IVD TECHNOLOGY
FOR IN VITRO DIAGNOSTICS DEVELOPMENT & MANUFACTURING

Originally Published IVD Technology March 2004

Assay Development

A new brain marker for laboratory assessment of TIA and stroke

Assays based on autoantibodies generated by NMDA receptors can provide highly sensitive and specific results quickly.

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Stroke is the most common devastating neurologic disease worldwide and the third-leading cause of death after heart disease and cancer. In the United States in 1997 there were 159,791 deaths that were attributed to stroke, a rate of 25.9 per 100,000 population. Of those who survive stroke, 40% may be permanently disabled and 10% may require costly long-term care.[1] Stroke risk increases with age; two-thirds of all strokes occur in people more than 65 years old. As the general population continues to age, this will place a major, and growing, medical and economic burden on the healthcare system. Progress has recently been made in understanding stroke mechanisms, yet stroke management remains suboptimal for a number of reasons.

First, the medical community currently faces several diagnostic challenges in managing stroke. One is to determine definitively in the emergency room or outpatient setting whether a patient has suffered a transient ischemic attack (TIA), which dramatically increases the likelihood of a later acute stroke.[2] Accurate differentiation of ischemic stroke from intracerebral hemorrhagic stroke (ICH) also is important.3 Furthermore, clinical, laboratory, and imaging data now routinely available allow the progression of stroke to be predicted only partially.

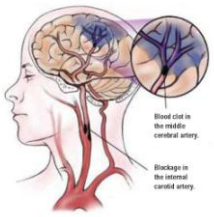
Second, many people who suffer a stroke either do not seek immediate medical care or experience delays in care provision even in countries where stroke care is advanced, such as the United States and Europe. Several clinical criteria can be employed to diagnose whether a patient may be having a stroke, but even these do not always enable differentiation from other disorders such as epilepsy, syncope, and migraine.

Finally, the importance of prompt stroke diagnosis and treatment after symptoms appear cannot be overstated. Delay in diagnosis and medical intervention beyond three hours of stroke onset may contribute to clinical deterioration and disability by forfeiting the limited amount of time during which the brain can respond to reperfusion and significantly increases the risk of hemorrhage after most of the permanent injury has occurred.[3] An early diagnosis, on the other hand, enables the physician to select the most effective emergency intervention, including neuroprotective therapy, and to predict clinical outcome more accurately.

This article presents an overview of in vitro diagnostic tests in development for risk assessment of TIA and stroke. It outlines predevelopmental research suggesting the molecular properties of N-methyl-D-aspartate (NMDA) receptors involved in stroke, preclinical studies of NMDA receptor (NMDAR) markers in animal models, and clinical evaluation of patients with TIA or stroke. On the basis of these investigations, several serological assays were designed and proposed, which are described here.

Prospects for Selective Testing for Stroke

During the past five years, a number of molecular and immunochemical assays have been evaluated for clinical use in neurology—for example, the Thrombx Evaluation Profile I and II tests, available from **Athena Diagnostics** (Worcester, MA), for diagnosing stroke and thrombosis. The Profile I test evaluates deep-vein thrombosis and hypercoagulation states resulting from a thrombotic event, such as stroke venous thrombosis, in order to determine a patient's need for intravenous anticoagulant therapy. The Thrombogene V portion of the test detects the Factor V Leiden mutation by polymerase chain reaction (PCR). Other blood coagulation markers monitored include antithrombin III, protein C, protein S, Factor IX antigen (in Profile II, for patients on anticoagulant therapy), and anticardiolipin antibodies (IgG, IgM, IgA) by enzyme-linked immunosorbent assay (ELISA) technique.



Ischemic stroke occurs when oxygen-rich blood flow to the brain is restricted by a clot or other blockage. Click to enlarge.

Stroke is related to different types of venous thromboembolism. The biochemical marker D-dimer, a breakdown product of a cross-linked fibrin blood clot that indicates occurrence of plasmin-mediated lysis of cross-linked fibrin, has been extensively evaluated for use in diagnostic tests for acute venous thromboembolism. A fully automated semiquantitative latex agglutination assay that uses turbidimetric or agglutination endpoints has been developed that provides results within 20 minutes, with a high sensitivity of 89–95%.[4]

However, the amount of D-dimer, like the other thromboembolic markers mentioned above, was increased in other circumstances that result in fibrin generation, including recent surgery, hemorrhage, trauma, cancer, and pregnancy. Also, this test does not elucidate the mechanisms of TIA and stroke actually responsible for the damage associated with neurotoxic molecular events.

Two of the three leading causes of death in humans—cardiovascular disease and stroke—are the consequence of atherosclerosis. Therefore, it is not surprising that several biochemical markers implicated in thromboembolic processes are also reported to be associated with stroke and stroke risk. Among these are homocysteine, cholesterol, and low-density lipoprotein (LDL, known as bad cholesterol), which are also classified as risk factors for cardiovascular and cerebrovascular diseases.

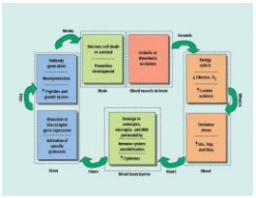


Figure 1. The ischemic cascade of neurotoxicity underlying stroke. Click to enlarge.

One of the promising laboratory tests for cerebral ischemia now being studied is the thrombotic marker homocysteine, the sulfinic analog of aspartate.[5] Approximately 25% of patients with symptomatic atherosclerosis have elevated plasma homocysteine levels caused by various factors. High levels of homocysteine may run in families, lending genetic heirs an increased susceptibility to heart attack and stroke.[6] Elevated plasma homocysteine may be a causal and modifiable risk factor for ischemic stroke: homocysteine abnormalities have been found in 20 to 40% of patients presenting with premature peripheral vascular disease or stroke.[7,8]

The pathogenicity of other thrombotic markers, such as antiphospholipid (aPL) protein antibodies, also has been investigated over the past two decades.[9] The role of aPL as a potentially important marker or cause of increased vascular risk associated with ischemic stroke is now recognized. As a component of excitatory membranes containing glutamate receptors, aPL may also be involved in neurotoxicity.

The neurotoxic effect of excitatory amino acids such as glutamate and aspartate in the brain also has been well documented (see Figure 1).[10] The research has shown a correlation between glutamate content in the blood and severity of acute ischemia. Cerebral damage and its association with progressing stroke have been attributed to increased glutamate release, or low glutamate reuptake, both in animals and in humans. However, only 56% of patients with progressing stroke are reported to have high glutamate content in blood sera.[10] Furthermore, even though glutamate is considered the strongest biochemical predictor of progressing stroke, this marker remains nonspecific for TIA.

Glutamate as a marker is very labile, and the same profile is seen with brain injury, Alzheimer's disease, epilepsy, and even changes in diet.[5]

The first attempt to use simultaneously neurospecific and thrombotic markers for differentiation of stroke type was undertaken by Canadian investigators.[11] They proposed a new diagnostic laboratory assay and designed a preliminary prospective cohort study to test a panel of biochemical markers—neuron-specific enolase (NSE), myelin basic protein (MBP), S-100b protein, and thrombomodulin (Tm)—in blood samples from 28 patients with acute ischemic stroke. In this study, four biochemical markers were assayed using a standard ELISA technique. Results demonstrated elevated levels of NSE in 89%, Tm in 43%, MBP in 39%, and S-100b beta in 32% of patients. At least one of the markers was elevated at the time of admission in 93% of the acute stroke patients. However, it is not yet clear how these markers are implicated in processes creating the neurotoxicity underlying stroke mechanisms.

Biomarkers That Have Been Discovered

In the past 30 years, substantial progress has been made in elucidating the mechanisms by which cerebral ischemia leads to brain damage. The cellular and molecular mechanisms of cerebral ischemic abnormalities have been better defined in terms of the role of glutamate and glutamate receptors, among the most widely distributed excitatory neuroreceptors in the central nervous system. According to a leading hypothesis, ischemia-induced glutamate release activates glutamate receptors (see Figure 2). It has been shown that glutamate and homocysteine activate the glutamate-binding site of NMDA receptors and participate in neurotoxic processes.[12]

As indicated in Figure 2, NMDA receptors are heteromeric pentamers or tetramers (the red bars) of NR1 and NR2 receptor subunits that determine the biophysical and pharmacological properties of the receptor. It has been shown that the NR1 subunit contains three transmembrane domains (TM1, TM3, and TM4) and two extracellular domains (S1 and S2, the yellow bars), which form the glutamate (or homocysteine) and glycine binding sites, respectively, and a hydrophobic domain (TM2) that forms the pore of the ion channel.[13] The NR2 subunit has four further subunits—NR2A, NR2B, NR2C, and NR2D—that are responsible for Na+- and Ca++-permeability regulation. The yellow extracellular loops in the figure are N-terminus fragments of NMDA receptors that are cleaved by thrombin-activated serine proteases during the neurotoxic cascade underlying stroke.

In clinical study, NMDA biomarkers were found to provide real-time evidence of neurotoxicity, with a decrease in levels of circulating NR2A/2B receptor subunits correlating well with reductions in neurotoxic conditions.[14]

Predevelopmental Research. Research elucidated properties of NMDA receptors that were isolated from human/rat synaptic membranes and purified by affinity chromatography on glutamate-Sepharose 4B.[15] Sodium disulfate (SDS) denaturing electrophoresis of purified NMDAR material revealed protein bands with molecular weights of 14, 68, and about 190 kD. Isolated NMDAR exhibits glutamate-binding activity with a single type of binding site. The analysis of 3H-L-glutamate binding to purified NMDAR in the presence of argiopin, a peptide blocker of NMDAR, demonstrated the dose-dependent inhibition, with a constant of inhibition (Ki) of 2.54 µg/ml.

Monoclonal antibodies (mAb) producing immunoglobulins G and M were obtained by hybridoma technique and used for identification of purified NMDAR. Colloidal gold-labeled mAb IgG revealed NMDAR-like immunoreactivity in synaptic terminals of glutamate-receptive neuronal axons of the cells in rat sensomotor cortex and their organotypic primary cell culture.[14]

The appearance of NR2A/2B peptide fragments of about 2 and 6 kD was observed in human plasma. Concurrently, high concentrations of autoantibodies to the NMDAR fragment were revealed in serum samples from stroke patients.

The approach to identifying the peptide sequence of the NMDAR fragment included isolation and investigation of the specific complementary DNA strand (cDNA) encoding this fragment. The immunoscreening of 106 clones in the human-brain cDNA library lGT11 by mAb and total IgG from ischemic stroke patients revealed one recombinant phage lNMDAR clone with a positive immunological signal. The restriction map obtained by use of standard endonucleases showed the

existence of a 0.5-kb cDNA insert. After cDNA nucleotide sequence determination by standard molecular biological procedures, a corresponding peptide consisting of 157 amino acid residues with a molecular weight of 19 kD was found.

An immunoactive epitope of the NR2A/2B peptide consisting of 21 amino acids was designed, produced by solid-phase synthesis, and purified up to 98% by preparative high-performance liquid chromatography. Standard 96-well microplates covered by peptide were used for assessment of stroke by ELISA.

Preclinical Research. Based on this predevelopmental research delineating the N-terminal peptide fragment of NMDAR as a possible biomarker of neurotoxicity underlying cerebral ischemia, the investigators performed a preclinical evaluation of the biomarker's utility for stroke assessment. A transient model of cerebral ischemia by middle cerebral artery occlusion (MCAo) and a model of ICH by blood cell infusion into cortex were used.[16] The principal hypothesis was that neurotoxicity affects NMDA receptors through cleavage of receptor subunits, generating peptide fragments that penetrate the blood-brain barrier, appear in the bloodstream, and generate autoantibodies.

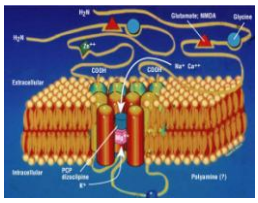


Figure 2. Scheme of N-methyl-D-aspartate receptors. Click to enlarge.

The researchers found up-regulated expression of NMDAR messenger RNA (mRNA) in cortical tissue damaged by infarct during ischemic conditions, by contrast with down-regulation under ICH. Simultaneously, NR2A/2B immunoreactivities were significantly reduced at all reperfusion time points in MCAo rats, reaching the lowest point of 4.6% at 24 hours after occlusion. Immunoreactivities of NR2A/2B showed no change at any time point of the experiment in rats with induced hemorrhage.[16]

The appearance and accumulation of NR2A/2B peptide was detected in the blood of rats with induced cerebral ischemia and hemorrhage. In MCAo rats, significantly increased concentrations of NR2A/2B peptide correlated with increased expression of NR2A/2B mRNA detected within the first 24 hours of reperfusion. Abnormally high concentrations of NR2A/2B, which act as foreign antigens on exiting the brain, initiate an immune response by generating autoantibodies in the blood. Significantly elevated levels of NR2A/2B autoantibodies were measured to the third day of induced cerebral ischemia.

Low concentrations of NR2A/2B peptide were observed in the blood samples of ICH rats, accompanied by a reduced immune response. It was hypothesized that thrombin activated serine proteases not implicated in mechanisms of necrosis underlying ICH but which caused cleavage of the NR2 receptor under cerebral ischemic conditions.[17] Alternatively, other proteases may be cutting the NR2 receptor to produce peptides with nonimmunoactive epitopes.

Clinical Research. The subsequent clinical study was conducted in the Department of Neurology and Neurosurgery at Pavlov's State Medical University in St. Petersburg, Russia. The objectives of the study were to determine the performance characteristics of NR2A/2B autoantibodies, glutamate, and homocysteine in identifying TIA and ischemic stroke and in distinguishing cerebral ischemia from ICH.[18, 19]

Following routine protocol, physicians in the emergency department recorded clinical data from the primary evaluation, including histories, neurological examination results (National Institutes of Health Stroke Scale, or NIHSS, scores), and computed tomography (CT) or magnetic resonance imaging (MRI) results. Of the 105 patients with cerebrovascular accidents, 87 were determined to have cerebral ischemia and 18 ICH. The former group consisted of 56 TIA sufferers and 31 with acute ischemic stroke. The control groups were 230 healthy individuals and 25 with controlled hypertension/atherosclerosis.

Glutamate concentrations were significantly elevated above the control levels of healthy individuals only in patients with TIA, while homocysteine levels increased in patients with hypertension and atherosclerosis, TIA, and ischemic stroke, in that ascending order with a correlation coefficient, r, of 0.97 (see Figure 3). Positive predictive value for TIA and ischemic stroke calculated for each test separately was not higher than 33%, with sensitivities of 37% for glutamate (cutoff of 32.0 µmol/L) and 61% for homocysteine (cutoff of 8.3 µmol/L).[18] Glutamate and homocysteine concentrations detected

in 23 of the 31 ischemic stroke patients correlated significantly with lesion volumes defined by MRI (r ~ 0.90) and with severity of symptoms as determined by NIHSS scores (r ~ 0.70).

The serum NR2A/2B autoantibody concentrations detected in all subject groups studied were increasingly higher for patients with hypertension and atherosclerosis, for those with TIA, and for those suffering acute ischemic stroke. The nine patients with severe cases of acute ischemic stroke and NIHSS scores of 30–34 had NR2A/2B autoantibody levels approximately six times higher than that of control subjects. The levels of NR2A/2B autoantibodies for the 18 patients with mild to moderate ischemic stroke and NIHSS scores of 16–22 were lower than for severe cases. Routine therapies, including nootropics, vasoactive substances, antihypertensive medication, and anticoagulants, improved neurological symptoms—that is, increased NIHSS scores by 10 to 12—within seven days in 58% of patients with acute ischemic stroke and produced a reduction in NR2A/2B autoantibodies to levels comparable to those of healthy individuals.[20]

Analysis of the correlation between NIHSS scores with NR2A/2B autoantibodies in patients with TIA (numbering 56) and ischemic stroke (27) demonstrated statistically significant correlations: the coefficients were 0.81 and 0.91, respectively. In nine patients with a known TIA onset time, comparison of diffusion-weighted-imaging lesion volumes detected within 3 hours of event revealed a correlation coefficient of 0.87 and a significant positive association with NR2A/2B autoantibody values. The analysis of correlation of lesion volumes by multimodal MRI scans and NR2A/2B autoantibodies of the same group of patients with ischemic stroke showed r = 0.79. The calculations of NR2A/2B autoantibody test performance characteristics indicate that this marker is a strong diagnostic parameter. It demonstrates positive predictive value of 91% for TIA diagnosis (with a sensitivity of 95% and specificity of 98%) and 86% for ischemic stroke diagnosis (with a sensitivity of 97% and specificity of 98%) at the cutoff point of 2.0 µg/L.[19]

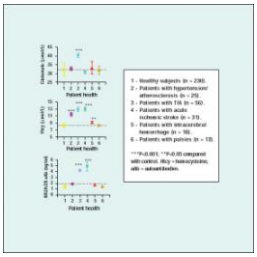


Figure 3. Glutamate, homocysteine, and NR2A/2B autoantibodies in the blood of patients. Blood samples were taken within 3 hours of cerebrovascular incident. Dotted lines show cutoffs at 32 µmol/L for glutamate, 8.3 µmol/L for homocysteine, and 2.0 µg/L for NR2A/2B autoantibodies. Click to enlarge.

The clinically predetermined cutoff for NR2A/2B autoantibodies allowed acute ischemic stroke to be differentiated from ICH. The monitoring of NR2A/2B autoantibodies within the first 6 hours of stroke event revealed patients with acute ischemic stroke only. These results were correlated with data obtained from CT and MRI scans.

An attempt was made to group patients with acute ischemic stroke according to the size of the damaged vessel, as determined by magnetic resonance angiography. Elevated plasma glutamate correlated primarily with blockage in the internal carotid artery, with the highest plasma homocysteine corresponding to blood clotting in the middle cerebral arteries, whereas NR2A/2B autoantibodies had a tendency to increase mostly when microvessels were damaged. The simultaneous detection of all three markers increased the positive predictive value for a diagnosis of TIA and ischemic stroke to 99%.

A Proposal for Stroke Assessment

On the basis of these predevelopment, preclinical, and clinical studies, CIS Biotech Inc. (Atlanta) has patented several IVD tests in several formats. These tests will be scientifically and clinically validated in clinical trials to suit FDA clearance regulations.

TIA Test. The NeuroTest-TIA kit for detecting NR2A/2B autoantibodies consists of an immunosorbent for NR2A/2B peptide and an indicator reagent comprising secondary antibodies attached to a signal-generating compound. The test is intended to be used to assess patients experiencing cerebral ischemia and TIA. Sensitivity is 97% and specificity 98% in the former case; respective performance figures for TIA are 95 and 98%. Predictive values are reported at 86 and 91%, respectively, at a cutoff point of 2.0 µg/L.[19]

A rapid multiple panel of biomarkers for stroke based on a latex agglutination technique also is being developed to detect NR2A/2B peptide, glutamate, and homocysteine. The main hypothesis behind its development is that simultaneous detection of these markers would improve the power of laboratory assay testing.

Stroke Tests. The multiple NeuroTest-Stroke assay employs triple concave slides with a built-in magnification device to detect the reaction visually, providing an immediate yes or no response. In this assay, plasma samples are mixed with antibody coupled with colored latex particles, and agglutination is indicated in 2–5 minutes. The reaction occurs in a homogeneous phase as follows:

antigen + latex-antibody ÆÆÆ {latex-antibody¨antigen}

CIS Biotech also is developing the NeuroTest-Stroke microassay, which is based on a lateral-flow technique using colored latex particles containing antibodies to NR2A/2B, glutamate, and homocysteine. The blood or plasma reconstitutes the latex-reagent and transports it to the detection line. In most cases, sandwich assays are performed. This test is a heterogeneous assay; that is, reactions in both solution and solid phase occur.

This test procedure involves four steps:

1. Blood is dropped on a specific site on the lateral-flow device.

2. Blood reconstitutes the colored latex reagent.

3. If the analyte in question is in blood, then the first reaction takes place: Antigen is bound to the antibody on the latex particleÆÆ{antigenÆantibody-latex} complex.

4. In parallel with the reaction in Step 3, transport to the detection line of the {antigenÆantibody-latex} complex with another antibody occurs. The following reaction then takes place:

   {antigenÆantibody-latex} ÆÆ alignment with second antibody.

The concentration of this complex can be quite high at the detection line and may be visually detected by color or measured by device using a fluorometric method. The analytical sensitivity is high because of the concentration process of the colored particle, or the catching principle. Healthy people generally have an NR2A/2B peptide concentration of 50 pg/ml.

In preliminary clinical studies, both NeuroTest-Stroke tests demonstrated a sensitivity of 98% and a specificity of 99%, with a positive predictive value of 99% at cutoffs of 100 pg/ml for NR2A/2B peptide, 32.0 µmol/L for glutamate, and 8.3 µmol/L for homocysteine.[18]

Technology Application. Data obtained from NMDAR biomarkers, especially when combined with data from other biomarkers such as glutamate and homocysteine, can be used to assess risk of TIA and stroke, to rule in ischemic stroke, to rule out hemorrhagic stroke and strokelike disorders, to guide treatment, to monitor rehabilitation following stroke, and to predict whether stroke will recur.

The latex agglutination method is especially well suited for use in emergency department and field settings because NR2A/2B levels are elevated at a very early stage of ischemic insult and thus provide real-time indication of neurotoxic events. In addition, results can be processed in less than 10 minutes, allowing for timely and appropriate intervention.

This method provides reliable data in a format that is simple to interpret. Application of the latex agglutination technique to analysis of brain biomarkers for stroke can reduce the cost of analysis, provide an opportunity to monitor the progress of a treatment procedure in real time, and allow physicians to determine the efficacy of medication administered in the treatment of stroke.

Conclusion

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 blood vessels, activating the immune system and generating autoantibodies to brain-specific antigens. The NR2A/2B peptide fragment and its autoantibodies represent a new marker of neurotoxicity underlying cerebral ischemia and stroke. In conjunction with a neurological examination, this marker can assist in the differential diagnosis of TIA or stroke and, as a complement to neuroimaging, distinguish ischemic from hemorrhagic stroke.

Given that three hours is an outside limit for administering appropriate therapies for stroke, improving outcomes depends upon a fast initial response—one measured in minutes. The NeuroTest-TIA and NeuroTest-Stroke assays are anticipated to improve risk assessment for TIA and stroke. The simultaneous detection of glutamate, homocysteine, and NR2A/2B autoantibodies should help assess stroke progression much as cardiac markers now can be used to identify a heart attack and assess risk of infarction.

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