Published in final edited form as:

J Neuroimmunol. 2005 August ; 165(1-2): 166–171. doi:10.1016/j.jneuroim.2005.03.020.

Anti-glia nuclear antibody: Marker of lung cancer-related paraneoplastic neurological syndromes

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Abstract

We describe a new antibody, called anti-glia nuclear antibody (AGNA), in patients with paraneoplastic neurological syndromes (PNS) and small-cell lung carcinoma (SCLC). AGNA was initially identified in 24 sera of our archives by immunohistochemistry on rat cerebellum. AGNA positive sera showed a characteristic nuclear staining of the Bergmann glia in the Purkinje cell layer. Immunoblots and probing a cerebellar expression library with AGNA sera did not identify the antigen. Twenty of the 24 patients with AGNA had PNS and all but two had lung cancer. AGNA was identified in 13/113 (11.5%) patients with SCLC compared with 0/122 with other types of cancer ($p < 0.0001$). The frequency of AGNA was not higher than expected for the presence of SCLC in the different PNS subtypes except in LEMS ($p = 0.0002$). AGNA was present in 13/30 (43%) of LEMS patients with SCLC, compared with 0/19 of LEMS patients without cancer ($p = 0.0006$). We conclude that the recognition of AGNA is helpful since this antibody is found in PNS associated with SCLC, particularly LEMS, in which other onconeural antibodies are absent.

Keywords

Paraneoplastic neurological syndromes; Lung cancer; Autoantibody

1. Introduction

The detection of onconeural antibodies has been very useful in helping to define the paraneoplastic etiology of a given neurological syndrome. Onconeural antibodies are detected in many laboratories by screening of sera on frozen sections of rat or mouse cerebellum (Moll et al., 1995; Vincent et al., 1998). Positive immunoreactivities are usually confirmed by immunoblot of recombinant proteins or neuronal homogenates.

Sera from patients with small-cell lung carcinoma (SCLC), the most common tumor associated with paraneoplastic neurological syndromes (PNS), sometimes harbor antibodies against neural antigens that have not previously been recognized as associated with PNS (Gure et al., 2000). When this happens, it is important first to establish whether the antibody is related to the presence of a specific type of cancer, and second to investigate whether the antibody is associated with a particular PNS. Even if the antibody is not directly related to the immune

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response that causes a PNS, it may indicate the presence of an underlying cancer undiagnosed at the time of the antibody determination.

Here we describe a new antibody specificity, and show that it is a marker for SCLC. This antibody may help in the diagnosis of SCLC-related PNS, particularly the Lambert–Eaton myasthenic syndrome (LEMS).

2. Methods

2.1. Patients

We retrieved from the archives of one of the investigators (FG) those samples which had shown immunoreactivity restricted to the nuclei of the Bergmann glia (Yamada and Watanabe, 2002) of the Purkinje cell layer and to subpopulations of glial cell nuclei in the white matter during routine immunohistochemistry of rat cerebellum to detect onconeural antibodies, as previously described (Fig. 1) (Graus et al., 1997). The reactivity was defined as anti-glial nuclear antibody (AGNA).

To define the clinical associations with AGNA, we studied the serum of 113 patients without PNS and SCLC, 122 with other cancer types, and 19 with idiopathic LEMS, defined by the absence of cancer after at least 3 years of follow-up (O'Neill et al., 1988). To ascertain the frequency of AGNA among different PNS associated with SCLC, we reviewed the presence of AGNA immunoreactivity in 30 patients with paraneoplastic LEMS, 27 with paraneoplastic cerebellar degeneration (PCD), 10 with sensory neuropathy, 10 with opsoclonus, and 8 with limbic encephalitis. All these patients had definite SCLC, except six who had X-ray evidence only of lung cancer. None of the sera harbor anti-Hu, anti-Ri, anti-Zic4 (Bataller et al., 2004b) or other associated antibodies that could obscure the presence of AGNA immunoreactivity.

2.2. AGNA immunohistochemistry

AGNA immunoreactivity was analyzed by immunohistochemistry (serum screening dilution 1:500; biotinylated IgG from AGNA positive serum at 50 µg/ml) using an avidin–biotin technique on paraformaldehyde-fixed frozen rat tissues or acetone-fixed human cerebellum as described previously (Graus et al., 1997). To show if AGNA of different patients recognized similar epitopes, rat cerebellar sections were pre-incubated with undiluted normal serum, an anti-Hu-positive serum, or AGNA-positive serum for 3 h followed by a biotinylated IgG obtained from a positive AGNA serum and developed with a standard avidin–biotin technique as described (Bernal et al., 2003). To study if there was AGNA immunoreactivity in samples of SCLC, paraffin sections were deparaffinised in xylene, rehydrated in alcohol, washed in tap water and heated for 2 min in a pressure cooking oven in 0.1 M sodium citrate buffer (pH 6.0). After inhibition of endogenous peroxidase with 0.3% H₂O₂, sections were sequentially incubated with undiluted normal human serum, biotinylated AGNA or control IgG in 10% normal human serum overnight at 4 °C, and developed with the avidin–biotin immunoperoxidase technique (Bernal et al., 2003).

For double-labeling experiments, frozen sections of rat brain were simultaneously incubated with a combination of NeuN (dilution 1:200) mouse monoclonal (Chemicon, Germany), a specific marker of neuronal nuclei (Mullen et al., 1992), and biotinylated AGNA IgG (1:50) overnight at 4 °C. Thereafter, sections were incubated with a combination of fluorescein-conjugated horse anti-mouse antibody (1:200) (Vector, Burlingame, CA) and rhodamine isothiocyanate-conjugated avidin (1:1000) (Sigma, St. Louis, MO) for 2 h at room temperature. Optical sections and digitally generated stereopairs were obtained using a Leica TCS 4D confocal laser-scanning microscope.

2.3. Western blot analysis

Proteins extracted from tissue homogenates of human cerebellar granule cells, frontal cortical neurons and white matter, rat cerebellum, and HeLa and LAN-1 neuroblastoma cell lines were separated by electrophoresis on a 4–12% polyacrylamide gel, transferred to nitrocellulose paper and subjected to standard Western blot procedures using an avidin–biotin method as described (Cunningham et al., 1986; Saiz et al., 1997).

2.4. Screening of a cerebellar cDNA expression library

A Uni-ZAP XR Library (Stratagene, La Jolla, CA) from human cerebellum was immunoscreened with a pool of five AGNA sera (each diluted 1:1000) as previously reported (Bataller et al., 2004a). Several rounds of antibody screening were performed to reach a yield of 100% positive plaques. Phage clones were subcloned using the *in vivo* phage rescue protocol (Stratagene, La Jolla, CA) and sequenced.

To ascertain if positive clones were recognized by all five AGNA sera of the pool, we prepared nitrocellulose filters with mixed phage plaques (50% of plaques from positive clones and 50% from irrelevant clones). Filters were cut into strips and incubated with each of the five AGNA sera or control serum and developed by an avidin–biotin immunoperoxidase technique as described (Bataller et al., 2003).

3. Results

3.1. Diagnostic criteria of AGNA

By definition, AGNA was characterized by a highly restricted, and easily recognized, reactivity against the nuclei of Bergmann glia of the rat cerebellum (Fig. 1). Other cells of the cerebellum were negative except for isolated glial nuclei in the white matter. Double labeling experiments confirmed that the nuclei stained around the Purkinje cells were those of glial rather than neurons of the granular cell layer (Fig. 1). The antibody-binding pattern in rat cerebellum was abolished by preincubation with 16 (76.2%) of the 21 sera used in competitive inhibition experiments. AGNA immunoreactivity was also reproduced in mouse and human cerebellum.

3.2. Reactivity of AGNA in rat tissues and SCLC

In addition to the cerebellum immunoreactivity, AGNA labeled a subgroup of glial nuclei throughout the brain, ependymal cells, and satellite cells of dorsal root ganglia. Choroid plexus, endothelial cells and meninges were negative. AGNA positive isolated neuronal nuclei, defined by the nuclear size and shape of the cell and immune reaction with NeuN antibody, were observed in cortex, hippocampus, basal ganglia, hypothalamus and upper brainstem (Fig. 2). In some neuronal groups in the basal forebrain and diencephalon, all nuclei were AGNA positive. Unlike adult rat brain, AGNA labeled the majority of neuronal nuclei in the brain of early (P1 to P3) postnatal rats. Systemic rat tissues (thymus, lung, heart, stomach, colon, liver, spleen, kidney, and testis) probed with biotinylated AGNA IgG were negative (data not shown). A SCLC from a patient without PNS incubated with the same antibody showed a strong immunoreactivity that was abolished with preincubation with an AGNA positive serum (Fig. 3).

3.3. Antigen analysis

Immunoblots of different tissue homogenates probed with several AGNA positive sera failed to produce a common band to identify AGNA more precisely. Screening of the cerebellar library produced 13 reactive clones coding for eight proteins. Only two of them were brain specific and localize in the nucleus and none was recognized by more than one AGNA serum.

3.4. Clinical and immunological associations of AGNA

AGNA was identified in 24 patients, 19 had SCLC, three had radiological evidence of lung cancer, and two had no cancer but one of them had LEMS with a follow-up of less than a year. Thus, detection of this antibody was highly predictive of a SCLC. Nine patients had LEMS, five PCD, one of them also with LEMS (Mason et al., 1997), three sensory neuronopathy, two limbic encephalitis, and one sensorimotor neuropathy. Four patients did not have a PNS. Two of them had SCLC and one X-ray evidence of lung cancer; the final neurological diagnoses were chemotherapy-related sensory–neuropathy, diabetic neuropathy, and undefined encephalopathy in the setting of widespread, progressive, metastatic SCLC. The fourth patient had a slowly progressive dementia and rheumatoid arthritis with no evidence of cancer after 3 years of follow-up.

To analyze the presence of intrathecal synthesis, rat cerebellar sections were incubated with similar amounts of IgG from paired serum and CSF of five AGNA-positive patients (PCD: 2, LEMS: 2; non-PNS: 1). In four patients, AGNA reactivity was lost in the CSF at IgG concentrations higher than that of the paired serum. In the fifth patient, AGNA reactivity was lost at IgG concentrations that were half of the paired serum IgG concentration. These experiments suggested that intrathecal synthesis of AGNA was not a common feature in the population studied.

Due to the observed association with SCLC, we analyzed the presence of AGNA in sera from 113 patients without neurological disease and SCLC, and in 122 with other cancer types. AGNA was only detected in 13 (11.5%) patients all of them with SCLC ($p < 0.0001$). To ascertain if the frequency of AGNA in SCLC patients with PNS was higher than that observed in SCLC patients without neurological disorders, we reviewed the frequency of AGNA in patients with PNS and SCLC. The analysis was restricted to those who did not harbor other onconeural antibodies (Hu, Ri, Zic4) since their presence conceals AGNA immunoreactivity. The results are summarized in the table. Compared with the frequency of AGNA in patients with isolated SCLC, 23 (27%) of 85 patients who had SCLC associated with a variety of PNS had AGNA ($p = 0.009$). However, the higher frequency of AGNA in PNS was mainly due to the relative frequency of LEMS patients (see below), while other PNS patients showed a frequency of AGNA not different from that in patients with SCLC alone (see Table 1). Interestingly, all five patients with PCD and AGNA also had voltage-gated calcium channel (VGCC) antibodies (Graus et al., 2002).

To ascertain if AGNA was linked to LEMS, rather than to an associated SCLC, we analyzed the presence of AGNA in 49 patients with LEMS with or without SCLC. Thirteen of the 30 (43%) LEMS patients with SCLC were positive for AGNA, whereas none of the 19 LEMS patients without cancer had AGNA ($p = 0.0006$).

4. Discussion

This study describes a new antibody, designated as AGNA, that may be helpful in the diagnosis of PNS associated with SCLC. Some PNS, like LEMS, are not associated with onconeural antibodies and may also occur in the absence of cancer. We show that AGNA is a good marker for the presence of an underlying SCLC and may help to identify which patients with clinical features of classical PNS (Graus et al., 2004), such as LEMS or PCD, without anti-Hu or other well-characterized onconeural antibodies, are at risk for the presence of a SCLC. However, the presence of AGNA alone cannot be considered as indication that the neurological syndrome under study is paraneoplastic because four (16.7%) of the 24 patients with AGNA had neurological diagnoses other than well-defined PNS (Graus et al., 2004).

AGNA produces a characteristic immunoreactivity in paraformaldehyde-fixed rat cerebellum but it was not detected by immunoblot. The negative immunoblot and unsuccessful attempt to define target antigen(s) using a cerebellar expression library suggest that the epitopes recognized by AGNA may be conformational, a finding also observed in anti-Tr antibodies of patients with PCD and Hodgkin disease (Graus et al., 1997). Until the identification of the AGNA antigen(s), which will allow the unambiguous diagnosis of this antibody, the very characteristic immunohistochemical pattern shown in Fig. 1 may be used to identify AGNA.

In order to determine that the immunohistochemical pattern defined by AGNA represented immune reaction with the same antigens we did an immunohistochemical competition assay in which 76.2% of AGNA sera completely abrogated the binding of biotinylated AGNA IgG to cerebellar sections. This finding indicates (1) that AGNA positive sera probably recognize the same antigens, but (2) they recognize more than one epitope, and (3) not all epitopes are recognized, at least with the same intensity, with all the AGNA sera. These data agree with those found in other onconeural antibodies such as anti-Hu (Manley et al., 1995; Graus et al., 1998).

The pattern of immunoreactivity of AGNA in newborn and adult rat brain has similarities with that of anti-CV2, also called CRMP5, antibodies observed in patients with various PNS and SCLC (Honnorat et al., 1996; Yu et al., 2001). Both antibodies recognize antigens which are widely expressed in the developing nervous system and then down regulated and expressed in the adult brain only in a subpopulation of glial cells, oligodendrocytes in the case of CV2 antibodies, and neurons. Other antigens that are preferentially expressed at developmental stages have been identified by serological analysis of expression cDNA libraries derived from SCLC, using sera of SCLC patients without PNS (Gure et al., 2000). Therefore, these developmentally regulated antigens seem highly immunogenic when expressed in SCLC and the triggered immune response may associate with PNS in some cases.

We could not ascertain the coincident expression of AGNA in SCLC patients with PNS and anti-Hu, Ri or Zic4 antibodies. This data will remain unknown until we can determine AGNA by more specific methods. Even if anti-Hu immunoreactivity, the most common antibody, is eliminated from the immunohistochemistry assay with preabsorption of sera with HuD, the high titers of anti-Hu antibodies usually required to absorb the sera at high dilutions where AGNA immunoreactivity may be lost. Despite this drawback, our data suggest that immunity against the AGNA antigen expressed in SCLC may not be relevant in the pathogenesis of the associated PNS because, except for patients with LEMS, the frequency of AGNA in PNS is not higher than that in SCLC without PNS.

The distribution of PNS associated with AGNA differs from that seen with anti-Hu, anti-CV2/CRMP5, or other onconeural antibodies associated with SCLC and PNS in that AGNA is overrepresented in LEMS (Yu et al., 2001; Graus et al., 2001). Although a few SCLC patients with LEMS may harbor anti-Hu (Dropcho et al., 1989; Lucchinetti et al., 1998), Ri (Pittock et al., 2004), CV2/CRMP5 (Yu et al., 2001), or Zic4 (Bataller et al., 2003) antibodies, AGNA was detected in 43% of our series of LEMS and SCLC. We do not think this figure was biased because patients were selected by the presence of AGNA immunoreactivity without previous knowledge of the clinical features. Interestingly, all five patients with PCD, SCLC, and AGNA also had anti-VGCC antibodies suggesting that the immune responses to the tumor antigens might be linked in some way. However, the absence of AGNA in the serum of patients with non-paraneoplastic LEMS suggests that AGNA is induced by the SCLC independently of VGCC antibodies.

Acknowledgements

The authors wish to thank Mercè Bonastre, Eva Caballero, and Tracey Newman for their excellent technical assistance. Supported in part by grants QLGI-CT-2002-01756 of the European Union and PI030028 Fondo de Investigaciones Sanitarias, Madrid, Spain (FG), and RO1CA107192 (JD). The authors thank all the neurologists who provided clinical information on their patients.

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Fig. 1.

(A) Frozen section of paraformaldehyde-fixed rat cerebellum immunoreacted with AGNA positive serum. There is intense labeling of the nuclei of Bergmann glia in the Purkinje cell layer. (B) Higher magnifications of the same section incubated with AGNA. (C) Double labeling study showing that AGNA reacts with the nuclei (red) of Bergmann glia, which are not labeled with NeuN a neuronal nucleus-specific monoclonal antibody (green). Note that NeuN labels the nuclei of granular cells and other cerebellar neurons except Purkinje cells, as previously described (Mullen et al., 1992). Bar=38 μ m (A), 18 μ m (B), and 24 μ m (C). Sections counterstained with hematoxylin in (A) and (B).

Fig. 2. Frozen section paraformaldehyde-fixed rat hippocampus double labeled with biotinylated AGNA IgG (red) and NeuN antibody (green). The nucleus of one neuron coexpress both antigens (yellow) whereas the nucleolus is only labeled with AGNA. Bar=11 μ m.

Fig. 3. Paraffin embedded sections of small-cell lung carcinoma (SCLC) immunoreacted with biotinylated AGNA IgG. The robust reactivity of the biotinylated AGNA IgG (A) is abolished by preincubation with an AGNA positive serum (B). Sections counterstained with hematoxylin.

Table 1

Frequency of AGNA in patients with paraneoplastic neurological syndromes (PNS) and small-cell lung carcinoma (SCLC) without anti-Hu, Ri or Zic4 antibodies

Syndrome	Number of patients with PNS	AGNA (%)	<i>p</i> value ^a
LEMS ^b	30	13 (43.3)	0.0002
Cerebellar degeneration	27	5 (18.5)	0.34
Sensory neuropathy	10	3 (30.0)	0.12
Opsoclonus	10	0 (0.0)	0.60
Limbic encephalitis	8	2 (25.0)	0.26
Total PNS	85	23 (27.1)	0.009
No PNS	113	13 (11.5)	

^a All syndromes compared with the series of SCLC without neurological disease by the Fisher's exact test.

^b Paraneoplastic LEMS patients were collected from three laboratories (JD, FG, AV); all the other patients are from the Barcelona database only. Analysis remains statistically significant ($p=0.01$) if only the 23 paraneoplastic LEMS patients (eight (35%) were AGNA positive) from the Barcelona database are analyzed.