Neurofibromin Expression and Astroglialosis in Neurofibromatosis (Type 1) Brains

MICHAEL L. NORDLUND, PH.D., TILAT A. RIZVI, PH.D., CAMILYNN I. BRANNAN, PH.D., AND NANCY RATNER, PH.D.

Abstract. Patients with type 1 neurofibromatosis (NF1) have mutations in the gene encoding the protein neurofibromin. Immunocytochemistry on sections of cortex and cerebellum of unaffected and NF1 individuals and wild-type and NF1-deficient mice showed that the distribution of neurofibromin was similar to that reported for rat. However, dystrophic neurofibromin-expressing neurons were found in human but not rodent brain. Intensity of anti-neurofibromin reactivity was reduced in NF1-deficient mice but not in human brains. GFAP was upregulated in three NF1 brains studied by immunocytochemistry; a 4-18-fold increase in GFAP levels was documented by Western blot analysis in three brains. GFAP content/cell and the number of GFAP-immunoreactive astrocytes was increased in NF1 brains as compared to the controls. These results suggest that mutations in the NFI gene do not grossly alter the pattern of neurofibromin expression, but activation of astrocytes may be common in NF1. Presence of degenerative debris in one of two brains using the cupric silver method suggests that degeneration is not always detectable in NF1 brains.

Key Words: Astrocyte; Degeneration; GFAP; Neurofibromin; NF1; Ras.

INTRODUCTION

Von Recklinghausen’s, or type 1, neurofibromatosis (NF1) is a disease that is inherited as a dominant trait and affects approximately 1:3,500 individuals (1, 2). The diagnostic criteria for NF1 include neurofibromas (3–5), café au lait spots (6, 7), axillary or inguinal freckling (8), iris Lisch nodules (9–11), characteristic bone abnormalities and/or a first degree relative with the disease. NF1 patients are also predisposed to a variety of CNS abnormalities. Tumors of the optic nerve (astrocytomas) affect up to 30% of children with NF1 (12, 13). The most common neurologic disorder in childhood of NF1 patients is specific learning disability (SLD), which occurs in 30–45% of children with NF1 (14–16). In addition, some of the areas in the brain (basal ganglia, brainstem and subcortical white matter) from NF1 patients show an increased signal intensity on T2-weighted MRI (17–21). Frequency of these areas of increased T2 signal intensity on MRI varies from 43–79% (17–22). These have been called small hamartomas, heterotopias or unidentified bright objects (UBO) (22–24). According to a recent report (25), there are two populations of NF1 patients, 1) with areas of increased T2 signal intensity (UBO+) and 2) without areas of increased T2 signal intensity (UBO−). These authors also hypothesized that frequent occurrence of SLD in NF1 patients may be related to UBO. The cellular and molecular changes resulting in brain abnormalities in NF1 are unknown. The only available study of NF1 brain described Nissl staining of the cerebral cortex. NF1 brains had increased incidence of abnormal cortical lamination, heterotopic neurons in cortical white and gray matter and glial nodules that most closely resembled astrocytes (26). The contribution of neuronal and/or glial abnormalities to CNS manifestations of NF1 is not known.

The NF1 gene on human chromosome 17 has been cloned, the NF1 cDNA has been sequenced (27–29) and the protein product, neurofibromin, has been partially characterized. Neurofibromin is a 220 kD protein containing a central domain of approximately 300 amino acids that shares homology to the mammalian GTPase activating protein (GAP) and to the yeast homologs, IRA1 and IRA2 (30, 31). These proteins bind the intracellular signaling molecule ras and stimulate its intrinsic GTPase activity (32–34). In vitro, ras has been shown to induce growth and/or differentiation in a number of cell types, including neurons (35–39). It has been proposed that neurofibromin may be important in the regulation of these mechanisms. Neurofibromin has been reported to stimulate the endogenous GTPase activity of both N-ras (40) and H-ras in vitro (41) while microinjection of the GAP-related domain (GRD) can restore the ability to proliferate in mitotic-deficient yeast strains, which lack IRA proteins (41, 42).

Neurofibromin is present at 10 times higher levels in the adult nervous system than in other adult rodent tissues (43–46). We reported earlier (47) that in adult rat, neurofibromin is predominantly expressed in the cell bodies and dendrites of large neurons which project their axons long distances. Ultrastructural analysis of neurofibromin distribution in Purkinje cells revealed that neurofibromin is localized to the smooth endoplasmic reticulum (47). Neurofibromin is also expressed by oligodendrocytes but is not detectable in astrocytes (43). The distribution of
neurofibromin in the human spinal cord is similar to that observed in the rat (43), but the distribution in the human brain has not been studied.

In this paper, we demonstrate the distribution of neurofibromin in cortex and cerebellum of the adult normal and NF1 brains as well as in mice heterozygous at the NF1 locus. We report the presence of profound astrocytic gliosis in three NF1 brains; astrogliosis is the first cellular abnormality reported in NF1 brains.

MATERIALS AND METHODS

Brain Tissue

Brains from NF1 Patients: Three NF1 brains were obtained from patients diagnosed with NF1 based on NIH guidelines; two had a family history of NF1. None of these patients showed any evidence of neurological disease other than NF1.

Patient 1 was a 37 year old male who died from neurofibrosarcoma with rhabdomyoblastic differentiation (malignant triton tumor) affecting bladder, lungs, liver, colon, prostate and kidney. He had a family history of NF1 and SLD in childhood (school performance problems). This patient had multiple extensive neurofibromas, multiple small lumbar nerve root masses, extensive bronchopneumonia and one non-functioning kidney. He was treated once by chemotherapy (Hosfamidine for 3 days) 3 months before death. The autopsy report documented no gross lesions, but hydropneumatic kidney and triton tumor of lung, liver and colon. The postmortem interval for this patient was 3.5 hours.

Patient 2 was a 31 year old male who died of paraspinal retroperitoneal neurofibrosarcoma in the left cerebellar hemisphere and brainstem. He had been given three cycles of chemotherapy (Hosfamidine 2 g/day for 4 days) and four cycles of radiotherapy. He had multiple café au lait macules, cutaneous neurofibromas and low thoracic dystrophic scoliosis. He had undergone occipital craniotomy with associated mass lesions. At autopsy he had lungs with innumerable metastases, a hypertrophic heart and one kidney showed radiotherapy effect. Postmortem interval for this patient was 6 hours.

Patient 3 was a 30 year old female who died of occlusion of esophagus and trachea, resulting from a paravertebral neurofibrosarcoma. The patient had a family history of NF1. She had been diagnosed for malignant neoplasm of bronchus and lung, secondary neoplasm of bone marrow and bone, ascites dyschromia, anemia, abnormal weight loss, large masses on the spine, extensive erosion of bone, hepatosplenomegaly, adenopathy, soft tissue nodule in lung parenchyma, compression of one kidney, retroperitoneal mass and pneumonia. She had learning disabilities and delayed motor skills in childhood (was unable to walk until the age of 2 and could not run until 6 years). Neither chemotherapy nor radiation therapy were given to the patient. We do not have an autopsy report on this patient. Postmortem interval for this patient was 3.5 hours.

Control Brains: In total we studied three fixed and three frozen control brains. Fixed specimens were obtained from a 36 year old female who died of ligature strangulation, a 38 year old female who died of drug overdose and a 68 year old male who died of self-inflicted injuries. Though this patient had a history of depression, no other known neurological disease was diagnosed. No fresh frozen tissue was available from these three patients. None of these patients had any history of neurological disease. The brains from these patients were harvested within 8 hours after death.

Two frozen control brains were harvested 12-14 hours postmortem. One brain was from a 65 year old female who died of lung cancer; the second patient was a 59 year old male who died of myocardial infarction. A third patient was a 67 year old female who died of anterior myocardial infarction and ventricular tachycardia. This brain was harvested 23 hours postmortem. None of these brain tissues were fixed at the time of harvest.

Brains from Heterozygote and Wild-type NF1 Transgenic Mice: Eight month old male heterozygotes (n = 3) and wild-type (n = 3) NF1 C57BL6 transgenic mice were used (48). Mice were genotyped by polymerase chain reaction (PCR) as described by Brannan et al (48).

Tissue Preparation

Human Brains: Right and left hemispheres were separated from NF1 brains. One hemisphere was quickly dissected into 8 cm3 cubes, frozen in liquid nitrogen and then stored at −80°C. The second hemisphere was sliced in 1-2 cm slabs and placed in 4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4, for 24 hours. The slabs were then placed in fresh fixative for an additional 48 hours before the slices were cut into 4 cm3 blocks. Alternate blocks of tissue were labeled and prepared for either paraffin embedding or for frozen storage. Cubes prepared for frozen storage were incubated through two more changes of fixative and cryoprotected by 24 hour incubations in 10, 20 and 30% glycerol in PB containing 2% DMSO. After equilibration in the 30% glycerol cryoprotectant, the blocks were individually frozen at −80°C.

Fixed control brains (n = 2) were placed in 10% formaldehyde within 6-8 hours of death and then stored at 4°C for 2 years. After we received these brains, they were incubated in 20% sucrose in PB for cryoprotection. Brain samples from the third control patient were harvested within 18 hours after death and placed in 4% paraformaldehyde in PB and prepared for frozen storage as described for NF1 brains.

Frozen control brains (n = 3) dissected into 8 cm3 cubes were frozen in liquid nitrogen and stored at −80°C.

Mice Brains: Heterozygote and wild-type NF1 transgenic mice were perfused with 4% paraformaldehyde, postfixed in the same fixative and then incubated in 20% sucrose in PB for cryoprotection. Cortex and cerebellum from these brains were analyzed for neurofibromin staining.

Antibodies

Neurofibromin: Previously characterized polyclonal antibodies (43, 47) raised against two different domains of neurofibromin, PC 772-1085 and PC 2435-2745, were used at 1-3 µg/ml to study the expression of neurofibromin in the cortex and cerebellum of human and mouse brains.

Gial Filibrillary Acidic Protein: Monoclonal (mouse, Sigma Catalog #G-3893) and polyclonal (rabbit, Dako-Catalog #Z334) antibodies to gial fibrillary acidic protein (GFAP) were used. Monoclonal antibody was used at a dilution of 1:1,000 for both immunohistochemistry on the human material and for Western
analysis of the human tissue extracts. In addition, polyclonal antibody to GFAP was used at a dilution of 1:20,000–1:100,000 for immunohistochemical study of human brains.

* Tubulin: Monoclonal antibody (mouse, Amersham-Catalog #N357) was used at a dilution of 1:2,500 for Western blotting.

** Immunocytochemistry**

Free-floating frozen sections from human and mouse brains were processed for immunostaining as previously described (47) using antibodies recognizing GFAP and neurofilament.

*** Western Analysis of GFAP Levels***

Tissue extracts of cortex and cerebellum, under conditions known to dissociate cytoskeletal proteins from NF1 and human control brains, were prepared by homogenizing tissue in 8 M urea in 0.1 M Tris, pH 8.0 (3 g/10 ml buffer). Extracts were incubated on ice for 30 minutes and the protein content was analyzed using the method of Markwell et al (49). Aliquots containing 0.3, 1, 3, and 10 μg of protein were diluted in Laemmli buffer and loaded onto 12% polyacrylamide gels. Electrophoresis and Western blot analysis were performed as previously described (43).

** Cupric Silver Degeneration Staining**

Blocks of tissue from cortex, cerebellum, thalamus, pons and medulla from two NF1 brains were equilibrated in 4% paraformaldehyde in 0.067 M cacodylate buffer, pH 7.4. The samples were embedded in gelatin and 35 μm sections were prepared as previously described (50). The cupric silver (CuAgt) degeneration reaction was performed according to de Olmos et al (51).

** RESULTS**

** Expression of Neurofibromin in Control and NF1 Brains**

To determine the distribution of neurofibromin in control and NF1 human brains, sections of cortex and cerebellum were immunostained with anti-neurofibromin. These regions of the brain were selected as they show the most prominent neurofibromin immunoreactivity in rat brain. Though both polyclonal antibodies effectively stained neurofibromin in the human, the best results were obtained with PC 2435–2745 and are demonstrated in Figure 1. High magnification views of sections of cortex from a control (Fig. 1A) and an NF1 (Fig. 1C) brain stained with PC 2435–2745 demonstrate that pyramidal neurons are intensely labeled; most if not all of the interneurons appear to remain unstained. Pyramidal cell staining in both control and NF1 brains was characterized by intense immunoreactivity in cell bodies and the apical dendrites. Basal dendrites also showed some staining while axonal labeling was visible only occasionally. The intensity of staining was roughly similar in control and NF1 brains. As described previously (43, 44, 47), use of preimmune sera produced only faint and nonspecific staining and preincubation of the antibodies with the appropriate fusion protein or full length neurofibromin (obtained from G. Bollag & F. McCormick) abolished staining (not shown).

Neurofibromin staining in human control (Fig. 1B) and NF1 (Fig. 1D) cerebellum was most prominent in Purkinje cells, where staining was localized predominantly to the soma and proximal dendrites. Other neuronal types, such as granule, basket and stellate cells, did not show any staining. However, we cannot exclude the possibility that these interneurons are weakly stained due to the relatively high background in these immunohistochemical experiments on human cerebellar (control and NF1) tissues. These data are consistent with the pattern of neurofibromin staining in the rat cortex and cerebellum (47).

In two of the control brains and all NF1 human brains, neurofibromin-positive cells were observed in the cortex in both the gray and white matter and had small cell bodies (approximately 25 μm) with multiple long processes and could be traced a few hundred micrometers (Fig. 2). Cell processes displayed multiple varicosities and coursed at oblique angles throughout the cortex. Similar cells were not detected in the cerebellum. The morphology of these cells corresponds to that of dystrophic neurons (52). Similar cells were never detected in rodent brains.

** Expression of Neurofibromin in Heterozygote and Wild-type NF1 Transgenic Mice**

To compare the distribution of neurofibromin in human brain and in a mouse model of NF1, cortex and cerebellum of wild-type and heterozygous mice were fixed and processed for immunostaining for neurofibromin. In the cortex, neurofibromin staining was detected in the apical dendrites and cell soma of pyramidal cells in both wild-type (Fig. 1E) and heterozygote (Fig. 1G) mice. Intense neurofibromin staining was also visualized in the apical dendrites and cell bodies of the Purkinje cells of wild-type (Fig. 1F) and heterozygote (Fig. 1H) cerebellum. In addition, axonal and cell staining was also observed in these mice. In contrast to the human tissue, the cortex and cerebellum from heterozygote NF1 mice demonstrated decreased intensity of immunostaining for neurofibromin compared to the immunostaining in the same regions of wild-type animals.

** Expression of Neurofibromin in Gila**

Prominent neurofibromin immunoreactivity has previously been reported in oligodendrocytes of the adult rat and human spinal cord (43). The present study also detected neurofibromin-positive immunostaining in oligodendrocytes in human brain (not shown). There was no staining of neurofibromin in astrocytes, microglia or endothelial cells in the cortex or cerebellum.
Fig. 1. Neurofibromin in the cortex and cerebellum from control and NFI human brains and from heterozygote and wild-type NFI transgenic mice: Photomicrographs from control (A) and NFI (C) human cerebral cortex demonstrate that neurofibromin is localized to the cell bodies and apical and basal dendrites of pyramidal cells. Similarly, neurofibromin staining is enriched in the apical dendrites and cell bodies of Purkinje cells in the cerebral cortex from control (B) and NFI (D) brains. These panels demonstrate the similarity of neurofibromin staining intensity and distribution of neurofibromin in these regions from control and NFI individuals. In both wild-type (E) and heterozygote (G) mouse cortex neurofibromin is enriched in the apical dendrites of pyramidal cells; some cell body staining is also present. In the cerebellum, apical dendrites and cell bodies of Purkinje cells are intensely stained from wild-type (F) and heterozygote (H) animals. In addition, axonal staining and some granule cell staining are observed in the mice. In contrast to the human tissue, the cortex and cerebellum from heterozygote NFI mice demonstrate decreased staining intensity compared to the same regions from wild-type animals. Bar = 20 µm.
GFAP Expression in NF1 Brains

GFAP, a marker for astrocytes, was immunohistochemically localized in human control and NF1 brains. Frontal, temporal and occipital cortex and cerebellum from NF1 and control brains were processed for immunostaining using mouse monoclonal anti-GFAP at a dilution of 1:1,000 and rabbit polyclonal anti-GFAP at a dilution of 1:20,000. Both antibodies gave similar results except that sections stained with monoclonal anti-GFAP had some background staining while sections reacted with the polyclonal antibody did not. GFAP-expressing cells were present in the gray and white matter of control cortex (Fig. 3A, C), however, an intense network of astrocytes was stained in NF1 brains (Fig. 3B, D). Increased GFAP staining was visualized throughout the gray and white matter of all three NF1 brains compared to the three control brains. GFAP staining in both control and NF1 cortex was most highly enriched underlying the cortical plate (Fig. 3A, B, E, F) and around the ependymal zone (Fig. 3C, D, G, H) of the ventricle. The morphology of GFAP-labeled astrocytes in the cortex of human control and NF1 brains appeared similar (Fig. 3E-H). Overstaining of control tissue neither revealed additional staining of astrocytes nor did it significantly alter the intensity of staining, suggesting that more GFAP was present in NF1 brains than in the control brains.

The numbers of GFAP-labeled cells/field from the gray and white matter of the cortex of one of the control and one of NF1 brains were counted (Fig. 4). Three fields per section were counted and three sections from control and NF1 brains were taken into consideration. Mean number of cells/field for gray and white matter from control and NF1 brains are graphically represented in Figure 4; 40% more GFAP-expressing cells were present in this NF1 brain as compared to the control processed at the same time. The mean numbers of cells per field analyzed were 280 (control brain) and 392 (NF1 brain) cells for white matter and 80 (control brain) and 112 (NF1 brain) cells for gray matter. Thus, the increase in number of GFAP-expressing cells in this NF1 brain was apparent in both gray and white matter of the cortex. The unpaired t-test was performed for these values; the increase in number of GFAP cells in NF1 brain was significant with p = 0.03 for white matter and p = 0.02 for gray matter.

To determine whether hypertrophy of GFAP-labeled astrocytes occurs in NF1 brains, the mean area of GFAP-
immunoreactive cells was also measured (Fig. 5) from the same control and NFI brains used for cell counts. These measurements were carried out on 50 cells each in white and gray matter from control and NFI brain. GFAP-expressing cells from the cortex of the NFI brain were increased in size in gray matter (19%); increase in white matter was 31% as compared to astrocytes in control brain. Unpaired t-test showed that in white matter the increase was significant with p = 0.0001. In the gray matter the increase in the cell area of astrocytes gave a p value of 0.005.

To determine whether the amount of GFAP per astrocyte was increased in NFI brains, we reduced the concentration of the polyclonal anti-GFAP antibody to 1:100,000 as described by Oh et al. (53). At this dilution, GFAP-immunoreactive cells in the control brain were mostly undetectable (Fig. 6A, C). In contrast, all NFI brains showed a large number of intensely labeled cells (Fig. 6B, D). The gray matter of the control brain showed very few GFAP-immunoreactive cells; both the number and intensity of reaction was increased in NFI brains (Fig. 6A, B). White matter of the control did not show even a single stained cell in contrast to the NFI brain which was robustly stained (Fig. 6C, D). These data suggest that the amount of GFAP/astrocyte is likely to be increased in NFI; increase in cell number and size also appear to contribute to increased GFAP expression.

Quantification of GFAP Levels in NFI and Control Brains

To quantitate the difference in GFAP levels between control and NFI brains we prepared tissue extracts under conditions known to dissociate cytoskeletal proteins of control (n = 3) and NFI (n = 3) frontal cortex and cerebellum (n = 2). Varying amounts of protein were loaded onto gels and GFAP and β-tubulin were visualized using Western analysis. Blots stained for GFAP or tubulin were scanned and the density of the bands was compared in control and NFI extracts. Both controls and NFI blots showed multiple bands after GFAP staining (Fig. 7); similar multiple bands stained with GFAP were shown by Griffin et al. (54) in control and Alzheimer brain. All the bands from each lane were integrated into one for measuring signal density. Three blots were quantified by densitometry from each control and NFI brain sample; mean values were calculated for each control and NFI blot and are graphically presented in Figure 8. All three NFI brains contained much higher concentrations of GFAP than normal human controls. The concentration of GFAP differed among the three NFI brains studied. All three controls showed similar concentrations of GFAP that were 4–18-fold lower than that in NFI samples (Fig. 8). In contrast, tubulin concentration in control and NFI brains differed no more than twofold between normal and NFI extracts (not shown). The NFI brain which showed maximum concentration of GFAP was used in detail for analysis of cell number and cell area discussed above and presented in Figures 4 and 5.

Neuronal Degeneration

As increased GFAP is often correlated with neuronal degeneration (55), tissue from the two NFI brains with maximally increased GFAP were evaluated for evidence of neurodegeneration using the CuAg staining method (51). Degenerative debris was observed predominantly in subcortical white matter in both the cerebral and cerebellar cortex in one of these brains. Figure 9A demonstrates argyrophilia in the form of black precipitate in  

cortical white matter of the occipital cortex. Little or no degenerative debris was observed in white matter tracts coursing through the thalamus (Fig. 9B), demonstrating that degeneration was region-specific. However, in the second NF1 brain, no degeneration was evident.

**DISCUSSION**

**Neurofibromin Expression in Normal Human and Control Mice Brains**

In this study, we have demonstrated that the pattern of neurofibromin expression in human and mouse cortex and cerebellum is similar to that observed in the rat (47). Neurofibromin is enriched in large projection neurons, and within the neurons it has been localized in the dendrites and cell bodies. Oligodendrocytes are moderately stained, but microglia, astrocytes and endothelial cells did not show any staining. The distribution of neurofibromin in the spinal cord was also reported to be similar in rat and human (43). One of the striking features of NF1 is the high incidence of SLD. Disruption of the NF1 gene in mice revealed that these animals do not develop features of human NF1 such as café au lait macules or neu-
Fig. 7. GFAP levels are elevated in NF1 brains: GFAP levels were quantified with Western analysis of tissue extracts prepared from control (A) and NF1 (B) cerebral cortex. Western blots were immunostained with monoclonal anti-GFAP at a dilution of 1:10,000. Compare the intensity of the signal for GFAP in NF1 (B) and control (A). Lane 1 0.3 μg, Lane 2 1 μg, Lane 3 3 μg and Lane 4 10 μg protein loaded onto polyacrylamide gels.

Fig. 8. Expression of GFAP in human controls and NF1 brains: Graphic representation of the mean signal intensity from Western blots, stained for GFAP, from three control and three NF1 patients (NF1-A, NF1-B and NF1-C). There is a difference in the magnitude of the increase in GFAP among the three NF1 patients. Note that the difference in concentration of GFAP between control and NF1 is higher when the protein loaded was less as the signal begins to saturate.

Neurofibromin Expression in NF1 Brains

The intensity of neurofibromin immunoreactivity was similar in normal human brain and in NF1 brains. NF1 patients might have mutations that allow the production of non-functional forms of neurofibromin. As we do not know what type of mutations the patients in our study possessed, we cannot rule out the possibility that some of the detectable neurofibromin in these patients is non-functional. We also did not find any alterations in neurofibromin level in the skin in 9/10 NF1 patients using immunohistochemistry (58). Our immunocytochemical and Western blot analyses showed a reduction of neurofibromin in heterozygous mice in which the NF1 gene has been knocked out by homologous recombination (48). It remains possible that in human CNS, which contains less neurofibromin than the rodent (43), our techniques are not sensitive enough to detect a predicted maximal twofold change in neurofibromin expression.

Our data clearly demonstrate that gross abnormalities in the levels or distribution of neurofibromin are not present in NF1 patients, at least in cortex and cerebellum. This was true even though one patient had severe learning disabilities and demonstrated significantly delayed acquisition of motor skills (was unable to walk until the age of 2 and could not run until 6 years), and a second patient demonstrated significant school performance problems.

Elevated Neurofibromin Expression in a Subset of Neurons

Some intensely neurofibromin-immunoreactive cells were observed in the gray and white matter of the cerebral cortex in both control and NF1 brains. The significance of these cells, tentatively defined as neurons based solely on their long processes, is unclear. These cells may represent the "heterotopic neurons" observed by Rosman and Pearce (26) in the gray and white matter of NF1 and control patients. Heterotopic neurons were detected in the white matter of ten NF1 brains studied and in two of five control brains, suggesting that these neurons are more common in NF1 patients (26). Dys trophy neurons with similar morphology to those shown here have been reported in Alzheimer's disease (52). These cells may represent degenerating neurons. We observed dystrophic neurons in all three NF1 brains and in two of three control brains. Because of the limited number of brains in our study, we have not addressed the relative frequency of these neurons in NF1 as compared to control brains.

Gliosis in NF1 Brains

Reactive gliosis (astrogliosis) is a phenomenon which has been reported in many neurodegenerative diseases in-
including Alzheimer's disease, schizophrenia, Down syndrome, amyotrophic lateral sclerosis and Parkinson disease (59–65), and results from astrocyte proliferation and/or astrocytic hypertrophy with increased expression of GFAP (66–70). Levels of GFAP varied among the three brains; we do not know if all NF1 brains will show astrogliosis. However, all three NF1 brains evaluated contained significantly elevated GFAP levels as shown by both immunohistochemistry and by Western analysis, giving strong evidence of gliosis. Two of the three patients studied had undergone chemotherapy. We cannot rule out a contribution of the treatment to GFAP elevation. However, the third patient never received either chemotherapy or radiotherapy, and in that brain increase in GFAP was highest. Therefore, astrogliosis can occur in NF1 patients regardless of therapeutic intervention.

It is probable that both astrocytic proliferation and increased synthesis of GFAP contribute to the increased GFAP levels in NF1 brains. The increase in the number of GFAP-labeled astrocytes in white and gray matter of NF1 brain is 40%, less than the twofold increase in Down syndrome and Alzheimer's disease (54). The increase in astrocyte size measured in Down syndrome and Alzheimer's disease was 320% (54), much more than the increase measured in NF1 brain (31% in white matter and 19% in gray matter). Our data likely represent a maximal size increase, as the NF1 brain analyzed for cell counting and measurement of cell area of GFAP-positive astrocytes showed maximal GFAP concentration as assessed by Western blotting. Therefore, it is likely that increases in cell number and cell size contribute less to the overall increase in GFAP levels than does an increase in GFAP per astrocyte. The intensity of GFAP immunoreactivity was always most prominent in the outer boundary of the cortex and around the ventricle (in controls and NF1 brains) but the increase in intensity was pronounced in these regions and throughout the NF1 cortex, consistent with previous findings in Down syndrome and Alzheimer's disease (62).

GFAP levels are increased in response to CNS injuries such as axotomies, stab wounds, pharmaceutical insults, transplantation and disease (70–74), suggesting that observed gliosis might be secondary to neuronal degeneration. Brains from patients with neurodegenerative diseases such as Alzheimer's, Down syndrome, and amyotrophic lateral sclerosis (59–62) all show in-
creased GFAP levels, particularly in regions of extensive neuronal degeneration. Therefore, two NFI1 brains were studied for neuronal degeneration using the CuAg staining reaction; the CuAg stain impregnates degenerating cell bodies, axons and dendrites (51). We observed degeneration in only one NFI1 brain, and only at restricted sites. Degeneration in this brain might be secondary to trauma induced by paraspinal tumors and cutaneous neurofibromas. Secondary degeneration is consistent with the lack of degeneration observed in a second NFI1 brain in which GFAP levels were high. However, it remains possible that mutations in the NFI1 gene induce neuronal degeneration that is transient and undetected by CuAg staining, and secondarily, astrocytic gliosis. Astrocytes are unstrained by anti-neurofibromin antibodies and thus contain little or no neurofibromin. It therefore seems unlikely that aberrant neurofibromin expression directly results in astrocytic activation.

Recently, Kaplan et al (75) detected abnormal focal areas of high signal intensity (FASI) on T2-weighted images in 60–70% of children with NFI1. Positron emission tomography (PET) scans of these patients obtained using fluorodeoxyglucose (FDG) showed that all large FASI lesions were metabolically inactive. In addition, visual inspection of PET images also showed inhomogeneity in cortical distribution. These authors showed a relationship between learning disability and UBO (22–24) and suggested that 1) children with NFI1 associated with FASI are at a greater risk for learning disability and 2) disturbances in FDG cortical metabolism may be predictive of intellectual deficits in these NFI1 patients. Astrogliosis in NFI1 brains could be related to learning disabilities in NFI1 patients. Although mice with targeted mutations at the NFI1 locus do not develop features of human NFI1 such as café au lait macules or neurofibromas (48, 74), Bourtchuladze et al (57) have demonstrated that, similar to humans, these mutant mice have subtle but significant learning disabilities.

ACKNOWLEDGMENTS

We thank Dr. R. C. Switzer, III, Neuroscience Associates, Knoxville, TN, for silver staining of NFI1 human brains, and Dr. Michael T. Shipley for many helpful discussions and for suggesting the dilution for the antibody for GFAP. We thank the National Neurofibromatosis Foundation for providing liaison with the NFI1 patients who generously donated their bodies for this investigation and to the staff of the hospitals who carried out the autopsies. We particularly thank Dr. Vincent Riccardi for carrying out one autopsy and Dr. J. Bonner and Ms. S. Ruscio for coordinating acquisition of other human samples.

REFERENCES

28. Visschot D, Buchberger AM, Xu G, et al. Deletions and a translo-
73. O’Callaghan JP. Quantification of glial fibrillary acidic protein: Comparison of slot immunoassays with a novel sandwich ELISA. Neurotoxicol Teratol 1991a;13:275–81


Received December 9, 1994
Revisions received January 19 and March 9, 1995
Accepted March 10, 1995