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Characterization of Naturally Occurring Cutaneous Neurofibromatosis in Holstein Cattle

A Disorder Resembling Neurofibromatosis Type 1 in Humans

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Neurofibromatosis in cattle is typically a noncutaneous disease. A small group of cows in a Holstein dairy herd developed cutaneous neurofibromatosis. This unique condition was investigated and compared with neurofibromatosis type 1 (NF1) in humans. All cutaneous lesions but one were consistent with neurofibromas in noncutaneous sites in cattle and neurofibromas in patients with NF1. One bovine lesion was classified as a neurofibrosarcoma. Immunohistochemistry and electron microscopy supported Schwannian differentiation in benign and malignant lesions. Linkage analysis with a polymorphism in the bovine NF1 gene confirmed that two affected animals from the same sire inherited the same paternal NF1 allele. Bovine cutaneous neurofibromatosis is a naturally occurring disease in this group of animals, characterized by skin tumors morphologically identical to those of NF1. An informative polymorphism at the NF1 locus of two animals and their sire suggests this disorder may be caused by hereditary mutations at the bovine NF1 locus. (Am J Pathol 1994, 145:1168–1174)

Neurofibromas are uncommon in domestic animals. These tumors occur more frequently in cattle than in other species and usually involve multiple sites of the brachial plexus, intercostal nerves, and cardiac nerves. Less common sites are retroserosal areas of the thorax and abdomen, nerves to skeletal muscles (especially the tongue), and roots and ganglia of cervical spinal nerves. Multiple cutaneous neurofibromas, like those seen in humans with neurofibromatosis (von Recklinghausen disease), are rare[1] and have not been reported in reviews of bovine peripheral nerve sheath tumors.[2,3]

Neurofibromatosis type 1 (NF1) is an autosomal dominant condition in humans that occurs in 1 in every 3000 births. It is considered to be completely penetrant with variable expressivity. Because clinical features of the disease are highly variable, it is sometimes difficult to diagnose in early childhood. Characteristically, however, an affected individual develops a triad of lesions including cutaneous neurofibromas, pigmented skin lesions (cafe au lait spots), and possibly one or more iris hamartomas. The neurofibromas usually appear in adolescence and increase in size and number with age. The progression of the disease may be accelerated by hormonal influences.[4] It has been difficult to find a naturally occurring animal model that reflects the multifaceted aspects of the disease.

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Recently, cutaneous neurofibromas were observed in four cows from a Holstein dairy herd. Three of these animals were from the same sire lineage. This report describes clinical, morphological, and genetic findings and discusses the similarities of this condition to NF1.

Materials and Methods

Animals

Four mature cows (3119, 3371, 3314, and 3724) from an Alabama Holstein dairy herd of 300 developed multiple cutaneous nodules located primarily on the face, neck, and head. Three of the 4 were available for detailed examination. One cow was sacrificed after histological diagnosis before the initiation of this study. The remaining three cows were given complete physical and ocular examinations. Eyes were examined using a Finnoff transilluminator and indirect ophthalmoscopy. Pupils were dilated with topical instillation of 1% tropicamide.

Pathology

The location, size, and shape of gross lesions were recorded. Biopsies of nodules were fixed in 10% neutral-buffered formalin embedded in paraffin sectioned at 4 to 5 μ and stained with hematoxylin and eosin (H&E). Adjacent sections were selected for immunohistochemical studies. The avidin-biotin complex immunoperoxidase method was used to stain deparaffinized sections with antibodies to S-100 protein (Dako, Carpinteria, CA; dilution 1:7200), myelin basic protein (Biogenex, San Ramon, CA; prediluted), vimentin (Dako; dilution 1:160), muscle specific actin (Enzo, Syosset, NY; dilution 1:1600), α_1-antichymotrypsin (Biogenex; dilution 1:3), desmin (Biogenex; dilution 1:400), and neuron-specific enolase (Dako; dilution 1:1200) following standard techniques.[5] The staining was performed using a Ventana 320 automated immunostainer.

Tissue fragments from selected areas were fixed in 3% glutaraldehyde in Millonig's buffer (pH 7.3), postfixed in 1% osmium tetroxide, dehydrated in graded alcohols, and embedded in Spurr resin. Ultrathin sections were stained with uranyl acetate and lead citrate and examined with a Philips 301 electron microscope.

Molecular Biology

Cryopreserved semen from the common sire (animal 3287) was obtained from the American Breeders Service (DeForest, WI) and DNA was extracted for Southern blot analysis. Fresh whole blood was obtained from affected animals (3119, 3371, and 3314) and their unaffected offspring (3718, 3915, 4075, and 4260) as well as six unrelated normal Holstein cows. DNA for Southern analysis and RNA for Northern analysis was extracted from these specimens. Animal 3724 had been sacrificed before initiation of molecular genetic analysis and no DNA or RNA was available.

Preparation of Genomic DNA and Southern Analysis

Fresh Whole Blood

Genomic DNA was obtained by lysing fresh whole blood with 20 mmol/L Tris, 20 mmol/L $MgCl_2$, 2% Triton X-100, and 0.6 M sucrose. Cells were then digested in 75 mmol/L NaCl, 25 mmol/L EDTA, 0.5% sodium dodecyl sulfate (SDS), and 250 μg/ml proteinase K, pH 8, overnight at room temperature. The solution was then extracted twice with phenol and once with chloroform and DNA was precipitated with 0.5 volume of 7.5 M ammonium acetate and 1.5 volume of isopropanol. DNA pellets were resuspended in TE buffer (10 mmol/L Tris, 1 mmol/L EDTA, pH 8).

Frozen Semen

Genomic DNA was prepared as described previously.[6] Semen was treated at 50 C with 2% β-mercaptoethanol in 0.01 M Tris, 0.1 M NaCl, 0.01 M EDTA, and 0.5% SDS for 30 minutes. Proteinase K was added to a final concentration of 200 μg/ml and incubated overnight at room temperature. The solution was extracted twice with phenol, twice with phenol:chloroform 1:1, and twice with chloroform. DNA was precipitated with 0.5 volume of 7.5 M ammonium acetate and 1.5 volume of isopropanol. DNA pellets were resuspended in TE buffer.

Southern Analysis

Five micrograms of each bovine DNA sample was digested with the restriction enzymes *Eco*RI, *Taq*I, *Pst*I, *Pvu*II, *Msp*I, and *Bgl*II according to the instructions supplied by the manufacturer (Bethesda Research Laboratories, Bethesda, MD), separated on 1.2% agarose gels, and transferred to Hybond N membranes using standard Southern blotting methods. Southern blots were hybridized at 65 C with oligo-labeled cDNA probes in 1 M NaCl, 10% dextran sulfate, 1× Denhardt's solution, 1% SDS, and 100

μg/ml sheared denatured salmon sperm DNA. Washes were to a final stringency of 0.2× standard saline citrate (SSC), 0.1% SDS at 65 C. The following NF1 cDNA probes were used: GE2, FF13, FF1, FB5D, AE25, and P5.[7,8]

RNA Extraction and Northern Analysis

From fresh whole blood, whole RNA was extracted using the RNAzol isolation solution (Tel-Test) according to the instructions supplied by the manufacturer. Five micrograms of each RNA sample was incubated for 15 minutes at 60 C in a mixture of 2 μl 10× Gel Running Buffer (0.4 M MOPS, 100 mmol/L NaAc, 10 mmol/L EDTA, pH 6.8), 3.5 μl formaldehyde, 10 μl formamide, and 1 μl EtBr (400 mg/L). The samples were separated by electrophoresis through 1% agarose gels containing formaldehyde and blotted overnight onto Hybond N membranes. Random priming and hybridization were as described for Southern analysis using the NF1 cDNA probe P5. Washes were to a final stringency of 2× SSC, 0.1% SDS at 50 C.

Results

Physical and Clinical Findings

Three animals (3371, 3119, and 3314) were available for detailed morphological and genetic characterization. A fourth animal (3724) was sacrificed before ocular examination and molecular genetic studies. The four animals were healthy and above herd average in milk production. Initial physical examination revealed no abnormalities other than multiple cutaneous nodules. The referring veterinarian initially assumed the nodules were painful because of the animals' avoidance response to palpation. However, this reaction decreased as the animals became accustomed to physical examinations. The lesions usually enlarged slowly and ranged from small (1 to 4 cm diameter) to large (up to 15 cm diameter) masses. Facial masses were most common and usually periocular (including the eyelids) and periauricular (Figure 1). Many of the smaller nodules were arranged as coalescent chains or clusters along the neck, brisket, and ventral thorax. Most nodules were in pigmented skin.

The pedigree of animals 3371 and 3119, 9- and 10-year-old daughters of the same sire (3287), and animal 3724 (a granddaughter of 3287) is shown in Figure 2. The cutaneous nodules of these three animals were similar, with a few exceptions. Mucocutaneous nodules were noted only in animal 3371, one at the lateral canthus of the left eye (Figure 1B) and the other at the anus. A neck lesion in animal 3119 was aggressive in behavior. The initial mass (4 × 4 × 1 cm) failed to heal completely after biopsy and began to grow rapidly. Subsequent biopsies of the ulcerated mass were followed by rapid regrowth. The mass attained an approximate size of 12 × 10 × 5 cm and extended into underlying tissues incorporating the jugular vein. Although the eyes of both cows were sighted, both had intraocular lesions. These consisted of iris abnormalities in two animals and retinal abnormalities in one. Iris abnormalities were mild bilateral (3119) and unilateral (3371) linear hypopigmented foci in the peripheral iris. The retinal lesions in animal 3119 consisted of three raised, nodular lesions adjacent to the optic disk of the left eye.

Animal 3314, a 9-year-old daughter of a Holstein bull with a pedigree distinct from that of sire 3287, had few tumors. These were small to medium sized (4 to 7 cm diameter) nodules primarily on the face. The eyes were normal.

Histology and Immunohistochemistry

The cutaneous nodules consisted of loosely cohesive spindle-shaped cells arranged in short interwoven bundles or swirling patterns interrupted by relatively acellular areas of dense collagen. Cells were usually well differentiated, had a moderate amount of poorly stained eosinophilic filamentous cytoplasm, and oval vesicular nuclei with marginated chromatin (Figure 3).

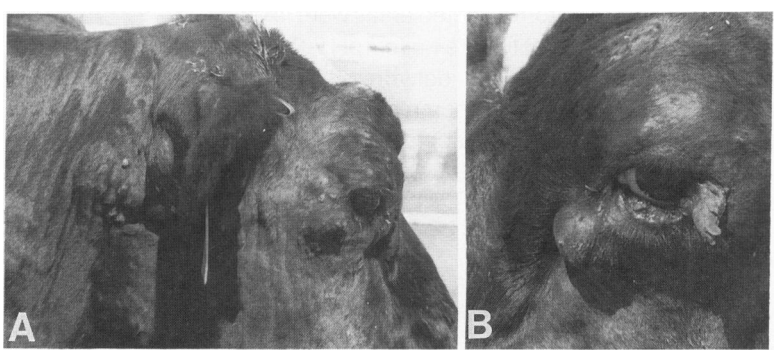

Figure 1. *Cutaneous neurofibromas in a Holstein dairy cow (animal 3371).* **A:** *Lesions usually grew slowly and were commonly periocular and periauricular.* **B:** *Mucocutaneous neurofibroma at the lateral canthus of the left eye of a Holstein cow with multiple cutaneous neurofibromas (animal 3371). The mass resulted in abrasion of the conjunctiva.*

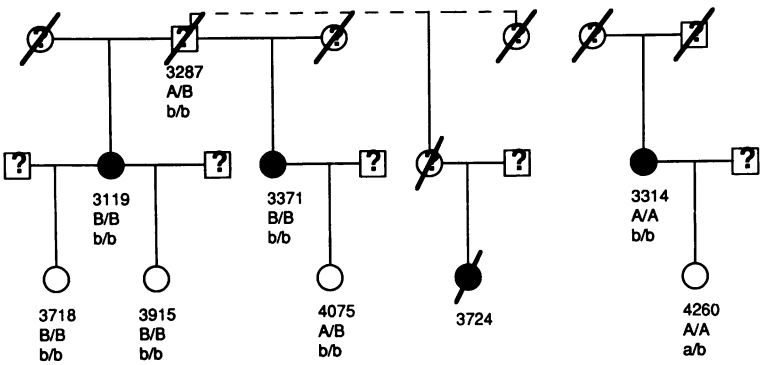

Figure 2. *Pedigree of Holstein cows with multiple cutaneous neurofibromas (animals 3119, 3371, 3724, and 3314). A "?" indicates that this animal was not available for examination. A and B refer to the alleles present at the GE2* TaqI *polymorphism and a and b are the alleles for the P5* TaqI *polymorphism. Animals 3119 and 3371 both inherit from their heterozygous sire (animal 3287) a B allele, as demonstrated by the GE2* TaqI *polymorphism. Animal 3724 was sacrificed before obtaining blood for Southern blot analysis.*

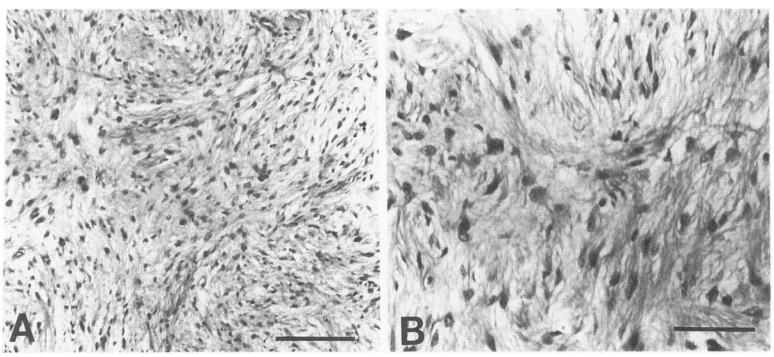

Figure 3. *Cutaneous neurofibroma from a Holstein cow.* A: *The nodules consist of loosely cohesive spindle-shaped cells arranged in short interwoven bundles or swirling patterns. (bar = 200 μ).* B: *Higher magnification demonstrating filamentous nature of cytoplasm (bar = 100 μ).*

Mitotic figures were usually rare. In collagen-rich areas cells were small and had indistinct cytoplasm and small hyperchromatic nuclei. Sections of nerve were common throughout all nodules, but no contiguous relationship of nerve sheath with tumor cells was demonstrable. All lesions, with the exception of the recurrent neck mass (3119), were consistent with non-cutaneous neurofibromas as seen in cattle and with neurofibromas as seen in NF1. Although the neck lesion was originally benign, subsequent biopsies contained pleomorphic cells with a high mitotic rate (up to 10 mitoses per high power field) and focally invasive characteristics. Histology of the recurrent tumor was consistent with neurofibrosarcoma. Neoplastic cells in benign and malignant tumors were positive for S-100 protein (Figure 4) and vimentin, weakly positive for neuron-specific enolase, and negative for myelin basic protein, smooth muscle actin, α_1-antichymotrypsin, and desmin.

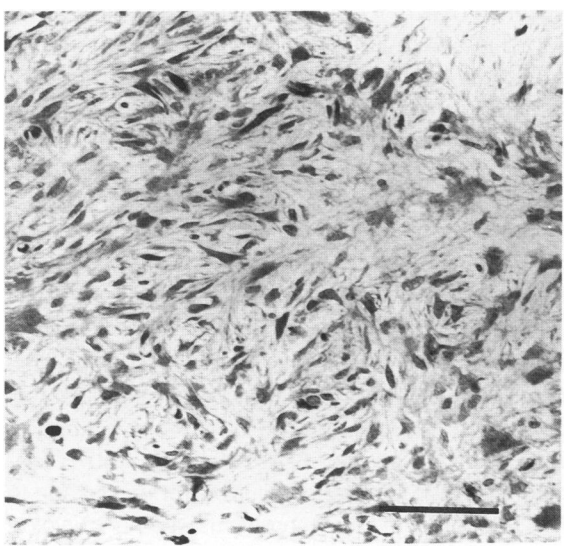

Figure 4. *Cutaneous neurofibroma from a Holstein cow. Many neoplastic cells are positive for cytoplasmic S-100 protein (bar = 50 μ).*

Electron Microscopy

Ultrastructurally, the neoplastic cells were spindle shaped and had long cytoplasmic processes that tended to wrap around collagen bundles and to interdigitate (Figure 5). Small intercellular junctions were noted in areas where cell-to-cell contacts occurred. Their cytoplasm contained the usual organelles including scattered mitochondria and cisternae of rough endoplasmic reticulum, ribosomes, and polysomes. Interrupted basal laminae material covered the cell surfaces.

Molecular Biology

Southern blot analysis was performed by hybridizing the human NF1 cDNA clones P5, AE25, FB5D, FF1,

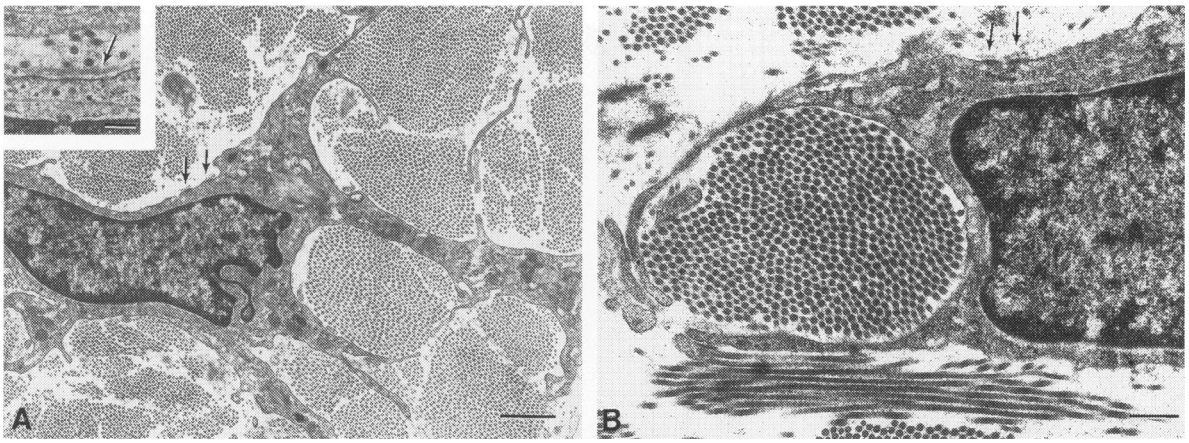

Figure 5. *Cutaneous neurofibroma from a Holstein cow. The neoplastic cells have long cytoplasmic processes that tend to wrap around collagen bundles and to interdigitate. Interrupted basal laminae material covered cell surfaces (arrows).* **A:** *bar = 1 μ, inset, bar = 200 nm,* **B:** *bar = 400 nm.*

FF13, and GE2 to bovine genomic DNA digested with the restriction enzymes *Taq*I, *Eco*RI, *Msp*I, *Bgl*II, *Pvu*II, and *Pst*I. No variants were detected with AE25, FB5D, FF1, and FF13. However, with GE2, a 5′ NF1 cDNA probe, a polymorphism was discovered using digestion with *Taq*I. Two alleles were identified. The absence of a *Taq*I site (designated GE2 allele A) resulted in a 6-kb fragment, whereas its presence resulted in a 4.1-kb fragment (designated GE2 allele B). In addition, with P5, a 3′ NF1 cDNA probe, another polymorphism was discovered using *Taq*I digestion. The absence of a *Taq*I site resulted in a 3.5-kb fragment (designated P5 allele a), whereas its presence resulted in a 1.8-kb fragment (designated P5 allele b). Figure 2 shows the genotypes for these two restriction fragment length polymorphisms in affected animals and their relatives. Notably, animals 3119 and 3371 were found to have inherited the same paternal allele from animal 3287, as demonstrated by the GE2/*Taq*I polymorphism. Unfortunately, the lack of availability of DNA from the sires of 3718, 3915, and 4075 precludes drawing further conclusions about linkage to NF1. Note that the polymorphisms used are uninformative in determining whether animals 3718 and 3915 inherited their mother's affected chromosome. The different putative NF1 haplotype in the two different pedigrees also is not surprising because these two pedigrees are unrelated and independent NF1 mutations would be expected.

Northern blot analysis was performed on peripheral blood RNA using the human NF1 cDNA clone P5. Detectable transcription of the NF1 gene was noted in all affected animals and normal controls (data not shown). No band size or quantitative abnormalities were noted in affected animals.

Discussion

This is the first report that describes the histology of cutaneous neurofibromas in cattle and documents the lesions by immunohistochemistry and electron microscopy. Cutaneous neurofibromas in this small group of animals are analogous to those seen in NF1. The histological and electron microscopic features of the bovine cutaneous neurofibromas were consistent with classical noncutaneous neurofibromas that occur in cattle[2,9] and with cutaneous neurofibromas that occur in NF1 patients.[10–13] The tendency of cytoplasmic processes to wrap around collagen bundles and the overall ultrastructural appearance of the neoplastic cellular elements with cytoplasmic processes covered by basal lamina material are characteristic of Schwannian differentiation. This is supported by S-100 protein positivity of the tumor cells. The recurrent neck mass in one animal was considered to have undergone malignant transformation, as occurs in some patients with NF1.[10,12] There has been no recurrence 6 months after repeated cryosurgeries.

There are rare, brief reports of cutaneous neurofibromas in cattle.[14–16] The most thorough report describes lesions in calves sired by a single bull on a Czechoslovakian farm.[14] Although the multiple cutaneous masses incorporated nerves, the neoplastic cells were identified as activated fibroblasts with abundant cytoplasmic organelles. This description contrasts with more recent descriptions of neurofibromas.[1,2,9,10] There has long been a controversy over the neoplastic cell type in neurofibromas.[10,17] Neurofibromas in humans and animals are composed of components of the peripheral nerve including

Schwann cells, perineural cells, fibroblasts, and collagen.[18] Schwann cells usually predominate and are presumably the neoplastic cellular element.[18–23]

The intraocular lesions in two related animals suggest that there may be additional clinical similarities between bovine cutaneous neurofibromatosis and NF1. It is well known that in addition to neurofibromas, NF1 patients nearly universally have tiny hamartomas of the iris (Lisch nodules). A smaller percentage of NF1 patients may have retinal lesions and malignancies (especially optic glioma or other tumors of the central nervous system).[24,25] The significance of the eye lesions cannot be definitively determined until complete histopathological examination is performed. This would require enucleation and was not considered a reasonable option with these productive animals.

Southern blot analysis failed to reveal any large mutations (ie, deletions, translocations) in affected animals using the human *NF1* cDNA probes P5, AE25, FB5D, FF13, FF1, and GE2. The cross-hybridization of the human probes to bovine DNA indicates the NF1 gene is strongly conserved. Informative polymorphisms were discovered using GE2 and P5 with *Taq*I restriction digestion. Animals 3119 and 3371 were found to have inherited the same paternal allele for GE2 at the *NF1* locus, suggesting (albeit weakly) that this disorder in Holstein cows may be due to mutations in the bovine *NF1* locus. Further linkage analysis was inconclusive given the small number of affected animals and the unavailability of DNA from all other related animals.

NF1 in humans is inherited as an autosomal dominant disorder with complete penetrance.[26] If these animals have a bovine analogue of NF1, one would expect more affected offspring of animal 3287 out of approximately 108,000 possible offspring (based on units of semen available, expected calving rate, and death loss). Several explanations could account for this observation. One obvious explanation is that bull calves (approximately 50% of the offspring) are sent to slaughter before they reach an age when lesions become phenotypically apparent. Thus, 50% (54,000) of the animals that might express the condition are eliminated from the population. Another explanation is that the common sire could represent an example of gonadal mosaicism. This would explain why his phenotype was not noted as abnormal and that only a relatively small number of mature offspring (2 of 54,000) have the characteristic phenotype of multiple cutaneous neurofibromas. A third possible explanation is that although NF1 in humans has nearly 100% penetrance, in the bovine model penetrance may be reduced and less severely affected offspring of animal 3287 could have escaped detection.

There is a significant need for an animal model of NF1. Despite major advances in the molecular biology of the disease, there remain many unanswered questions regarding the pathogenesis of the various presentations of NF1. A suitable animal model would facilitate *in vivo* and *in vitro* investigations by providing a model with a relatively short life span and a readily available source of research material. A recent attempt to construct a mouse model has been made by creating a germ line knockout of the NF1 gene[27]. These cattle may represent a naturally occurring animal model of NF1. The histology, immunohistochemistry, and electron microscopy of the lesions are consistent with those of NF1. However, to establish definitively whether the bovine morphological representation of NF1 in this report truly represents an animal model for NF1, it will be necessary to identify a specific mutation in the NF1 gene of affected animals.

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