Hereditary Pick’s disease with the G272V tau mutation shows predominant three-repeat tau pathology

I. F. Bronner,1 B. C. ter Meulen,2 A. Azmani,2 L. A. Severijnen,3 R. Willemsen,3 W. Kamphorst,4 R. Ravid,5 P. Heutink1 and J. C. van Swieten2

1Department of Human Genetics, Section Medical Genomics and Center for Neurogenomics and Cognitive Research, VU University Medical Center and VU University, Amsterdam, 2Department of Neurology and 3Department of Clinical Genetics, Erasmus Medical Center, Rotterdam, 4Department of Pathology, VU University Medical Center, Amsterdam and 5Netherlands Brain Bank, Amsterdam, The Netherlands

Correspondence to: Dr John C. van Swieten, Department of Neurology, Erasmus Medical Center, Dr Molewaterplein 40, 3015 GD, Rotterdam, The Netherlands
E-mail: j.c.vanswieten@erasmusmc.nl

Frontotemporal dementia and parkinsonism linked to chromosome 17 have been associated with mutations in the microtubule associated protein tau (MAPT or tau) gene. This disorder is characterized by a large spectrum of neuronal and glial tau lesions in different brain regions. Pick bodies were found in a family with hereditary Pick’s disease with the G272V mutation and in several families with other tau mutations in exons 9 and 11–13. The biochemical composition of Pick bodies varies between these mutations. Until recently, no detailed biochemical characterization of G272V brain material was done owing to unavailability of fresh frozen brain material. We now report a detailed study using the immunohistochemistry, western blots and electron microscopy of two brains with the G272V mutation that recently became available. Both brains showed severe neuronal loss in the temporal cortex, whereas in the frontal cortex the loss was less; and abundant Pick bodies in the dentate gyrus of the hippocampus, and caudate nucleus. The Pick bodies consisted exclusively of three-repeat (3R) isoforms, as was demonstrated by isoform-specific antibodies and supported by western blot analysis of sarkosyl-insoluble tau. These observations confirm that this family diagnosed with hereditary Pick disease meets all the criteria for this condition, including the presence of Pick bodies that are unphosphorylated at Ser262 and contain twisted filaments with long periodicity consisting only of 3R tau.

Keywords: brain; focal dementias; neuronal degeneration; tau expression

Abbreviations: NFT = neurofibrillary tangles; PSP = progressive supranuclear palsy; 3R = 3-repeat; 4R = 4-repeat


Introduction

Pick’s disease is one of the possible pathological entities of frontotemporal dementia, and is characterized by the presence of round argyrophilic inclusions (i.e. Pick bodies) in several regions of the brain. The major constituent of Pick bodies is the hyperphosphorylated protein tau. Several other diseases, such as Alzheimer’s disease or progressive supranuclear palsy (PSP), also contain tau aggregates and therefore, the general term tauopathies was introduced (Goedert et al., 1992; Spillantini et al., 1997). The observation, however, that disease-specific neuronal and glial inclusions showed selective aggregation of 3-repeat (3R) and 4-repeat (4R) tau isoforms, led to the further subclassification of tauopathies into 3R and 4R tauopathies. For example, the tau positive inclusions [neurofibrillary tangles (NFT)] in PSP mainly consist of 4R tau, whereas tau inclusions in sporadic Pick’s disease (Pick bodies) are predominantly composed of 3R tau. Therefore, these conditions are considered to be 4R and 3R tauopathies. Frontotemporal dementia, linked to chromosome 17q21–22, is a familial group of tauopathies in which neuronal and glial tau inclusions can vary in morphology and tau isoform composition depending on the type and localization of the mutation in the tau gene. In these families Pick bodies
are observed in patients with most exon 9 mutations and several mutations outside exon 10 and flanking regions: K257T (Rizzini et al., 2000), L266V (Hogg et al., 2003; Kobayashi et al., 2003), L315R (van Herpen et al., 2003), S320F (Rosso et al., 2002), Q336R (Pickering-Brown et al., 2004), G342V (Lippa et al., 2000), K369I (Neumann et al., 2001), G389R (Murrell et al., 1999; Ghetti et al., 2000). Interestingly, some variance is observed between different mutations in the composition of Pick bodies. Several mutations give rise to Pick bodies that consist of mainly 3R isoforms, whereas others consist of a mixture of both 3R and 4R tau, demonstrating that a detailed analysis (e.g. tau isoform specific antibodies) should supplement standard (immuno)-histochemistry results and western blotting.

Hereditary Pick disease as a clinicopathological phenotype of FTDP-17 has been associated with the G272V mutation in the tau gene (Sanders, 1939; Schenk, 1959; Groen and Endtz, 1982; Heutink et al., 1997; Spillantini et al., 1998). Biochemical and electron microscopic characterization of the Pick bodies associated with this mutation had not been performed owing to unavailability of fresh frozen brain material. Fresh frozen brain material of two patients from such a family has recently become available and is characterized in this paper by means of immunohistochemistry, western blot and electron microscopy. Using isoform-specific antibodies (de Silva et al., 2003) we were able to determine that the hereditary Pick disease in this family (Fig. 1), originally described as early as 1939 by Sanders et al. (1939) and later by Schenk (1959) and Groen and Endtz (1982) is a classical 3R tauopathy.

Materials and methods
Clinical history

Patient I
At the age of 45 years this patient increasingly created problems at work as a legal employee as a result of conflicts with her colleagues. At home she became increasingly apathetic, neglected household affairs and personal hygiene. Owing to impulsiveness and loss of attention, driving a car became dangerous. Her impaired financial judgement resulted in huge debts. Neurological examination of the patient showed intact language and memory functions, but impaired planning and abstract thinking. Cranial nerves were intact. Upper and lower extremities showed normal muscle tone and strength; tendon reflexes were symmetrically intact. MRI showed mild atrophy of the anterior and temporal lobes. In the next 2 years, she became socially withdrawn, and even avoided visits by family members. Her condition gradually deteriorated over the next 5 years. At the age of 50 years she was admitted to a psychogeriatric nursing home. She died at the age of 54 years. Family history showed that her father and her eldest brother had died from Pick’s disease at the age of 53 and 47 years, respectively, whereas a sister is currently affected and still alive.

Patient II
At the age of 52 years, this woman developed inappropriate and repetitive (verbal and motor) behaviour and became stubborn and emotionally blunt. At her first out-clinic visit, the patient showed impaired orientation and naming, recurring utterances and perseverations, but normal memory and visuoconstructive functions. Cranial nerves were intact. Muscle-tone, strength and reflexes of upper and lower extremities were normal. At that time CT scan showed mild symmetrical temporal atrophy. She was admitted to a psychogeriatric nursing home at the age of 58 years, where she died at the age of 67 years, 15 years after the onset of the disease. Family history showed that her father and her eldest brother had died from Pick’s disease at the age of 55 and 47 years, respectively, whereas her youngest son already developed dementia at the age of 44 years.

Brain pathology and staining
Routine staining with haematoxylin–eosin, Bodian, Gallyas and Congo red was performed. Immunohistochemistry for tau protein made use of phosphorylation-dependent (AT8 1:40, Innogenetics, Ghent, Belgium), AT180 (1:500, Innogenetics), AT270 (1:500, Innogenetics), PHF1 (1:500, donated by P. Davies), MC1 (1:25,}

Fig. 1 Family tree of the G272V family with hereditary Pick’s disease. Males are depicted as squares, females as circles. Affected members are shown in half black, members suspected to be affected as quarter black. I and II represent patients I and II, respectively.
G272V patients show 3R tau pathology

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donated by P. Davies) and 12E8 (1:500, donated by P. Seubert, Elan Pharmaceuticals, San Francisco, CA) and phosphorylation-independent [BR01 (1:500, Innogenetics), Tau 2 (1:100, Sigma Fine Chemicals, St Louis, MO)] antibodies. Antibodies directed against ubiquitin (1:500, Dako, Glostrup, Denmark), β-amyloid (1:100, Dako), α-synuclein (1:500, Chemicon, Temecula, CA) and glial fibrillary acidic protein (GFAP; 1:500, Dako) were also used. Staining was revealed using the biotin/avidin Vectastain system (Vector Laboratories, Burlingame, CA), as described (Rosso et al., 2001).

For staining with specific anti-tau phospho-specific Ser181 antibody (577814, Calbiochem, 1:500), tissue sections were boiled in citrate buffer for 5 min, treated with 99% formic acid for 20 s and stored overnight at room temperature. Tau antibodies specific against 3R (RD3, Upstate, Charlesville) and 4R tau isoforms (RD4, Upstate, Charlesville) were used (de Silva et al., 2003) to investigate the tau isoform composition of Pick bodies. Sections from the two G272V cases as well as an Alzheimer’s disease and a PSP control were pretreated by pressure-cooking in sodium citrate buffer (pH 6) for 5 min, followed by incubation with RD3 (1:6000) and RD4 (1:100) at 4°C overnight.

**Tau haplotyping**

MAPT H1/H2 haplotypes were determined by SNPs rs9468, rs916793 and rs1800547 using Taqman SNP genotyping assay and 20 ng of genomic DNA according to the manufacturer’s protocol.

**Tau extraction, immunoblotting and electron microscopy**

Sarkosyl-soluble and insoluble tau proteins were extracted from the gyrus frontalis inferior, gyrus frontalis medialis, gyrus temporalis medialis, cerebellum, hippocampus and caudate of both patients as described (Goedert et al., 1992). Dephosphorylation and immunoblotting using the phosphorylation-independent anti-tau antibody H-7 (1:2000) (Mercken et al., 1992) or the 3R specific anti-tau antibody RD3 (1:2000) were performed as described (Rizzu et al., 2000; Rosso et al., 2001). Aliquots of sarkosyl-insoluble tau extracted from the gyrus frontalis medialis were processed for electron microscopy as described (Goedert et al., 1992; Lee et al., 2001). The phosphorylation dependent anti-tau antibody AT8 was gold conjugated and used at a dilution of 1:100.

**Results**

**Pathology**

Severe frontal and temporal atrophy with enlarged lateral ventricles was present in both brains. Brain weights were 872 and 897 g for brain I and brain II, respectively. The hippocampus and amygdala showed atrophy in both brains, but much more severe in brain II. The anterior part of the caudate nucleus and putamen were severely atrophic in brain II. Substantia nigra and locus coeruleus were normally pigmented in both brains. In both brains, neuronal loss and gliosis were severe in all layers, except layer IV, of the temporal and insular cortices, although more pronounced in brain II. The frontal cortex showed a mild neuronal loss and gliosis in brain I and a moderate loss in brain II. The cingular cortex, the parietal and occipital cortices were normal, except for some senile plaques in the occipital cortex of brain II.

Both brains showed moderate neuronal loss in CA1 and CA2, and severe loss in subiculum, entorhinal cortex and amygdala. The caudate nuclei had a normal appearance, but showed severe loss of large neurones in both brains. Neuronal loss in the substantia nigra was severe in brain I and mild in brain II.

Bodian and Gallyas staining revealed a few NFT in the frontal cortex in brain I and in both patients many round inclusions (Pick bodies) in the granular cells of the dentate gyrus and the small neurones of the caudate nucleus.

Immunohistochemical staining with AT8 showed numerous Pick bodies, which were abundant in the granular cells of the dentate gyrus of brain I, whereas the dentate gyrus of brain II showed more curvilinear deposits and a few Pick bodies (Fig. 2A and B). NFT were present in a few neurones of the CA, the subiculum and the entorhinal cortex, the amygdala and the thalamus of both brains. Some tau-positive neurones and a few glial cells were present in the frontal cortex (Fig. 3C), whereas almost no tau staining was seen in the temporal cortex. Abundant tau inclusions were present in small neurones of the caudate nucleus (Fig. 3A). Tau staining was positive in several neurones of the substantia nigra of brain II, but was absent in brain I. A few tau-positive neurones were present in locus ceruleus, pons and brainstem in both brains. The cingular, parietal and occipital cortices in both brains did not show tau-positive inclusions. A few tau-positive neuronal inclusions and some dystrophic neurites were seen in the dentate nucleus of the cerebellum (Fig. 3D). Immunohistochemistry with PHF1 and AT180 gave a similar, although less intense, staining. Staining with the 3R specific antibody, RD3, showed many Pick bodies in the granular cells of the dentate gyrus of brain I (Fig. 2C), whereas more diffuse cytoplasmatic staining and a few Pick bodies were found in the granular cells of brain II (Fig. 2D). Abundant Pick bodies were present in the small neurones of the caudate nucleus of both brains (Fig. 3B). RD4 staining was negative for all Pick bodies in both brains (Fig. 2E), but gave positive staining of a few NFT in CA1 and CA2 of brain I (Fig. 2G) and a diffuse cytoplasmatic staining. In addition, RD4 gave positive staining of a few NFT in CA1 and CA2 of brain I (Fig. 2G) and a diffuse cytoplasmatic staining of some granular cells in the dentate gyrus of brain II (Fig. 2F). A faint cytoplasmatic staining of a few small neurones in the caudate nucleus was seen in both brains (data not shown). The RD4 staining of Alzheimer’s disease and PSP controls showed NFT in Alzheimer’s disease, and gave NFT and tau-positive glial inclusions in the PSP brain (Fig. 2H). Pick bodies did not stain with the antibodies 12E8 and PS262, but weak staining was observed in some Pick bodies when stained for ubiquitin. Staining for α-synuclein and β-amyloid was negative in both brains.

**Mutation analysis and haplotyping**

Mutation analysis of both patients had already been done as described before (Spillantini et al., 1998). In addition,
Immunohistochemistry with AT8 antibody shows numerous Pick bodies in the granular cells of the dentate gyrus of brain I (A) and some Pick bodies and curvilinear cytoplasmatic deposits in the same cells in brain II (B). The RD3 antibody gives positive staining of numerous Pick bodies (although less than with AT8) in brain I (C), and some Pick bodies and more diffuse cytoplasmatic staining of many granular cells in brain II (D). Staining with RD4 antibody is negative for Pick bodies (E), but shows diffuse cytoplasmatic staining in a few granular cells in brain II (F), and of a few NFT in CA1 of brain I (G). NFT and glial inclusions in a PSP brain were positive for RD4 staining (H). Scale bar A through H = 100 μm.
tau haplotypes of both mutant and wild-type alleles were determined. Since both patients showed a H1/H1 haplotype, both mutant alleles were expressed in the H1 background.

Characterization of frozen brain material

Western blots of the sarkosyl-insoluble fraction of tau from several regions (including the frontal and temporal cortices) mainly showed 60 and 64 kDa bands. In some regions (i.e. frontal cortex and caudate) an extra band of 68 kDa band was also present (Fig. 4A). This pattern is typical for Pick's disease pathology (Delacourte et al., 1998). Interestingly, the sarkosyl-insoluble tau from the cerebellum in brain II also showed 60 and 64 kDa bands, which is in accordance with the presence of tau inclusions observed in the immunohistochemistry.

After dephosphorylation, sarkosyl-insoluble tau consisted predominantly of 3R tau, and the amount of 3R0N and 3R1N was much higher than 3R2N. The 3R0N isoform ran somewhat lower on gel than the corresponding recombinant tau band (Fig. 4B), as a result of the high amount of alkaline phosphatase used in this dephosphorylation step, which was confirmed by using the 3R-specific antibody (Fig. 5). The small amount of 4R tau observed consisted mainly of 4R1N (Fig. 4B), although, probably owing to the sensitivity of the monoclonal antibody, some other 4R bands could also be detected.

Western blots of the sarkosyl-soluble fraction from the frontal and temporal cortices showed all isoforms (Fig. 4C).

Sarkosyl-insoluble tau filaments

When the sarkosyl-insoluble fractions of both patients were evaluated under the EM, it contained AT-8 positive tau filaments (Fig. 6A) that resembled ribbon twisted filaments (Fig. 6B). They had however a longer periodicity (>110 nm) than those found in Alzheimer’s disease patients, but their width was similar (20 nm).

Discussion

The present study shows abundant Pick bodies in the caudate nucleus and dentate gyrus of the hippocampus in two patients with the G272V mutation in exon 9 of the tau gene. Furthermore, we showed, that sarkosyl-insoluble tau extracted from Pick bodies consists predominantly of 3R tau isoforms. Sarkosyl-insoluble tau filaments were shown to be ribbon twisted, with long periodicity.

The presence of Pick bodies in the two G272V brains could be detected using both Bodian and Gallyas silver staining, as has been also observed in the L266V mutation (Hogg et al., 2003). This is in contrast to other tau mutations and sporadic Pick cases, in which Pick bodies are Bodian positive and Gallyas negative (Neumann et al., 2001; Ikeda et al., 2002;
Fig. 4 Western blots of tau protein extracted from patients’ brains. (A) Sarkosyl-insoluble tau extracted from several brain regions of patients I and II. AD, sarkosyl-insoluble tau extracted from Alzheimer’s disease brain. (B) Dephosphorylated sarkosyl-insoluble tau from several brain regions of patients I and II. (C) Dephosphorylated sarkosyl-soluble tau from several brain regions of patients I and II. AD, sarkosyl-soluble tau extracted from the same region of Alzheimer’s disease brain; Rec, recombinant tau consisting of all six isoforms. Names are according to tau isoforms. Sizes are in kDa.

Fig. 5 Western blots of tau protein extracted from patients’ brains stained with RD3. (A) Hyperphosphorylated sarkosyl-insoluble tau stained with the 3R specific antibody RD3. II, sarkosyl-insoluble tau extracted from brain material of patient II; PID, sarkosyl-insoluble tau extracted from a classical Pick’s disease patient; AD, sarkosyl-insoluble tau extracted from brain material of an Alzheimer’s disease patient. Sizes are in kDa. (B) Dephosphorylated sarkosyl-insoluble tau stained with the 3R specific antibody RD3. Rec, recombinant tau consisting of all six isoforms. Since the antibody is 3R tau specific, only the 3R isoforms are stained. Names are according to tau isoforms.

Fig. 6 Electron micrographs of G272V tau filaments. Electron microscopic examination of sarkosyl-insoluble tau extracted from the frontal lobe of patients I and II. (A) A tau filament taken from patient I. Both patients showed similar filaments. (B) Sarkosyl-insoluble tau labelled with gold conjugated AT8. In both micrographs the black bar depicts 50 nm.
Rosso et al., 2002; Kobayashi et al., 2004). Although it has been suggested that this difference could be explained by the increased affinity of the Gallyas method for 4R tau (Dickson, 2001), in our experiments, by using isoform specific antibodies, no 4R tau was detected in these Pick bodies. Therefore, an alternate explanation for this observation must be found.

The distribution of the Pick bodies to the caudate nucleus differs from most sporadic Pick’s disease cases and several tau mutations, in which Pick bodies are abundant in the frontal and temporal cortices (Kosaka et al., 1991; Murrell et al., 1999; Lippa et al., 2000; Neumann et al., 2001; Zhukareva et al., 2002; Pickering-Brown et al., 2004). The absence of Pick bodies in the temporal cortex may be related to the severe neuronal loss; however Pick bodies were also absent in the frontal cortex, which was much less affected. The presence of tau-positive lesions in the dentate nucleus of the cerebellum is in agreement with the presence of insoluble tau protein on western blot (Fig. 4A).

The mild glial or astrocytic tau pathology in the present G272V brains contrasts with several other mutations with Pick bodies, in which glial or astrocytic tau pathology was much more pronounced (Murrell et al., 1999; Lippa et al., 2000; Neumann et al., 2001; Kobayashi et al., 2003, 2004; van Herpen et al., 2003). This variable extent of glial or astrocytic tau pathology in conjunction with the presence of Pick bodies might reflect an intermediate step in the neurodegenerative process comparable with the astrocytic reaction that is specific for 4R tau isoforms.

The positive RD3 (Fig. 2B and D) and negative RD4 staining of Pick bodies reflects the exclusive accumulation of 3R tau isoforms in the Pick bodies of the G272V patients. Our western blot data, which show the presence of 60 and 64 kDa bands in the sarkosyl-insoluble tau fraction, and mainly 3R isoforms after dephosphorylation confirm these immunohistochemical findings. We reason that the low levels of 4R tau observed on these western blots correspond to the presence of some NFT in the CA1 and CA2 in brain I; the diffuse or faint positive cytoplasmatic staining of some granular cells and of few small neurones in the caudate nucleus of brain II that could also be detected by our sensitive monoclonal RD4 antibody in the immunostaining.

With ageing, tau proteins can become insoluble, and sometimes contaminate the preparation of the so-called ‘pathological tau aggregates’. We reason, therefore, that the presence of NFT in our patients is a part of the normal ageing process and not a part of the disease. This contamination has also been described in several other neurodegenerative diseases (Hof et al., 1992a, b, c). The diffuse cytoplasmatic staining in some granular cells of the other brain, however, is interesting and should be investigated for other mutations.

Therefore, our observations are in accordance with the generally accepted concept of Pick bodies, which is, to be exclusively composed of 3R tau isoforms (Delacourte et al., 1996, 1998). Other FTDP-17 mutations with a Pick-like phenotype have also shown the presence of 3R and 4R tau isoforms on western blots of sarkosyl-insoluble tau. In these studies, this might also be explained by the co-occurrence of giall pathology or NFT (Murrell et al., 1999; Lippa et al., 2000; Neumann et al., 2001; Rosso et al., 2002; van Herpen et al., 2003), as the composition of Pick bodies was not tested using isoform-specific antibodies.

The absence of staining, using the antibodies 12E8 and PS262 with and without formic acid pretreatment, indicates the lack of phosphorylation of the serine at position 262, and agrees with the observations of most other studies of Pick’s disease (Delacourte et al., 1996, 1998; Probst et al., 1996; Arai et al., 2001; Ikeda et al., 2002). It is, however, in contrast with the more recent observations, where Pick bodies, following formic acid pretreatment, showed some immunoreactivity with the antibody 12E8 and strong staining with PS262 (Ferrer et al., 2002; Arai et al., 2003). Since this immunoreactivity was even observed in patients showing Pick bodies that consisted predominantly of 3R tau isoforms (Arai et al., 2003), we reason that this observation cannot be explained by a specific reaction with phosphorylated Ser262 of 4R tau.

Since Pick bodies in the G272V and L266V mutations consist only of 3R tau, it is an interesting question whether exon 9 mutations induce a more efficient aggregation of 3R tau compared with 4R tau. However, in vitro studies showed a higher aggregation rate of 3R and 4R L266V tau (Hogg et al., 2003). In addition, another exon 9 mutation (i.e. I260V) causes a 4R tauopathy with NFT (PSP) and shows a higher aggregation rate for 4R tau compared with 3R tau (Hasegawa et al., 1998; Barghorn et al., 2000; Grover et al., 2003).

The tau positive filaments from both patients were shown to have a very long periodicity. Although most filaments described in sporadic Pick’s disease are straight filaments, several cases described PHF-like filaments with a long periodicity of 120–160 nm, whereas the filament width has been shown to vary between 13 and 26 nm (Kato and Nakamura, 1990; Murayama et al., 1990; Dickson, 2001). This means that the observed width of 13 nm is also according to the filaments described in the literature.

The present immunohistochemical and biochemical investigations show that our patients meet all of the criteria to be diagnosed as hereditary Pick’s disease. However, pathology-proven cases of Pick’s disease are clinically indistinguishable from FTD cases without Pick bodies. In addition, Pick’s disease is etiologically heterogeneous by the fact that it has been associated with different types of tauopathy (3R, combined 3R-4R or even 4R) (Zhukareva et al., 2002) as well as with combined 3R-4R FTDP-17 cases (Pickering-Brown et al., 2000, 2004; van Herpen et al., 2003). Conversely, other tau mutations that also have a combined 3R-4R isoform profile [V337M and R406W (Hutton et al., 1998; Poorkaj et al., 1998)] are not classified as Pick’s disease because these patients show only presence of neurofibrillary tangles. This means that Pick’s disease is nothing more than another connotation for the presence of Pick bodies. We would suggest to bring the term Pick’s disease back into clinical practice and to use the pathological classification of 3R, 4R or mixed type of tauopathy for etiological refinement.
We also suggest to include immunohistochemistry using isoform specific tau antibodies to supplement the results of western blots of other FTDP-17 and atypical tauopathy cases in order to refine tau pathology. This detailed analysis will allow us to include also the somewhat atypical Pick cases and will lead to the identification of new subgroups. Possibly these groups will help us to reveal new and distinct disease pathways that could help us in understanding the pathogenesis of all neurodegenerative diseases better.

Acknowledgements

We wish to thank Dr M. Goedert for providing us with recombinant tau, Dr P. Seubert for donating the antibody 12E8, Dr P. Davies for providing us with the antibodies PHF1 and MC1, and T. de Vries Lentsch for photography and artwork. This work was supported in part by the ‘Nederlandse Organisatie voor Wetenschappelijk Onderzoek’ (NWO).

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