Demonstration of natural autoantibodies against the neurofilament protein α-internexin in sera of patients with endocrine autoimmunity and healthy individuals

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Abstract

Serum anti-pituitary antibodies (APAs) to cytosolic antigens have been found in association with autoimmune hypophysitis, idiopathic hypopituitarism, and other autoimmune endocrinopathies. Here, an immunoblot method was used to search for serum autoantibody (AAb) reactivities against pituitary antigens, including nuclear and cytoskeletal proteins, in six patients with idiopathic hypopituitarism, 60 patients with type 1 diabetes, nine patients with autoimmune polyglandular syndrome (APS) type 1, and in 74 healthy controls. Frequent patient serum IgG reactivity was observed against a 60 kDa human pituitary antigen, and the cross-reactive 62 kDa protein from rat brain was identified as α-internexin (α-INX) by proteomic methods. IgG and IgM AAbs to this neuron-specific type IV intermediate filament (IF) protein were found in most sera of patients with endocrine autoimmunity as well as healthy subjects with no significant differences in frequencies between the groups, but the levels of IgM α-INX AAbs were higher in patients with hypopituitarism as compared to healthy controls (P = 0.032, Mann–Whitney U-test). These findings suggest that α-INX AAbs are not specifically related to autoimmune endocrine diseases and most probably are a part of the natural AAb repertoire. This is the first demonstration of α-INX AAbs as one of the predominant neuronal IF AAbs in human sera.

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1. Introduction

Autoimmunity to pituitary gland, the central endocrine organ, is considered to be the hallmark of autoimmune hypophysitis [1]. This rare disease is one possible cause for unexplained hypopituitarism [2]. Anti-pituitary antibodies (APAs) against multiple antigens have been detected by immunoblot studies in patients with autoimmune hypophysitis or idiopathic hypopituitarism [1–3] and the main autoantigens identified as α-enzolase [4] and growth hormone [5] by different groups. APAs to these or other cytosolic antigens are not highly specific to autoimmune pituitary disease and have also been found in other endocrinopathies, including type 1 diabetes [6] and Addison’s disease [1]. However, it is not known whether APAs are indicative of pituitary autoimmunity in these endocrine disorders or reflect the AAb responses to the same or similar antigens in other tissues. The associations between different autoimmune diseases are well known and also AAbs related to associated diseases can be detected with higher frequency as, for example, shown in type 1 diabetes [7]. The more tight clustering of endocrine autoimmune diseases is characteristic for type 1 autoimmune polyglandular syndrome (APS 1), also known as autoimmune-polyendocrinopathy-candidiasis-ectodermal-dystrophy syndrome. APS 1 is caused by mutations in the autoimmune regulator (AIRE) gene and manifests typically in autoimmune destruction of adrenal and parathyroid glands, but the process can affect other endocrine organs including gonads, β-cells of the pancreas, thyroid and pituitary gland [8,9].

In previous studies, APAs have been detected against antigens in cytosolic and membrane fractions of the pituitary, whereas crude nuclear fractions containing cytoskeletal
components have not been further studied [1–6]. However, the serum immunoactivity to nuclear and cytoskeletal proteins from neuronal tissue has been described in different disease conditions. For example, high titer AAbs against neuronal nucleoproteins (Hu antigens) are the hallmark of paraneoplastic neurological syndromes associated with small cell carcinoma of the lung [10]. AAbs recognizing neuronal cytoskeletal proteins are found in association with various autoimmune and nonautoimmune neurologic diseases [11] or are even detected in healthy individuals where they most probably represent naturally occurring AAbs [12]. Among cytoskeletal proteins expressed in neuronal tissues, intermediate filament (IF) proteins are considered as one of the main targets for human serum AAbs [11,12].

In this study, we searched for AAb reactivities against pituitary antigens, including nuclear and cytoskeletal proteins, among patients with idiopathic hypopituitarism, type 1 diabetes, and APS 1. Here, we report the first data on the frequent occurrence of humoral autoimmunity to the neuronal IF protein α-internexin (α-INX) in patients with endocrine autoimmunity as well as in healthy subjects and compare the levels of α-INX AAbs between the patient and control groups.

2. Materials and methods

2.1. Subjects

Serum samples were obtained from 60 patients with type 1 diabetes (31 females, mean age 28.0 years, range 4–59 years), from nine patients with APS 1 (four females, mean age 19.0 years, range 12–31), from six patients with idiopathic hypopituitarism (three females, mean age 31.0 years, range 17–72 years) and from 74 healthy controls (blood donors and healthy volunteers; 39 females, mean age 32.3 years, range 3–63 years). Type 1 diabetes was diagnosed according to clinical criteria: hyperglycemia, ketosis, ketonuria, polydipsia, polyuria, and weight loss. Patients with hypopituitarism had deficiency of two or more pituitary hormones and all major causes of hypopituitarism (pituitary tumor, irradiation, trauma or apoplexy) had been excluded. The patients with APS 1 were of Finnish origin and their sera were kindly provided by Dr. P. Peterson from the University of Tampere, Finland.

2.2. Preparation of antigens

Adult human autopsy pituitary tissue (10h post mortem) and adult rat pituitaries and brain cortex were obtained and kept at −70 °C until use. Tissues were homogenized in phosphate buffered saline pH 7.4 (containing 1 mM Pefabloc SC, 1.5 mM pepstatin, 2 μM leupeptin and 3 μg/ml aprotinin) using a Dounce homogenizer. The same mixture of protease inhibitors was included in all buffers during the further steps of fractionation, performed at 0–4 °C. Accord-

ing to previously described method [3], the homogenates were first centrifuged at 400 × g for 20 min to pellet nuclei and cell debris (400 g sediment) followed by centrifugation at 100,000 × g for 60 min to separate the cytosolic and membrane fractions. The control tissues (rat liver, adrenal, kidney, cardiac and skeletal muscle) were handled in the same manner. In order to characterize the immunoreactive antigen of 62 kDa in rat pituitary and brain cortex, further fractionation of the rat brain cortex 400 g sediment was performed by resuspending it in 4 volumes of buffer A (10 mM Tris–HCl, pH 7.4, 10 mM NaCl, 1.5 mM MgCl2) containing 0.5% Triton X-100, and centrifuging through a layer of 30% sucrose in buffer A at 2000 × g for 30 min. This step was repeated two more times. The resulting pellet was extracted with 8 volumes of buffer B (20 mM Tris–HCl, pH 7.4, 0.5 M NaCl, 5 mM EDTA, 1% Triton X-100) for 30 min followed by centrifugation at 20,000 × g, 30 min. The remaining detergent insoluble fraction was dissolved in buffer C (10 mM Tris–HCl, pH 8.0, 8 M urea, 1 mM EDTA, 10 mM DTT) overnight and clarified by centrifugation at 100,000 × g for 1 h. The supernatant was enriched in the 62 kDa antigen. Neurofilament triplet proteins (NFTPs) from bovine spinal cord were purified as described earlier [13]. Protein concentrations in extracts were measured by the Bradford method using bovine serum albumin as a standard [14].

2.3. SDS–PAGE and immunoblotting

Protein samples were subjected to SDS–PAGE on 8–10% gels, and separated proteins were stained with colloidal Coomassie blue [15] or transferred onto nitrocellulose membranes (0.2 μm, Bio-Rad, Hercules, CA, USA) for immunoblotting. After blocking membranes with 3% skimmed milk and 0.05% Tween-20 in Tris-buffered saline (TBS-TM) for 1 h, the control Abs used were mouse monoclonal anti-α-INX (clone 2E3; Novus Biologicals, Littleton, USA) and anti-neurofilament 200 (clone NS2; Sigma, St. Louis, USA). Then incubation with alkaline phosphatase conjugates of rabbit anti-human IgG, anti-human IgM or anti-mouse Ig (diluted 1:1000; Dako, Glostrup, Denmark) and substrate reaction with nitro blue tetrazolium/5-bromo-4-chloro-indolyl phosphate followed. Two-dimensional electrophoresis (2-D) of the rat 62 kDa antigen extract proteins (50 μg) was carried out using Immobiline Dry Strips (pH 4–7L, 13 cm) in the isoelectric focusing step and 15 cm × 16 cm gels in SDS–PAGE according to the manufacturer’s instructions (Amersham Pharmacia Biotech AB, Uppsala, Sweden) followed by immunoblot on Immobilon-P membranes (Millipore, Bedford, USA) as described above. For preabsorption experiments two sera positive for both human 60 kDa and rat 62 kDa antigens were precubated either with 100 μg rat brain or liver proteins (dissolved in 8 M urea) before immunoblotting human pituitary proteins.
containing the 60 kDa antigen. Preabsorption was performed with sera diluted 1:50 in TBS-TM for 5 h at +4 °C. Similarly, three sera were preabsorbed with the mixture of NFTPs from bovine spinal cord or with the 62 kDa antigen extract from rat brain cortex at concentrations of 5 and 25 μg/ml prior to immunoblotting with rat 62 kDa antigen.

### 2.5. Statistical analysis

Fisher’s exact test and the Mann–Whitney U-test were used for comparison of parameters between the groups. Spearman rank correlation test was performed for correlation analysis. P-values below 0.05 were considered to be statistically significant.

### 3. Results

#### 3.1. Detection of serum immunoreactivity to the 60 and 62 kDa proteins in human and rat brain tissues

By using a limited number of sera from the patients with endocrine autoimmunity in immunoblot experiments, the major IgG immunoreactivity appeared against a 60 kDa antigen in human pituitary crude homogenate and its 400 g sediment fraction whereas it was undetectable in membrane and cytosolic fractions (Fig. 1A). Other reactions, including those with proteins from cytosolic and membrane fractions were less frequent and were not subjected to further investigation. Sera containing AAbs to the 60 kDa antigen of human pituitary also demonstrated reactivity at 62 kDa on immunoblots of rat pituitary and brain cortex homogenates (Fig. 1B). The sera reactive with 60 kDa antigen were subjected for analysis using MALDI-TOF MS. By searching the protein database SwissProt, the determined peptide sequence was found to match the partial sequence of α-internexin.

#### 3.2. Identification of the rat 62 kDa antigen as α-internexin

The 62 kDa antigen was soluble only in denaturing conditions of high-molar (4–8 M) urea, indicating possible association with cell nucleo- or cytoskeleton. As shown in Fig. 2, extraction resulted in significant enrichment of the 62 kDa antigen.
mass data matched with the highest score to rat α-INX (SwissProt accession no. P23565, theoretical M<sub>r</sub> = 56115.6 and pI = 5.20), covered by peptides over 31% of its protein sequence. The identity of α-INX and the 62 kDa antigen was further confirmed by specific binding of α-INX monoclonal Abs to the same set of 62 kDa antigen pI isoforms as recognized by human sera (Fig. 3, lower panel). Monoclonal anti-α-INX Abs also recognized the 60 kDa antigen on immunoblots of human pituitary homogenate (data not shown).

Rat α-INX extract contained several additional proteins with major bands at 200, 143, 65 and 53 kDa (Fig. 2). As these proteins were soluble only under denaturing conditions, they may represent other neuronal intermediate filament proteins. Considering the appropriate molecular weights and pI, the possible candidates were the NFTPs (50 kDa) — NF-H (heavy), NF-M (medium), NF-L (light) and glial fibrillary acidic protein (GFAP), respectively. Consistently, monoclonal Abs to NF-200 (NF-H) recognized the 200 kDa antigen on immunoblots (Fig. 4).

### 3.3. Human serum AAbs to α-INX and other neuronal cytoskeletal proteins

In order to determine the frequency of α-INX AAbs in patients with endocrine autoimmunity and in healthy subjects, the rat α-INX extract was used in further immunoblot experiments for broad screening of serum IgG and IgM AAbs to α-INX and, for comparison, to other neuronal cytoskeletal proteins present in the extract. A serum dilution of 1:200 was determined to be optimal for screening of positive serum α-INX AAb reactivities of both Ig classes. A serum that produced an intensive staining of α-INX on the blot was chosen as a positive reference and included in all experiments. Serum reactivities to α-INX were analyzed densitometrically (Multianalyst software, version 1.1; Bio-Rad).

Hercules, USA), and the band intensity of at least 10% of that of the reference serum was considered to be positive.

Representative serum IgG and IgM immunoblots are shown in Fig. 4. Both serum IgG and IgM AAbs recognized the 62 kDa α-INX and the 200 kDa NF-H proteins with highest frequency among patient groups and healthy controls (Table 1). IgG AAbs to α-INX were present in most patients with type 1 diabetes (63.3%), hypopituitarism, and APS 1 (both 66.7%), as well as in healthy controls (56.8%). Differences between patient and control groups were statistically not significant (Fisher’s exact test, Mann–Whitney U-test; P > 0.05). In the same lane with IgG results, IgM AAbs to α-INX were slightly more frequent in patients with hypopituitarism (83.3%), APS 1 (88.9%) and type 1 diabetes (70%) than in the healthy subjects (63.8%). When the IgM α-INX AAb levels were compared between the groups, a significant difference was obtained between the patients with hypopituitarism and healthy controls (P = 0.032, Mann–Whitney U-test). Assessing the distribution of IgG and IgM α-INX AAbs in different age groups of
healthy controls (years 1–15, 16–30, 31–45, 46 and older), the highest frequency was found among young people of 16–30 years (70% for IgG and 80% for IgM) although this was not statistically different from other groups. While α-INX and NF-H were predominant AAb targets in all studied groups, the AAbs to other putative neuronal IF proteins on immunoblots were less frequently detected in sera and had similar occurrence among the groups. In decreasing frequency, both IgG and IgM AAbs were found against proteins of 143, 53 and 65 kDa by immunoblot (data not shown).

3.4. Correlation between anti-NF AAbs and specificity of α-INX AAbs

No correlation was observed between the levels of α-INX and NF-H AAbs of both Ig classes neither between IgG and IgM AAbs to α-INX or NF-H in sera of healthy subjects. Among patients with endocrine autoimmunity, a correlation was present between IgM α-INX and NF-H AAbs ($r = 0.42$, $P < 0.05$; Spearman rank correlation). Comparative immunoblotting of purified bovine NFTPs and rat α-INX extract with monoclonal Abs and selected human sera demonstrated an Ab cross-reactivity between the main neuronal IF proteins within two samples (Fig. 5A). However, only scarce amounts of α-INX could be detected in bovine NFTP preparations by α-INX monoclonal Abs, and reactivity was undetectable for sera with high α-INX AAb levels. As revealed from preadsorption experiments with three sera, the preincubation with α-INX extract inhibited the serum AAb reactivity to α-INX in a concentration-dependent manner whereas the solution of NFTPs did not (Fig. 5B).

4. Discussion

The present study is the first report of frequent humoral autoimmune reactivity to the neuronal IF protein α-internexin in humans. Alpha-INX was first described in 1985 as a 66 kDa protein that copurifies with rat neuronal IF proteins [16]. Along with NFTPs — NF-L, NF-M and NF-H with apparent molecular weights of 68, 145 and 200 kDa, respectively — α-INX forms the group of type IV IF. Alpha-INX is expressed before NFTPs throughout most of the developing nervous system, and its expression is mainly restricted to the central nervous system in the adult [17–19]. It is an evolutionarily highly conserved protein, displaying 94% identity between rat and human α-INX amino acid sequences [20]. The functions of α-INX have been suggested to be as a scaffold for neuronal IFs during

Table 1

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>IgG AAbs to α-INX</th>
<th>IgG AAbs to NF-H</th>
<th>IgM AAbs to α-INX</th>
<th>IgM AAbs to NF-H</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type 1 diabetes</td>
<td>60</td>
<td>38 (63.3%)</td>
<td>31 (51.7%)</td>
<td>42 (70.0%)</td>
<td>50 (83.3%)</td>
</tr>
<tr>
<td>Hypopituitarism</td>
<td>6</td>
<td>4 (66.7%)</td>
<td>2 (33.3%)</td>
<td>5 (83.3%)</td>
<td>3 (50.0%)</td>
</tr>
<tr>
<td>APS 1</td>
<td>9</td>
<td>6 (66.7%)</td>
<td>3 (33.3%)</td>
<td>8 (88.9%)</td>
<td>9 (100%)</td>
</tr>
<tr>
<td>Healthy controls</td>
<td>7490$^a$</td>
<td>42 (56.8%)</td>
<td>47 (65.5%)</td>
<td>44 (63.8%)</td>
<td>58 (84.1%)</td>
</tr>
</tbody>
</table>

$a$ 74 and 69 sera from healthy controls were tested for IgG and IgM Abs, respectively.
The clear distinction between NAABs and AAbs arising during autoimmune diseases remains still elusive. It has been shown that the levels and reactivity profiles of NAABs are prone to alterations in human disease, both autoimmune and nonautoimmune [24]. On the other hand, AAbs associated with autoimmune disease have been also demonstrated at low levels in healthy subjects [25]. In this study, despite the high overall frequency of serum α-INX AAbs, variable AAb levels were detected in individual sera, and even more, IgM α-INX AAb levels were increased in patients with idiopathic hypopituitarism. The last finding can reflect the involvement of autoimmune mechanisms in development of idiopathic hypopituitarism.

Toh et al. [26] demonstrated by an immunoblot approach already in 1995 that most sera from patients with spongiform encephalopathies react with NF subunits from brain tissues. Serum IgG most frequently recognized 200 and 150 kDa NF proteins and less frequently the 70 kDa NF subunit and 62 kDa NF-associated protein. In the same year, Stefansson et al. [12] detected in over 90% of patients with different neurological diseases and in healthy subjects IgG and IgM AAbs against the 200 kDa NF-H protein in homogenates of neural tissues, while other immunoreactivities were relatively rare. These findings were supported by others, confirming that AAbs to NF-H are present in most of the normal human sera, whereas AAbs to NF-M and NF-L are found more rarely [27]. Consistent with previous results we found both IgG and IgM NF-H AAbs to be frequent in healthy individuals (Table 1). AAbs to other neuronal cytoskeletal antigens, most probably corresponding to NF-M, NF-L and GFAP, were also relatively rare in our study. In contrast, both IgG and IgM α-INX AAbs were revealed in most sera suggesting that α-INX AAbs are one of the predominant anti-NF AAbs in human sera. Preabsorption experiments and antibody reactivity patterns suggested no significant cross-reactivity of α-INX AAbs with other neuronal IF proteins. It is very probable that in the study of Toh et al. [26] the 62 kDa NF-associated protein, recognized by serum IgG from patients with infectious and noninfectious neurodegenerative diseases, actually corresponds to NF-H antigen. The same apparent molecular weight, association with other NFs and reactivity profiles of NAAbs are prone to alterations in human disease, both autoimmune and nonautoimmune [24]. On the other hand, AAbs associated with autoimmune disease have been also demonstrated at low levels in healthy subjects [25]. In this study, despite the high overall frequency of serum α-INX AAbs, variable AAb levels were detected in individual sera, and even more, IgM α-INX AAb levels were increased in patients with idiopathic hypopituitarism. The last finding can reflect the involvement of autoimmune mechanisms in development of idiopathic hypopituitarism.

The apparent molecular weight of 62 kDa for rat α-INX in our study is very close to that obtained previously [28]. Also the main isoforms of rat α-INX in the pI range of 5.2–5.5 found by 2-DE are compatible with both computed pI value for α-INX (5.2) and that obtained experimentally [18].
Different pI isoforms of α-INX probably correspond to differentially phosphorylated forms of the protein as the neuronal IF are well known for their high level of phosphorylation [19,29]. It has been shown that some serum AAb binding to NF-H is dependent on the phosphorylation state of the protein [27,30]. In our study, some sera recognized also more acidic isoforms of α-INX with pI up to 4.3 while α-INX monoclonal Abs reacted only modestly with this subset of antigens. These differences could be related to AAB recognition of phosphorylation dependent epitopes on α-INX.

The questions about the origin and the physiologic role of the naturally occurring α-INX AAbs as well as other neuronal AAbs are mostly unanswered. However, in more recent studies increased Ab levels against NFTPs or their individual subunits have been found in association with various inflammatory or neurodegenerative neurologic diseases [11,31–34]. These findings may lead to the speculation that humoral reactivity to α-INX can be altered in these diseases in a similar manner. In the rat experimental model it has been shown that α-INX protein expression increases dramatically after axonal injury and decreases after reinnervation in the facial motoneurons [35]. Recently, human immune reactivity to α-INX was described as the humoral response of a neuroblastoma patient to a novel product of neuromuscular system [36]. Considering these data, the questions concerning the possible role of α-INX AAbs in various human neurological and neoplastic diseases could be of interest to address in further studies.

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