistochemical, ultrastructural, and fluorescent criteria do not differentiate between HDLS and POLD, because both share the same defining features. This suggests again, and more convincingly, that HDLS and POLD may represent 2 faces of the same disease rather than 2 distinct entities. In addition, we provide evidence supporting the notion that oxidative damage is a pathogenic mechanism in adult-onset leukodystrophy with neuroaxonal spheroids and pigmented glia (i.e. HDLS and POLD).

MATERIALS AND METHODS

Source Material

We obtained limited clinical records and formalin-fixed, paraffin sections or blocks of frontal lobe from 5 HDLS and 10 POLD cases from our colleagues in outside institutions (see Acknowledgments). Brainstem or spinal cord sections were also available for 5 POLD cases. Two positive (i.e. adult ALD cases) (26) and 3 negative “normal” age-matched controls were included in all morphologic staining runs and analyses. After the blinded morphologic analyses were completed, the available clinical histories were reviewed.

Morphologic Protocol

All samples were given a letter code, and the slides were labeled accordingly. Three white matter fields (zones 1, 2, and 3) were chosen at 200× magnification in 1 section per case and control. Zone 1 was defined as an area of intact myelinated fibers, whereas zones 2 and 3 displayed moderate or more severe demyelination, respectively, compared with controls and zone 1 of the same slide. This protocol permitted comparison of normal and affected areas within the same white matter section. Each zone was analyzed in a blinded fashion for all histologic, histochemical, and immunohistochemical stains, as well as for ultrastructural and fluorescent microscopic analyses. The sclerotic burned-out areas were not evaluated. The presence and number of spheroids, macrophages, oligodendrocytes, and astrocytes and the presence and degree of myelin loss, axonal loss, vascular fibrosis, and inflammation were assessed in all zones. These changes were rated individually for each variable by 1 unbiased observer (ZSA) on a scale of increasing severity, from 0 to 3, in intervals of 0.5, compared with controls and zone 1 on the same slide. Oligodendrocytes were usually identified morphologically as small, round, dark blue nuclei with clear perinuclear halos in hematoxylin and eosin (H&E) sections. The lack of lymphocytes in these same areas with the antibodies described below justifies our confidence in oligodendrocyte...
identification. The color and tinctorial properties of macrophages in the H&E slides were also noted. Immunohistochemical labeling of macrophages, astrocytes, and, occasionally, for oligodendrocytes, staining with Prussian blue, D-PAS, Ziehl-Nielsen, Sudan black, and cresyl violet, and immunostaining for ferritin and oxidative stress/damage markers were rated in the same blinded fashion, compared with controls. Immunostaining for MAG, proteolipid protein, and myelin basic protein (MBP) was also performed and analyzed similarly. Finally, after finding that no morphologic differences could be identified between the various samples by 2 observers (ZSA and JMP), the code was broken and group comparisons (i.e. POLD, POLD with neurologic family history, and HDLS) were conducted by the same observers. To characterize the biochemical phenotype of axons displaying spheroids, supplementary immunohistochemical runs for parvalbumin, glutamic acid decarboxylase, choline acetyltransferase (CHAT), somatostatin, and substance P were performed on the 2 cases that displayed the most prominent spheroids in each group. These samples were also evaluated in a blinded fashion as possible. Data are represented as means ± SEM. Statistical significance was determined by 1-way analysis of variance.

**Histologic and Histochemical Staining**

Representative sections of frontal white matter were routinely stained with H&E, cresyl violet, Luxol fast blue-PAS (LFB-PAS) for myelin, Bodian for axons, Perl’s Prussian blue for ferric iron (Fe3+), Sudan black, D-PAS, and prolonged Ziehl-Nielsen acid fast stains. The latter 3 stains label ceroid effectively.

**Immunohistochemical Staining**

All immunohistochemical stainings were done in batches of 20 by hand or, when stipulated, with an automated system. Appropriate positive and negative (omitting the primary antibody) controls were included in each batch. The oxidative positive controls were ALD brain sections (26). Spheroids were initially characterized by using standard techniques (H&E and Bodian) and commercially available antibodies targeting neurofilament protein (monoclonal, 1:1000, #M762; DAKO Autostainer, DAKO, Carpenteria, CA), ubiquitin (monoclonal, 1:15K, #MAB1510; Chemicon, Temecula, CA), and amyloid precursor protein (APP A4; monoclonal, 1:20K with heat retrieval, #MAB348; Chemicon International). Astrogliosis was assessed with a glial fibrillar acidic protein antibody (polyclonal, 1:10K, #Z334; Dako). Occasional slides were immunostained for carboxic anhydrase II (polyclonal, 1:15,000; Rockland, Gilbertsville, PA) to identify oligodendrocytes. Antibodies targeting myelin proteins, including MAG (polyclonal, 1:500, #sc-16598; Santa Cruz Biotechnology, Santa Cruz, CA), proteolipid protein (monoclonal, 1:12K, #MAB388; Chemicon), and MBP (monoclonal, 1:800, #NCL-MBP; Novocastra Laboratories, Inc.; Vector Laboratories, Burlingame, CA) were also used. Markers of inflammation were identified using the DAKO Autostainer with EnVision Plus, AEC Plus, EDTA at pH 8.8, or citrate buffer at pH 6.1 for antigen retrieval and antibodies to CD68 (MO814 citrate, 1:200), CD3 (MO254, 1:50 EDTA), CD8 (M7103, 1:100 EDTA), CD20 (M0755, 1:800 EDTA), tumor necrosis factor-α (monoclonal, 1:150, #sc-7317; Abcam, Cambridge, MA), and interleukin-1α (polyclonal, 1:150, #80-3054-01; Abcam). CD4 immunostaining was performed on the Ventana XT system (Ventana, Tuscon, AZ) with CCI [a tris(hydroxymethyl)aminomethane-based buffer at a slightly basic pH] for antigen retrieval (1:50; Novocastra). Cell death was evaluated by performing terminal deoxynucleotidyl transferase biotin dUTP nick-end labeling (TUNEL) (In Situ Cell Death Detection Kit, POD; Roche Diagnostics, Indianapolis, IN) and activated caspase-3 immunostaining (polyclonal, 1:1000, #AF835; Research and Diagnostic Systems, Inc., Minneapolis, MN).

To determine whether a particular subset of axons were more vulnerable to spheroid formation, antibodies to parvalbumin (monoclonal, 1:3000, #P-3088; Sigma Chemical Company, St. Louis MO), glutamic acid decarboxylase (glutamic acid decarboxylase 65/67 [C-20], polyclonal, 1:100 with retrieval, SC 7513; Santa Cruz Biotechnology), CHAT (polyclonal, 1:2000, #AB143, Chemicon), somatostatin (polycional, A0566, 1:600, AEC plus; DAKO), and substance P (polyclonal, 1:10,000, #20064; Immunostar, Hudson, WI) were also be applied to 2 cases of each group with the most spheroids.

**Ultrastructural Examination**

“Pop-off” sections of frontal white matter from each HDLS-POLD case, as well as positive and negative controls, were subjected to modified processing for electron microscopy (29). The field to be examined ultrastructurally displayed at least 1 macrophage, 1 spheroid, and several glial cells. It was selected from an H&E-stained slide.

**Fluorescence Microscopy**

Unstained and deparaffinized sections were used to detect autofluorescent pigment with a Nikon fluorescent microscope, coupled to an RT slider SPOT camera (SPOT Diagnostic Instruments, Inc., Sterling Heights, MI). A band pass filter exciting at 465 to 495 nm and emitting at 515 to 555 nm was used to assess green autofluorescence. Excitation was also performed with a 340- to 380-nm-dichroic interference filter (400 nm), and blue autofluorescent images were acquired through a 435- to 485-nm barrier filter. For
Presentation, images were processed with SPOT RT software version 3.5.

RESULTS

Source Material
We examined 4 groups of subjects: negative (n = 3) and positive ALD (n = 2) controls, “sporadic” POLD (n = 4), POLD with a neurologic family history (n = 6), and HDLS (n = 5). Of the 6 POLD cases with a neurologic family history: 1 male had a living maternal uncle with a similar clinical phenotype diagnosed as a “leukodystrophy,” and a maternal aunt who died at an early age in a nursing home (familial POLD); 1 male had a paternal grandmother who died at 72 of “Alzheimer’s disease,” a paternal cousin and uncle with “bipolar disorder,” a paternal cousin who had “multiple sclerosis,” and a maternal grandmother who died of a “stroke”; 1 male’s mother died in her 60’s of “multiple sclerosis”; 1 female’s mother died at 62 of “Alzheimer disease”; 1 male’s mother and maternal aunt developed dementia (clinically suspected to be Alzheimer disease) in their 70’s; and 1 female’s maternal grandmother died at 63 of a “strange brain disease.” All, except the last 3, have a probable to definite family history of a CNS white matter disorder; the first almost certainly has the same disease (familial POLD). It is noteworthy that the last patient with POLD with a family history had the clinical diagnosis in our institution of a frontotemporal dementia, probably Pick disease, as did 2 of the patients with autosomal dominant HDLS. Another patient with sporadic POLD had the premorbid diagnosis of “progressive terminal multiple sclerosis.” All of the patients with HDLS had an autosomal dominant adult-onset leukodystrophy with abundant spheroids in cerebral white matter at biopsy or autopsy; 4 have been previously reported (case 3 in Reference 8; patient 3 in Reference 13; and 2 in a poster at the International Congress of Neuropathology, San Francisco, CA, September 2006, by N. Cairns et al). The fifth patient with HDLS was a female who died at 39 years with her onset of disease at 22 years. The mean age at onset (in years) of symptoms is earlier for our HDLS cases (36.2 [range 22–44]) compared with that for our sporadic POLD cases (48.3 [range 41–65]) or POLD with a neurologic family history (43.2 [range 23–59]) (p > 0.05). Mean duration of disease is 9.0 ± 1.9 and 9.6 ± 5.6 years for our patients with HDLS and sporadic POLD, respectively, but 5.0 ± 4.9 years for those with POLD with a neurologic history (p > 0.05). Age at death in our study is later for sporadic POLD: sporadic POLD (57.3 [range 46–74]), POLD with a neurologic family history (48.2 [range 32–61]), HDLS (45.8 [range 39–51]), and controls (38.6 [range 25–61]) (p > 0.05). Patients in all disease groups presented with various mental and motor symptoms, including behavioral and personality changes, depression, speech difficulties, confusion, cognitive impairment, memory problems, rigidity, and epilepsy. As their disease progressed, irritability, anxiety, hypersexuality, acalculia, gait problems, spasticity, chorea, difficulty swallowing, tremor, grip reflexes, oral hyperkinesia, dystonia, cogwheeling, visual problems, incontinence, and sensory disturbances were reported in no particular sequence and with variation between groups.

Histologic and Histochemical Staining
H&E-stained frontal sections revealed small, but variable, numbers of amphophilic to gray to brown pigmented macrophages and axonal spheroids in all cases (Fig. 1A–C). Macrophages were commonly observed in a
random distribution in frontal white matter but were also noted perivascularly or along the periphery of the U-fibers. The number of pigmented macrophages was associated with the severity of lesion (e.g., zone 1, mean rating 1.4 ± 0.2; zone 2, mean rating 2.1 ± 0.2; and zone 3, mean rating 2.2 ± 0.2; p < 0.05 for zone 1 vs zone 3) in all disease groups. H&E staining also revealed increased oligodendrocytic loss with severity of demyelination, in addition to loss in normal areas in some cases (zone 1, mean rating 0.6 ± 0.02; zone 2, mean rating 1.4 ± 0.3; and zone 3, mean rating 2.3 ± 0.2; p < 0.05). Rare pigmented macrophages and axonal spheroids were also present occasionally in normal areas. Astrogliosis (HDLS, mean rating 1.1 ± 0.3; POLD, mean rating 1.1 ± 0.3; POLD with family history, mean rating 1.3 ± 0.3; and ALD, mean rating 2.0 ± 0.7; p > 0.05) and macrophage infiltration (HDLS, mean rating 1.7 ± 0.3; POLD, mean rating 2.0 ± 0.2; POLD with family history, mean rating 2.0 ± 0.2; and ALD, mean rating 2.8 ± 0.1; p > 0.05) were moderate, compared with ALD cases. Vascular fibrosis was present in more than half of the cases (8 of 16), whereas mild to moderate lymphocytic vascular cuffing was observed in only a few (HDLS, mean rating 0.8 ± 0.2; zone 2, mean rating 1.4 ± 0.3; and zone 3, mean rating 2.3 ± 0.2; p < 0.05). Rare pigmented macrophages and axonal spheroids were also present occasionally in normal areas.

Immunohistochemical Staining

Spheroids were intensely and diffusely immunoreactive for APP (zone 1, mean number 13.9 ± 5.1; zone 2, mean number 22 ± 5.2; and zone 3, mean number 24 ± 4.2; p > 0.05) (Fig. 5A, B) and neurofilament protein (zone 1, mean number 18.5 ± 5.1; zone 2, mean number 18.8 ± 4.4; and
zone 3, mean number 20.8 ± 3.1; p > 0.05) and less consistently for ubiquitin (zone 1, mean rating 0.7 ± 0.2; zone 2, mean rating 1 ± 0.3; and zone 3, mean rating 1.2 ± 0.3; p > 0.05). Spheroids were present in the deep cortical

FIGURE 4. Sporadic pigmentary type of orthochromatic leukodystrophy, 46-year-old female. Recently phagocytosed myelin debris (blue) in an otherwise red macrophage (arrow) within an area of decreased myelinated fibers; zone 2. Luxol fast blue-periodic acid-Schiff, 300×.

FIGURE 3. Iron-filled macrophages (arrows). Perl’s stain; original magnification: 200×. (A) Sporadic pigmentary type of orthochromatic leukodystrophy, same 74-year-old male; zone 2. (B) Hereditary diffuse leukoencephalopathy with spheroids, same 48-year-old female as in Figure 1; zone 3.

FIGURE 5. Axonal swellings. (A) Pigmentary type of orthochromatic leukodystrophy (POLD) with neurologic family history, 49-year-old female; zone 2. Anti-amyloid precursor protein (APP) immunostaining; original magnification: ×100. (B) Sporadic POLD, same 74-year-old male as illustrated earlier; zone 3. APP, original magnification: 100×. (C) Hereditary diffuse leukoencephalopathy with spheroids, 48-year-old female, same patient as in Figures 1 and 3. Immunoreactive spheroids (arrows), anti-choline acetyltransferase; original magnification: 100×.

gray matter or normal U-fibers in over one half of the cases (8 of 15), irrespective of disease group. There were no detectable differences in the size, texture or staining characteristics of the spheroids in HDLS and POLD. There was, however, a detectable difference in their number. For example, with APP immunostaining, the average number of
spheroids in zones 2 and 3 for HDLS was 23.7 (range 4–56), for POLD with a family history it was 29 (range 4–74), and for sporadic POLD it was 14.3 (range 5–25) (p > 0.05). Glial fibrillary acidic protein-immunoreactive astrogliosis was seen in all cases, predominantly along the U-fiber periphery, and sometimes even in normal (zone 1) or mildly affected areas (zone 1, mean rating 0.8 ± 0.2; zone 2, mean rating 1.1 ± 0.3; and zone 3, mean rating 1.6 ± 0.3; p > 0.05), but much less than in the ALD controls (HDLS, mean rating 1.1 ± 0.3; POLD, mean rating 1.1 ± 0.3; POLD with family history, mean rating 1.3 ± 0.3; and ALD, mean rating 2.0 ± 0.7; p < 0.05). The degree of gliosis was associated with myelin and oligodendrocytic loss in the same zone, but not with vascular fibrosis. Antibodies targeting myelin proteins (i.e. MAG, proteolipid protein, and MBP) failed to reveal any selective losses. The small and infrequent lymphocytic cuffs were
with iron in macrophages (0+); astrocytes and macrophages: astrocytes (HO-1: zone 2, mean rating 2.3 ± 0.2; zone 3, mean rating 2.3 ± 0.2; p > 0.05). Occasionally, pigmented cells with typical morphologic features of macrophages did not stain with anti-CDD68. In these few cases, CA II staining was performed to exclude the possibility that these cells were actually oligodendrocytes. Cell death evaluated by TUNEL revealed only a modest labeling of oligodendrocytes and astrocytes. A moderate number of macrophages and astrocytes immunostained for activated caspase-3, particularly evident in the cytoplasm of macrophages, whereas astrocytes and a few oligodendrocytes displayed nuclear and cytoplasmic staining.

Spheroids in each group demonstrated immunoreactivity for parvalbumin and CHAT (Fig. 5C) and less so for somatostatin. Only 1 HDLS case and 1 POLD case displayed immunoreactivity for activated caspase-3, particularly evident in the cytoplasm of macrophages, whereas astrocytes and a few oligodendrocytes displayed nuclear and cytoplasmic staining.

In most cases, compared with normal controls and zone 1 of the same slide, there was increased immunoreactivity for HO-1 (Fig. 6A–C) and SOD2 (Fig. 6D, E) (markers of oxidative stress) and for HNE, MAL, and NT (Fig. 6F–I) (markers of oxidative damage), primarily in astrocytes and macrophages: astrocytes (HO-1: zone 2, mean rating 1.2 ± 0.3; and zone 3, mean rating 1.2 ± 0.3; SOD2: zone 2, mean rating 2.0 ± 0.2 and zone 3, mean rating 1.8 ± 0.3; HNE: zone 2, mean rating 0.6 ± 0.2 and zone 3, mean rating 0.9 ± 0.3); macrophages (HO-1: zone 2, mean rating 1.0 ± 0.3 and zone 3, mean rating 1.2 ± 0.2; SOD2: zone 2, mean rating 1.2 ± 0.3 and zone 3, mean rating 1.1 ± 0.3; HNE: zone 2, mean rating 0.3 ± 0.1 and zone 3, mean rating 0.5 ± 0.1) of zones 2 and 3; weaker and less frequent immunoreactivity for HO-1 and SOD2 was also seen in oligodendrocytes and spheroids. Immunoreactivity for MAL and NT (Fig. 6F, H, I) was not quantitatively analyzed but was much less impressive and more restricted to macrophages. Ferritin immunoreactivity was mainly increased in macrophages (zone 1, mean rating 0.7 ± 0.3; zone 2, mean rating 1.3 ± 0.3; and zone 3, mean rating 1.6 ± 0.3; p > 0.05), probably reflecting their increased cell size and numbers, but not in oligodendrocytes. These were observed in all disease groups and were similar but less intense to those seen in the ALD cases (HDLS, mean rating 1.6 ± 0.4; POLD, mean rating 0.9 ± 0.4; POLD with family history, mean rating 0.6 ± 0.2; and ALD, mean rating 1.8 ± 0.4; p > 0.05). Staining intensities of oxidative markers variably correlated with one another and, sometimes, directly with iron (Fig. 3A, B) and ferritin staining.

**Ultrastructural Examination**

The preservation of the samples was poor, which hampered cell identification. However, the natural insolvability of the lipopigments, ceroid and lipofuscin, allowed us to compare and contrast their appearance between groups. Pleomorphic cytoplasmic inclusions were present in macrophages > astrocytes > oligodendrocytes in all abnormal groups, which differs from classical lipofuscin (30) but is similar to that reported in cases of POLD (3–7, 31) and NCL (19). Some of these materials consisted of small, irregular clumps of electron-dense granular material (Fig. 7A), whereas others consisted of large masses of variable electron density and granularity (Fig. 7B, C). One large inclusion had a rather characteristic lobulated to bosselated appearance (Fig. 7C, D). The 61-year-old control had the latter material in 1 cell of undetermined origin. Within these inclusions one could occasionally discern a lamellar substructure, thin clear clefts, or rarely a cholesterol crystal (Fig. 7A), but not fingerprint patterns. Various stages of myelin breakdown, including fingerprint profiles, were identified in macrophages and some astrocytes. Spheroids contained numerous neurofilaments and a few mitochondria.

**Fluorescence Microscopy**

Pigment in neurons, macrophages, and other glia displayed strong green autofluorescence with a band pass filter exciting at 465 to 495 nm and emitting at 515 to 555 nm.

**FIGURE 6.** Oxidative stress (A–E) and damage (F–I). (A) Hereditary diffuse leukoencephalopathy with spheroids (HDLS), 51-year-old female with diffuse iron in macrophages (0–1+), astrocytes (1+), and oligodendrocytes (2–3+); immunoreactivity predominantly in astrocytes (arrows); zone 3. Anti-hemoglobin-1 (HO-1): original magnification: 200×. (B) Sporadic pigmentary type of orthochromatic leukodystrophy (POLD), 53-year-old female without iron; immunoreactivity predominantly in astrocytes (arrows); zone 3. Anti-HO-1: original magnification: 200×. (C) Sporadic POLD, 46-year-old female with iron in macrophages (2+) and astrocyte/oligodendrocytes (0–1+); immunoreactivity predominantly in astrocytes (arrow); zone 2. Anti-HO-1: original magnification: 200×. (D) HDLS, 48-year-old female, same patient as in Figures 1 and 3, with iron in macrophages (2+); immunoreactivity predominantly in astrocytes (arrows); zone 3. Anti-manganese-superoxide dismutase (SOD2): original magnification: ×100. (E) Familial POLD, 50-year-old male without iron; immunoreactivity in large macrophage (m) and astrocytes (arrows); zone 3. Anti-SOD2: original magnification: 200×. (F) HDLS, 48-year-old female, same patient as in Figures 1, 3, and 6D, with iron in macrophages (2–3+) and astrocytes (0–1+); immunoreactivity predominantly in macrophages (arrows); zone 2. Anti-malondialdehyde (MAL): original magnification: ×200. (G) POLD, 53-year-old female with neurologic family history and iron in macrophages (2+), astrocytes (2+), and oligodendrocytes (0–1+); immunoreactivity predominantly in astrocytes (arrows); zone 3. Anti-4-hydroxynonenal: original magnification: 200×. (H) HDLS, 51-year-old female, same patient as in Figure 6A, with iron in macrophages (0–1+), astrocytes (1+), and oligodendrocytes (2–3+); immunoreactivity in macrophages (arrows) and astrocytes; zone 3. Anti-nitrotyrosine: original magnification: 200×. (I) Sporadic POLD, 46-year-old female, same patient as in Figures 4 and 6C, with iron in macrophages (2+) and astrocyte/oligodendrocytes (0–1+); immunoreactivity predominantly in macrophages (arrows); zone 2. Anti-NF: original magnification: 200×.
However, when excitation was performed with a 340- to 380-nm dichroic filter and images were acquired through a 435- to 485-nm barrier filter, strong blue autofluorescence was present only in macrophages and weak autofluorescence was present in the other glia and neurons (Fig. 8B), suggesting that this filter allows for more specific identification of ceroid.

**DISCUSSION**

Extensive morphologic analyses of 5 HDLS cases (diagnosed primarily on the basis of an autosomal dominant, adult-onset leukodystrophy with spheroids in biopsy or autopsy samples) and 10 POLD cases (diagnosed neuro-pathologically primarily on the basis of a leukodystrophy with pigmented glia, 6 of the latter having a family history of neurologic illness), failed to reveal any consistent clinical or morphologic differences between these “diseases,” confirming previous reports that they may represent variations within a disease spectrum (8, 9). We found that iron and ceroid accumulation is as characteristic of the white matter lesion in HDLS as it has been for POLD. We also present the first direct demonstration, to our knowledge, of oxidative damage in either HDLS or POLD.

HDLS is defined as an autosomal dominant, adult-onset white matter disorder, but a similar inheritance pattern is seen in some cases of adult-onset POLD (5, 7–9). In other families with POLD, individual cases are reported or siblings are affected, whereas parents do not display neurologic dysfunction (1, 4), suggesting the possibility of autosomal recessive transmission or that incomplete penetrance may accompany autosomal dominance. In our 10 cases of POLD, we identified 6 individuals who had a family history of neurologic illnesses, including Alzheimer disease, an adult-onset leukodystrophy, multiple sclerosis, and Alzheimer disease-multiple sclerosis, all of which have been reported to mimic clinically cases of a leukoencephalopathy (16, 17) or leukodystrophy. Indeed, 1 of the POLD cases in this study was clinically diagnosed in our medical center as a frontotemporal dementia, perhaps Pick disease. Given the absence of autopsy reports for the other family members, we cannot exclude the possibility that these relatives were affected with the same disease, thereby supporting an autosomal dominant family history in some of our POLD cases. It is not uncommon for single cases of rare genetic diseases to be reported as sporadic and only later does a heritable defect become apparent (e.g. adrenoleukodystrophy; J.M. Powers, unpublished observation, 1973).

In addition to the similar inheritance pattern, the predominant clinical features of HDLS and POLD also overlap (reviewed in Reference 8). Both diseases are
characterized by early behavioral and psychiatric changes, as well as neurologic signs. Although our cohorts suggest that the onset of symptoms in HDLS may be earlier than in POLD, there is again a significant overlap. Reported cases of HDLS have a later mean age of onset than ours (36.2 years), probably because 1 of 5 cases had an onset at 22 years, but approximately the same onset (44 years) as our POLD cases (reviewed in References 8 and 17). The mean age of onset for the family reported by Axelsson et al (11), however, also was 36 years. The rate of progression of both diseases is highly variable. Our HDLS and sporadic POLD subgroups had slightly longer disease duration than our POLD patients with a neurologic history (9 years vs 5 years), but the same duration (approximately 9 years) reported for cases of HDLS (reviewed in References 8 and 17). Women accounted for 60% to 75% of our cases of HDLS and sporadic POLD, whereas a male predominance (4:2) was seen in our POLD cases with a positive family history. In the literature HDLS has a slight female predominance (55%) (reviewed in Reference 17), as does familial POLD (66%) (32). Definite conclusions about the role of gender in HDLS-POLD are limited by the relatively few number of cases reported.

Given the similar clinical and nonspecific neuroimaging features of HDLS and POLD (8, 13, 32), diagnosis at present relies on histopathologic findings. Our detailed morphologic analyses revealed no consistent or obvious differences between HDLS and POLD cases. Both diseases display prominent cerebral white matter demyelination, moderate astrogliosis, moderate macrophage infiltration, and loss of oligodendrocytes, with relative sparing of axons and U-fibers. Reactive astrogliosis is most prominent in mild or moderately affected areas in some cases. Loss of oligodendrocytes in normal areas suggests that oligodendrocytes may be the initial target of this disease, with subsequent myelin and axonal degeneration and compensatory astrocytic reactivity. A previous report implicated abnormal MAG and decreased MBP in a familial case of OLD (10), which was not apparent in our cases. A sparse presence of T cells, inflammatory cytokines, and vascular fibrosis is noted in some cases, but they are not consistently associated with each other or any group.

Numerous sudanophilic, autofluorescent macrophages and some glia contain lipopigment. In our study, macrophages and glial lipopigment stain most intensely with D-PAS and a prolonged Ziehl-Nielsen, modestly with cresyl violet and Sudan black, but only variably with Perl’s Prussian blue for iron. Ultrastructural examination of macrophages reveals various stages of myelin breakdown (33) as well as lipopigment bodies. Macrophages are most numerous in actively demyelinating areas (zones 2 and 3), suggesting that their presence is associated with the severity or duration of the disease.

Spheroids are apparent in the affected cerebral white matter, as well as in the relatively spared subcortical white matter and more infrequently in deep cortex in all groups. There was no obvious difference between the mean number of spheroids in zones 2 and 3 in cases of HDLS versus POLD with a neurologic family history (23.7 vs 29, respectively), further supporting the notion that the histopathologic features of these diseases are the same. Those with sporadic POLD, however, had fewer spheroids (14.3), but those patients also were the oldest at death. A statistically significant difference between the average numbers of spheroids between groups is lacking, but this may be due to the relatively few number of samples studied. The observed trend of patients with sporadic POLD having fewer spheroids than patients with HDLS or POLD with a neurologic history may still be relevant. The number and distribution of spheroids may relate to different stages of the disease, as previously suggested (8). For example, Case 2 of Marotti et al (8) displayed many more spheroids in the biopsy (36 years) than at autopsy (39 years). This observation, coupled with their demonstration in deep cortex and the relatively spared arcuate fibers in other reports (17) has suggested a primary axonal lesion in HDLS (8, 12, 13, 17).

Further evidence for this postulate might be the bilateral and symmetrical pyramidal tract degeneration in cases reported as HDLS (12–14, 16, 17), and particularly for the presence of spheroids within the pyramidal tracts of the medulla and spinal cord (9, 11, 12, 17). This postulated pathomechanism may be true; but, if so, these same findings also have been
described in cases reported as POLD, including van Bogaert’s and Nyssen’s original report (1, 3, 5, 6). The primary pathogenic lesion in POLD, however, is assumed to reside in a myelin or oligodendrocytic abnormality, a theory that recently has gained some support (10). Our study may shed some light on these issues. The decreased number of oligodendrocytes in the normal white matter (zone 1), the relative axonal sparing, and the absence of spheroids at the “growing” edges of the myelin lesions opposite the burned-out gliotic areas (zones 2 and 3) support a primary lesion of oligodendrocytes. The reactive rather than dystrophic fine structure of the spheroids (34) seen in this study and also consistently reported by others in cases of POLD or HDLS (2, 8, 9, 11–13, 15, 17) suggests a secondary axonal lesion, perhaps due to upstream axonal destruction in the cerebral white matter (e.g. the internal capsule), and provides yet another morphologic link between HDLS and POLD. In addition to APP, neurofilament protein, and ubiquitin immunoreactivity, spheroids often stained for parvalbumin and CHAT. Axons that arise from interneurons coexpressing parvalbumin and CHAT in the neostriatum or cortex may be more vulnerable to axonal injury in HDLS-POLD.

The lipopigment observed in our cases of both POLD and HDLS is ultrastructurally distinct from normal lipofuscin (30) that usually accumulates in aging postmitotic cells, such as neurons (18). More importantly, it is identical to some of the deposits illustrated in cases of POLD (Fig. 7C, D of Reference 2, Fig. 2E of Reference 4, and Fig. 5A of Reference 31) and NCL (Figs. 6 and 7 of Reference 35, Fig. 9 of Reference 36, Fig. 4 of Reference 37, Fig. 3C of Reference 38, and Figs. 39, 46, and 47 of Reference 39). Therefore, from its ultrastructural features, this lipopigment is most consistent with ceroid. Other authors have reported fingerprint patterns in the lipopigment inclusions of POLD (2, 3), which are characteristic of juvenile NCL (Batten’s disease), but are not typically seen in lipofuscin (30, 39, 40). The inability to identify any fingerprint patterns in our samples might be due to postmortem autolysis and the harsh conditions of the pop-off technique (29). The staining and autofluorescent characteristics of this material, particularly those in macrophages, is also distinct from neuronal lipofuscin in the adjacent cortex and fulfills some of the histochemical criteria for ceroid: intense staining with acid-fast and PAS stains (41, 42). It is presently impossible to prove definitively that a lipopigment is either ceroid or lipofuscin (40), but all of the characteristics listed above provide compelling evidence that it is ceroid, as previously suggested (2). Although the pathogenic role of oxidative damage in the formation of lipofuscin remains controversial, there is abundant evidence that ceroid is an end product of oxidative damage (reviewed in References 18 and 42). The oxidative origin of the ceroid in aged murine adrenal cells was supported by the presence of HNE and MAL, toxic aldehydes formed by oxidative damage to polyunsaturated fatty acids, in ceroid-containing cells (41). Likewise, many of the ceroid-containing macrophages in HDLS-POLD demonstrate immunoreactivity for HNE and MAL, particularly MAL.

It has been known for decades that free radical reactions cause damage and polymerization of lipids and proteins to form autofluorescent age pigment-like residues (43, reviewed in References 18 and 42). Recently, a role for free radical damage has been implicated in several neurologic diseases, including primary diseases of myelin, such as ALD (26) and multiple sclerosis (reviewed in Reference 44). We now provide morphologic evidence for an oxidative insult in the pathogenesis of HDLS-POLD. The oxidative damage in ALD was restricted to affected white matter, and its intensity correlated directly with the degree of inflammation (26). In HDLS-POLD the distribution is the same, but the morphologic intensity of oxidative damage is much less than that in ALD, which correlates with the lack of inflammation and relative chronicity of the leukodystrophy in HDLS-POLD. Immunoactivity for markers of both oxidative stress (HO-1 and SOD2) and damage in both HDLS and POLD is found primarily in astrocytes (particularly HNE) and macrophages (particularly MAL), but oligodendrocytes are also variably affected. The presence of iron in some of these cases suggests that ROS formation and oxidative injury may be the result of iron-induced oxidative mechanisms via the Fenton and Haber-Weiss reactions (22, 23). Brain tissue is deficient in protective antioxidant mechanisms and rich in polyunsaturated fatty acids, so it is especially sensitive to ROS-mediated damage (27), perhaps ultimately manifesting as accumulations of ceroid lipopigment in HDLS-POLD.

The predominant mode of oligodendroglial cell death in HDLS-POLD remains undetermined. The traditional apoptotic marker TUNEL and caspase-3 activation are infrequently identified in any disease group. In ALD, activated caspase-3 immunoreactivity also was infrequently noted in oligodendrocytes; a cytolytic pathomechanism mediated by the CD8-granule exocytosis pathway and HNE/peroxynitrite (26) was considered most likely. A definitive marker for cytosis in such samples, however, is currently unavailable. On the other hand, in a chronic disease such as HDLS-POLD and in contrast to a fulminant disease such as ALD, even a few apoptotic signs can be significant. Peroxynitrite, which cannot be assayed directly in this type of system, can be implicitly demonstrated through a surrogate marker: the abnormal nitration of proteins (45). However, given the relatively reduced immunoreactivity for NT, compared with those of HNE or MAL, peroxynitrite would appear to be not as likely a candidate for the oxidative damage as HNE or MAL. On the other hand, these variations may merely reflect a difference in the sensitivity of the immunoreagents or may be due to the selective upregulation of these oxidative and antioxidant proteins, depending on the molecular pathway involved. In other leukodystrophies, the identification of oxidative stress/damage in macrophages and glia may relate to the production of a wide variety of cytokines by these cells, as seen in ALD (26). Both may contribute to the severity and chronicity of the demyelination that ensues. However, more important is the fact that all of our markers provide evidence for oxidative damage.

In summary, our extensive morphologic analyses indicate that HDLS and POLD share similar clinical features, and no consistent morphologic differences are noted between these 2 diseases. As a result of these studies,
we recommend that 1 inclusive neuropathologic diagnosis be used for both diseases, at least for the present: adult onset leukodystrophy with neuroaxonal spheroids and pigmented glia (8). However, the basic molecular or genetic cause(s) of these diseases must be identified to conclusively determine whether they truly represent a single disease entity or a final common morphologic pathway for 2 or more diseases. Oxidative damage has been shown previously to be causative in ceroid pigment accumulation (reviewed in References 18 and 42), which is a morphologic hallmark of both HDLS and POLD. Our direct demonstration of oxidative damage markers in both diseases confirms these data. Oxidative damage may be either a primary or secondary phenomenon. From a therapeutic standpoint, this question is somewhat irrelevant: oxidative damage, whether primary or secondary, needs to be minimized or neutralized. Antioxidant therapy was recommended for ALD (26), and N-acetyl-l-cysteine was chosen as the agent, which has demonstrated some success (46). Such therapeutic successes, even though limited, suggest that antioxidant therapy might also be effective in HDLS-POLD, for which causes are currently unknown and no rational therapeutic options are available.

ACKNOWLEDGMENTS

The authors thank the following individuals for generously providing HDLS-POLD brain tissue: Mark W. Becher and Ty W. Abel, Vanderbilt University Medical Center, Nashville, TN; Melvyn J. Ball, Oregon Health and Sciences University, Portland, OR; Eileen H. Biggio and Bruce C. Quinn, Northwestern University Medical School, Evanston, IL; Nigel Cairns, Washington University School of Medicine, St. Louis, MO; Barbara J. Crain, The Johns Hopkins Medical Institutions, Baltimore, MD; Dikran S. Horoupian, Stanford University, Palo Alto, CA; Richard W. Leech and Kar-Ming Fung, University of Oklahoma, Norman, OK; Carol K. Petito, University of Miami School of Medicine, Miami, FL; and The Brain and Tissue Bank of the National Institute of Child Health and Development (N01-HD-4-3368, 3383); C. Harker Rhodes, Dartmouth-Hitchcock Medical Center, Lebanon, NH; Gary Ross, Geisinger Medical Center, Danville, PA; Ira Shoulson, Strong Memorial Hospital, Rochester, NY; and Marjo S. van der Knaap and Wouter Kamphorst, Free University Hospital and Brain Bank, Amsterdam, the Netherlands. The authors also acknowledge the ultrastructural knowledge of Cedric Raine, PhD, DSc; the technical expertise of Karen Bentley, Karen Vanderbint, Frances Vito and Patricia Bourne; and the usual outstanding secretarial assistance of Tina Blazey.

REFERENCES

1. van Bogaert L, Nyssen R. Le type tardif de la leucodystrophie progressive familiale. Rev Neurol (Paris) 1936;65:21–45
29. di Sant’Agnese PA, Mesy-Jensen D. Diagnostic electron microscopy on reembedded (“popped off”) areas of large Spurr epoxy sections. Ultrastruct Pathol 1984;6:247–53
34. Lampert PW. A comparative electron microscopic study of reactive, degenerating, regenerating, and dystrophic axons. J Neuropathol Exp Neurol 1967;26:345–68