

The humoral response in the pathogenesis of gluten ataxia

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Abstract—Objective: To characterize humoral response to cerebellum in patients with gluten ataxia. **Background:** Gluten ataxia is a common neurologic manifestation of gluten sensitivity. **Methods:** The authors assessed the reactivity of sera from patients with gluten ataxia (13), newly diagnosed patients with celiac disease without neurologic dysfunction (24), patients with other causes of cerebellar degeneration (11), and healthy control subjects (17) using indirect immunocytochemistry on human cerebellar and rat CNS tissue. Cross-reactivity of a commercial IgG antigliadin antibody with human cerebellar tissue also was studied. **Results:** Sera from 12 of 13 patients with gluten ataxia stained Purkinje cells strongly. Less intense staining was seen in some but not all sera from patients with newly diagnosed celiac disease without neurologic dysfunction. At high dilutions (1:800) staining was seen only with sera from patients with gluten ataxia but not in control subjects. Sera from patients with gluten ataxia also stained some brainstem and cortical neurons in rat CNS tissue. Commercial anti-gliadin antibody stained human Purkinje cells in a similar manner. Adsorption of the antigliadin antibodies using crude gliadin abolished the staining in patients with celiac disease without neurologic dysfunction, but not in those with gluten ataxia. **Conclusions:** Patients with gluten ataxia have antibodies against Purkinje cells. Antigliadin antibodies cross-react with epitopes on Purkinje cells.

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Gluten sensitivity is an immune-mediated disease triggered by the ingestion of gluten in genetically susceptible individuals.¹ Genetic susceptibility in gluten sensitivity is apparent by the fact that up to 90% of patients with celiac disease (CD) express the HLA class II molecules DQ2. The remaining 10% express HLA DQ8.

Gastrointestinal symptoms because of involvement of the small bowel (CD or gluten sensitive enteropathy) are a common presenting feature. However clinical manifestations can be diverse, for example, the pruritic vesicular rash of dermatitis herpetiformis.² Neurologic manifestations were first reported in 16 patients with established CD who had gait ataxia, with involvement of other areas of the central and peripheral nervous systems.³ We have suggested that gluten sensitivity can be presented solely with neurologic dysfunction,⁴ with ataxia (gluten ataxia) being the most frequent presentation.⁵ Up to 90% of patients with dermatitis herpetiformis and 33% of patients presenting with neurologic dysfunction associated with gluten sensitivity also have CD.⁴ The remaining patients have no histologic evidence of small bowel involvement but have serologic markers (serum anti-gliadin antibodies) and genetic susceptibility (HLA DQ2) consistent with gluten sensitivity.

Based on a large epidemiologic study (>200 patients), gluten ataxia was found to account for 40% of cases with idiopathic sporadic cerebellar degeneration.⁶ This figure was lower in two smaller studies.^{7,8} Another study⁹ found similarly high prevalence, but in both sporadic and familial ataxias. The number of patients (26 sporadic and 24 familial) was too small to derive any meaningful conclusions about the prevalence of gluten ataxia among familial ataxias. A larger study (117 sporadic and 55 familial)⁶ found the prevalence of gluten sensitivity among familial ataxias to be the same as healthy control subjects. Possible reasons for these differences include that the prevalence of antigliadin antibodies in the population varies depending on the antigliadin assays used (5%⁸ to 13%⁶) and that there is a possible geographic variability in the prevalence of gluten ataxia as there is in the prevalence of CD.

Neuropathologic findings found in patients with gluten ataxia when autopsied showed perivascular cuffing with inflammatory cells, predominantly affecting the cerebellum, and resulting in loss of Purkinje cells implying that the neurologic insult may be immune mediated. It is yet unknown whether such immune-mediated damage is primarily cellular or antibody driven. This article presents a study of the

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Table Summary of immunocytochemistry results using human and rat cerebellum

Tissue substrate	Group sera (n)	Dilutions and percentage of strong positive staining					
		1:200	1:400	1:800	1:100	1:300	1:600
Human cerebellum	Gluten ataxia (5)	100%	100%	60%			
	Celiac disease no neurology (5)	40%	40%	0%			
	Other ataxias (5)	20%	0%	0%			
	Healthy control subjects (5)	20%	0%	0%			
Rat cerebellum	Gluten ataxia (10)				90%	90%	90%
	Celiac disease no neurology (19)				26%	26%	0%
	Other ataxias (6)				0%	0%	0%
	Normal control subjects (12)				0%	0%	0%

humoral response that may be implicated in such damage.

Methods. *Patient and control sera.* This was a dual center study carried out in Sheffield, UK, and Trieste, Italy. Sera from gluten ataxia and ataxia control patients were collected in Sheffield and used by both groups. Sera from healthy control subjects and patients with CD without neurologic dysfunction were collected at each center after informed consent was obtained. The South Sheffield Research Ethics Committee approved the study protocol. Sera were collected from 13 patients upon diagnosis with gluten ataxia (defined by the presence of IgG antigliadin antibodies and the absence of any other cause of ataxia including paraneoplastic cerebellar degeneration) attending the gluten sensitivity/neurology clinic at the Department of Clinical Neurology, The Royal Hallamshire Hospital, Sheffield. Sera from 24 patients with newly diagnosed CD but no evidence of neurologic dysfunction and 11 patients with other causes of cerebellar degeneration (2 Friedreich ataxia, 5 autosomal dominant ataxia, 3 sporadic ataxia, 1 ataxia postcerebellitis) were used as disease control patients. Finally, samples from 17 healthy individuals were used as healthy control subjects. All samples were stored at -70°C immediately after collection.

CNS tissue. Immunocytochemistry using human cerebellum was performed in Sheffield, UK. Immunocytochemistry using rat cerebellum was performed in Trieste, Italy. Human cerebellar tissue from a patient with motor neuron disease was obtained at autopsy after consent from relatives. The tissue was snap-frozen in liquid nitrogen and stored in an airtight container at -70°C . It was then sectioned in a cryostat (Bright Instruments, UK), and $10\text{-}\mu\text{m}$ -thick sections were collected on Polysine-coated slides (BDH, UK).

Rat CNS tissue was obtained from adult Sprague-Dawley rats. Procedures involving animals and their husbandry were performed in accordance with European Community directives. Rat brains were fresh-frozen on dry ice, sectioned in a cryostat (Leica), and $10\text{-}\mu\text{m}$ sagittal sections were collected on gelatin-coated slides (Sigma, UK). The slides were dried overnight at room temperature and stored at -80°C until required.

Immunocytochemistry on rat and human CNS tissue. On human CNS tissue, sera from 5 patients from each group were used at dilutions of 1:100, 1:200, 1:400, 1:800,

1:1,600, and 1:3,200. Anti-calbindin D28k antibody (Sigma) diluted 1:200 with phosphate buffered saline/0.05% Tween 20 (PBST) was used as a positive control for Purkinje cell staining; negative controls included sections incubated without either serum or antihuman IgG secondary antibody.

On rat CNS tissue, sera from 11 patients with gluten ataxia, 19 patients with CD but no neurologic illness, 6 patients with other causes of ataxia, and 12 healthy control subjects were analyzed. All sera were used at dilutions of 1:100, 1:300, and 1:600. In addition, a subset of 3 sera from patients with gluten ataxia also was tested at 1:1,200 and 1:1,500.

CNS sections were warmed to room temperature and then washed in PBST for 10 minutes at room temperature. Sections were incubated for 1 hour at room temperature with either patient or healthy control sera. After incubation with sera, sections were washed in PBST and incubated for 30 minutes at room temperature with a horseradish peroxidase-conjugated goat anti-human IgG antibody (Jackson ImmunoResearch Laboratories) diluted 1:200 (for rat tissue) or 1:500 (for human tissue) with PBST. After a second wash in PBST, sections were developed for 20 minutes at room temperature using a peroxidase substrate solution of 0.4 mg/mL 3,3'-diaminobenzidine tetrahydrochloride (DAB) with 0.0135% hydrogen peroxide (Sigma). Sections were dehydrated in methanol, cleared in methanol/xylene (1:1) followed by xylene, and then mounted.

Semiquantitative evaluation of the signal intensity of the rat and human CNS sections was performed independently by two blinded observers. Weak or strong positive staining was recorded if Purkinje cell staining went above background levels, and negative staining was recorded if Purkinje cell staining did not exceed background levels. Patient and control samples were run simultaneously with a concordance rate of 75% between the observers.

Based on the optimal serum dilution for rat CNS tissue, 1:600, reactivity with other rat brain structures using sera from 10 patients with gluten ataxia (and also 6 patients with CD but no neurologic dysfunction and 12 healthy control subjects) was investigated using the above method.

Immunocytochemistry on human CNS tissue using anti-gliadin antibody and patient sera before and after adsorption with crude gliadin. Sera (four patients with gluten ataxia, three patients with CD without neurologic dysfunction, and two healthy control subjects) were diluted to

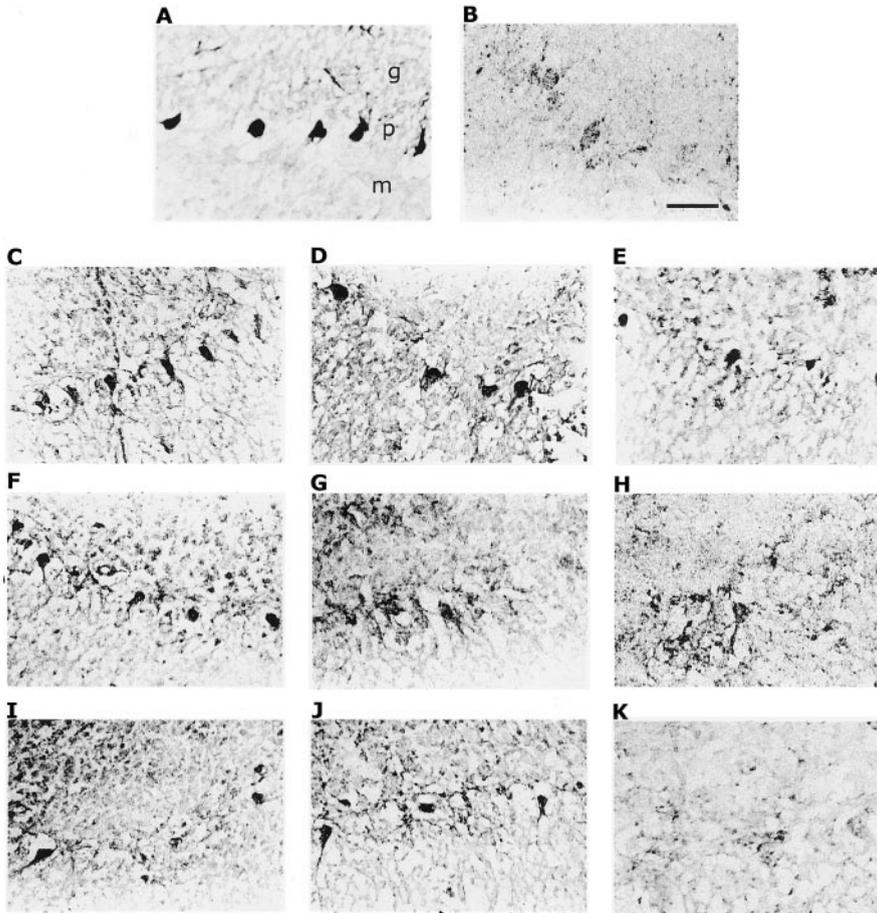


Figure 1. Immunohistochemical staining of human cerebellar Purkinje cells using anti-calbindin D_{28K} antibody (A, positive control), anti-human IgG only (B, negative control), and sera at different dilutions from a patient with gluten ataxia (C-E), a healthy blood donor (F-H), and a patient with celiac disease without neurologic dysfunction (I-K). Dilutions used were 1:200 (C,F,I), 1:400 (D,G,J) and 1:800 (E,H,K). Calibration bar = 65 μ m. g = granular layer; p = Purkinje cell layer; m = molecular layer.

1:200 and 1:800 in PBST or in a saturated crude gliadin (Sigma) solution (~20 mg/mL) made up in PBST. Commercial rabbit polyclonal peroxidase-conjugated predominantly IgG anti-gliadin antibody (Sigma) was diluted from 1:100 to 1:256,000 in either PBST or a saturated crude gliadin (Sigma) solution (~20 mg/mL) in PBST. Once diluted, sera and commercial anti-gliadin antibody were incubated for 1 hour at room temperature to allow adsorption of anti-gliadin antibodies by the crude gliadin. The effectiveness of blocking of anti-gliadin antibodies was tested by an anti-gliadin ELISA assay on the sera after adsorption. Immunocytochemistry was performed as above with adsorbed and nonadsorbed samples.

Results. *Characteristics of patients with gluten ataxia.* The mean age of the 13 patients with gluten ataxia was 61 years (range 46–79). The mean duration of the ataxia was 11 years (range 3–25). Ten patients had the HLA DQ2, one had DQ8, and two had DQ1. All patients were by definition positive for IgG anti-gliadin antibodies; four also had IgA anti-gliadin antibodies. Screening for antiendomysium (AEA) immunoreactivity and reaction with purified human tissue transglutaminase¹⁰ (tTG) revealed only one patient with gluten ataxia that was strongly positive for AEA and tTG. Duodenal biopsies revealed CD in five patients, results were normal in six patients, and the procedure was not performed in two patients.

Immunocytochemistry on human CNS tissue. Consistently strong staining of Purkinje cells was seen using anti-calbindin D_{28K} (figure 1A) as a positive control. Sec-

tions incubated either without primary or without secondary antibody showed consistently absent Purkinje cell staining (see figure 1B).

In the 5 patients with gluten ataxia, positive staining of Purkinje cells was seen at all dilutions up to 1:800 (see figure 1, C, D, and E) with strong staining up to 1:400. The staining persisted at higher dilutions in three patients. At dilutions of 1:800, sera from the control groups showed no staining (see figure 1, H and K). However, at dilutions of 1:200, staining was seen in 2 of 5 patients with CD but no neurologic illness (see figure 1I), in 1 of 5 patients with ataxia of a different cause, and in 1 of 5 healthy control subjects (see figure 1F).

Immunocytochemistry on rat CNS tissue. Sera from 12 of 13 patients with gluten ataxia showed strong staining of Purkinje cells at 1:100, 1:300, and 1:600 dilutions (figure 2, A through C), with marked staining particularly of cell somas and dendritic processes even at dilutions of 1:600. None of the subset of sera from three patients with gluten ataxia tested at 1:1,200 dilution showed any recognizable staining. In contrast, sera from 4 of 12 healthy control subjects showed only weak staining at 1:100 dilution (see figure 2D), with no further staining detected at higher dilutions (see figure 2, E and F). No staining was seen using serum from six control patients with ataxia. The sera from 5 of 19 patients with CD without neurologic dysfunction showed strong staining of Purkinje cells at 1:100 and 1:300 dilutions (see figure 2, G and H) but only weak staining at a dilution of 1:600 (see figure 2I). Weak staining was seen in nine patients and no staining was

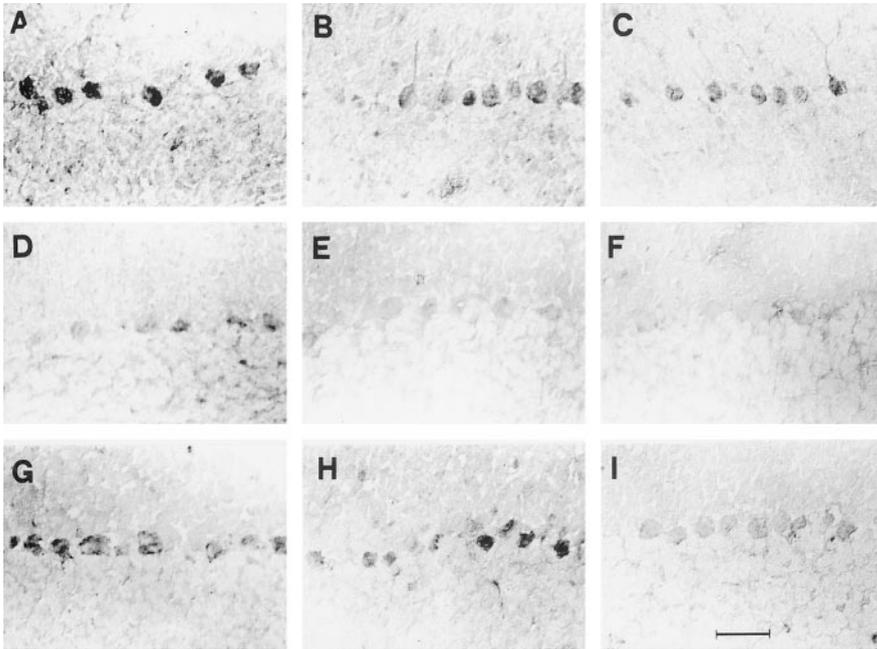


Figure 2. Immunohistochemical staining of rat cerebellar Purkinje cells using sera at different dilutions from a patient with gluten ataxia (A-C), a healthy blood donor (D-F), and a patient with celiac disease without neurologic dysfunction (G-I). Dilutions used were 1:100 (A,D,G), 1:300 (B,E,H) or 1:600 (C,F,I). Calibration bar = 65 μ m.

seen in six. Sera from 10 patients with gluten ataxia showed weak or absent staining of the striatum, corpus callosum, hippocampus, brain blood vessels, choroid plexus, and ependyma. The more consistently stained cells in the cortex were large stellate-like cells (not pyramidal neurons) in cortical layers IV and V (figure 3A) and small polymorphic cells in the layer VI (see figure 3B), especially in the frontal cortex. Large neurons of the deep cerebellar nuclei (see figure 3C) and neurons of the reticular formation and pontine gray in the brainstem (see figure 3D) also stained. Sera from two of six patients with CD but no neurologic illness showed staining of cortical layer VI but no staining of any other structures. The sera from healthy control subjects did not stain cells in any brain area except for one case with weak staining in cortical layer VI.

Reactivity of sera and anti-gliadin antibody with human cerebellar tissue after adsorption with crude gliadin. Commercial rabbit anti-gliadin antibody stained human cerebellar Purkinje cells, at an optimal dilution of 1:800, with a pattern identical to that seen using sera from patients with gluten ataxia.

Staining with commercial anti-gliadin antibody at a dilution of 1:1,600 (figure 4A) was abolished after adsorption of the antibody for 1 hour at room temperature with crude gliadin (see figure 4B). Comparative adsorption experiments were performed using sera (dilution 1:200) from patients with gluten ataxia (see figure 4, C and D) and patients with CD but no neurologic dysfunction (see figure 4, E and F). The staining persisted but was weaker even after adsorption with gliadin when using sera from patients with gluten ataxia. The staining was eliminated when using sera from patients with CD and no neurologic dysfunction. None of the postadsorption sera tested positive for the presence of antigliadin antibodies.

A summary of all immunocytochemistry results can be seen in the table.

Discussion. These results suggest that patients with gluten ataxia have circulating antibodies di-

rected against cerebellar Purkinje cells, with some reactivity seen with deep cerebellar nuclei brainstem and cortical neurons. Some weak staining with both rat and human cerebellum was seen using sera from other patient groups. At low dilutions some sera from normal control subjects also showed weak staining. Such staining of Purkinje cells at low dilutions is thought to be nonspecific and has been described before.¹¹ Strong and persistent staining at higher dilutions was only seen when using sera from patients with gluten ataxia. The absence of staining using sera from the group of patients with other causes of ataxia suggests that this is not just an epiphenomenon of Purkinje cell loss. However, the presence of similar although weaker staining with sera from only some patients with CD without neurologic dysfunction implies that these antibodies are not necessarily neurotoxic. The demonstration of reactivity of commercial IgG antigliadin antibody with human Purkinje cells may be the reason why sera from patients with CD but no neurologic dysfunction stain Purkinje cells. However, patients with gluten ataxia possess additional antibodies against Purkinje cells as demonstrated by the adsorption experiments. These antibodies are different to antigliadin antibodies and appear to be present exclusively in patients with gluten ataxia. Antigliadin antibodies are present in almost all patients with celiac disease with gastrointestinal symptoms. Yet only 6% to 10% of patients with established CD develop neurologic dysfunction. One hypothesis may be that antigliadin antibodies only become neurotoxic if they gain access to the CNS. Antigliadin antibodies have been found in the CSF in patients with gluten sensitivity and neurologic dysfunction.¹² The mechanism by which and reason why antigliadin antibodies may gain access to the CNS remains obscure. The presence of

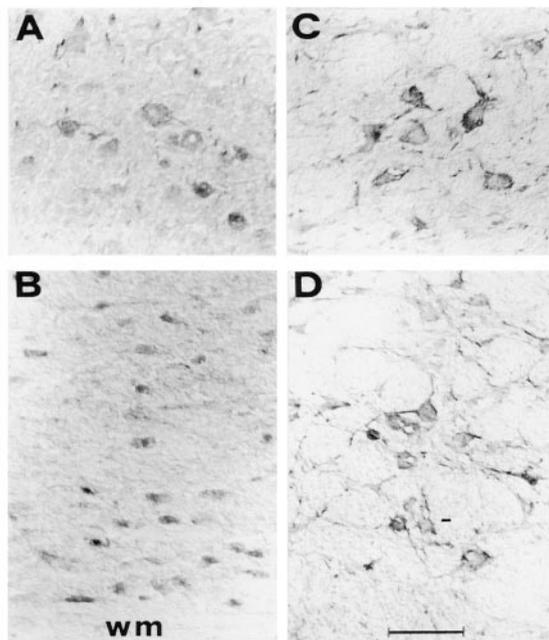
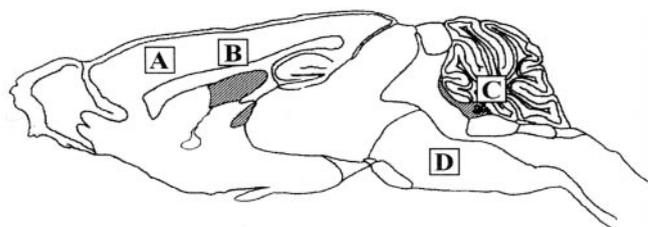


Figure 3. Immunohistochemical staining obtained using serum from a newly diagnosed untreated patient with gluten ataxia in different brain areas (dilution 1:600). Purkinje cell staining is shown in figure 2A. The approximate position of the high magnification photographs is indicated by the letters marked on the camera lucida drawing of a sagittal section of rat brain (top panel). In the cortex, only a subset of cells was stained, comprising principally horizontal or polymorphic cells in layers V (A) and VI (B). The deep cerebellar nuclei stained strongly at the level of neuronal cell somas and processes (C), as did the pontine gray nuclei (D). Calibration bar = 65 μ m. wm = white matter.

lymphocytic infiltration of the perivascular space and the neuropil by CD4 and CD8 T cells¹³ in cases of gluten ataxia suggests cell-mediated responses may also play a part. Cell-mediated inflammation may compromise the blood-brain barrier allowing for the entry of antigliadin antibodies into the CNS.

It remains unclear why some patients with gluten sensitivity present solely with neurologic dysfunction whereas others have gastrointestinal symptoms or a pruritic rash. Genetic susceptibility may have a small role. Although 70% of patients with gluten ataxia have the HLA DQ2 (found in 90% of patients with CD) and 10% have the HLA DQ8 (usually found in the remaining 10% of patients with CD), 20% have the HLA DQ1. HLA DQ1 has not yet been reported in association with celiac disease but may be an important difference in genetic susceptibility to gluten ataxia compared with CD.

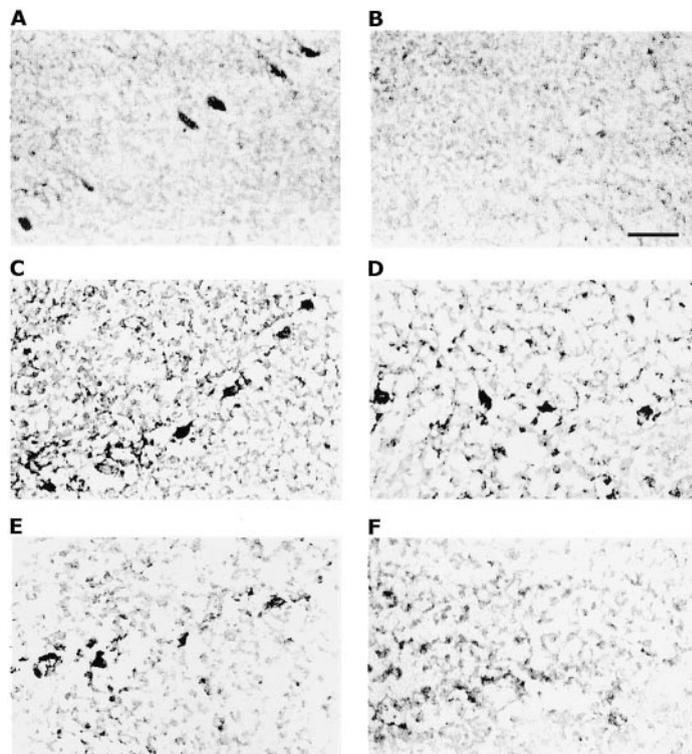


Figure 4. Immunohistochemical staining of human Purkinje cells using a commercial rabbit anti-gliadin antibody at a dilution of 1:1600 (A), serum from a patient with gluten ataxia at dilution 1:200 (C), and serum from a patient with celiac disease without neurologic dysfunction at dilution 1:200 (E), both before (A,C,E) and after adsorption (B,D,F) of antigliadin antibodies by incubation with crude gliadin. Calibration bar = 65 μ m.

The staining of Purkinje cells using sera from patients with gluten ataxia agrees with the neuropathologic findings from postmortem examination of patients with gluten ataxia.^{3,5} The most consistent finding is the loss of Purkinje cells. Although the cerebellum and in particular the Purkinje cells appear to be most susceptible to damage in patients with gluten ataxia, other areas of the brain are not spared. Our results demonstrate that sera from patients with gluten ataxia react with both brainstem and cortical neurones in rat CNS tissue. Such involvement has been reported both clinically and neuropathologically: patients with gluten ataxia may exhibit myoclonus (possibly cortical in origin¹⁴), dementia,¹⁵ and brainstem signs.¹⁶

The cross-reactivity of anti-gliadin antibodies with cerebellar Purkinje cells suggests that gliadin proteins and cerebellar Purkinje cells share common epitopes. Such common epitopes also have been demonstrated to exist between gliadin proteins and enterocytes.¹⁷ A recent study investigating human genome search in CD using gliadin cDNA as probe demonstrated that several genes have partial gliadin homology.¹⁸ The nature of the antigens recognized by sera from patients with gluten ataxia is still unknown. A specific large scale screening approach of

human brain expression libraries is envisaged to achieve the identification of antigens recognized by the gluten ataxia antibodies. Characterization of these antibodies by immunoblotting may provide a useful marker for the diagnosis of gluten ataxia in a manner analogous to the use of specific antibodies as markers of paraneoplastic cerebellar degeneration.

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