

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

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Paraneoplastic syndromes of the central nervous system (PNS) consist of a variety of neurologic disorders including encephalomyelitis (limbic encephalitis, brainstem encephalitis, and acute myelitis), sensory neuronopathy, subacute cerebellar degeneration, visual paraneoplastic syndrome, stiff-man syndrome, and motor neuron disease.¹⁻³

For the clinician, it is often difficult to arrive at a reliable diagnosis of the neurologic syndrome and to detect the underlying tumor. Neurologic signs and symptoms, although often stereotypic, are not specific. In two-thirds of cases the underlying tumor is not discovered until after the first neurologic symptoms.⁴ Furthermore, the neoplasm may be small and difficult to detect.⁵⁻⁷ PNS are frequently associated with serum autoantibodies reactive with neuronal antigens that are also present in the underlying tumor. These antibodies include (1) the anti-Yo antibody, also called "type 1 anti-Purkinje cell antibody" (PCA-1), "APCA-1," or "type I antibody"; (2) the anti-Hu antibody, also called "type 1 anti-neuronal nuclear antibody" (ANNA-1) or "type IIa antibody"; and (3) the anti-Ri antibody, also called "type 2 anti-neuronal nuclear antibody" (ANNA-2) or "type IIb antibody" (table). A clinical diagnosis of PNS is supported by the finding of these specific anti-neuronal antibodies. The detection of these antibodies should also lead to a fo-

cused search for specific underlying neoplasms.⁸⁻¹²

A major problem in comparing results obtained by different groups of investigators has been that individual laboratories have employed different laboratory techniques for detection of these antibodies. Although identification of these antibodies has rested on immunohistochemistry and Western blot analysis, not all laboratories have used Western blot analysis to identify the molecular weight of detected antigens. Furthermore, material from a number of animal species has been used, methods of preparing substrates for Western blot are not always spelled out, and concentrations of serum and end-point staining have not always been defined. In some cases, this has resulted in substantial differences in the sensitivity and specificity of these techniques for antibody detection.¹²⁻¹⁷ Some early publications and recent reports on several incompletely characterized novel anti-neuronal antibodies have complicated this situation and make it even more difficult for the clinician to interpret the diagnostic value of these antibodies.¹⁸⁻²²

Approach to consensus. In view of these uncertainties and the existing controversy between some research groups on the techniques for proper detection and on the terminology of anti-neuronal antibodies in paraneoplastic neurologic disease, a

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Table. Criteria for identification of paraneoplastic anti-neuronal antibodies

Name of antibody	First description of antibody	Paraneoplastic neurologic syndrome	Most frequently associated tumors	Immunohistochemical criteria for detection	Western immunoblot criteria for detection	Comments
Anti-Hu ANNA-1 Type IIa	Graus et al, 1986 ²⁸ Lennon, 1989 ¹² Greenlee et al, 1991 ³²	Encephalomyelitis, sensory neuronopathy, autonomic neuropathy	Small-cell lung cancer, neuroblastoma; rarely non-small-cell lung cancer, prostate cancer, seminoma	Strong staining of all CNS and peripheral nervous system neuronal nuclei, sparing of nucleoli and weaker fine granular staining of cytoplasm; negative on systemic tissues	35- to 40-kD reactive bands on extracts of isolated CNS neurons or (optional) reactivity with recombinant protein (HuD, HuC, Hel-N1)	Anti-Hu: both immunohistochemistry and Western immunoblot identification required ANNA-1 or type IIa: immunohistochemically identical to anti-Hu, initial description used no Western immunoblot identification, but Western immunoblot identification now required for diagnostic purposes For Western blot identification, proteins from isolated CNS neurons are sufficiently reliable when 37- to 40-kD protein complex is identified; use of recombinant proteins is optional (licensed in US)
Anti-Yo PCA-1 Type I APCA-1	Furueux et al, 1990 ²⁶ Lennon, 1994 ¹⁵ Jaekle et al, 1985 ²⁵ Greenlee, 1982 ²⁴	Subacute cerebellar syndrome, dysarthria and nystagmus	Ovarian cancer, breast cancer, other gynecologic cancer	Coarse granular staining of Purkinje cell cytoplasm and proximal axon and dendrites, negative nuclei; negative on systemic tissues	62-kD band and mostly 34-kD band on purified Purkinje cell extracts or (optional) reactivity with CDR34 and CDR62, p52/PCD17 recombinant proteins	Anti-Yo: both immunohistochemical and Western immunoblot required APCA-1, PCA-1, or type I: initial description used no Western immunoblot identification, but for diagnostic purposes it is now required For Western blot confirmation, proteins from isolated Purkinje cells reliable when 34- and 62-kD bands are identified; use of recombinant proteins is optional (licensed in US)
Anti-Ri ANNA-2 Type IIb	Luque et al, 1991 ¹¹ Lennon, 1994 ¹⁵ Brashear et al, 1994 ³⁰	Opsoclonus, ataxia, nystagmus, dizziness, dysarthria	Breast cancer (total number of patients described still small), small-cell lung cancer (one case)	Strong staining of all CNS but not of peripheral nervous system neuronal nuclei, with sparing of nucleoli and weaker fine granular staining of cytoplasm; negative on systemic tissues	55- and 80-kD bands on neuronal proteins or (optional) reactivity with 55-kD recombinant protein Nova	Anti-Ri: both immunohistochemical and Western immunoblot identification required ANNA-2 or type IIb: initial description used no Western immunoblot identification, but now it is required for diagnostic purposes For Western blot identification, proteins from isolated CNS neurons are sufficiently reliable when 55- and 80-kD bands are identified; use of recombinant protein Nova is optional (licensed in US)

workshop on this topic was organized during an international symposium on paraneoplastic neurologic disease held October 22-23, 1994, in Rotterdam, The Netherlands. The primary aim of the workshop was to develop a consensus report that contained generally accepted guidelines in order to make laboratory testing of anti-neuronal antibody detection more uniform and easily interpretable and to clarify the terminology.

This paper reports on the work of 11 international research groups that formed a consensus group to discuss the terminology, methodology of detection, and interpretation of paraneoplastic anti-neuronal antibodies.

Terminology. Since the initial description of a paraneoplastic anti-neuronal antibody by Wilkinson and Zeromski in 1965,²³ different terminologies for paraneoplastic antibodies have been proposed by different groups working in this field.

It is important to realize that during a relatively short period of time both the designations and the methods of detection have been refined and the techniques and disclosure of antibody characteristics have improved. We first present a brief survey of the terminology.

In 1982 an anti-Purkinje cell antibody was identified by Greenlee and later by Jaekle et al and by

Greenlee and Brashear in patients with paraneoplastic cerebellar degeneration and gynecologic malignancies.^{8,10,24,25} Neither group designated a specific name but both described the antibody as an anti-Purkinje cell antibody. Various names were designated later. The name "anti-Yo" was introduced in 1990 for those anti-Purkinje cell antibodies reacting with 34- and 62-kD proteins from isolated Purkinje cells on Western blot.²⁶ The abbreviations "PCA" and "PCAb" were used by Lennon in 1989¹² using immunohistochemical criteria and were modified to "PCA-1" in 1994 solely on the basis of immunohistochemical criteria.^{15,16} The term "APCA-1" was introduced in 1989.²⁷

The original description of possible anti-neuronal nuclear antibodies mainly emphasized the cytoplasmic staining by the antibody.²³ Graus et al noted the nuclear reactivity and described reactivity with 37- to 40-kD molecular weight proteins on Western blotting.^{9,28} In 1986, this antibody was designated "anti-Hu" (after the first patient in whom it was discovered).²⁸

The anti-Ri antibody was first described in 1988 using both immunohistochemical and Western immunoblot characterization,²⁹ and its name was introduced in 1991.¹¹

The term "ANNA" was introduced by Lennon in

1989¹² and was modified to "ANNA-1" and "ANNA-2" in 1994 on the basis of immunohistochemistry alone.^{15,16} Earlier others already identified differences in ANNA reactivity as "anti-Hu" and "anti-Ri" by additional testing on Western blots.^{28,29}

The designations "type I" for anti-Purkinje cell antibodies and "type II" for the anti-neuronal nuclear antibodies were assigned in 1983 and in 1991 respectively, and the type II antibodies were further specified as "type IIa" and "type IIb" in 1993, based on a combination of immunohistochemistry and Western blotting.^{10,25,30-32}

Although each of these teams of investigators has made valuable contributions to current understanding of this area, it is clear that unequivocal definition and terminology is necessary to enable comparison of results from different laboratories and to avoid confusion on the part of the clinician. Obviously, the main point is not so much how these antibodies are being named as how to formulate criteria for the proper methods of detection and classification of these antibodies.

Methods of detection. The nomenclatures with numerical designations, such as ANNA-1 and -2, PCA-1, and types I, IIa, and IIb (table), were originally given to those antibodies determined by immunohistochemical assays only.^{12,25,30-32} The terms "anti-Hu," "anti-Ri," and "anti-Yo" are based on determination by both immunohistochemistry and Western blotting.^{11,26,28,33} Hence, strictly speaking, these terminologies cannot be considered interchangeable. Nevertheless, all nomenclatures are intended to refer to equivalent antibodies and should therefore preferably be regarded as synonymous, as recommended in the December 1994 issue of **Neurology**.^{15-17,34} However, to allow them to be regarded as such, identical methods and criteria should be used, which implies redefinition of the criteria in such a way that both immunohistochemical and Western immunoblot assays are required for proper detection of all three PNS-associated anti-neuronal antibodies. The consensus group agrees that identification by immunohistochemistry alone, even when performed in experienced laboratories and according to previously issued recommendations,^{15,16} cannot be considered a sufficiently reliable test for the characterization of these antibodies. If anti-Purkinje cell cytoplasmic antibodies or anti-neuronal nuclear antibodies are detected solely by immunohistochemistry, one should speak of anti-Purkinje cell antibody (APCA or PCA) or anti-neuronal nuclear antibody (ANNA) without further specification. In line with the recommendations of **Neurology**, we consider the anti-Yo antibody synonymous with APCA-1 or PCA-1, anti-Hu synonymous with ANNA-1, and anti-Ri synonymous with ANNA-2, provided that both immunohistochemical and Western immunoblot assays are used.

A specimen yielding no reactivity on immunohistochemistry is interpreted as negative for anti-neuronal antibodies, although Western blot analysis

may detect low titers of anti-Hu antibodies in serum samples from neurologically normal patients with small-cell lung cancer using highly affinity-purified recombinant proteins.

To provide noncontroversial and accurate criteria for the definition of these antibodies, we outline here some general guidelines on the appropriate methods as well as some technical details. We expect that following these guidelines will make identification of novel antibodies more easily comparable and interpretable.

Immunohistochemistry. The first screening for anti-neuronal antibodies in serum or CSF consists of an immunohistochemical assay. Immunohistochemical screening can also detect other atypical (non-anti-Yo, non-anti-Hu, and non-anti-Ri) anti-neuronal antibodies. Patients' serum samples should be sent overnight and tested within 48 hours or can be stored at 4 °C for several weeks. For longer delays, samples should preferably be stored at -80 °C until tested.

Human cerebellar and cerebral cortex are preferentially used as tissue substrates for immunohistochemistry. Occasionally, dorsal root ganglia, myenteric plexus, medulla, spinal cord, and control tissues such as liver, muscle, and kidney can be used for special interests, although on indirect immunohistochemistry some background staining may be found because of the presence of irrelevant IgG in systemic tissues. If obtaining human tissue is difficult, mouse or rat tissue can be used for avidin-biotin or peroxidase-antiperoxidase methods since these species express most human PNS antigens. However, misleading results may be obtained when fluorescence staining is used in nonhuman tissue.³⁵ For novel antibody screening, one should always employ human tissue, and it may well prove valuable to use nonhuman tissue as well.

Criteria for positive immunohistochemical identification of antibodies are presented in the table.

It is possible to discriminate immunohistochemically between anti-Hu and anti-Ri by using peripheral neurons—for example, myenteric plexus or dorsal root ganglion cells—as substrate.^{15,36} In contrast to the Hu antigen, the Ri antigen is not expressed in peripheral neurons.

Antibody titers should be determined by serial dilutional titration. In each series of tests, parallel control sections should include a section without use of primary serum, a section using a known positive serum, and a section with a known negative serum. The secondary antibody can be labeled with either fluorescein isothiocyanate (FITC), horseradish peroxidase, alkaline phosphatase, biotin, or other enzymes to visualize bound antibodies. Either polyclonal or monoclonal secondary antibodies against total human Ig or IgG may be chosen. Titers of antibodies depend on many factors, among which are the purity of the antigen, concentration of the secondary antibody, time of incubation, quality of tissues, and type of secondary antibody. Dilution and diluent of the secondary antibody are of

great importance in this respect. The secondary antibody should be diluted in the same medium used as blocking agent. Initial assays from the early groups used the FITC-labeled secondary antibody,^{8-10,37} which is easy to work with and reliable. Other frequently used techniques are avidin-biotin-peroxidase and streptavidin-peroxidase methods.³⁸ Different labeling techniques will often lead to differences in end-point staining. In general, immunoperoxidase and avidin-biotin methods give higher end-point titers than does indirect immunofluorescence. Therefore, each laboratory should critically establish dilution and method of visualization of secondary antibody, using appropriate positive and negative controls.

Ideally, a set of positive reference control serum samples is assembled by exchange of various serum samples between experienced laboratories.

End-point staining titers for positive identification of antibody should be determined using large series of controls and positive standards to prevent false-positive detection. In most immunohistochemical assays using FITC-labeled secondary antibody, end-point titers of >1:500 can be regarded as positive, yielding high specificities. In some patients, however, lower titers can represent true-positive reactivity.¹⁴

With high serum IgG concentrations, background staining can occasionally make the assay difficult to interpret. Isolation and direct labeling of serum IgG may eliminate background reactivity and may improve the quality of the assay.³⁸

Western immunoblot methods. Essential and optional criteria for positive identification on Western immunoblot are provided in the table.

With the availability of recombinant antigens (HuD, Hel-N1/ple21, HuC, Ri [Nova], CDR34, CDR62, p52/PCD17, and CZF) it may be useful, although not obligatory, to use these complementary to neuronal extracts.³⁹⁻⁴⁵ One should realize, especially in the United States, that the commercial diagnostic use of the recombinant proteins HuD, Yo (CDR62), and Ri (Nova) is licensed by Genica (Boston, MA).

When using neuronal proteins as antigenic substrate, an important factor for the diagnostic reliability of this technique is the nature and concentration of the protein extracts. Collected tissues should be processed as rapidly as possible (preferably within 6 to 8 hours, maximally 24 hours) after death.

For anti-Hu detection on Western blots, a protein extract from isolated neurons or neuronal nuclei from normal human, rat, or murine cerebral or cerebellar cortex is used.³⁸ If available, the recombinant HuD protein can also be used. For recognition of anti-Yo and other anti-Purkinje cell antibodies on Western blots, a protein extract of isolated human or murine Purkinje cells, separated on a sucrose/Ficoll gradient, is used. Optionally, the Yo recombinant protein (CDR62) or the p52 recombinant protein of Sakai et al can be used next to the Pur-

kinje cell proteins.^{39,41}

For identification of anti-Ri antibodies, the same guidelines as used for anti-Hu antibodies are applicable. For the reliable detection of anti-Ri antibodies, Western blotting on neuronal proteins is required. The Ri recombinant protein may also be used for proper recognition of anti-Ri antibodies. However, immunohistochemistry and Western blot on neuronal proteins are sufficiently reliable for demonstrating the anti-Ri antibody.

As a control antigenic substrate, protein extracts from human and murine liver, kidney, or muscle can be used.

Diagnostic value. Although no large clinicopathologic studies are available to accurately establish sensitivity and specificity of these assays for detection of PNS-associated anti-neuronal antibodies, it is clear that the presence of these antibodies has high diagnostic value.^{4,11,14,25} However, in a minority of patients with characteristic PNS and high antibody titers no tumor could be found despite extensive evaluation.^{5,46} One presumes that in these cases the tumor either is too small to be detected or may have spontaneously regressed.^{4,5} On the other hand, failure to detect anti-Yo, anti-Hu, or anti-Ri antibodies in patients with suspected paraneoplastic syndromes does not exclude a paraneoplastic neurologic disease, as it does not exclude an underlying malignancy.

If, following the guidelines above, anti-Yo (APCA-1), anti-Hu (ANNA-1), or anti-Ri (ANNA-2) antibody is identified, a sufficiently reliable diagnosis of PNS can be made despite a lack of proof of the presence of cancer.

Finally, some cases with a clear clinical diagnosis of PNS are antibody-negative in the presence of cancer. Whether these patients harbor paraneoplastic antibodies that cannot be detected with currently available methods remains unclear.

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