Serum Anticholinergic Activity as an Index of Anticholinergic Activity Load in Alzheimer’s Disease

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Key Words
Alzheimer’s disease · Serum anticholinergic activity · Inflammation · Anticholinergic activity

Abstract
We reported a procedure of serum anticholinergic activity (SAA) measurement and the reliability and reproducibility of the receptor binding assay, and we also described the usefulness of SAA measurement reflecting the anticholinergic activity (AA) in the central nervous system (CNS). According to the results of a 10 times repeated measurement of standard atropine binding, the relative error was between –5.5 and +3.7%, and we considered that measurement of SAA in our studies is accurate and validated. Downregulation of acetylcholine activates inflammation in both CNS and peripheral tissue, which causes AA in both sites. Therefore, changes of AA in the CNS link with SAA in the peripheral system even if a substance having AA does not penetrate through the blood-brain barrier. Then we redescribe issues that require attention in the measurement of SAA. It is generally defined that any SAA greater than the detection limit of a quantitative atropine equivalent level (≥1.95 nM in our study) is positive. According to previous studies, SAA is considered to be positive when its atropine equivalent is ≥1.95 nM and undetectable when this is <1.95 nM. Nevertheless, as a low SAA can act as AA in the CNS, we should assume that SAA might also be positive if its marker concentration is between 0 and 1.95 nM. In addition, SAA should be measured around 11 a.m. or somewhat later because of the diurnal rhythm of cortisol in humans.

Introduction
Alzheimer’s disease (AD) is a progressive neurodegenerative disorder with the extracellular deposit of β-amyloid in senile plaques, intracellular formation of neurofibrillary tangles and loss of neuronal synapses and pyramidal neurons. Among the neural degeneration, that of acetylcholinergic neurons is evident in the nucle-
us basalis of Meynert [1]. Deterioration of acetylcholinergic neurons is hypothesized with a reduction of choline acetyltransferase activity and levels of acetylcholine (ACh) in AD [2]. By the extensive effort of new drug development, the acetylcholine esterase inhibitors tacrine, donepezil, galantamine and rivastigmine are successfully marketed and clinically used to reduce progression of AD symptoms. ACh neurons from the nucleus basalis of Meynert projecting to the cortex, hippocampus and thalamus serve important functional roles in conscious awareness, attention, working memory and cognitive functions [3, 4]. Moreover, as ACh regulates not only cognitive function, but also inflammation [5, 6], downregulation of ACh activity activates the inflammatory system, and then a substance with anticholinergic activity (AA) appears [7]. Therefore we speculate that AA appears endogenously in AD in which downregulation of ACh is essential [1, 8]. This hypothesis is derived from our two reports [9, 10] and is reported in our review papers [11–13]. We undertook to measure the serum anticholinergic activity (SAA) [14] as a marker of AA appearing in the central nervous system (CNS). In this review, firstly we report a procedure of SAA measurement, and its reliability and reproducibility in a receptor binding assay to measure SAA. Secondly, we discuss the usefulness of measuring SAA as an index of AA in the AD brain. Thirdly, we describe issues that require attention in the measurement of SAA.

**Procedure of SAA Measurement and Its Reliability and Reproducibility in the Receptor Binding Assay**

SAA is measured based on a conventional competitive radioreceptor binding assay reported by Tune and Coyle [14]. 3H-QNB (quinuclidinyl benzate), a specific muscarinic antagonist, was used as a radioactive ligand, and atropine, also a specific muscarinic antagonist, was used to obtain a standard dose-response curve (Mitsubishi Chemical Medience, Uto, Japan). Rat brain homogenized membrane fraction was used as a source of muscarinic receptors. Drug-free human serum was used in each measurement as blank. To measure the SAA, 100 μl patient serum was mixed with 200 μl rat brain membrane fraction and 200 μl of 3 nmol/l 3H-QNB, then diluted with 1,500 μl of 50 mmol/l Na-phosphate buffer (pH 7.7) adjusted to a total volume of 2,000 μl. The mixture was incubated for 60 min at 22°C. Then, it was filtered with a cell harvester-provided polyethylene imine-treated glass filter, and the filter was washed 3 times with 5 ml of 10 mmol/l Na-phosphate buffer (pH 7.7). The trapped radioactive glass filter was put in a vial with a scintillator and measured in a liquid scintillation counter (Tri-Carb 2500TR, Perkin Elmer Life Science, USA) for 5 min each. The receptor binding assay was made in duplicate.

The rat brain homogenate membrane fraction was prepared as follows. Rats were decapitated under pento-
barbital anesthesia, and the brains were removed. The frontal part of the brain including the frontal cortex and basal forebrain was dissected and homogenized in ice-cold Tris-EDTA buffer (5 mmol/l Tris-HCl, 1 mmol/l EDTA, 1 mmol/l dithiothreitol) containing 10% sucrose. The homogenate was centrifuged at 105,000 g for 1 h at 4°C, and the aliquot of the supernatant was used for the receptor binding assay.

We express SAA as the concentration of atropine. So, reproducibility of the standard atropine binding curve is important to see the reliability. Therefore, we show the results of a 10 times repeated measurement of standard atropine binding in table 1 (offered by Ms. Kinuko Zaizen of Mitsubishi Chemical Medience). A relative error was between −5.5 and +3.7%. The percent relative error is calculated by the equation of \(\frac{\text{back-calculated value}}{\text{theoretical value}} - 1\) × 100, and it meant trueness. The
coefficient of variation was between 1.8 and 18%. Therefore, the values show good accuracy with a small variance in each concentration of atropine and are close to the theoretical dose response curve of atropine (table 1). The minimum binding activity of atropine is 1.95 nM which is the detection limit, and the maximum binding activity is 50 nM as shown in table 1. Therefore, we considered that measurement of SAA in our studies is accurate and validated.

Does SAA Reflect AA in the Brain?

SAA is accepted as a total peripheral AA load at least. We would like to emphasize that SAA reflects AA load in the CNS, at least in AD which is deficient in ACh (fig. 1; courtesy of Hori et al. [11]). In AD which is thought to involve inflammation [15], the permeability of AA at the blood-brain barrier (BBB) might be increased [16], therefore, AA in the CNS can permeate through the BBB into peripheral tissue and appear as SAA. The group of Frey [16, 17] reported that AA is conferred by a small molecule between 100 and 1,000 Da and produced by the oxidative stress in AD. Although this molecule is able to penetrate the BBB, even though the AA in the CNS is a high molecule like protein possibly reflected as SAA, through the inflammation mechanism described below.

Downregulation of ACh causes not only cognitive dysfunction but also hyperactivity of the inflammatory system both in the CNS and peripheral tissue, causing AA in both the CNS and peripheral tissue. Therefore, changes of AA in the CNS link with SAA in the peripheral system, even if AA does not penetrate through the BBB. The reason why changes of AA in the CNS link with that of SAA is considered to be the following; SAA inhibits muscarinic ACh activity of parasympathetic neurons and produces cytokine activation with macrophages; then, the proinflammatory cytokines penetrate into the brain and possibly activate the microglia. The activated microglia in the brain produces proinflammatory cytokines accelerating AD [16, 18, 19]. Therefore, any AA produced in the brain or peripheral tissue causes inflammation activating microglia or macrophages, respectively; the produced proinflammatory cytokines penetrate the BBB both ways, and then the AA reflects each region. From these points of view, we speculated that SAA reflects AA in the CNS at least in AD which is related with a deficiency in ACh.

Issues That Require Attention in Measuring SAA in AD Therapy

Finally we describe two issues which require attention in the measurement of SAA. We have already commented on these two issues in a previous article [12].

Tune and Coyle [14] showed that SAA of ≥3.5 nM atropine equivalents is necessary for beneficial effects of antipsychotics in schizophrenic patients in order to avoid extrapyramidal side effects. We reported, however, that SAA at 2.38 nM caused adverse effects on cognitive function in an AD patient [20]. We thought that it was quite probable that even low values of SAA could affect cognition and behavioral/psychological symptoms of dementia in AD, albeit with no effect on cognition in patients without dementia [21, 22]. We generally defined SAA as pos-
itive at any level greater than the detection limit of our quantitative atropine equivalent level ($\geq 1.95$ nM in our study). According to previous studies, SAA is considered to be positive when its atropine equivalent is $\geq 1.95$ nM and also undetectable when this is $< 1.95$ nM. Nevertheless, because a low SAA can cause AA in the CNS, we should assume that SAA might also be positive if its atropine concentration is under the detection limit between 0 and 1.95 nM (fig. 2; courtesy of Hori et al. [12]).

The next important issue on the agenda is when SAA should be measured. If inflammatory processes cause AA, it is natural to think that adrenal corticosteroids should inhibit AA because of anti-inflammatory properties. On the other hand, adrenal corticosteroids have been reported to induce or increase AA [23]. The diurnal rhythm of adrenal corticosteroid levels in plasma is high early in the morning and rapidly declines after that [24]. We assumed that this rapid decline might cause disinhibition of the immune system; thus, the immune system may be activated in the afternoon and after that until nighttime. If the blood level of adrenal corticosteroids rises above the usual values, the decline of the steroid levels should also become larger, which is expected to cause a more active inflammatory state and to produce AA (fig. 3; courtesy of Hori et al. [12]). Although we have never studied the diurnal rhythm of SAA, from our logic AA might increase in the late morning to around noon after the peak of adrenal corticosteroids in the early morning. This mechanism might explain why deliriums are subdued in the morning and occur more often in the late afternoon or at night. We assume SAA should be measured after 11 a.m. or somewhat later [12], though there is no data of any SAA diurnal rhythm. In our previous articles [9, 10], the time to withdraw blood to measure SAA was between 11 a.m. and noon, because we started to see the patient at 10 a.m., and it took about 1 h or more to make a diagnosis.

This diurnal rhythm of AA and the relationship between AA and adrenal corticosteroids need more research. We speculate that the relationship between the diurnal rhythm of AA and adrenal corticosteroids is related with the clinical features of delirium and the appearance of symptoms for Lewy body disease.

Conclusion

In this review article, we reported the procedure of SAA measurement, the reliability and reproductibility of the receptor binding assay and emphasized the usefulness of SAA measurement. However, further research is needed to confirm the basic science before progressing to the clinical usefulness. It is still necessary to identify the SAA and AA in the CNS, and the pharmacokinetics and pharmacodynamics of their marker substances using experimentally and basically medical approaches in the future.

References


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