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Antibodies to Cerebellar Nerve Fibres in Two Patients with Paraneoplastic Cerebellar Ataxia

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Abstract. *The aim of this study was to characterize two new atypical anti-neuronal antibodies using an immunohistochemical method on rat cerebellum and Western blot techniques with primate cerebellar tissue and with recombinant neuronal proteins. Atypical sera from two patients with paraneoplastic neurological syndromes associated with different tumours were detected. Case number 1 presented cerebellar degeneration and Merkel cell carcinoma and case number 2 paraneoplastic brainstem encephalitis and malignant fibrous histiocytoma. By immunohistochemistry, the two new atypical antibodies showed a similar fibrillar positivity in the molecular and granular layers and around the Purkinje cells. The dot blot with recombinant neuronal proteins (HuD, NOVA-1, CDR62/Yo, Amphiphysin) was negative, whereas the Western blot with neuronal antigens of primate cerebellum identified two different proteins with molecular weights (64 kD in case number 1, and 70 kD in case number 2). In conclusion, the two new antibody reactivities against nerve fibres should be integrated into the diagnostic paraneoplastic neurological syndromes guidelines.*

In patients affected by paraneoplastic neurological syndromes (PNS) and by other autoimmune diseases of the central nervous system, the anti-neuronal-specific autoantibodies are recognized to be biological markers of tumours (1-4). Usually, these PNS are rare conditions, that often allow the early detection of an underlying tumour (5,6). Anti-neuronal antibodies play a diagnostic role and may disappear when the tumour is removed, with favourable prognostic significance for the neurological disease. The significance of these autoantibodies for PNS is still being debated (7,8). The access of anti-neuronal-specific antibodies to the central nervous system and into the neurons, as well as their mode of action in the neurons, are still missing links between the appearance of anti-neuronal antibodies in the serum of tumour patients and the clinical symptoms of PNS (9,10).

For diagnostic purposes, the reactivity of these antibodies is tested on human/rat cerebral/cerebellar tissues, generally yielding either nuclear, or cytoplasmic staining (11). An antibody reactivity that is restricted to cerebellar dendrites and axons (cerebellar nerve fibres) is not included into PNS guidelines (11). We report the study of two atypical anti-neuronal antibodies showing a similar immunohistochemical pattern on rat cerebellar tissue, related to different tumours and different PNS.

Materials and Methods

Sera and cerebrospinal fluids. Atypical sera were detected from a routine screening and identified as follows: serum number 1 from a patient with malignant fibrous histiocytoma associated with paraneoplastic brainstem encephalitis; serum number 2 from a patient with Merkel cell carcinoma associated with cerebellar degeneration. Negative control sera were collected from 10 healthy subjects and positive control sera from 2 patients with definite PNS associated with tumours (anti-Hu antibodies). All controls and atypical sera were kept frozen at –80˚C until used.

Frozen sections of rat brain. Adult Sprague-Dawley rats were anaesthetized by ether and killed. The cerebral and cerebellar hemispheres were washed in saline solution and fixed with 4% paraformaldehyde in phosphate buffer solution (PBS) for 4 hours. The tissues were then kept overnight in 10% sucrose solution.

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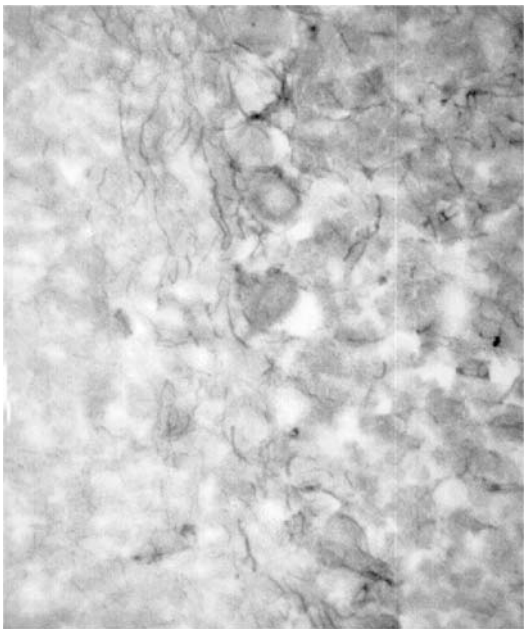

Figure 1. *Atypical antibody present in the serum of a patient with paraneoplastic brainstem encephalitis associated with malignant fibrous histiocytoma: avidin-biotin immunoperoxidase reaction on frozen section of rat cerebellum. Reactivity is evident in the nerve fibres in the molecular and granular layers and around the soma of Purkinje cells. (400x)*

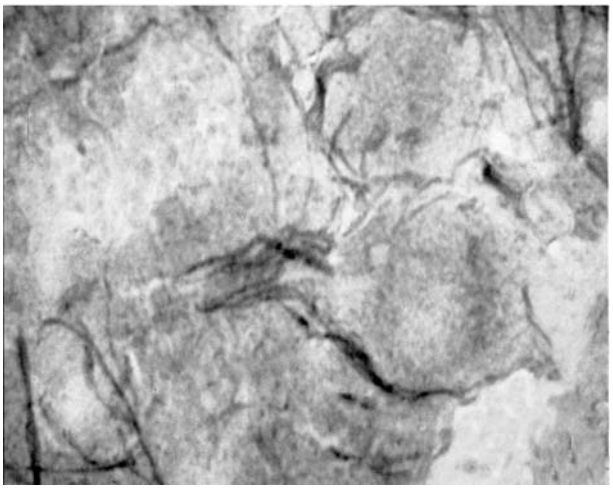

Figure 2. *Atypical antibody present in the serum of a patient with paraneoplastic brainstem encephalitis associated with malignant fibrous histiocytoma: avidin-biotin immunoperoxidase reaction on frozen section of rat cerebellum. Dendrites and axons in molecular and granular layers and nerve fibres around Purkinje cells were stained. (1200x)*

Frozen cerebral and cerebellum were cut and sections placed on poly-L-lysine-coated slides.

Avidin-biotin immunoperoxidase on frozen sections of rat brain and cerebellum. Tissue samples were treated with 3% H_2O_2 in 10% methanol-PBS solution for 20 minutes, followed by 0.05% trypsin digestion for 10 minutes at 37˚C. After washing with PBS, the tissues were first incubated with 10% rabbit serum for 30 minutes at room temperature and then with atypical sera for 1 hour at room temperature. The sera were diluted 1:500 in PBS. The secondary biotinylated rabbit anti-human IgG antibody (DAKO, Glostrup, Denmark) was applied at a 1:200 dilution in 1% BSA-PBS solution for 45 minutes at room temperature. After 3 washes with PBS, the frozen sections were incubated for 45 minutes at room temperature with an avidin-biotin complex. Finally, the antibody binding was visualized with 3,3'-diaminobenzidine tetrahydro- chloride solution (DAB), and then the sections were mounted in Entellan, as previously described (12).

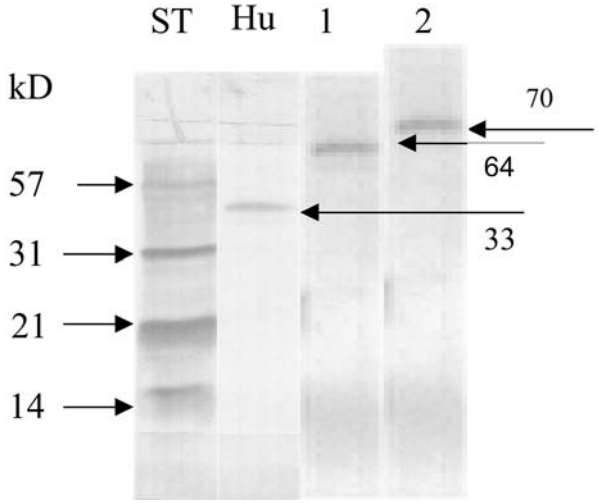

Figure 3. *Western blot of primate cerebellar antigens: STD, standard molecular weights; Hu, serum of a patient with anti-Hu antibody; 1, serum of the patient with malignant fibrous histiocytoma which labels a protein of 64 kDa; 2, serum of the patient with Merkel cell carcinoma which labels a protein of 70 kDa.*

Immuno dot blot with the recombinant proteins HuD, NOVA-1, CDR62/Yo and Amphiphysin (Milenia Biotec, Bad Nauheim, Germany). The nitrocellulose strips, previously blotted with the recombinant antigens, were hydrated with buffer solution for 5 minutes at room temperature. The patients' sera and positive and negative control sera, diluted 1:20000, were incubated on each strip for 90 minutes at room temperature on a shaker. After 3 washes with buffer solution, the nitrocellulose strips were incubated with the polyclonal goat anti-h-IgG-immunoglobulin, labelled with alkaline phosphate, for 1 hour at room temperature on a shaker. Finally the reaction was revealed with the substrate solution (5'bromo-4'chlor-3'indolyl-phosphate-toluidine and 4'-nitro-tetrazolium chloride).

Western blot with primate cerebellar antigens (Euroimmun, Lubeck, Germany). The nitrocellulose strips, previously blotted with neuronal antigen extract from primate cerebellum, were incubated first with goat serum for 15 minutes and then with patients' sera, diluted 1:51 in goat serum, according to the manufacturer's

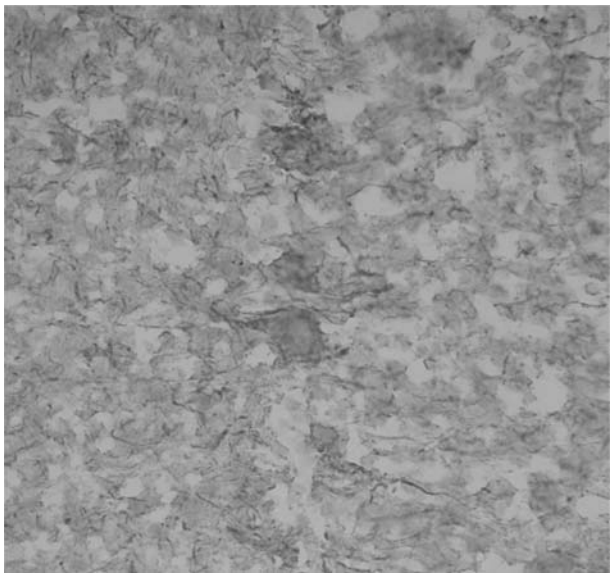

Figure 4. *Atypical antibody present in the serum of a patient with Merkel cell carcinoma associated with cerebellar degeneration: avidin-biotin immunoperoxidase reaction on frozen section of rat cerebellum. Dendrites and axons in molecular and granular layers and nerve fibres around Purkinje cells were positive to the reaction. (400x)*

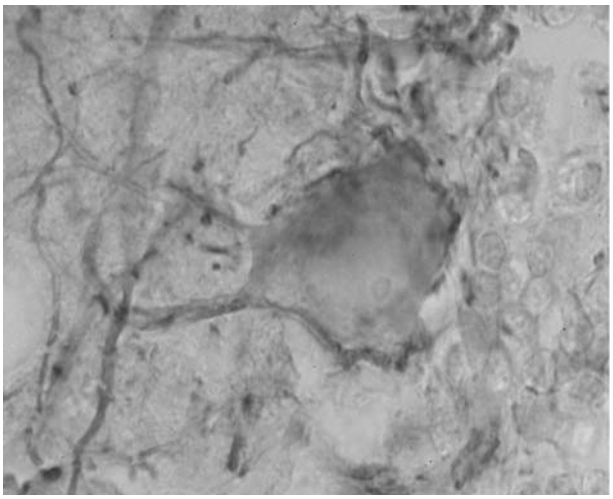

Figure 5. *Atypical antibody present in the serum of a patient with Merkel cell carcinoma associated with cerebellar degeneration: avidin-biotin immunoperoxidase reaction on frozen section of rat cerebellum. Detail of the product of reaction in the nerve fibres in molecular and granular layers and around Purkinje cells. (1200x)*

instructions, for 1 hour. After many washes with goat serum, the strips were incubated with secondary antibody for 1 hour. The reaction was developed with substrate solution.

Results

Patients' sera yielded a nerve fibre-restricted staining pattern only on rat cerebellum. In samples of rat brain, the immunohistochemical pattern of the 2 atypical neuronal antibodies was always negative.

The clinical characteristics of the patient positive for antibody 1 have been previously described (13). An evident positivity was observed in the cerebellar nerve fibres in the molecular and granular layers and around the soma of Purkinje cells (Figure 1). Figure 2 shows the same reactivity pattern at higher magnification. The antibody titre was 1:2000 in serum and titre 1:10 in cerebrospinal fluid (CSF). The immuno dot blot with recombinant proteins was negative and the Western blot with primate cerebellar antigens revealed a molecular weight of 64 kD (Figure 3, line 1).

Antibody 2 was present in the serum of a patient with cerebellar degeneration associated with Merkel cell carcinoma. Dendrites and axons in molecular and granular layers and nerve fibres around Purkinje cells were positive to the reaction (Figure 4; Figure 5, the same in detail). The antibody titre was 1:8000. Following the treatment with radio-chemotherapy, which improved both the neoplastic and neurological picture, the serum sample was weakly positive (titre, 1:100). The immuno dot blot with recombinant proteins was negative and the Western blot with primate cerebellar antigens revealed a molecular weight of 70 kD (Figure 3, line 2).

Discussion

Among typical immunoreactivities described in the PNS field to date (11), staining at the axon hillock and outlining perikarions and dendrites characterize anti-GAD autoantibodies, and might resemble the reactivity that we found in the two patients' sera. However, both sera were anti-GAD-negative (data not shown). A patient with Merkel cell carcinoma and brainstem encephalitis, which occurred 5 years after the tumour diagnosis, has been reported (14).The patient's serum had antibodies to filamentous structures in the brain and cerebellum. This reactivity seemed not to be nerve fibre-restricted, as Western blot of isolated Purkinje cells was positive for 3 bands. Moreover, no figure was shown (14). Anti-neurofilament antibodies were also reported to be part of the anti-neural reactivity in sera of patients with small cell lung carcinoma and visual PNS (15).

The reactivity that we found to be nerve fibre-restricted was superimposable in our two PNS patients, and was probably directed to neurofilament/neurotubule structures. Both malignant fibrous histiocytomas (16) and Merkel cell carcinomas (17) can express proteins associated with such

structures. The unavailability of both tumours prevented us from testing the sera for the presence of reactivity against antigens shared by rat/primate cerebellum and tumours themselves. Merkel cell carcinoma can express the Hu antigen, as reported in a patient with multifaceted neurological disorder and this kind of tumour (18). Our sera recognized proteins that were different from Hu.

Our findings draw attention to a cerebellar nerve fibre-restricted pattern of autoantibody reactivity that associates with PNS. Such a pattern might integrate the diagnostic PNS guidelines (19).

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References

1 Vincent A, Honnorat J, Antoine JC, Giometto B, Dalmau J and Lang B: Autoimmunity in paraneoplastic neurological disorders. J Neuroimmunol *84*: 105-109, 1998.

2 Albert ML and Darnell RB: Paraneoplastic neurological degenerations: keys to tumour immunity. Nat Rev Cancer *4(1)*: 36-44, 2004.

3 Bataller L and Dalmau JO: Paraneoplastic disorders of the central nervous system: update on diagnostic criteria and treatment. Sem Neurol *24(4)*: 461-471, 2004.

4 Giometto B, Taraloto B and Graus F: Autoimmunity in paraneoplastic neurological syndromes. Brain Pathol *9*: 261-273, 1999.

5 Scheid R, Honnorat J *et al*: A new anti-neuronal antibody in a case of paraneoplastic limbic encephalitis associated with breast cancer. J Neurol Neurosurg Psychiatry *75(2)*: 338-340, 2004.

6 Lorusso L, Hart IK *et al*: Immunological features of neurological paraneoplastic syndromes. Int J Immunopathol Pharmacol *17(2)*: 135-144, 2004.

7 Dalmau J and Posner J: Neurologic paraneoplastic antibodies (anti-Yo; anti-Hu; anti-Ri): the case for a nomenclature based on antibody and antigen specificity. Neurology *44*: 2241-2246, 1994.

8 Vianello M, Giometto B *et al*: The spectrum of antineuronal autoantibodies in a series of neurological patients. J Neurolog Sci *220*: 29-36, 2004.

9 Sutton I and Winer JB: The immunopathogenesis of paraneoplastic neurological syndromes. Clin Sci *102*: 475-486, 2002.

10 Drlicek M, Liszka U *et al*: Circulating antineuronal antibody reach neurons *in vivo*: an autopsy study. J Neurol *239*: 407-410, 1992.

11 Moll JW, Antoine JC, Brashear HR *et al*: Guidelines on the detection of paraneoplastic anti-neuronal-specific antibodies: report from the Workshop to the Fourth Meeting of the International Society of Neuro-Immunology on paraneoplastic neurological disease, held October 22-23, 1994, in Rotterdam, The Netherlands. Neurology *45*: 1937-1941, 1995.

12 Nano R, Balegno S, Vaccarone R, Corato M and Ceroni M: Detection of paraneoplastic anti-neuronal-specific antibodies: comparison of different immunohistochemical techniques. Anticancer Res *23(3B)*: 2377-2381, 2003.

13 Corato M, Marinou-Aktipi K, Nano R *et al*: Paraneoplastic brainstem encephalitis in a patient with malignant fibrous histiocytoma and atypical anti-neuronal antibodies. J Neurol (in press).

14 Cher LM, Hochberg FH, Teruya J *et al*: Therapy for paraneoplastic neurologic syndromes in six patients with protein A column immunoadsorption. Cancer *75*: 1678-1683, 1995.

15 Hasegawa T, Seki K, Hasegawa F *et al*: Dedifferentiated liposarcoma of retroperitoneum and mesentery: varied growth patterns and histological grades: a clinicopathologic study of 32 cases. Hum Pathol *31*: 717-727, 2000.

16 Schmidt U, Müller U, Metz A and Leder L-D: Cytokeratin and neurofilament protein staining in Merkel cell carcinoma of the small cell type and small cell carcinoma of the lung. Am J Dermatopathol *20*: 346-351, 1998.

17 Greenlee JE, Steffens JD, Clawson SA, Hill K and Dalmau JO: Anti-Hu antibodies in Merkel cell carcinoma. Ann Neurol *52*: 111-115, 2002.

18 Kornguth SE, Kalinke T, Grunwald GB, Schutta H and Dahl D: Anti-neurofilament antibodies in the sera of patients with small cell carcinoma of the lung and with visual paraneoplastic syndrome. Cancer Res *46*: 2588-2595, 1986.

19 Graus F, Delattre JY, Antoine JC *et al*: Recommended diagnostic criteria for paraneoplastic neurological syndromes. J Neurol Neurosurg Psychiatry *75*: 1135-1140, 2004.

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