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Neurological Disease-Associated Autoantibodies against an Unknown Protein Encoded by a RES4-22 Homologous Gene

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Screening a human small intestinal library with human serum yielded a clone which encoded a protein *res4-22* the gene of which was highly homologous to a recently described gene located in the Huntington's disease locus. Autoantibodies against *res4-22* (anti-*res4-22*), mainly of the immunoglobulin (Ig)A type, were detected in patients with neurological disorders at a higher frequency (18.4%) than in healthy blood donors (8.0%). In neurological patients with cerebral ischaemia anti-*res4-22* was found significantly more often (47.4%) than in the total group of neurological patients. Anti-*res4-22* positive sera showed significantly more frequently myelin staining in cerebellum and nerve sections than anti-*res4-22* negative sera. Our findings demonstrate a new species of human autoantibodies against a newly described protein the function of which is still unknown.

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INTRODUCTION

Autoantibodies (AAbs) can be found in patients with autoimmune diseases. In these disorders, AAbs may play a predominantly pathogenic role (e.g. acetylcholine receptor blockade in myasthenia gravis [1]), or may be associated with disease without proven causal connection (transglutaminase antibodies in coeliac disease [2]). Irrespective of a pathogenic role, AAbs related to disease are of diagnostic importance. However, AAbs are also found in healthy individuals in the absence of overt antigenic stimulation (autoantibodies to interferon (IFN)-α [3]). The latter antibodies are designated as natural autoantibodies (NAAbs) and are reported to constitute a substantial part of the normal B-cell repertoire [4]. NAAbs recognize both self and nonself antigens like infectious agents and are mostly polyreactive [5]. NAAbs have been postulated to play a biological role in several physiological processes [4].

Genes coding for autoantigens may be detected by screening cDNA expression phage libraries using patients antisera. Searching a human intestinal library for autoantigens of coeliac disease, a disorder characterized by damage of the small intestinal mucosa and IgA AAbs, by use of serum of a coeliac patient yielded, however, only one positive phage clone which did not show any reactivity with other coeliac sera. This clone encoded a protein *res4-22*, the gene RES4-22 of which was highly homologous to a transcript recently isolated from a human brain cDNA library. The gene product of RES4-22 was not isolated until now. Its predicted structure does not show any significant similarity to known protein sequences or obvious domain/motif structures. RES4-22 is located in the Huntington's disease gene region on chromosome 4p16.3 [6].

Because the coeliac patient used for isolation of the *res4-22* clone also suffered from polyneuropathy, sera of other patients with neurological diseases were tested for reactivity with *res4-22*. A significant association of AAbs against *res4-22* (anti-*res4-22*) to neurological diseases was detected.

MATERIALS AND METHODS

cDNA library screening. A human small intestinal cDNA library cloned in the vector λgt11 (HL1133b, Clontech, Heidelberg, Germany) was used. The cDNA is contained in the β-galactosidase gene and expressed as a fusion protein with the N-terminal part of the enzyme. A total of

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800 000 clones were screened. Overnight cultures of *E. coli* Y1090r^{-} (200 μl) were incubated with about 7000 phage of the library and plated on LB agar plates for 4 h at 37 °C. A nitrocellulose membrane saturated in 10 mmol/l isopropyl-β-D-thiogalactopyranoside (IPTG) was placed on the plates for additional 4 h at 37 °C. Filters were removed from the plates, washed once in washing buffer (50 mmol/l Tris pH 8.0, 150 mmol/l NaCl, 0.05% Tween 20), blocked for 5 min in washing buffer containing 2% Tween and incubated with serum of patient 1184 (1 : 100 in washing buffer) over night. After three washing steps bound IgA was detected with antihuman IgA (conjugated with alkaline phosphatase, 1 : 1000 in washing buffer, DAKO Diagnostika GmbH, Hamburg, Germany). Positive phage clones were isolated from the plates and used for further analysis.

Determination of insert size. The size of the insert in the λ-phage clone was determined electrophoretically after polymerase chain reaction (PCR) using λgt11 forward and reverse primer (Promega GmbH, Mannheim, Germany).

Homology search. The database of the National Center of Biotechnology Information (NCBI Bethesda, MD, USA) was searched using Advanced BLAST programme [7].

Sequencing. Sequence analysis was performed by automated didesoxy chain terminating sequencing [8,9] using ThermoSequenase (Amersham Pharmacia Biotech Europe GmbH, Freiburg, Germany) and 5′-fluorescently labelled λgt11 forward primer. The products were separated and determined on ALFexpress sequencer (Amersham Pharmacia Biotech Europe GmbH).

Subcloning of the cDNA insert. In order to avoid reaction of naturally occurring human antibodies against β-galactosidase [10] and to obtain a higher yield of the expressed protein, the coding region of λ-phage clone 1037 containing the RES4-22 fragment was amplified using PCR with forward and reverse primers designed according to the obtained gene sequence of the clone. The stop codon and the region downstream from this codon were omitted. The PCR product was subcloned into the vector pTrcHis2-TOPO using the pTrcHis2-TOPO TA® Cloning kit (K4400-01, Invitrogen, Leek, the Netherlands) according to the manufacturer's instructions. In this vector the cDNA insert from the λ-phage is contained in an *E. coli* plasmid and expressed as a fusion protein with a C-terminal 6 × histidine tag (HisTag). Because the insertion of the cDNA fragment is not unidirectional, resulting *E. coli* clones were sequenced to determine the direction of the fragment. Two clones were isolated: MYS9 with the insert in sense direction to the HisTag and MYS7 in antisense direction for control.

Sera. For screening of the cDNA library serum of a patient (No. 1184) was used who suffered from coeliac disease and polyneuropathy. For testing of proteins derived from clone MYS9, 100 sera of healthy blood donors, 158 sera of patients with various neurological diseases were used.

Preparation of proteins for immunoblotting. After overnight culture of the clones MYS9 and MYS7, 400 ml LB medium (10 g bactotryptone, 5 g yeast extract, 50 mg ampicilline/e, pH 7.5, autoclaved) were inoculated. Cells were grown under vigorous shaking for 2 h at 37 °C. IPTG was added to a final concentration of 1 mmol/l and the cells were grown for an additional 2 h at 37 °C. After harvesting by centrifugation 5 000 × ***g*** cells were disrupted using 7 ml BugBuster™ Protein Extraction Reagent (Novagen, Calbiochem-Novabiochem GmbH, Schwalbach, Germany) and the suspension was centrifuged for 15 min at 15 000 × ***g***. The sediment containing the expressed protein in inclusion bodies was dissolved in 20 ml 50 mmol/l phosphate buffer pH 7.0 with 300 mmol/l NaCl (buffer A) containing 8 mol/l urea for 1 h. After centrifugation for 15 min at 15 000 × ***g*** supernatant was

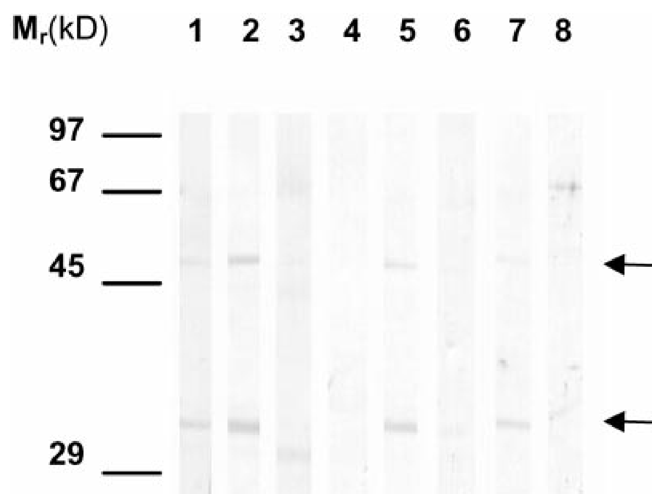

Fig. 1. Reactivity of different immunoglobulin (Ig) subclasses with purified HisTag protein from clone MYS9 in immunoblot. Lane 1: anti-HisTag antibody (POD-labelled); lanes 2, 3, 4: serum of patient no. 1184; lanes 5 and 6: serum of a neurological patient; lanes 7 and 8: serum of a blood donor. Staining for IgA: lanes 2, 5, 7; for IgG: lanes 3, 6, 8; for IgM: lane 4. Arrows indicate the positions of protein bands at 32 kDa and 48 kDa stained by HisTag-antibody and patients auto-antibodies (Aabs).

incubated with 1 ml TALON™ Metal Affinity Resin (Clontech Laboratories GmbH, Heidelberg, Germany) gel for 1 h while shaking. The gel was washed 5 times with buffer A and 5 times with buffer A containing 5 mmol/l imidazole. Elution was performed using 1 ml buffer A containing 100 mmol/l imidazole for 20 min. The protein was diluted with an equal volume of 2 times concentrated sample buffer for reducing sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) [11]. In addition to the expected protein band in clone MYS9 at about 32 kDa a second band was found at about 48 kDa also reactive with anti-HisTag-antibody and patients AAbs (Fig. 1) which could arise from covalent binding of *res4-22* to bacterial proteins.

Immunoblotting. Samples were incubated in reducing buffer for 10 min at 95 °C and subsequently applied for SDS-PAGE. Subsequently, proteins were transferred to nitrocellulose [12]. After incubation with human sera (1 : 100) for 2 h bound antibodies were detected by antihuman IgA or IgG (peroxidase conjugated; 1 : 1000), antihuman IgE (unlabelled; 1 : 1000) and antirabbit Igs (peroxidase conjugated, 1 : 700), or antihuman IgM (biotinylated; 1 : 1000) and peroxidase conjugated streptavidin (1 : 1000) (all from DAKO Diagnostika GmbH). Antibody against HisTag (peroxidase labelled) was applied at dilution of 1 : 1000 (Seramun Diagnostika GmbH, Dolgenbrodt, Germany).

Immunofluorescence. Undiluted sera were prescreened on slides carrying sections of 30 different tissues (Biochip-Mosaik, No. FA 1599–0001–2, EUROIMMUN GmbH, Groß Grönau, Germany) by indirect immunofluorescence. Further screening was carried out on monkey jejunum, peripheral nerve (*n.* suralis) and cerebellum sections (Biochip, No. FA 1111–1005–1, EUROIMMIUN). For this, sera were diluted 1 : 5 and incubated 30 min at 37 °C on the tissue sections. After washing, bound antibodies were detected using fluorescein isothiocyanate labelled antihuman IgA (DAKO).

Statistics. Rates of reactive sera in immunoblot and in different

Table 1. Reactivity of IgA of different sera with the HisTag fusion protein encoded by clone MYS9 in immunoblot

Subjects	Number of negative sera	Number of positive sera	Percent of positive sera (95% Confidence interval)
Healthy blood donors	92[a]	8[b]	8.0 (2.7–13.3)
Neurological diseases	129[c]	29[d]	18.4 (12.3–24.4)*
From these:			
Cerebral ischaemic conditions[§]	10[e]	9[f]	47.4 (24.9–69.9)**
Epilepsy, fits	9	3	25.0 (0.5–49.5)
Brain tumours	8	2	20.0 (0.0–44.8)
Polyneuropathy	14	2	12.5 (0.0–28.7)
Multiple sclerosis	13	1	7.1 (0.0–20.6)
Parkinson's disease	6	1	14.3 (0.0–40.2)
Chorea Huntington	8	1	11.1 (0.0–31.6)
Myopathies	7	2	22.2 (0.0–49.4)
Metabolic diseases[§§]	9	0	0.0 (0.0–0.0)
Other peripheral nerve	23	1	4.2 (0.0–12.2)
Other neurological diseases	26	7	21.2 (7.3–35.1)

Four sera refer to patients with diseases belonging to two different subgroups and were counted separately in each. *$P < 0.05$ compared with healthy blood donors, ** $P < 0.001$ compared with healthy blood donors, $P < 0.01$ compared total number of neurological patients and group of patients with other diseases of the nervous system, $P < 0.05$ compared with patients with polyneuropathy, multiple sclerosis, and metabolic diseases, mean age (± SD) in the different patient groups: [a] 38.0 ± 12.0, [b] 43.0 ± 17.7, [c] 51.3 ± 15.9, [d] 54.2 ± 16.8, [e] 65.9 ± 14.4, [f] 68.0 ± 8.2. Patient groups comprising [§]perfusion deficits and haemorrhage, [§§]Wilson's disease and Gaucher's disease, [§§§]paresis and inflammation etc.

groups of neurological diseases were presented with 95% confidence intervals. Differences of reactivity between different groups were analyzed by means of Chi^2- or 2-sided Fisher exact test (Statistica 5.1, StatSoft, Tulsa, USA).

RESULTS

Screening of the human small intestinal library with serum of patient 1184 suffering from coeliac disease and polyneuropathy resulted in detection of λ-phage clone 1037. This clone contained a cDNA insert of 1.2 kb. A data base search indicated 99% homology ($P < 1.6 \times 10^{-190}$) to regions in gene loci AB000459, AB 000460, AB000461, and HSL196E3 (human mRNA clones RES4-22A, B, C comprising 4.6–4.8 kb cDNA and DNA sequence from cosmid L196E3) [13,14]. These genes encode unknown gene products located in the gene locus for Huntington's disease.

Sera of eight other coeliac patients, however, were negative for IgA AAbs against *res4-22* (data not shown). Thus, anti-*res4-22* should have a specificity different from coeliac specific AAbs. Therefore, it was examined if the antibodies recognizing *res4-22* were related to neurological diseases. A significant difference in the frequency of IgA positive sera could be observed between healthy blood donors and patients with neurological diseases (Table 1). Those neurological patients with cerebral ischaemia had IgA antibodies against *res4-22* with significantly higher frequency than the mean frequency in the group of neurological patients. Anti-*res-4-22* positives did not differ significantly in age from anti-*res4-22* negatives in the group of blood donors, of neurological patients, and in the subgroup of neurological patients with cerebral ischaemia. IgA positive sera were tested for the presence of other Ig classes reactive with *res4-22*. Contrary to IgA, there was only faint or no reactivity of IgG, IgM (Fig. 1), and IgE (data not shown).

Table 2. Immunofluorescence reactivity of IgA anti*res4-22* positive and negative sera on cerebellum, nerve, and gut sections

	Number of sera with positive reactivity in immunoflourescence		
Nerve myelin	Cerebellum	Myelin	Gut smooth muscle
13 *res4-22* positive sera	6*	8**	6
12 *res4-22* negative sera	0	0	2

* $P < 0.05$ or **$P < 0.005$ compared with negative controls.

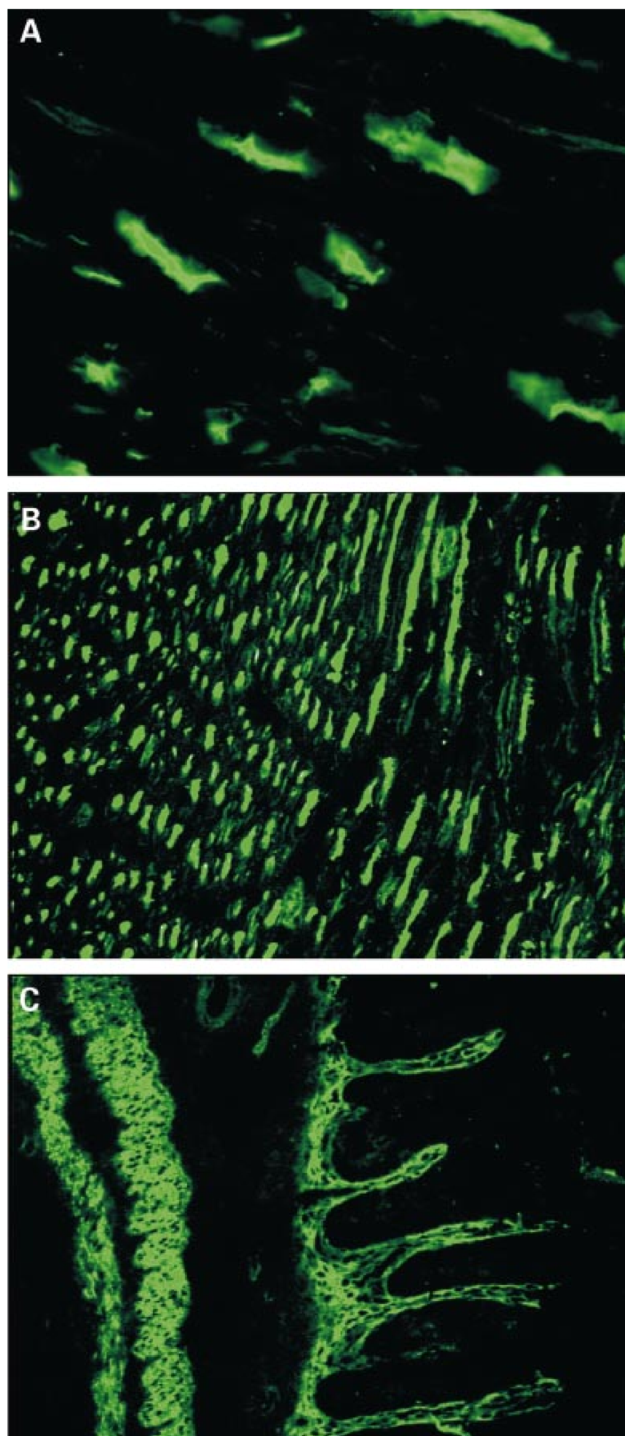

Fig. 2. Staining pattern of anti-*res4-22* IgA positive sera. Myelin staining pattern of (A) Schwann cells of the human nerve (400 times) and (B) of cerebellum (100 times). (C) Staining of smooth muscle cells of small intestinal lamina propria and tunica muscularis (100 times).

Binding to sections of 30 different tissues was investigated with 3 anti-*res4-22* IgA positive sera. The most prominent patterns were staining of myelin of primate central and peripheral nervous system and of smooth muscle preferentially of intestinal origin (Fig. 2). Subsequently, 13 *res4-22* positive and 12 negative sera were tested on cerebellum, nerve and gut sections (Table 2). Depending on the tissue, 6–8 of 13 *res4-22* positive sera were also positive in immunofluorescence with antihuman-IgA as secondary antibody. Two *res4-22* negative sera showed also an immunofluorescence staining.

DISCUSSION

Our findings demonstrate a new, hitherto unidentified species of human antibodies binding to a protein with unknown function encoded by a recently discovered gene on chromosome 4p16.3 [6]. This gene was originally isolated from a human brain cDNA library. Its presence in intestinal cDNA libraries is consistent with an ubiquitous expression as suggested [6].

The serum used for cDNA screening was from a patient with both coeliac disease and neurological disorder. Because a relation to coeliac disease was missing, further samples of neurological patients were investigated. AAbs were found significantly more often in these patients than in blood donors. Furthermore, the frequency in patients with cerebral ischaemia was significantly above the mean frequency in neurological patients. The age of anti-*res4-22* positive patients in the group of blood donors, of neurological patients, and in the subgroup of neurological patients with cerebral ischaemic conditions did not differ significantly from that of anti-*res4-22* negatives, thus suggesting a relation to disease.

The reason for the appearance of anti-*res4-22* is not clear until now. These AAbs might not only be secondarily involved in pathogenesis, i.e. as a consequence of brain or nervous tissue damage, but might as well play a causative role as it was described for antiantiphospholipid antibodies also found to be associated with cerebral ischaemia [15–17]. Anti-phospholipid antibodies are known to interfere as β_2-glycoprotein I (GPI) antibodies with the inhibitory action of GPI on coagulation [18]. GPI antibodies have been shown to bind to cells of the central nervous system [19,20]. Anti-*res4-22* can also be found in healthy subjects but only at a frequency of about 8%. The existence of neuronal associated AAbs at a low frequency in the normal population has been reported [21,22]. The importance of such findings is not determined until now.

Although the gene coding *res4-22* is localized in the locus for Huntington's disease, no association with this disorder could be verified.

AAbs against *res4-22* are mainly of the IgA type. This may argue for a mucosal origin, where IgA-producing immunocells predominate [23]. In accordance with this, elevated IgA AAb levels of suspected mucosal origin against brain components, i.e. neurofilaments and myelin, are also reported to be present in animals with bovine spongiform encephalopathy [24]. Furthermore, IgA antibodies from coeliac patients are reported to be reactive with brain blood-vessel structures [25].

Reactivity of antibodies with tissue sections is not congruent with immunoblot data. Only a part of *res4-22* positive sera recognized the tissue antigens. This may indicate either different

antigenic density in both assay systems or that antibodies against *res4-22* are coexistent with other antibody specificities.

The brain gene coding for *res4-22* is composed of at least 21 exons and may be spliced alternatively giving rise to three different transcripts consisting of 792–1265 amino acids. The DNA insert of clone 1037 comprised only one third of this gene out of the last three exons. Thus, there may be further epitopes for AAbs which could not be detected with the fragment used for the present analysis. Use of the complete antigen might enhance the fraction of antibody positive patients and improve the sensitivity to detect a pathological condition. Therefore, the determination of the total frequency of AAbs against *res4-22* awaits cloning and expression of the complete protein.

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