Neural cell adhesion molecule in human serum. Increased levels in dementia of the Alzheimer type

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Memory impairment is a process associated with alterations in neuronal plasticity, synapses formation, and stabilization. As the neural cell adhesion molecule (NCAM) plays a key role in synaptic bond stabilization, we analyzed the usefulness of soluble NCAM isoforms in the diagnosis of patients with dementia of the Alzheimer type (DAT).

NCAM was measured in the sera of 70 control subjects and 43 DAT patients (with different severity of cognitive impairment, GDS), employing Western blot and densitometric quantification. LMW-NCAM bands (100–130 kDa) decreased significantly with age independently of sex. DAT patients presented values of LMW-NCAM and HMW-NCAM significantly higher than healthy controls of similar age (higher than 130 kDa). Only LMW-NCAM was associated with GDS. Our results suggest that NCAM could be involved in the pathogenesis of DAT disorder and that serum NCAM levels could be useful as differential diagnostic markers of the disease.

Keywords: Serum NCAM; Dementia of the Alzheimer type; Serum marker

Introduction

Alzheimer’s disease is the most prevalent cause of dementia worldwide and affects 5–10% of the population aged over 65 years old. Still, the diagnosis of the pathology is only defined after autopsy or brain biopsy, by observation of histological manifestations, found predominantly in the hippocampus amygdalus and the cerebral cortex, such as neurofibrillar degeneration, intraneuronal fibrillary tangles, extracellular senile plaques, and vascular deposits of amyloid (Meches et al., 1977). Because of the difficulty of its diagnosis and to avoid implicit assumptions about the pathophysiology of this heterogeneous syndrome, the disease is called dementia of the Alzheimer type (DAT) (Schellenberg et al., 1992). Therefore, the ante mortem diagnosis of the disease is based on clinical history, physical and neurological examination, together with the exclusion of other causes of dementia by neuroimaging. Biochemical markers to assist diagnosis and help the treatment would be clinically helpful but currently are unavailable.

Glycoproteins involved in the adhesion of neural cells are believed to regulate the stabilization of synaptic junctions, neurite outgrowth, wound repair, functional plasticity, and control release of neurotransmitter biosynthetic enzymes. These molecules belong to one of three major structural adhesion families: the immunoglobulin superfamily, cadherins, and integrins, which are expressed at high levels on neurons and non-neuronal cells (Hampel et al., 1996). Recent evidence suggests that members of the immunoglobulin superfamily are involved in functional neuronal plasticity in the adult animal, such as that associated with learning (Fields and Itoh, 1996; Rose, 1995), and that both short- and long-term memory requires some adhesion molecules to allow synapses formation (Ranheim et al., 1996). One of such options is the glycoprotein NCAM, which mediates homophilic binding between neighboring cells such as neurons, astrocytes, and muscle cells and heterophilic interactions between cells and extracellular matrix components (Ronn et al., 2000).

A single gene encodes NCAM, but alternative splicing of the pre-mRNA produces at least three major isoforms, NCAM 120, NCAM 140, and NCAM 180 (Ricard et al., 2000; Ronn et al., 2000). While NCAM 120 lacks a cytoplasmatic domain and is attached to the membrane by a covalent-bound phosphatidylinositol anchor, NCAM 140 and 180 present transmembrane and intracytoplasmatic domains. In the vertebrate nervous system, NCAM is the dominant carrier of an unusual carbohydrate consisting of long homopolymers of sialic acid (PSA), as isoforms of 200–250 kDa (PSA-NCAM). PSA-NCAM is abundantly expressed in the central nervous system during development and in early postnatal brain (Hampel et al., 1996; Ni Dhuill et al., 1999; Seki and Arai, 1993). In adult brain, PSA-NCAM is down-regulated but confined to a few restricted areas, such as the olfactory system and the mossy fiber system in the hippocampal formation, characterized by a high level of structural remodeling. It was proposed that the down-regulation of PSA-NCAM accompa-
nies a change in NCAM function from a plasticity-promoting to a stability-promoting molecule. According to this hypothesis, the presence of long PSA chains on NCAM inhibits cell adhesion therefore allowing structural remodeling to occur (Ronn et al., 2000).

Besides, it was found that NCAM of high molecular weight is up-regulated in astrocytes exposed to elevated hydrostatic pressure in vitro, in neurodegenerative disease and after neural trauma (Cotman et al., 1998; Ricard et al., 2000; Roche et al., 1997). Nearly all biochemical analysis on NCAM has been performed on membrane-bound forms of the molecule. However, soluble forms of NCAM have been observed in conditioned media from cultured neural or muscle cells (Bock et al., 1987; Thomaidou et al., 2001) and in different body fluids (Lynch et al., 1997; Poltorak et al., 1995), although its role in different pathological process is unknown.

In the present work, we study the pattern of NCAM isoforms found in human serum. We demonstrate that circulating NCAM is significantly increased in DAT patients, suggesting that the measurement of serum NCAM could be useful to improve the diagnosis of neurological diseases with cognitive deficit.

Materials and methods

Patients and healthy control subjects

Serum NCAM was measured in patients with dementia of the Alzheimer type (DAT) and healthy controls. The control population consisted of 70 subjects without evidence of neurological pathology [40 women and 30 men; median (Md) age: 55 years old, range: 19–87 years]. The DAT group included 43 subjects (32 women and 11 men; Md age: 82 years; range 59–95 years). Patients included in this study never suffered head trauma, seizures before the onset of the dementia features, uncontrolled hypertension, congestive heart failure, cerebral stroke, thyroid dysfunction, electroconvulsive therapy, exposure to neuroleptics, sleep disorders, mental retardation, psychosis, or depression. Patients suffering from systemic or other neurological disorders such as Parkinson’s, hydrocephalus, or Huntington’s disease causing cognitive impairment were excluded. All subjects underwent neurological, psychiatric, and physical examinations, imaging scans of the brain, as well as a comprehensive set of neuropsychological tests selected for sensitivity to cognitive deficiencies in aging and dementia. It included tests to evaluate all different types and processes of memory, attention, psychomotor speed, language, vision spatial function, and praxis. The severity of cognitive impairments was assessed using the Global Deterioration Scale (GDS: 2–3, 4–5, and 6–7 degrees corresponding to mild, moderate, and advanced, respectively) (Reisberg et al., 1982). The Ethical Committees of the Institute of Oncology “Angel H. Roffo” and the Italian Hospital of Buenos Aires approved this study. The Helsinki Declaration was carefully followed.

Serum specimens

Patients and controls were sampled before breakfast, in the morning, from the antecubital vein. The serum specimens from healthy donors and those obtained from dementia patients were separated from blood, collected, and frozen immediately in aliquots of 100 µl at −80 °C until processing. Serum aliquots were stored for less than 3 months and were used only once after thawing.

Western blot analysis and sample conditions

Serum samples were diluted 1:30 in PBS and 100 µl of each one were mixed with concentrated sample buffer under reducing (plus 5% β-mercaptoethanol; condition A) or nonreducing conditions (condition B). Then, samples were heated for 2 min at 100 °C. Western blot analyses for NCAM were carried out using a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) that was performed as described by Laemmli (1970), using 7.5% separating and 4% stacking gels. A concentrated sample buffer (fourfold) contained 100 mM Tris, 10% v/v glycerol, 50 mg/ml SDS, and 0.5 mg/ml bromophenol blue (pH: 6.8). The presence or absence of 5% β-mercaptoethanol marks condition A or B, respectively. All reagents for electrophoresis were purchased from Sigma, USA. In each SDS-PAGE, as an internal standard, a pool of serum samples was run. For molecular weight determination, Kaleidoscope molecular weight markers were used (Bio-Rad Laboratories, USA). Separated proteins were transferred to nylon membranes (PVDF, Amersham) using an aqueous electrical transfer (Bio-Rad Lab). Nonspecific binding sites were blocked for 1 h at room temperature in PBS–Tween 20 (0.1%) with 5% fat-free dry milk. Membranes from gels in condition A were incubated with a polyclonal IgG goat antibody (dilution 1:500; Santa Cruz Biotechnology) whose epitope mapped at the carboxy terminus of human NCAM. The membranes from PAGE in condition B were revealed with a monoclonal antibody (dilution 1:250, MAB 2122 Chemicon International Inc., USA), which recognized proximal PSA regions. Both antibodies were used at 4 °C overnight. Then, membranes from gels run in condition A were exposed to a biotinylated antigen IgG (1:500, Gibco), as second antibody, for 1 h at room temperature (RT), followed by horseradish-peroxidase-conjugated streptavidin (1:1000, Gibco) for 1 h at RT. Membranes from condition B were exposed to an antimouse IgG peroxidase (1:1000, Amersham) for 1 h at RT. Both primary and second antibodies were diluted in 50% PBS-blocking buffer. Three washings were performed with PBS–Twee 20 (0.1%) in each step. The chemoluminescence revelator system employed was ECL (Amersham). Membranes were exposed to a film BioMax Light (Kodak).

Measure of bands from Western blot analysis

Membranes were then analyzed with an image densitometer Bio-Rad (model GS 700) and the Molecular Analysis software was used to determine intensity differences among bands. NCAM values were expressed as folds of the pooled serum sample used as standard (value 1) that was included in every gel. Intraexperiment error was about 15%.

Statistical methods

Differences in the level of NCAM among the groups were compared using the Mann–Whitney test, appropriate median tests for even skewed data. We used linear regression to summarize the joint effects of sex and age on NCAM data. A Receiver Operator Characteristic (ROC) curve (Fletcher, 1988) was developed to determine the optimal reference value to differentiate patients from controls. Sensitivity, specificity, positive predictive value (PV+), and negative predictive value (PV−) were calculated employing...
the optimal cutoff point. The chi-square test was used to assess statistical significance in bivariate comparisons. A difference of $P < 0.05$ was considered to be significant. SPSSPC+ statistical package was used for the aforementioned analyses.

**Results**

This study was carried out to determine the diagnostic value of the soluble forms of NCAM in DAT disease. But, as only few data are reported about serum NCAM isoforms in healthy populations, we first analyzed the expression of this circulating adhesion molecule in 70 controls of different age and sex.

As shown in Material and methods, we employed Western blot in two experimental conditions. When SDS-PAGE were performed under reducing conditions and revealed by NCAM polyclonal antibody (condition A), we detected a unique band of 80 kDa (Fig. 1A). On the contrary, when nonreducing conditions were employed and the Western blot was performed with the monoclonal antibody (condition B), we observed a pattern of bands between 100 and 180 kDa (Fig. 1B), being the more conspicuous of 110, 120, and 145 kDa. For densitometric analysis, bands of molecular weight higher than 130 kDa (HMW-NCAM) and lower than 130 kDa (LMW-NCAM) were clustered.

**NCAM levels in the control population**

As the distribution of NCAM 80 kDa, HMW-NCAM, and LMW-NCAM values in the control population was skewed toward high values, a nonparametric test was employed to analyze differences between groups.

The values of serum NCAM found in the control population were stratified according to age. As shown in Table 1, the band of 80 kDa did not change with age. On the other hand, LMW-NCAM bands decreased significantly with age (linear regression, Pearson coefficient 15.31, $P < 0.01$), while HMW-NCAM bands increased in elderly humans (MW test, $P < 0.01$).

As the scope of this work was to study NCAM in DAT patients, a disease predominantly found in people older than 55 years, we chose this age to perform a cutoff to differentiate young from old people. It was found that LMW-NCAM in people older than 55 years had Md values of 0.35 (0–1.34), while young people showed significantly higher values [0.70 (0–1.96)] (MW test, $P < 0.01$). On the other hand, HMW-NCAM was significantly increased in people older than 55 years [0.82 (0–2.00)] with respect to the young-people group [(0.27 (0–2.47)] (MW test, $P < 0.01$).

Also, the levels of serum NCAM were analyzed according to sex. Table 2 shows that only HMW-NCAM were significantly different, showing higher values in women.

Then, the effect of age and sex on the distribution of NCAM values was analyzed together. When data were adjusted by sex and age, only LMW-NCAM differed with age ($P < 0.01$); the other differences are nonsignificant (data not shown, multivariate analysis).

![Fig. 1. Western blots for NCAM in the sera of healthy individuals and DAT patients with similar age. (A) Western blot developed by NCAM polyclonal antibody (condition A). A unique band of 80 kDa was detected. (B) Western blot developed by NCAM monoclonal antibody (condition B). A pattern of bands between 100 and 180 kDa was observed. Patients with high values of NCAM were selected for better illustration.](image-url)
We also analyzed possible associations between serum LMW-NCAM and HMW-NCAM from each subject. No correlation was observed between these values.

**Soluble NCAM analysis in patients with DAT syndrome**

According to the differences observed in the control population, the levels of serum NCAM of DAT patients were compared with the subgroup of control population older than 55 years (n = 35) (Md age 70, range 55–87).

As shown in Table 3, no difference was observed in the circulating levels of NCAM 80 kDa between patients with brain degenerative disease and controls subjects. On the other hand, densitometric analysis of Western blot revealed that both LMW-NCAM and HMW-NCAM values were higher in DAT than in controls; these differences are statistically significant.

In Figs. 2 and 3, we show the distribution of LMW-NCAM and HMW-NCAM in the serum of DAT patients and controls. Similar to controls, no correlation between LMW-NCAM and HMW-NCAM values was found in patients. The reference values of 0.63 for LMW-NCAM and 1.06 for HMW-NCAM were close to the inflection point on each ROC curve (data not shown), thereby maximizing sensitivity and specificity. These points corresponded to the 70% percentile value in normal control subjects for controls over 55 years both for the low- and high-MW isoforms of the circulating adhesion protein. Then, in each case, values over this concentration of NCAM were defined as elevated.

As shown in Figs. 2 and 3, from the optimal cutoff point, the number of DAT patients with elevated serum NCAM is higher than in the control population; these differences are statistically significant. Besides, the percentage of patients with elevated NCAM values is almost identical for LMW or HMW isoforms. Further analysis indicated that 18 DAT patients presented both increased isoforms, 4 patients showed only elevated values for LMW-NCAM, and 5 patients only for HMW-NCAM.

The assay of NCAM levels in the studied DAT population showed a sensitivity of 51.2 % (LMW-NCAM) and 53.5 % (HMW-NCAM), and a specificity of 71.4%. The PV+ was about 70% and the PV− was 54.3% for both LMW and HMW-NCAM.

To further shed light on the pathogenesis of the disease, we analyzed whether variables such as the time of DAT syndrome evolution or the severity of cognitive impairment, measured by the GDS scale, were associated with serum NCAM levels. No significant association was observed between HMW-NCAM levels and these pathological parameters. However, when DAT patients were grouped according to cognitive impairment, elevated LMW-NCAM were found in 33.0% of those with mild GDS and in 55.6% with high GDS. This way, all DAT patients with values of LMW-NCAM higher than 2 AU, shown in Fig. 2, presented high GDS and/or a long time of evolution of their disease.

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Values of circulating NCAM according sex in control subjects</th>
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<tr>
<td>NCAM</td>
<td>Feminine</td>
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<tr>
<td>80 kDa</td>
<td>1.01 (0.22–1.93)</td>
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<tr>
<td>LMW-NCAM</td>
<td>0.55 (0.00–1.73)</td>
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<tr>
<td>HMW-NCAM</td>
<td>0.67 (0.03–2.48)</td>
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Values are expressed as median and range.

*MW test.

Fig. 2. Distribution of the LMW-NCAM in serum from DAT patients and controls. The line indicates the optimal cutoff point (0.63 AU). A higher number of DAT patients showed elevated values as compared with healthy controls. *P < 0.05, chi-square test.

<table>
<thead>
<tr>
<th>Table 3</th>
<th>Serum NCAM values in patients suffering from dementia of the Alzheimer type (DAT) compared with controls older than 55 years</th>
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<tr>
<td>NCAM</td>
<td>Controls</td>
</tr>
<tr>
<td>80 kDa</td>
<td>0.97 (0.22–1.93)</td>
</tr>
<tr>
<td>LMW NCAM</td>
<td>0.35 (0.00–1.34)</td>
</tr>
<tr>
<td>HMW NCAM</td>
<td>0.82 (0.00–2.00)</td>
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Values are expressed as median and range.

*MW test.

Fig. 3. Distribution of the HMW-NCAM in serum from DAT patients and controls. The line indicates the optimal cutoff point (1.06 AU). A higher number of subjects with elevated HMW-NCAM values were observed in the DAT group, compared with controls. *P < 0.05, chi-square test.
Discussion

Cell adhesion molecules, such as NCAM, are cell surface macromolecules that control cell–cell interactions during development of the nervous system by regulating processes such as neuronal adhesion and migration, neurite outgrowth, synaptogenesis, and intracellular signaling (Hampel et al., 1996). Various CAMs have been abundantly found in the pre- and postsynaptic membrane of hippocampal neurons (Schuster et al., 2001). NCAM, specially those isoforms of high molecular weight, has been implicated in synaptic plasticity, learning, and memory (Lüthi et al., 1994; Solonimia et al., 1998). In this sense, Rusakov et al. (1995) described a high immunopositivity for high molecular weight NCAM in a learning-related region of the chick forebrain, and Schuster et al. (1998) observed increasing levels of NCAM 180 kDa associated with long-term potentiation (a model widely used to study synaptic plasticity) of learning tasks in an animal model (Rusakov et al., 1995; Schuster et al., 1998). Moreover, treatment of hippocampal slices or cultures with antibodies to NCAM or modifying NCAM by enzymatic removal of its polysialic acid resulted in decreased LTP-related synaptic response and learning (Becker et al., 1996; Kramer et al., 1997) and intraventricular injection of NCAM antibodies impairs learning in rats and chicks (Doyle et al., 1992; Scholey et al., 1993). Taking these data together, it is not surprising that this molecule exhibits alterations related with age or with diseases with cognitive deficit.

Almost all the analyses of NCAM have been performed on membrane-bound forms of the molecule. However, more recent evidence shows that soluble forms also exist and are shed from the cell surface (Bock et al., 1987; Lynch et al., 1997). While Takamatsu et al. (1994) found only one NCAM faint band of 110–130 kDa in the serum of six healthy controls, up to our knowledge, no systematic study was performed on circulating NCAM expression in a larger population of healthy individuals. We employed the Western blot technique with two commercial antibodies. When SDS-PAGE was performed under reducing condition and a polyclonal antibody that recognizes the carboxy terminus of human NCAM was used, we detected a unique band of 80 kDa. However, when samples were run under nonreducing conditions, and revealed with a monoclonal antibody, a different pattern of bands was observed; the sum for each subject did not account for the only band of 80 kDa. This fact is difficult to explain, but we must have in mind that both SDS-PAGE were performed in different conditions and with antibodies that recognize different epitopes. It is possible that serum bands of NCAM detected with the monoclonal antibody correspond to NCAM protein core carrying different quantities of oligosaccharides chains because of posttranslational modifications. Other possibility is that LMW bands represent fragments generated through proteolytic processing of isoforms of higher MW, as it is known that several proteases and their inhibitors are increased in DAT patients (Sacerdote de Lustig et al., 1994).

Our results indicated that LMW bands (lower than 130 kDa) decreased significantly with age and more abruptly after 55 years old. These alterations may be related with structural and functional changes in the nervous system such as neuronal loss or reduced dendritic arbor attributed to age. Histological studies determined that polysialylated NCAM of the dentate granule cells exhibits an age-related decline, although it has not been possible to directly correlate the age-dependent decrease in polysialylation with an age-related cognitive decline (Fox et al., 1995). On the other hand, Wei et al. (1999) found no alteration in the expression of NCAM 180, employing RT-PCR, in the brain of senescence-accelerated mice. Recent findings suggest that age-related structural changes may be not degenerative, but they most likely represent continuing functional plasticity coupled with enduring structural changes at the level of the synapse or optimization of successful learning strategies (Mikkonen et al., 2001). Therefore, the continued expression of a polysialylated NCAM population in brain throughout life may reflect their involvement in functions other than neurogenesis. However, more studies are needed to elucidate the exact relationship between circulating and cell-associated NCAM and their role in the central nervous system.

Our main purpose was to study whether the levels of circulating NCAM are modified in a pathological disease with a cognitive decline, such as the dementia of the Alzheimer type (DAT). Because neurons apparently are a major source of NCAM, it is not surprising that serum samples from individuals with severe neuronal loss would show altered levels of NCAM. During recent years, many reports have indicated that in addition to the progressive neuropathology observed in patients with confirmed Alzheimer’s disease (AD), there are also plasticity-related changes in the brain of these patients. It is thought that these plastic events are an attempt of the brain to restore structure and function or to compensate for the damage caused by the disease. However, it cannot be discarded that these changes are a part of the pathological development of the disease (Mikkonen et al., 1999, 2001). No molecular marker is available to help in the early detection of AD and the disease is usually diagnosed at advanced stages. We found significantly increased levels of circulating NCAM in a group of 43 patients with DAT in relation to neurologically normal older individuals. These increased levels of serum NCAM may be associated with alterations in the expression of this protein in brain tissue. In this sense, Yew et al. (1999) determined that although there was little difference in the expression of NCAM in the occipital cortex and hippocampus of Alzheimer patients (AD) compared with healthy subjects of the same age, there were significantly fewer positive NCAM neurons in the frontal cortex. Besides, Mikkonen et al. (1999) also found alteration in the PSA-NCAM immunoreactivity in the dentate gyrus of AD.

Up to now, the neuropathological diagnosis of Alzheimer’s disease is mainly performed on the recognition of a pattern of changes in brain tissue obtained at autopsy. However, some authors have suggested that AD disease had manifestations outside the central nervous system that might be clinically useful (Nordenson et al., 1980). But, up to now, none of the studied alterations in nonneural tissues from DAT patients were useful as diagnostic markers for DAT (Blass and Gibson, 1993; Merched et al., 1977). It is an attractive hypothesis that alterations in circulating NCAM may be useful to diagnose Alzheimer disease. We found that both LMW and HMW-NCAM were elevated in the sera of more than 50% of DAT patients, showing similar power for the diagnosis of the dementia of the Alzheimer type. However, in the present study, there was considerable overlap between serum levels of NCAM in samples from healthy individuals and DAT patients. Although the cutoff criterion employed yields a good specificity (71.4%), approximately half of the DAT patients had NCAM levels below this cutoff (false-negative results); thus, sensitivity was poor (about 50%). Therefore, the utility of serum NCAM to distinguish patients with DAT from healthy older people is limited, although with potential clinical interest, because as mentioned before, no marker to diagnose Alzheimer’s disease is available.
Regarding differential diagnosis with other diseases of the nervous system, we have found that circulating NCAM values were similar to controls in a few patients with amyotrophic lateral sclerosis, a neurodegenerative process without cognitive deficit (unpublished results). Besides, NCAM levels were also found elevated in the serum and cerebrospinal fluid of patients with schizophrenia (Lyons et al., 1988; Poltorak et al., 1995), being proposed as an etiologic factor of this syndrome (Ni Dhuill et al., 1999).

Interestingly, LMW-NCAM seems to be associated with the severity of cognitive impairment, as elevated LMW-NCAM was more frequently found in patients with high GDS. Moreover, we observed that those DAT patients with the highest values of LMW-NCAM had advanced GDS and/or presented a longer evolution of their disease. Perhaps, LMW-NCAM would help to define biologically and clinically meaningful subgroups, and even differentiate AD from other dementias such as those of the vascular type, thus helping in the early detection of the disease, guide care, and eventual treatment.

In conclusion, to the best of our knowledge, this is the first report to describe serum levels of NCAM in the healthy population and its modulation by sex and age. We also found that circulating LMW- and HMW-NCAM increase with the presence of a DAT clinical–pathological entity, showing a good specificity though a mild sensitivity. On the other hand, as an increase in the number of patients with elevated levels of LMW-NCAM was observed in advanced stages of the disease, this adhesion molecule could be potentially useful as a marker of DAT progression. Future studies in a higher number of DAT patients from the beginning of the disease and along the follow-up period could clarify the real value of serum NCAM measurement.

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