Markers of Neurotoxicity: From Behavior to Autoantibodies Against Brain Proteins

Hugh L. Evans

Evidence of potentially neurotoxic exposures may be obtained in peripheral indicator media, but molecular or cellular evidence of neurotoxic effects has not been as readily available, primarily because the nervous system of living humans is beyond the reach of direct measures. Although there is limited evidence that molecular changes in the blood can provide information about neurotoxicity, several new approaches are being investigated. One is that the immune system may preserve evidence of damage to the nervous system. Debris from damaged cells in the nervous system may present as antigens, giving rise to autoantibodies, which may be detectable in blood for a long time after injury. Progress will depend on more control experiments and clarification of confounding variables. Validation of new molecular markers must go hand in hand with documentation of impaired function, most commonly measured as behavioral or neuropsychological changes. These noninvasive markers will be measured with greater sensitivity and precision, thanks to innovative computer technology. The practical advantages of the new markers may be as important as their contribution to our understanding of the mechanisms by which the nervous system defends against chemical insult.

**Indexing Terms:** nervous system/behavioral effects/biomarkers/glial fibrillary acidic protein

The aim of this paper is to survey markers of chemically induced injury to the nervous system. The purpose of identifying markers is to permit early remediation or therapy while effects may still be reversible, hopefully before overt signs of toxicity occur. If successful, markers of neurotoxicity will permit realistic exposure limits to be set on the basis of very early changes in the nervous system, and we no longer will need to count the sick workers or dead laboratory animals.

Most of the information to be reviewed involves molecular markers that can be measured in peripheral media such as blood. Since these markers are several steps removed from the functional sites in the nervous system, they must be evaluated by comparison with electrophysiological and imaging measures, as well as behavioral indices. These integrated techniques are needed to develop the neuro-epidemiology of low-level exposures as a critical component in the detection of adverse health effects.

Considerable work is needed to develop and validate new markers of neurotoxicity in humans with long-term, low-level exposure to neurotoxicants. A neglected but important subject is the apparently healthy individual whose behavioral and molecular measures may indicate subtle degrees of nervous system impairment. To establish the worker's health, normative data on the markers must be available. This currently is a major obstacle to wider use of markers. We also need to determine the specificity for neurotoxicity of many candidate markers. The markers should be related to standard indices of neurotoxicity and also should be determined with persons exposed to chemicals thought not to be specifically neurotoxic.

**Why are biomarkers of nervous system injury so difficult to identify?** There currently is a serious lack of readily accessible chemical or cellular markers of nervous system injury in humans. Several obstacles to surveillance of biomarkers of neurotoxicity exist: The brain is protected from physical injury by the skull and cerebrospinal fluid (CSF) cushion, and from toxicants by the blood–brain barrier. These protective features prevent collection of specimens of nervous system tissue or the easy passage of molecules from the nervous system to the peripheral circulation. Not only is the direct examination of brain material impossible in health surveillance of humans, but indirect evidence of neurotransmission, e.g., concentrations of neurotransmitters or metabolites in body fluids, have proved quite variable as indicators of neurotoxicity at realistic doses (1, 2). Measures of peripheral cholinergic markers do not correlate very well with toxicant-induced changes in brain cholinergic transmission (3). Other markers, based upon second messengers (4), have proved no more sensitive and reliable for early detection than routine screening tests such as electroencephalogram, computed tomography, or tests of motor reflexes (5).

**Molecular Markers**

**CSF Markers**

In searching for new ideas about molecular markers in peripheral media, one might first identify markers at the site of nervous system disease or damage (6). Once identified, these central markers (or their analogs and derivatives) could be sought in peripheral media. However, since assessment of markers in human brain is possible only after autopsy, one must recognize problems associated with attempts to identify markers from postmortem material, or from people who are living but seriously ill (6, 7).

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1 Nonstandard abbreviations: CSF, cerebral spinal fluid; NSE, neuron specific enolase; GFAP, glial fibrillary acidic protein; TMT, trimethyltin; and NF, neurofilament.
Many constituents of CSF have been suggested as markers of brain dysfunction (Table 1). However, most of these have not yet been found consistently in peripheral blood (e.g., 8, 9). Negative results have been reported in surveys of workers exposed to occupational chemicals at subclinical concentrations (10) or in Alzheimer disease (11), and highlight the importance of experiments with animals in developing positive evidence on the basis of sufficient toxicity being detectable in exploratory research.

**Table 1. Constituents of CSF proposed as markers of brain injury or dysfunction.**

<table>
<thead>
<tr>
<th>Marker</th>
<th>Neuropathology</th>
<th>References</th>
</tr>
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<tbody>
<tr>
<td>β2-Microglobulin</td>
<td>HIV dementia</td>
<td>93, 94</td>
</tr>
<tr>
<td></td>
<td>Various diseases</td>
<td>94</td>
</tr>
<tr>
<td>Cell adhesion molecule</td>
<td>HIV dementia</td>
<td>96</td>
</tr>
<tr>
<td>Microglial antibodies</td>
<td>Alzheimer disease</td>
<td>34</td>
</tr>
<tr>
<td>GFAP</td>
<td>Neurology patients</td>
<td>97, 98</td>
</tr>
<tr>
<td>Myelin basic protein</td>
<td>AIDS dementia</td>
<td>99</td>
</tr>
<tr>
<td></td>
<td>Asphyxiation</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Head injuries</td>
<td>98, 99, 101</td>
</tr>
<tr>
<td>NSE</td>
<td>Various</td>
<td>12-14, 100, 102, 103</td>
</tr>
<tr>
<td>Paired helical filaments</td>
<td>Alzheimer disease</td>
<td>104</td>
</tr>
</tbody>
</table>

Autoantibodies

A new type of biochemical marker may be found in serum titers of autoantibodies to cell-specific proteins of neuronal origin. Many chemicals directly attack immune cells, thus causing immune suppression, and other chemicals target the nervous systems (27), and there are significant interactions between the nervous and immune systems (28, 29). Despite all of this work, it is not yet clear how these complex relations play out in peripheral markers. For example, it is not clear as to why healthy people may have autoantibodies against brain antigens (30, 31). Despite unsuccessful attempts to relate immune system markers to nervous system disorders (32, 33), others have reported positive findings (34). The appearance in serum of autoantibodies against proteins of nervous system origin was associated with several diseases and injuries; these are summarized in Table 2.

Table isimade by text

There is at present enough evidence to invite speculation that toxicant-induced neuronal injury also might cause autoantibody formation, resulting in autoimmune disease (35). Theoretically, environmental agents can act as autoantigens (36–38). Current researchers hope that the immune system may preserve and make visible the evidence of neurotoxicity that has disappeared from the brain. If chemically induced neurotoxicity involves an autoimmune disorder, then autoantibodies might be measured as biomarkers. Measurement of autoantibodies also might provide clues as to whether the toxicity of some chemicals, particularly metals, might involve autoimmune disorders similar to those that may contribute to Alzheimer disease.

The following scheme suggests how autoantibodies may appear in serum as a consequence of chemically induced brain injury:

1. Changes in cell-specific proteins of neuronal origin may precede or accompany toxicant-induced neuropathy (39, 40). A prominent example is the increased concentration of GFAP in the brain, indicative of astrogliosis (41), in early stages of neurotoxicity (21, 22, 42).

2. Protein fragments from injured cells can make

**Table 2. Constituents of serum or plasma proposed as markers of brain injury or dysfunction.**

<table>
<thead>
<tr>
<th>Marker</th>
<th>Neuropathology</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myelin basic protein</td>
<td>Demyelinating diseases</td>
<td>105</td>
</tr>
<tr>
<td></td>
<td>Head injury</td>
<td>101</td>
</tr>
<tr>
<td>Anti-NF</td>
<td>Neurological patients</td>
<td>106–109</td>
</tr>
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<td>Anti-neuronal antibody</td>
<td>Cerebellar damage</td>
<td>110</td>
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<tr>
<td>Anti-myelin</td>
<td>Neuropathy</td>
<td>111</td>
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<tr>
<td></td>
<td>Population density</td>
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<td></td>
<td>Chlorpyrifos</td>
<td>59, 60</td>
</tr>
<tr>
<td>Various autoantibodies</td>
<td>Myasthenic syndrome</td>
<td>112</td>
</tr>
<tr>
<td></td>
<td>Multiple sclerosis</td>
<td>113</td>
</tr>
<tr>
<td></td>
<td>Hearing loss</td>
<td>114</td>
</tr>
<tr>
<td>Anti-GFAP</td>
<td>Alzheimer disease</td>
<td>51, 52</td>
</tr>
<tr>
<td>Anti-nerve growth factor</td>
<td>Lupus, arthritis</td>
<td>116</td>
</tr>
</tbody>
</table>
their way to the CSF (43) and on into the systemic circulation. Protein of neuronal origin can be detected in CSF as a marker of head injury or nervous system diseases (Table 1). A route for passage of central proteins from the CSF into the lymph system and then into the periphery has been identified (44). Nervous system injury caused by toxicants probably involves impairment of the blood–brain barrier (45, 46) as well as impairment of the scavenging function of microglia (42, 47), thus increasing opportunities for proteins to come into contact with the systemic blood.

3) Protein fragments from the nervous system probably do not survive long enough in the blood to be practical markers. GFAP, a representative protein constituent of the injured nervous system, was detectable in CSF but not in serum, probably because it was degraded too rapidly (O’Callaghan et al., unpublished results).

4) Autoantibodies can be formed against protein fragments from the nervous system (48). The early appearance and long survival of autoantibodies to these proteins might permit practical surveillance of exposure and toxicity. These autoantibodies also may be used to monitor the progression of injury and recovery. Since they identify specific cells, autoantibodies may help to identify the cellular mechanisms of neurotoxicity. Exposure to Pb can increase the antigenicity of neuronal proteins in rodents under some circumstances (49), but reports have not been consistent in defining the effects of Pb exposure on immune indicators in rodents at exposure levels that cause neurobehavioral toxicity (e.g., 50). This indicates that there is still much work to be done in identifying the variables that influence these phenomena.

Assessment of autoantibodies. Autoantibodies to GFAP, to the neurofilament (NF) proteins of the axon (NF68, NF160, and NF200), to the synaptic proteins (synaptotagmin and neurexin), and to myelin basic protein can be detected with an ELISA as described by Tanaka et al. (51–53) and modified in this laboratory by El-Fawal et al. (unpublished).

The ELISA appears to be useful for screening serum specimens because it requires <300 μL of serum and detects titers as low as 2 ng/L. The preferred medium is serum (rather than plasma) that has been divided into separate aliquots (to avoid thawing of the sample with each replicate assay) and then stored at −70 °C. Methodological work by El-Fawal in this laboratory (as yet unpublished) indicates that several Ig isotypes can be measured and that repeated assays of the same specimen within a short time frame (1 week) yield test–retest correlations from 0.7 to 0.9, with CVs between 10% and 20%. These methodological results are encouraging because they are similar to validation statistics of other biomarkers, including nonneuronal autoantibodies (54). However, much difficult work remains to demonstrate degree of specificity of binding, and the extent to which natural polyclonal antibodies react against “self” antigens (55). Also, attempts should be made to produce more specific autoantibodies.

Validity of serum autoantibodies as biomarkers. Limited results available to date do not resolve whether any of these autoantibodies will provide a good marker of exposure. In theory, autoantibodies should be formed only as a consequence to cell damage or cell death. Time-effect experiments with laboratory rats during subacute exposure support this conclusion, because the increase in autoantibody titers was not as closely correlated with the rising concentration of neurotoxic metal in the blood as with the subsequent appearance of signs of neurotoxicity such as behavioral changes.

The detailed examination of cellular changes in the brain, possible only with laboratory animals, is essential for understanding the mechanisms behind the appearance of molecular markers in serum. Changes in the concentration of GFAP have been shown to mark neurotoxicity in rats chronically exposed to methylmercury (56, 57) and to trimethyltin (TMT) (40). An increase in GFAP was associated with a TMT-induced decline in neuronal density. Changes in the rat’s home cage behavior provided the evidence of neurotoxicity needed to validate the new biomarkers.

One could detect GFAP in CSF of intoxicated monkeys and rats, indicating that this biomarker might find its way into the peripheral circulation. I did not pursue studies of CSF because that medium is not readily available for surveillance of humans. One could not detect GFAP in blood of experimental animals or humans, suggesting that GFAP is metabolized too rapidly to provide a useful marker in blood. For these reasons, I speculated that injury to the nervous system caused a release of structural proteins that could trigger autoantibody formation in the brain or in the peripheral circulation.

Autoantibodies in serum as markers of neurotoxicity.

Consistent with the view that autoantibodies should be formed only as a consequence to cell damage or cell death are data showing that antibody titers from the serum of 7-year-old children had no relation to blood Hg concentration in a population of Faroe Islanders studied by Grandjean et al. (58; Grandjean and El-Fawal, unpublished), which contained no instances of neurotoxic signs and presumably contained few cases of nervous system damage. Actual data on nervous system impairment were not available at the time of serum sampling.

Clinical studies have detected increased autoantibody concentrations in people exposed to chemicals. Individuals with chronic exposure to chlorpyrifos developed autoantibodies that could have originated from damage at a variety of sites, including peripheral nervous system myelin (59, 60). People near a spill of ureaformaldehyde had increased autoantibody concentrations to serum albumin (61). There were no obvious signs of toxicity in these people, but rigorous measures of neurotoxicity were not used.

Work in progress at my laboratory indicates that, under some circumstances, increased serum autoantibody concentrations to gliotypic and neuronotypic pro-
teins can be found during exposure to the neurotoxic metals methylmercury (56, 57, 62), Pb (49, 63–66), and Cd (67). Our most comprehensive body of results reflects the effects of exposure to Pb in rats and humans. Taken together, these data indicate that (a) autoantibodies to neuronal marker proteins are detectable in serum; (b) autoantibodies may appear in serum before the appearance of conventional indices of neurotoxicity such as behavioral or histopathological changes. The appearance of serum autoantibodies accompanies changes in the biomarker of Pb-induced brain injury (GFAP in the brain of rats). The same rats had increased serum autoantibody titers, detected long before clinical deficits appeared. These findings are compatible with the hypothesis that early, subclinical damage to the central nervous system causes the release of protein fragments, which can function as antigens for raising autoantibodies that are detectable in serum.

Serum autoantibodies from workers at a Cd/Ni battery plant in Poznan, Poland, also acted as potential biomarkers of exposure (54, 67). Workers were divided into three exposure levels according to job category and ambient concentrations of Cd and Ni: high (n = 12), medium (n = 5), and low (n = 10). Autoantibody titers tended to be higher in workers with high exposure levels, the predominating titers being of the IgG isotype. Anti-NF titers were positively correlated with ambient concentrations of Cd but not with ambient Ni. It is tempting to speculate that the degree of positive correlation between antibody titer and exposure reflects a metal’s specific effects upon the nervous system, as opposed to effects upon other organ systems.

Questions for further research. The demonstration that these autoantibodies are effective biomarkers of neurotoxicity will require the demonstration of their very low incidence in healthy persons, and their significant positive correlation with indices of neurotoxicity such as behavioral and electrophysiological measures in humans (see below) or neuropathology in animals. How does one interpret the presence of autoantibodies in people who appear to be normal and healthy? One must expect that a long-past injury may leave a long-lasting legacy in the form of autoantibodies. The toxicologist must do two things: First, focus upon autoantibody titers that are far higher than the range in “normal” individuals, as is currently done with molecular markers of medical problems in clinical chemistry. Second, look for a profile of markers (antibodies and other types of markers) that together point to nervous system injury.

Data also are needed to understand (a) how early in the evolution of toxic signs can the autoantibody markers provide useful information? (b) How long do the markers persist during chronic exposure or after cessation of exposure? (c) What structural changes in the nervous system give rise to the autoantibodies? This information should shed light on the mechanisms leading from initial penetration of toxicants into the brain and the appearance of autoantibodies in the serum.

We recognize that a percentage of healthy people may have measurable titers of autoantibodies (30, 31), including those against the neuronal proteins, and that some autoantibodies cross-react (55). Psychological or physiological stress may also increase autoantibody titers (68). Therefore, it is important to study serum markers from a control group known to be free of nervous system injury or stress. This population should be sufficiently heterogeneous to determine whether gender, racial, or dietary factors influence the proposed biomarkers.

Does each toxicant create a unique profile or “signature” of serum autoantibodies? The above human studies have raised some key questions that can only be addressed in animal models. The profile of autoantibodies appears to differ, depending on the nature of the exposure and the type of neurological impairment (Tables 1 and 2). This suggests that there may be a specific titer profile signature for each neurotoxicant. If precisely controlled exposures of animals confirm a unique autoantibody signature for each neurotoxicant, then the measurement of autoantibodies can provide evidence of exposure to a specific neurotoxicant as well as evidence of neurotoxic effect.

Behavior as an Index of Neurotoxicity

Evaluation of neurobehavioral function will continue to be an important component of monitoring and diagnosis of humans exposed to toxicants (69, 70). This important role for behavioral evaluation is based upon several factors. First, the impediments in establishing molecular or imaging markers of neurotoxicity, reviewed above, limit the number of alternative tests for evaluating neurotoxicity. Behavioral change is already recognized as a standard criterion for judging new biomarkers because behavioral change is one of the few proven indicators of effect in humans chronically exposed to low concentrations of potential neurotoxicants. Second, the information provided by molecular indices is only partly correlated with behavioral indices; i.e., information provided by tests at one level is not redundant with information provided at another level of investigation. Third, behavioral markers have an advantage over molecular markers in that behavior can be measured noninvasively from people in their natural environment (work site, clinic, or school). Thus, behavioral measures can be made repeatedly for surveillance or for monitoring recovery from exposure, even with populations who are not good candidates for venipuncture (small children, the elderly, or people opposed to venipuncture on religious or philosophical grounds). Fourth, behavioral measurement carries less risk to the health of researchers than working with human blood.

Advanced behavioral methods exploit portable computer technology to ensure objective evaluation and to improve consistency of test administration, which have been problems in large-scale surveillance where many test administrators must be trained and supervised (71–73). This permits the presentation of prerecorded instructions in both the auditory and visual modalities.
for testing of subjects who have limited education or speak only a language that is different from the test administrator’s. Computer algorithms can rapidly adjust test difficulty to match the subject’s performance, a significant advantage in reducing the time required for data collection (74).

Cognitive functions, e.g., learning, memory, and attention, are sensitive markers of subclinical neurotoxicity in persons (75–78), and these methods can be adapted for parallel experiments with animals (70, 74, 79). In all of these cases, behavioral change is one of the few types of conveniently available evidence for documenting cognitive impairment. However, the behavioral data for documenting neurotoxicity have seldom been integrated with biochemical measures from the same individuals (75, 80). Behavioral research has suffered from the same weaknesses that plague molecular research: lack of specificity (81), lack of a strong criterion of neuronal damage (82), weak evidence of exposure (83), and confounding variables (83).

**Neurophysiological Markers**

Evoked electrical potentials, based upon sophisticated research techniques, are sensitive to effects of Pb (84) or solvents (85). Electrophysiological techniques are subject to electrical “noise,” which can be a problem in field studies, but these problems may be overcome by advances in computer technology. Imaging techniques provide a nondestructive view of regional differences in brain metabolism and function, but currently are too expensive for large-scale screening and, at the current state of the art, cannot detect the insidious damage of long-term, low-level chemical exposures (5). Encouraging results have been obtained from recording while the subject is engaged in behavioral tests. This combined measurement of behavioral and imaging data, known as functional imaging, can be very powerful in linking brain anatomical regions with functional changes, a topic of great importance in neurotoxicology. Additional work is needed to understand why different results may be obtained from different imaging methods and how to improve the resolution of the image so that the cellular and anatomical detail can be seen.

Exhaled CO₂ proved to be an easily measured index of behavioral and metabolic changes during inhalation exposure to organic solvents (86–88). The technology is available to make similar measurements in the field with human subjects (89). Further work is needed to clarify the underlying mechanisms and potential confounding variables, e.g., level of motor activity and diet.

An intriguing recent finding is that the eye’s pupillary response to atropine may indicate cholinergic dysfunction and may be predictive of Alzheimer disease (90). Perhaps new markers of chemical neurotoxicity may be found in this approach (91).

**Recommendations**

1) There is a need for closer integration of neurobehavioral studies with research at the cellular and molecular levels of the nervous system. Neurobehavioral data have provided vital evidence of functional neurotoxicity. When direct examination of human brain is impossible, functional change becomes the gold standard for evaluation of biomarker sensitivity and validity.

2) There is a need for greater coordination between laboratory and field studies. Although laboratory work is vital to bringing potential markers into clear focus, the laboratory worker can benefit by understanding the clinical picture of neurotoxicity. The best use of molecular markers is to assist in the diagnosis and in monitoring of recovery from toxic injury.

3) Isolated changes in cellular and molecular markers should be interpreted cautiously, particularly in the absence of signs of neurotoxicity obtained from standard assays and clinical examinations. For example, the presence of autoantibodies does not, by itself, define an autoimmune disease. As indicated by Schulte’s review (92), our ability to measure phenomena far outstrips our ability to interpret these results. We need to develop a language that is appropriate for counseling individuals (i.e., patients, clients) about the meaning of their individual biomarker data. It currently is difficult to convey the meaning of a specific marker datum in terms of an individual’s statistical odds of experiencing a significant health hazard. It is equally difficult to convey the appropriate level of confidence in the reliability of the assay.

4) Experiments with laboratory animals play three indispensable roles in developing new biomarkers of chemically induced injury to the nervous system: (a) Histopathologic examination of the brain continues to be one of the major criteria of neuronal injury; (b) studies of molecular changes in localized areas of brain tissue are an important source of information linking peripheral markers to specific molecular and cellular changes in the brain; and (c) laboratory studies can begin with healthy animals, free of prior chemical exposure and other confounding factors commonly encountered in human populations. All three types of studies are needed for validating potential biomarkers, and direct examination of the brain during early stages of toxic exposure is possible only in experimental animals. Human autopsy material is rarely available for cases of chemical neurotoxicity, may not be fresh enough for some molecular assays, and interpretation of damage is often more complicated as to cause–effect and confounding influences than are specimens from experimental animals. Of course, human material is extremely valuable to gauge whether results from animals can be extrapolated to the human.

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