Blood Test Detecting Autoantibodies to N-Methyl-D-aspartate Neuroreceptors for Evaluation of Patients with Transient Ischemic Attack and Stroke

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Background: Stroke is a multisystemic disorder that includes mechanisms of thrombosis and neurotoxic coupling. Key metabolites of the molecular cascade following biochemical events appear simultaneously in brain tissue, the blood–brain barrier, and brain vessels, activating the immune system and generating autoantibodies (aAbs) to brain-specific antigens. We developed an ELISA blood test to measure aAbs to a subtype of N-methyl-D-aspartate (NMDA) receptors, which are the key markers of neurotoxicity underlying cerebral ischemia. We investigated the diagnostic accuracy of serum aAbs to NR2A/2B, a subtype of NMDA receptors, in assessing transient ischemic attack (TIA) and ischemic stroke (IS) and its ability to distinguish cerebral ischemia from intracerebral hemorrhage (ICH).

Methods: Autoantibodies to NR2A/2B were measured in 360 serum samples: 105 from TIA/stroke patients and 255 from controls, including patients with controlled hypertension/atherosclerosis and gender- and age-matched healthy individuals.

Results: Patients with TIA (n = 56) and acute IS (n = 31) had significantly higher NR2A/2B aAb concentrations than controls ($P < 0.0001$). The test sensitivities for TIA and IS were 95% and 97%, respectively, and predictive values were 86% and 91% at a cutoff point of 2.0 μg/L. The area under the ROC curve was 0.99. Monitoring NR2A/2B aAbs within 72 h differentiated IS and ICH ($P < 0.001$) and was confirmed by magnetic resonance imaging and computed tomography.

Conclusions: NR2A/2B aAbs are independent and sensitive serologic markers capable of detecting TIA with a high posttest probability and, in conjunction with neurologic observation and neuroimaging, ruling out ICH. The test may help assess risk of TIA in routine general practice and may potentially be useful in assisting diagnosis of acute IS in the emergency setting.
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Stroke is the leading cause of severe long-term disability and the third leading cause of death worldwide (1). The medical community currently faces several diagnostic challenges in the overall management of stroke. These include definitive determination of whether a patient has suffered a transient ischemic attack (TIA),[3] which dramatically increases the likelihood of a later acute stroke, and accurate differentiation of ischemic stroke (IS) from intracerebral hemorrhagic stroke (ICH) in the emergency department and in the outpatient setting. However, based on routinely available clinical, laboratory, and imaging data, progression of stroke can only partially be predicted. The search for serologic markers of stroke needs to progress such that these markers can be detected by fast and reliable blood tests for risk assessment and differential diagnosis. The existence of several such serologic markers and tests has been reported (2–6).

Embolic or thrombotic vascular occlusion stimulates

[3] Nonstandard abbreviations: TIA, transient ischemic attack; IS, ischemic stroke; ICH, intracerebral hemorrhage; NMDA, N-methyl-D-aspartate; NMDAR, NMDA receptor; aAb, autoantibody; MRI, magnetic resonance imaging; CT, computed tomography; NIHSS, National Institutes of Health Stroke Scale; DWI, diffusion weighted imaging; ROI, region of interest; mAb, monoclonal antibody; and CI, confidence interval.

the cascade of neurotoxicity, of which the excitatory *N*-methyl-D-aspartate (NMDA) receptor is one of the key regulators of nerve cell membrane functions, causing biochemical changes in brain tissue, the blood–brain barrier, and brain vessels. The loss of brain membrane integrity leads to damage to neurons, glia, and microvascular endothelial cells. Damaged or dying endothelial cells compromise the blood–brain barrier and increase its permeability. In addition, thrombin-activated serine proteases cause the cleavage of synaptic NMDA receptors (NMDARs) *(7)*. As a result, increased concentrations of the peptides produced by cleavage of brain-specific NMDAR fragments may enter the bloodstream *(8)*. Abnormally high concentrations of these peptide fragments, which act as foreign antigens once they leave the brain, initiate an immune response that generates autoantibodies (aAbs) in the blood *(9)*. Clinical reports have described the effects of stroke on the immune system *(10, 11)*. Patients with central nervous system disorders have been known to exhibit properties of autoimmunization to products of nerve cell degradation, in particular, damaged neuroreceptors *(9, 11–13)*.

This study was designed to investigate the utility of an ELISA serologic test that detects aAbs to NR2A/2B, a subtype of NMDARs, in identifying patients with cerebral ischemia. We also sought to determine the diagnostic accuracy of the test in determining TIA and acute IS in conjunction with a neurologic stroke scale and magnetic resonance imaging (MRI)/computed tomography (CT) and its ability to distinguish cerebral ischemia from ICH.

Materials and Methods

STUDY PROTOCOL

We conducted our study at the Neurology and Neurosurgery Department, I.P. Pavlov's State Medical University (February 1994 to November 1997) and the Clinic of Neurology, Institute of Human Brain (September 1998 to December 2000), which serve the same urban population of St. Petersburg. Local ethics committees of both the University and Institute approved the clinical protocols, and all patients, including controls, provided informed consent.

TIA is a brief episode of neurologic dysfunction caused by focal brain or retinal ischemia, with clinical symptoms typically lasting less than 1 h, and without evidence of acute infarction *(14)*. Patients (n = 64; 40% of 160 eligible patients) with a history of previous TIA and routine MRI examination within 24 h of symptom manifestation were recruited at the Clinic of Neurology, Institute of Human Brain. The following clinical data were compiled for patients: symptoms of TIA (side, motor, and/or sensory deficit and dysphasia) evaluated according to the National Institute of Health Stroke Scale (NIHSS); duration of symptoms; presence of vascular risk factors, including hypertension (systolic blood pressure >160 mmHg and/or diastolic blood pressure >95 mmHg); and athero-

sclerosis. All patients underwent duplex ultrasound of both carotid arteries. Patients (n = 8) with occlusion of the internal carotid artery on duplex ultrasound were excluded from the study. A total of 56 patients with TIA [32 women and 24 men; mean (SD) age, 59.9 (4.7) years] were studied.

Multislice (slice thickness, 5×10^{-4} m; 16 slices) whole-brain diffusion-weighted imaging (DWI) was performed in nine patients with TIA. The diffusion gradient was applied with two *b* values (0 and 830 s/mm²), 128 × 128 matrix size, and a 24-cm field of view. DWI scans were acquired in the *x*, *y*, and *z* directions, and the mean of all three diffusion directions was calculated to give the trace diffusion tensor and then process apparent diffusion coefficient maps. A DWI scan was considered positive if the scan revealed an area of hyperintensity on DWI and hypointensity on apparent diffusion coefficient maps relative to the normal brain, signifying acute cerebral ischemia. DWI lesion volumes were measured on the image of maximum contrast between lesion and normal brain regions (*b* = 830) by an experienced neuroradiologist blinded to clinical diagnosis. Volumes for regions of interest (ROI) drawn on the diffusion images were computed by multiplying the measured ROI per slice by section thickness, considering interslice gap.

We evaluated 110 consecutive patients (~ 10% of 1099 observed patients) 49–65 years of age admitted to the Intensive Stroke Unit of I.P. Pavlov's State Medical University Hospital within 3 h of cerebrovascular event (day 0) for participation. Inclusion criteria included (*a*) hemispheric cortical infarcts, (*b*) first episode of stroke, (*c*) persistence of neurologic deficit on admission, and (*d*) absence of previous treatment for psychiatric illness or serious disease. Patients (n = 44) with a history of atrial fibrillation, valvular heart disease (as shown on echocardiography), or connective tissue disease were excluded from the study, as were those with a previously undetected cardiac source of thrombus identified by clinical assessment. Other reasons for exclusion were noncortical stroke (n = 12), subarachnoid hemorrhage (n = 2), and death within 1–3 days of hospitalization (n = 4). A total of 49 patients with ICH and acute IS [16 women and 33 men; mean (SD) age, 53.8 (2.9) years] were studied.

According to standard protocol, physicians in the Intensive Stroke Unit recorded clinical data from the primary evaluation, including history and examination results. A final diagnosis was established by chart reviews from two independent investigators, as follows:

Acute IS was defined as neurologic deficit of sudden onset with focal rather than global neurologic dysfunction, with symptoms lasting more than 24 h and presumed to be of nontraumatic vascular origin *(15)*. In patients with cortical infarcts, neurologic deficit was evaluated using the NIHSS.

Patients with cerebral ischemia underwent brain MRI within 3 h (day 0) after their cerebrovascular event. The

imaging protocol included conventional T2-weighted images, perfusion-weighted imaging scan, and magnetic resonance angiography, study performed on a 1.5-Tesla MRI scanner (MR Vision; Siemens). All images were processed by proprietary programs written in interactive data language. The apparent mean transmit time, defined as the first moment of the measured tissue curve, was used. A neuroradiologist, blinded to clinical symptoms, drew three-dimensional ROIs by manual contouring of apparent mean transmit time images and computed infarct volumes by multiplying the measured ROI per slice by section thickness. Magnetic resonance angiography scans were graded as "occlusion" when there was an abrupt loss of all signal in a vessel.

Intracerebral hemorrhage was defined as that originating from the spontaneous rupture of small vessels damaged by chronic hypertension, causing bleeding into the cerebral parenchyma (16). On arrival at the hospital, a CT scan was performed with a matrix of 512×512 and a slice thickness of 8–10 mm on all patients. A radiologist blinded to clinical symptoms calculated lesion volumes on the radiographic plate, multiplying one-half the maximal values of longitudinal diameter by the transverse diameter by the number of 10-mm slices containing the hemorrhage. Those with evidence of ICH (right and left hemispheres) and infarct volume <0.03 L, comparable to that for IS patients, were assigned to the ICH group. Patients with ICH were also assessed by use of the Glasgow Coma Scale.

Control individuals at both clinical sites were age- and gender-matched healthy individuals (n = 230) without risk factors for IS (e.g., smoking, hypertension, diabetes mellitus, or hyperlipidemia). As a group at risk for TIA and stroke, individuals with controlled hypertension/atherosclerosis (n = 25) were used as additional controls. Several patients with hypertension/atherosclerosis suspected to have a "silent" stroke underwent DWI as described for patients with TIA.

SAMPLE COLLECTION, STORAGE, AND ASSAY

Before any intervention, blood samples and medical histories were taken, and CT or MRI, routine laboratory analyses, and diagnostic/therapeutic interventions were performed as appropriate. Blood samples were drawn from patients (hypertension/atherosclerosis, TIA, IS, ICH) admitted to the Intensive Stroke Unit within 3 h of onset and additionally at 6, 9, 12, 24, and 72 h after cerebrovascular accident for the ischemic and hemorrhagic stroke groups. Blood samples from age- and gender-matched healthy individuals were taken from the local blood bank at Pavlov's State Medical University. A sample (5 mL) was taken by venipuncture into vacuum tubes without anticoagulant (Becton Dickinson), and serum was separated by centrifugation at 4000g for 5 min at 4 °C and stored as 0.5-mL aliquots at −80 °C until an ELISA was performed.

ANTIGEN IDENTIFICATION AND CALIBRATOR ANTIBODIES

Total NMDAR was isolated from synaptic membranes of human cortex (autopsy material from Department of Pathology, University Hospital) and was purified by affinity chromatography on glutamate-Sepharose 4B and then by HPLC. NMDAR contains three major protein components and possesses glutamate-binding activity with a single type of binding site (17). The analysis of L-[³H]glutamate binding to purified NMDAR in the presence of argiopin, a blocker of NMDAR (18), demonstrated a dose-dependent inhibition with $K_i = 2.54$ mg/L.

The immunochemical identification of NMDAR was performed by use of commercial antibodies and antibodies kindly supplied by Dr. R. Wenthold (NIH, Bethesda, MD).

Monoclonal antibodies (mAbs) presenting IgG (7C5F2B2) or IgM (8E12G4C1) were obtained by the hybridoma method and used for identification of purified NMDAR (19). Colloidal gold-labeled mAbs and/or antibodies purified from patients (total IgG) with acute IS revealed NMDAR-like immunoreactivity, mostly in synaptic terminals of glutamate-receptive neuronal axons and, to a lesser extent, in the soma of cells in the rat sensomotor cortex and organotypic primary cell culture of the rat sensomotor cortex (19, 20). Peptide fragments of NR2A/2B (~2 kDa) were observed in the plasma of humans with acute cerebral ischemia by mAb IgM (20).

The approach to identifying the peptide sequence from NMDAR included isolation and investigation of the specific cDNA encoding NMDAR. The immunoscreening of 10^6 clones in the human brain cDNA library λGT11 by mAb and total IgG from IS patients revealed one recombinant phage λNMDAR clone with a positive immunologic signal. The restriction map obtained by use of standard endonucleases showed the existence of a 0.5-kb cDNA insert (21). After cDNA nucleotide sequence determination by a standard molecular biological procedure (22), a corresponding peptide consisting of 157 amino acid residues with a molecular mass of 19 kDa was found. A computer analysis of the known nucleotide sequence in the National Center for Biotechnology Information (NCBI) library revealed the existence of peptides homologous to the N-terminal domain of NR2A/2B receptors.

An immunoactive specific NR2A/2B peptide (21 amino acids) corresponding to the N-terminal sequence of NMDAR (23) was designed using the GCG sequence analysis program. The peptide was produced by solid-phase synthesis on methylbutyl-hydroacetyl resin, purified by HPLC, immobilized on matrix, and used as an antigen in an ELISA (12). Polyclonal antibodies against NR2A/2B peptide were raised by rabbit immunizations, and the IgG fraction of antibodies was purified according to standard techniques by use of chromatography on protein A-agarose and then on NR2A/2B-containing Sepharose 4B (20). These antibodies were used in an ELISA reagent to prepare eight calibrators, from 0.5 to 400

µg/L (Fig. 1A). The IgG concentrations in patient samples were expressed in µg/L according to a calibration curve. Sample diluent was included as the blank in each assay to calculate the zero unit value.

Western-blot analysis of plasma specimens from pa-

Fig. 1. Calibration curve for NR2A/2B aAbs by ELISA (*A*), and representative Western blot of peptide fragments of NMDAR by use of pooled plasma samples from individuals in the groups studied (*B*).

(*A*), the limit of detection for the ELISA was 0.5 µg/L. Detection is by absorbance at 492 nm. (*B*), plasma samples from healthy individuals (*lane 1*), patients with TIA (*lane 2*), and patients with an acute ischemic stroke (*lane 3*).

tients with TIA by use of polyclonal antibodies (IgG) against N-terminal domain NR2A/2B revealed several peptides of 68, 57, 45, 14, and 2 kDa. In the blood of patients with acute ischemic stroke, the accumulation of mostly low-molecular-mass peptides (2, 6, and 14 kDa) was found with the same polyclonal antibodies (Fig. 1B).
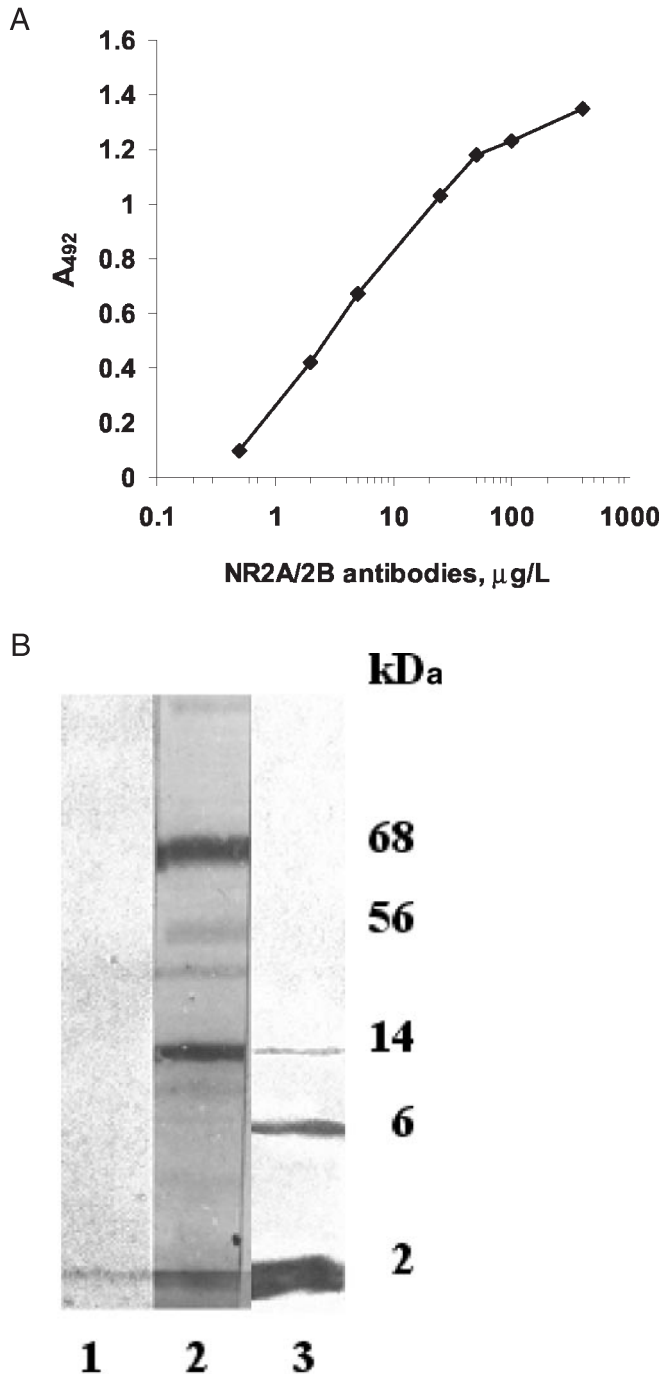
DETECTION OF aAbs TO NR2A/2B
A researcher blinded to the clinical and neuroimaging data performed the assays *(24)*. An ELISA (CIS-test; CIS Biotech, Inc.) was used for detection of NR2A/2B aAbs in the serum samples, according to the manufacturer's instruction manual.

We added 100 µL of diluted blood sera (1:50; 20 µL of serum sample + 980 µL of diluent) and sets of calibrators to NR2A/2B peptide-coated wells of microplates and incubated the plates for 1 h at 25 °C. After the wells were washed with buffer, we added 100 µL of rabbit anti-human horseradish peroxidase-labeled IgG (1:1000 dilution) to the wells and incubated them for 1 h. The reaction was revealed by *o*-phenylenediamine detection solution after additional washing. Detection solution (*o*-phenylenediamine in 0.05 mol/L citrate buffer, pH 4.3) was pipetted (100 µL) into each well of the microplate. The color reaction was developed for 20 min, stopped with acid solution (30 µL), and monitored at 490 nm on a microplate reader (Dynatech MR 4000). Sample buffer was also included as the blank in each assay to calculate the zero unit value. The NR2A/2B aAb titer in serum was determined by use of the calibration curve of absorbance units of aAbs vs concentration in the microplate wells.

STATISTICS
Statistical analysis was performed using the Statistica for Windows statistical package (1984–1999; StatSoft). Means (SD) and 95% confidence intervals (CIs) were used where appropriate. A ROC curve was used to calculate the cutoff value for optimal sensitivity and specificity *(25)*. Continuous variables were compared by use of the McNemar or Student *t*-test as appropriate. A two-sided *P* value <0.05 was considered significant. The Spearman rank correlation test was used to analyze the dependence of NR2A/2B aAb values on severity and volume of infarct.

Analysis of serial data from NR2A/2B aAb measurements in IS and ICH patients was performed by the method of summary weighted average areas under the response curve in an individual *(26)*. A logarithmic transformation of dependent variables was performed to achieve a gaussian distribution. In preparation of this report, the guidelines proposed by STARD Group were followed *(27)*.

Results
PATIENT CHARACTERISTICS
Of the 105 patients with cerebrovascular events, 87 were determined to have cerebral ischemia (TIA, n = 56; acute IS, n = 31) and 18 had ICH (Fig. 2). The control groups
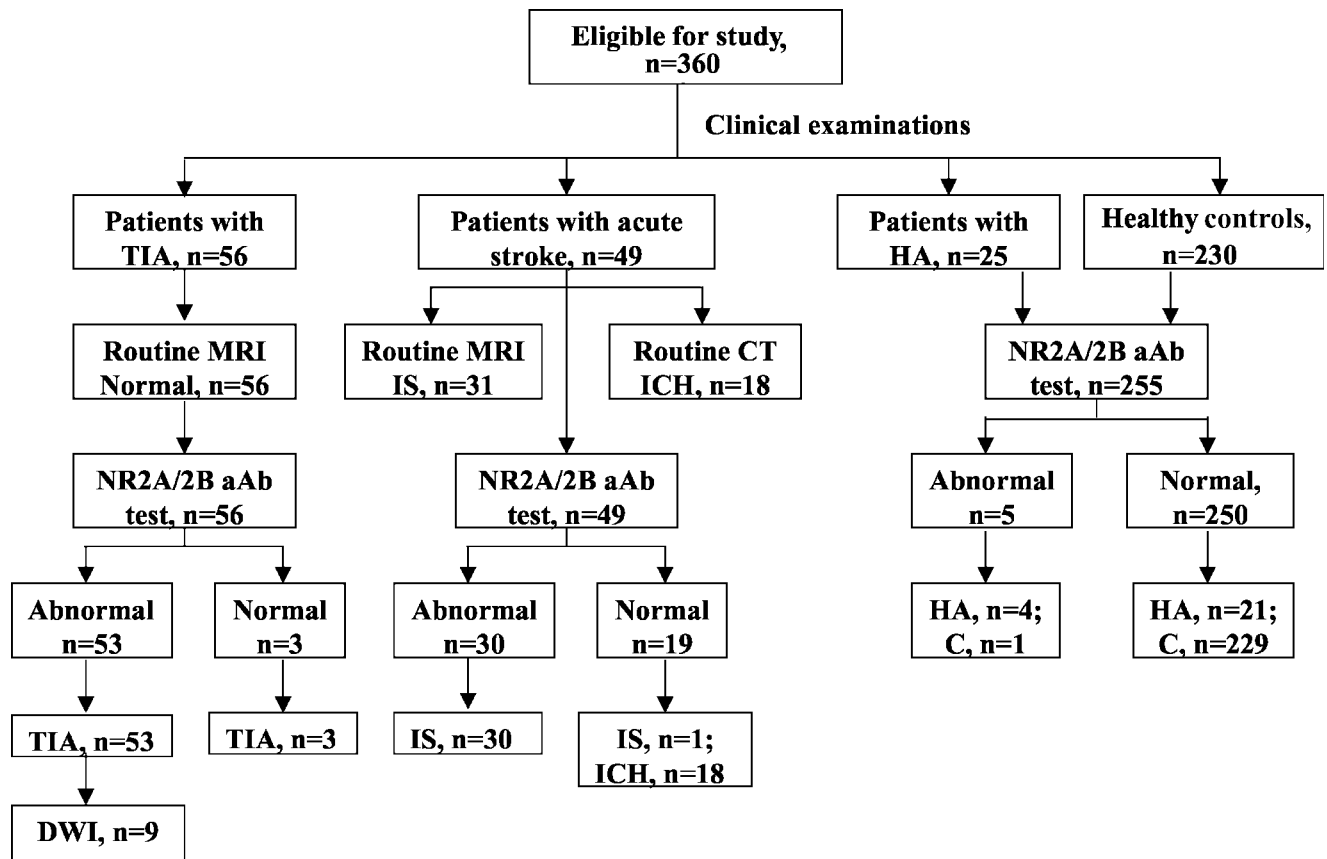
Fig. 2. Flow diagram for the diagnostic accuracy study for the NR2A/2B aAb test in healthy controls (*C*) and patients with hypertension/ atherosclerosis (*HA*), TIA, ICH, and acute IS.

included 230 healthy individuals and 25 with controlled hypertension/atherosclerosis (Fig. 2). All participants in the study were recruited between February 1994 and December 2000.

Patients with TIA had a history of hypertension in 86% of cases. The median NIHSS score on admission was 9 (range, 5–10). No abnormalities were detected on routine MRI in the previous history of any TIA patient. Detailed T2-weighted MRI and DWI in nine patients with recurrent TIA were analyzed at the third hour after admission. Regional ischemia was clearly depicted as hyperintensity on DWI, whereas T2-weighted imaging showed no changes. T2-weighted MRI showed that an area of infarction developed to day 7 of observation in four patients and was accompanied by neurologic worsening.

For the 31 patients with acute IS, a history of hypertension and atherosclerosis was recorded in 100% of cases. In this group, an acute occlusion of the internal carotid artery was observed in 94% of cases. Most patients (61%) had cortical lesions located in the left carotid artery of the left hemisphere. The median NIHSS score on admission was 25 (range, 16–34), and the median initial infarct volume was 19.2×10^{-3} L (12.1×10^{-3}–26.2×10^{-3} L). In seven patients, regional ischemia with a median volume of $<5 \times 10^{-3}$ L was depicted as hypoperfusion areas on

perfusion-weighted imaging scans within 3 h of symptom onset, whereas T2-weighted imaging showed expanded lesion areas to day 7 of hospitalization. The routine protocol of acute care, including magnesium (not tissue plasminogen activator) was used in patients with acute IS. Satisfactory outcome (improvement on the NIHSS score of 7 points or more) was recorded on day 7 in 18 patients (58%) and poor outcome (no or little improvement on the NIHSS score) in 13 (42%). Patients with good outcomes were moved to a rehabilitation clinic, where the treatment was continued.

The 18 patients with ICH included 9 with lobar and 9 with deep ICH. A history of hypertension was recorded in 94% of cases. The median Glasgow Coma Scale score on admission was 14 (range, 10–18), and the median initial volume of ICH was $22 \times 10^-$ L (10.2×10^{-3}–34×10^{-3} L). Satisfactory outcome was recorded in 10 patients (56%) and poor outcome in 8 (44%) according to the NIHSS score on day 7.

NR2A/2B aAb CONCENTRATION

NR2A/2B aAb-positive patients with TIA had a mean (SD) concentration of 4.02 (2.04) μg/L (range, 2.71–7.23 μg/L), and NR2A/2B aAb-positive patients with IS had a mean (SD) concentration of 5.01 (1.23) μg/L (range,
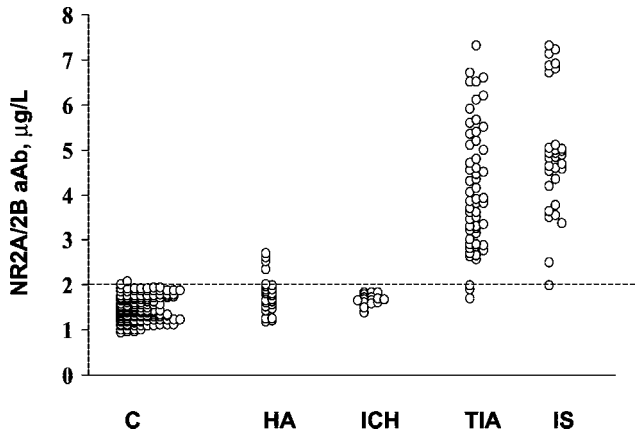
Fig. 3. Distribution of serum concentrations of NR2A/2B aAbs in healthy controls (*C*) and patients with hypertension/atherosclerosis (*HA*), ICH, TIA, and acute IS.

Blood samples were taken on admission. *Dashed line* shows cutoff.

3.24–7.21 μg/L). NR2A/2B aAb-negative patients with TIA or IS and those with ICH or hypertension/atherosclerosis had a mean (SD) value of 1.72 (0.23) μg/L, whereas controls had a mean (SD) concentration of 1.49 (0.22) μg/L (range, 1.02–1.98 μg/L).

The intraassay CV was 2.7–8.5%, and the interassay CV was 12–20%.

analysis of nr2a/2b aAb in groups studied

Fig. 3 shows the distribution of serum concentrations of NR2A/2B aAbs in controls (group C) and those with cerebrovascular diseases: hypertension/atherosclerosis (group HA), ICH, TIA, and IS as confirmed clinically and/or by MRI/CT. The comparison of mean values of NR2A/2B aAbs in independent age- and gender-matched groups demonstrated that aAb values for the control, hypertension/atherosclerosis, and ICH groups belong to the same distribution. Conversely, significant differences were observed for patients with cerebral ischemia (TIA/IS) compared with these three groups (Table 1). Median values for NR2A/2B aAbs increased in the following order: patients with hypertension/atherosclerosis, TIA, and acute IS.

Table 1. Comparison of NR2A/2B aAb concentrations in groups of age- and gender-matched individuals.

Groups compared[a]	n	McNemar test[b]	
		χ^2	*P*
C/HA	25	0.25	0.62
C/ICH	18	0	1.0
C/TIA	37	32.03	0.0001
HA/TIA	25	19.04	0.00001
C/IS	23	20.05	0.000008
HA/IS	23	17.05	0.00004

[a] C, control group; HA, hypertension and atherosclerosis.
[b] Degree of freedom is 1.

analysis of nr2a/2b aAbs in patients with tia

Analysis of the correlation between NIHSS score and NR2A/2B aAb concentrations in patients with TIA yielded a correlation coefficient of 0.81 and high statistical significance (Fig. 4A). In nine patients with known TIA onset time, the comparison of DWI lesion volumes detected within 3 h of the event yielded a correlation coefficient of 0.87 and a significant positive association with NR2A/2B aAb values (Fig. 4B).

The operating characteristics of NR2A/2B aAbs are
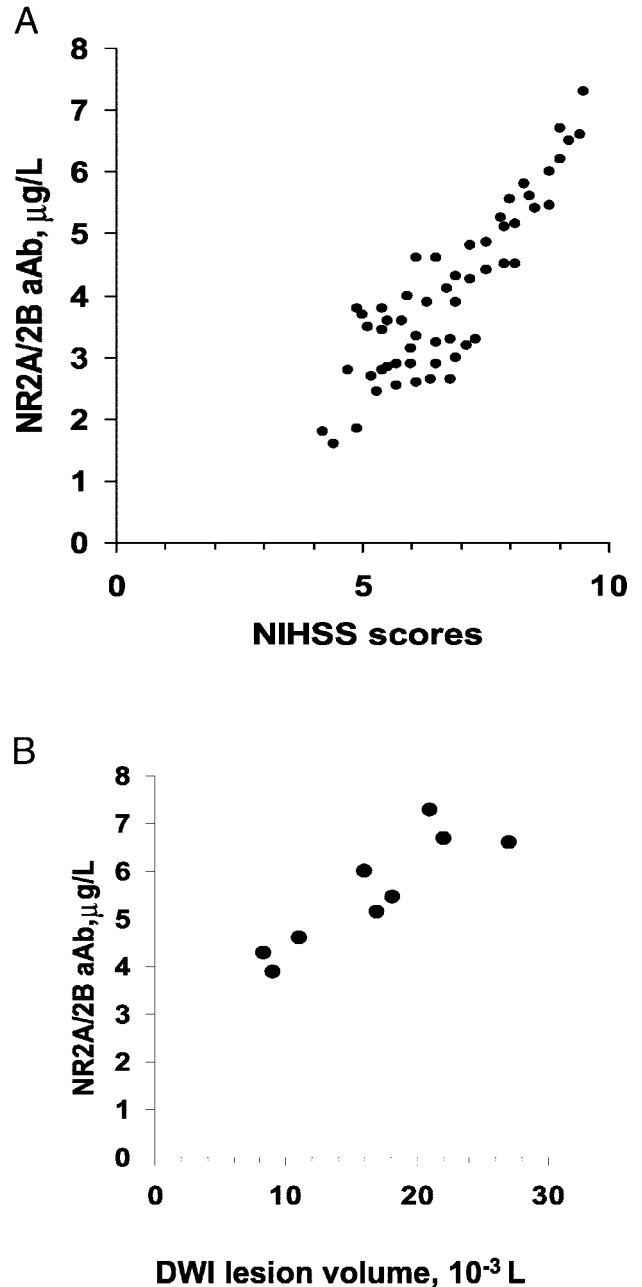

Fig. 4. Correlation of NR2A/2B aAb concentration with NIHSS score (*A*; n = 56) and DWI lesion volume in patients with TIA (*B*; n = 9).

Spearman coefficients: (*A*), $r_s = 0.81$ (*P* <0.0001); (*B*), $r_s = 0.87$ (*P* = 0.002).
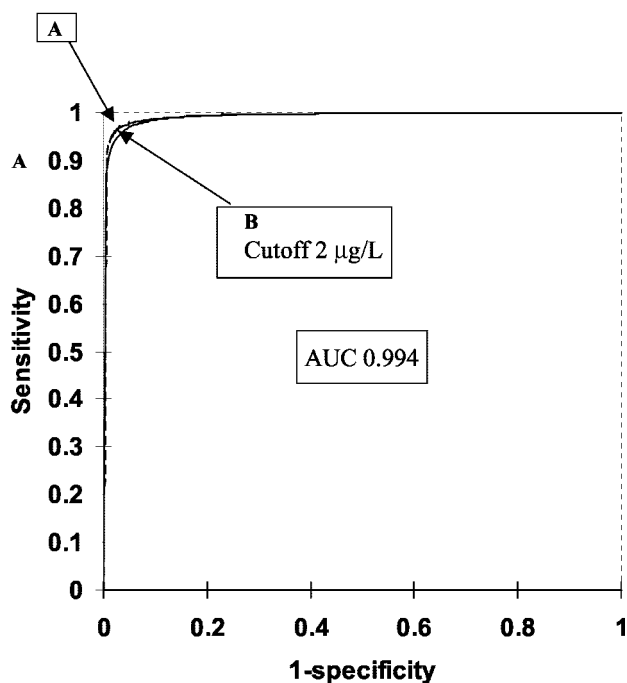
Fig. 5. ROC curves for serum NR2A/2B aAbs.
Line A, IS; *line B*, TIA. *AUC*, area under the curve.

depicted in Fig. 5 and Table 2. The tradeoffs between true-positive and false-positive rates is shown by presenting data as a traditional ROC curve (Fig. 5). The proportional area under the curve was 0.99. Table 2 shows the predictive values and likelihood ratios at specific cutoff points, chosen to approximate to sensitivities of 75%, 95%, and 98%. The best cutoff value for TIA diagnosis was the middle cutoff point shown (2.0 μg/L; sensitivity, 95%) at which a positive predictive value of 91% was achieved.

ANALYSIS OF NR2A/2B aAbs IN PATIENTS WITH ACUTE IS

There was a significant correlation (r_s = 0.91) between the NIHSS severity scores of acute IS and NR2A/2B aAb concentrations (Fig. 6A). Analysis of the correlation of lesion volumes defined by multimodal MRI scans and the NR2A/2B aAb concentrations for the same group of patients produced a correlation coefficient of 0.79 and showed statistical significance (Fig. 6B). Patients with severe cases of acute IS (n = 9) and a NIHSS score of 30–34 had NR2A/2B aAb concentrations approximately six times higher than that for controls. The NR2A/2B aAb concentrations for patients with mild to moderate IS (n = 18) and NIHSS score of 16–22 were lower than for severe cases. Intravenous magnesium treatment *(28)* followed by routine therapy (nootropics, vasoactive substances, anti-hypertensive medication, and anticoagulants) improved neurologic symptoms (NIHSS score decreased by 10–12) within 7 days in 58% of patients with acute IS and was accompanied by a decrease in NR2A/2B aAb concentrations to values comparable to those of healthy individuals.

The proportional area under the ROC curve for patients with acute IS was similar to that for TIA patients (Fig. 5). The predictive values and likelihood ratios at specific cutoff points were chosen to approximate to sensitivities of 94%, 97%, and 100%. The best cutoff value for IS diagnosis was the cutoff point at 2.0 μg/L (sensitivity, 97%) at which the positive predictive value was 86% (Table 2).

DYNAMIC CHANGES OF NR2A/2B aAbs IN PATIENTS WITH IS AND ICH

Significantly different NR2A/2B aAb profiles were observed in the blood of patients with acute IS and ICH (Fig. 7, A and B). Panels C and D of Fig. 7 show peak NR2A/2B

Table 2. Operating characteristics of different cutoff points for NR2A/2B aAb concentrations.

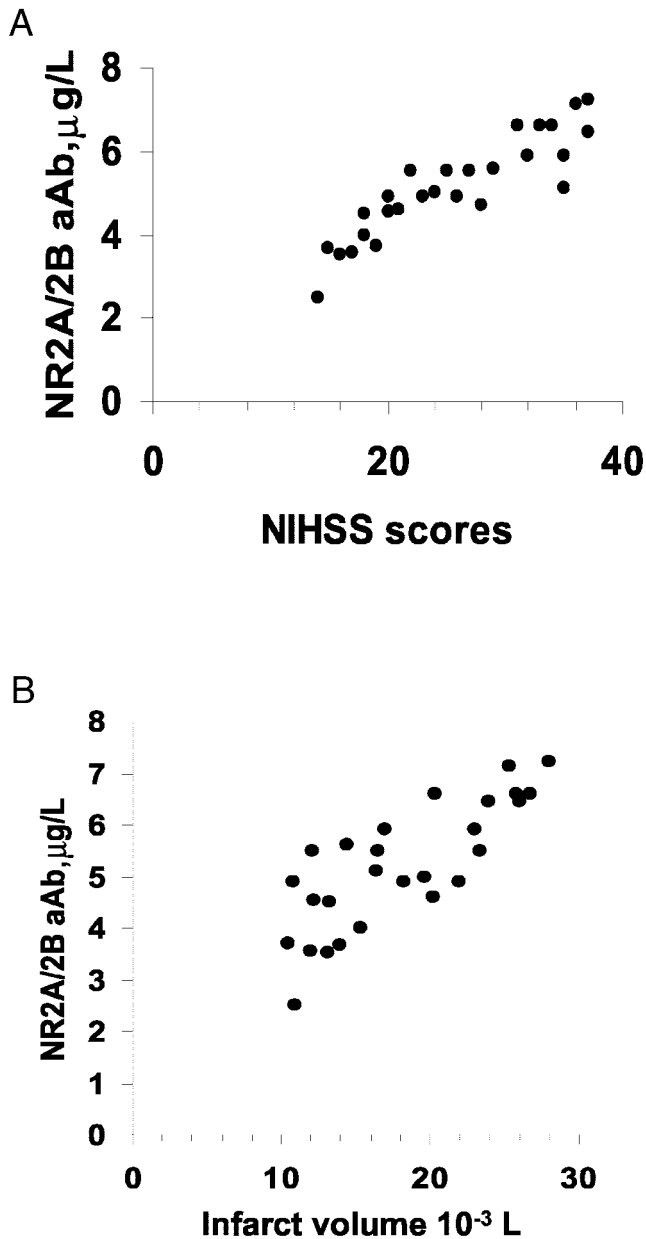| Characteristics	NR2A/2B aAb test performance at cutoff of		
	≥1.8 μg/L	≥2.0 μg/L	≥3.0 μg/L
Sensitivity, %			
TIA	98 (55/56)	95 (53/56)	75 (42/56)
IS	100 (31/31)	97 (30/31)	94 (29/31)
Specificity, %			
TIA and IS	89 (242/273)	98 (268/273)	100 (273/273)
Positive predictive value, %			
TIA	64 (56/86)	91 (53/58)	100 (42/42)
IS	50 (31/62)	86 (30/35)	100 (29/29)
Negative predictive value, %			
TIA and IS	99 (242/243)	98 (268/272)	100 (273/273)
Positive likelihood ratio (95% CI)			
TIA	8.6 (6.4–9.8)	47.5 (40.1–52.2)	
IS	8.8 (6.7–10.2)	48.5 (41.2–53.5)	
Negative likelihood ratio (95% CI)			
TIA	0.02 (0–0.05)	0.05 (0–0.07)	0.25 (0.1–0.35)
IS	0	0.03 (0–0.06)	0.06 (0.02–0.07)

Fig. 6. Correlation of NR2A/2B aAb concentration with NIHSS score (*A*) and infarct volume (*B*) in patients with acute IS (n = 27).

Spearman coefficients: (*A*), $r_s = 0.91$ ($P < 0.0001$); (*B*), $r_s = 0.79$ ($P < 0.0001$).

geometric means at the 95% CIs. There was strong evidence that there was both a greater peak value and higher overall serum concentration of NR2A/2B aAbs in patients with IS than those with ICH.

Discussion

Stroke and TIA are serious conditions involving brain ischemia. Both cause current or impending disability and a risk of death; however, timely diagnosis of TIAs offers a greater opportunity to initiate treatment that can forestall the possible onset of brain infarction (*14, 15*). After a first TIA, 10–20% of patients will likely have a stroke within the next 90 days; in 50% of these patients, the stroke occurs within the first 2 days after a TIA. TIAs are underrecognized, underreported, and undertreated (*14*). The development of symptoms of acute brain ischemia constitutes a medical emergency. New imaging techniques can accurately identify intracranial hemorrhage, tumors, and other disorders that can cause transient symptoms that might be otherwise misdiagnosed as TIAs (*14*).

During the past 5 years, several immunochemical assays have been proposed and partly evaluated for clinical use in neurology (*3, 4*). At present, the Thrombogene V and two Thrombix tests from Athena Diagnostics are available for differentiating stroke/thrombosis. These tests evaluate the frequent deep vein thrombosis and hypercoagulation conditions in patients to assess the need for intravenous anticoagulant therapy. Other tests use ELISAs to monitor changes in blood coagulation markers: anti-thrombin III protein C, factor IX, and anti-cardiolipin antibodies (IgG, IgM, and IgA). The biochemical marker D-dimer, a breakdown product of a cross-linked fibrin blood clot that indicates the occurrence of plasmin-mediated lysis of cross-linked fibrin, has been evaluated extensively for use in diagnostic tests to indicate acute venous thromboembolism. Indeed, one D-dimer latex agglutination assay provides results within 20 min with a sensitivity of 89–95% (*5*). Unfortunately, increased concentrations of D-dimer have been found in other settings that lead to fibrin generation, including recent surgery, hemorrhage, trauma, cancer, and pregnancy (*6*).

Several thrombotic (homocysteine, anti-phospholipid antibodies, anti-cardiolipin antibodies), neurotoxic (glutamate), and neurochemical markers (neuro-specific enolase, protein S100, and myelin) have been studied to date in an attempt to determine their reliability in the recognition of TIA/stroke (*2, 29, 30*). However, their role in the molecular cascade of neurotoxicity underlying stroke remains poorly understood, and these markers cannot yet be used for TIA or stroke identification.

On the basis of our predevelopmental and preclinical research delineating the utility of NMDAR in assessment of cerebral ischemia, we proposed a new serologic marker: aAb to NMDAR. We found up-regulated expression of NMDAR mRNA during ischemic conditions, in contrast to down-regulation under ICH (*31, 32*). A selective in-

aAb concentrations in patients with acute IS and ICH by the time the maximum occurs as derived from the data shown in panels A and B. This clearly shows that peak values tended to be lower and occurred somewhat earlier in the ICH patients than those with acute IS. The results of calculations of weighted average values of the area under the response curve for each patient with acute IS and ICH, expressed in summary measures that were then log-transformed, are presented in Table 3. The summary of maximum NR2A/2B aAb concentrations for the two groups compared is included in Table 3. The difference between the groups was expressed as the ratio of the
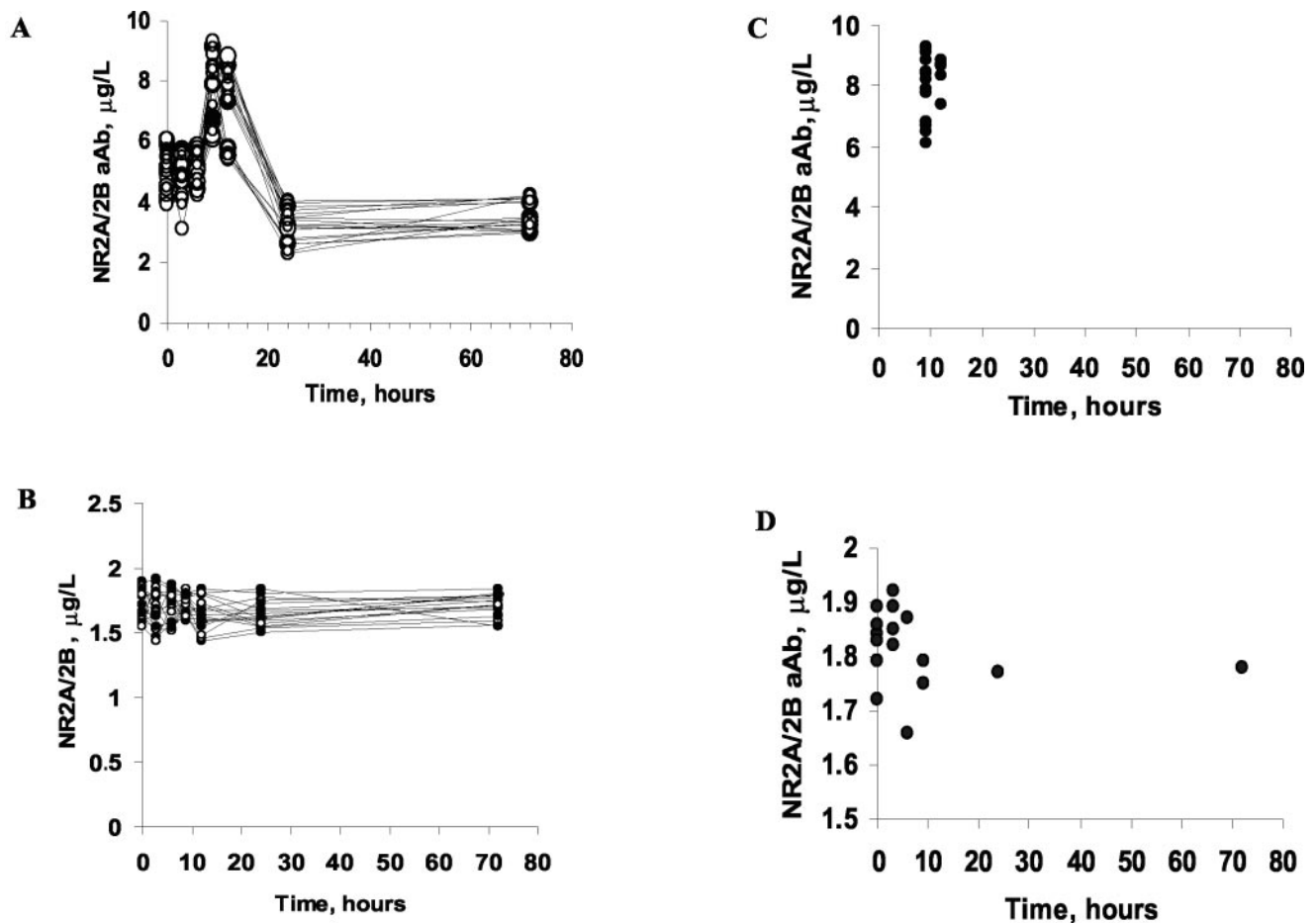
Fig. 7. NR2A/2B aAb concentrations measured in each patient with acute IS (*A*; n = 23) and ICH (*B*; n = 18) at hospital admission (time 0) and up to 72 h after admission.

(*C* and *D*), peak NR2A/2B aAb concentrations for patients with acute IS (*C*) and ICH (*D*).

crease in NR2A/2B aAbs reflected the appearance of excessive amounts of corresponding immunoactive peptide fragments in the bloodstream, causing aAb generation. Peptide appearance and accumulation in the blood correlated with high synthesis of glutamate receptors under ischemic conditions in the first hours of middle cerebral artery occlusion in rats (*31, 32*). In rats with

Table 3. Analysis of data from NR2A/2B aAb monitoring in IS and ICH patients.

Characteristics	Area under curve	Maximum concentration
IS patients (n = 23)		
Mean (SD), μg/L	123.66 (14.1)	8.20 (0.94)
Geometric mean, μg/L	122.88	8.15
ICH patients (n = 18)		
Mean (SD), μg/L	44.47 (2.28)	1.81 (0.07)
Geometric mean, μg/L	44.42	1.81
Ratio of geometric means (95% CI)	2.77 (2.52–2.93)	4.50 (4.37–4.65)
t-test	33.96 (df = 39)	93.33 (df = 39)
P value	<0.0001	<0.0001

induced cerebral ischemia, increased concentrations of NR2A/2B peptide fragments were detected within the first 24 h of reperfusion. The appearance of increased serum NR2A/2B aAb (IgG) was observed during the first 72 h of induced ischemia (*31*).

On the other hand, decreased mRNA expression of NR2A/2B receptor fragments was demonstrated in rats with induced ICH within the first 24 h after surgery (*32*). At the same time, the immunoreactivity of NR2A/2B (peptide) showed no changes in cortex and hippocampus compared with that in control animals. We found low concentrations of NR2A/2B peptide in the blood samples of ICH rats, accompanied by reduced immune response. In the case of ICH, thrombin-activated serine proteases that cleave NR2 receptor under conditions of cerebral ischemia (*7*) are probably not involved in the mechanisms of necrosis underlying the ICH. Perhaps other proteases cleave NR2 receptor to produce peptides with nonimmunoactive epitopes.

In this clinical study, in contrast to our preclinical research, the dramatic increase in NR2A/2B aAb concentrations was detected in nearly all patients with cerebral

ischemia within 3 h of onset. This result is possibly attributable to preexisting conditions in patients that may contribute to TIA, such as hypertension and atherosclerosis. In fact, we detected several individuals with increased NR2A/2B aAbs in the group of patients with controlled hypertension/atherosclerosis, indicating possible neurotoxic processes in nervous tissue and probable development of a silent stroke. These four patients (risk group) had low a NIHSS score (<3) with no abnormalities on DWI performed within 24 h of study admission.

Measurement of NR2A/2B aAbs may have two potential medical uses: (*a*) TIA risk assessment and (*b*) assisting in better clinical diagnosis of a patient with "stroke-like" symptoms. This premise is supported by the high predictive value of the test for recognizing individuals with previous and recurrent TIA (91% at 2.0 μg/L cutoff; likelihood ratio; 47.5) and by the significant correlations with neurologic deficit and lesion volumes. Conversely, the test may be used for ruling out individuals without TIA. If the test at a cutoff point of 2.0 μg/L were negative, the probability in a posttest for IS would be <2%.

With respect to laboratory diagnosis of acute hemispheric cortical IS, at about the same likelihood ratio of a positive test as for TIA (2.0 μg/L cutoff; likelihood ratio, 48.5), the slight decrease in posttest probability to 86% underscores the tendency for reduced test accuracy in cases with deeper infarcts. In another study that assessed patients with IS vs stroke-like disorders, we observed a significantly decreased correlation of NR2A/2B aAbs with infarcts >30 $\times$ 10^{-3} L, implying involvement of brain white matter (*28*). NR2A/2B aAb concentrations were also decreased to lower than that found in controls in extremely severe cases with lethal outcome (*28*). Other research devoted to the study of stroke effects on the immune system demonstrated that the different degrees of lateralized T-cell responses depended on location of brain lesion and neurologic deficit (*10*).

The highly increased NR2A/2B aAb concentrations measured in patients with TIA and acute IS may be indicative of early thrombosis and neurotoxicity in brain structures. Therefore, NR2A/2B aAb up-regulation could reflect neuroprotective effects of cortical tissue where NMDAR is primarily located and might serve as a fast immune reactant that traces the time of potentially salvageable "ischemic penumbra". Studies by During et al. (*33*) demonstrated that the oral administration of a single dose of a vaccine generating aAbs to NMDAR was associated with a strong neuroprotective effect in rats with induced cerebral ischemia and epilepsy.

There are possible explanations for abrupt increases in NR2A/2B aAb concentration in the blood of IS patients, based on the theory of immune response. NMDAR is cleaved by proteases into several endogenous brain peptides (Fig. 1B) with overlapping (continuous) epitopes (*34*). These peptides initiate immune response, generating high- and low-affinity aAbs with different latent periods. Perhaps the peak in NR2A/2B aAb concentration at 10–12

h after onset might be the result of aAb amplification over the previous days that coincided with severity of symptoms.

The evidence of natural aAbs to different endogenous peptides circulating in healthy organisms has been reported (*35*). It seems that these antibodies are capable of rapidly increasing their concentrations and differentiating their specificity to recognize certain epitopes prevalent in the blood.

Thus, the abrupt increase of NR2A/2B aAbs in IS patients is analogous to the immune reaction in patients with allergies. According to the theory of humoral immune response, many patients with stroke have a latent (undetected) TIA because antigens (peptides) have been introduced to the blood in sufficient time to develop the high-affinity antibodies first. The decrease in aAbs in the blood of patients might be attributable to (*a*) intravenous dilution from the magnesium therapy and/or (*b*) a loss of reactivity by antibodies bound by antigen in immune complexes.

In conclusion, the clinically predetermined cutoff for NR2A/2B aAbs allowed us to differentiate acute IS from ICH. Monitoring of NR2A/2B aAbs within 72 h after a stroke event allowed patients with acute IS to be identified; these results were statistically significant and correlated with data obtained from CT and MRI scans that were interpreted by neuroradiologists blinded to test results. The maximum concentrations of NR2A/2B aAbs detected in both patient groups indicated the probability of a clinically relevant therapeutic window; i.e., within the first 3–5 h for patients with ICH and up to 9–12 h for patients with acute IS. The latter observations may be important in an emergency setting when timely and appropriate use of tissue plasminogen activator can help increase the likelihood of a patient having a satisfactory outcome.

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