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Autoantibodies against Pituitary Peptides in Sera from Patients with Multiple Sclerosis

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Summary

Autoantibodies against pituitary peptides were demonstrated in sera from multiple sclerosis (MS) patients. Ten patients with lupus erythematosus disseminatus (SLE) and 97 healthy blood donors served as controls. The sera were used as primary antibodies in the indirect immuno-enzyme cytochemical (IEC) method, with fixed, paraffin-embedded rat brains and rat and hog pituitaries as antigen substrates. Eleven of the 33 MS sera reacted with peptides in the neural lobe/hypothalamic nuclei or distal lobe. The MS had a significantly higher incidence of peptide antibodies than sera from controls (11/33 vs 9/97). The mean antibody titers were significantly different (1577 vs 333). Comparison with rabbit reference antibodies specific to each of the 6 distal lobe hormones showed that the 9 distal lobe-positive MS sera reacted with cells harboring peptides of the somatotropin family. The presence of peptide autoantibodies was not related to clinical status or medical treatment. No antibodies against pituitary peptides were found in the SLE sera. One of the 11 positive MS sera showed antibodies against gastric parietal cells. None of the 11 sera showed antibodies against muscle, mitochondria, thyroid, adrenal, or parotid antigens.

We propose that in a proportion of patients with MS, these autoantibodies might be involved in the demyelinization process by interfering with the peptide/receptor

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interplay, thus placing MS as a disease in analogy with myasthenia gravis. Alternatively, these autoantibodies might be involved in the altered immunoregulation of MS or be secondary to the disease.

Key words: *Autoantibodies – Immunocytochemistry – Multiple sclerosis*

Introduction

Although the etiology of multiple sclerosis (MS) is not known, there is general agreement that the pathological manifestations of the disease are mainly of immunological character. Perivenular demyelination appears to be the first pathological sign (Adams 1975; Prineas 1978), and immunoglobulin (IgG) has been demonstrated in developing brain plaques by immunocytochemical and biochemical methods (Tavolata 1975; Mehta 1981). Further, there have been reports suggesting altered immunoregulation correlated to relapses of the disease (Goust et al. 1980; Reinherz et al. 1980) and observations of circulating immune complexes in the serum of these patients (Glikman et al. 1980; Jans and Sørensen 1980). In addition, the supporting laboratory diagnosis rests on the demonstration of a relative increase of IgG with oligoclonal bands in cerebrospinal fluid (CSF).

Recently, it has been reported that varying levels of the neuropeptide somatostatin in CSF correlated with relapses of the disease (Sørensen et al. 1980). In the light of this and the growing knowledge of peptide regulation of the blood–brain barrier function (Partridge et al. 1981; Zimmerman 1981) and of peptide modulation of the interplay between brain cells (Greengard 1981; Kriegler et al. 1981), we decided to investigate the possibility to demonstrate antibodies against pituitary peptides in sera from patients with MS.

The present study deals with an immunocytochemical demonstration of IgG antibodies directed against pituitary peptides in sera from MS patients. The sera were used as primary sera in the indirect immuno-enzyme cytochemical (IEC) method (Sternberger 1979) with fixed, paraffin-embedded sections of rat and hog pituitary glands and rat brains as antigen substrates. The results are compared with those obtained in a group of patients with systemic lupus erythematosus (SLE) and a group of healthy human subjects.

Materials and Methods

Material

Samples of freshly drawn peripheral blood were collected from the following groups of volunteers, including two groups of patients.

(i) Multiple sclerosis (MS). 33 patients (Table 1) examined and diagnosed at the Department of Neuromedicine, Frederiksborg County Hospital, Hillerød (24) and Hvidovre Hospital, Copenhagen (9) respectively, were chosen for evaluation. All

TABLE I

CLINICAL AND SEROLOGICAL DATA ON MS PATIENTS

Clinical type	No.	Sex	Age (yr)	Age (yr) at onset of MS	Duration of disease (yr)	Number of reactive sera (and sex) in	
						distal lobe	neural lobe
Relapsing type	24	6M, 18W	23–60 (mean 42.8)	16–57 (mean 33.1)	0.5–23 (mean 9.6)	5 (3M, 2W)	
Acute attack	20	5M, 15W	23–60 (mean 43.2)	20–57 (mean 34.4)	0.5–21 (mean 8.7)	4 (2M, 2W)	
Inactive	4	1M, 3W	28–55 (mean 40.8)	16–34 (mean 26.5)	4 –21 (mean 14.2)	1 (M)	1 (W)
Progressive type	9	2M, 7W	32–53 (mean 44.7)	20–44 (mean 34.8)	3 –21 (mean 9.9)	4 (2M, 2W)	1 (W)

patients (8 men, 25 women), aged 23–60 years (mean 43 years), fulfilled the criteria for a diagnosis of clinically defined MS confirmed by the CSF IgG index, found to be abnormal. Age at the onset of disease ranged from 16 to 57 years, and the duration of disease before the patients entered the study varied from 0.5 to 23 years. Twenty of the patients (with relapsing-type MS) displayed clinically acute attacks, manifested by an objective progression of previous symptoms, 9 patients displayed slowly progressive symptoms, and 4 patients were in silent phase at the time of blood sampling.

(ii) Lupus erythematosus disseminatus (SLE). Ten patients (2 men, 8 women) aged 24–76 years (mean 41 years) examined and diagnosed at the Department of Rheumatology, Hvidovre Hospital, Copenhagen in whom SLE had been diagnosed on clinical grounds (JAMA 1973) and confirmed by the presence in serum of anti-nuclear antibodies (ANA) (tested by indirect immunofluorescence on unfixed cryostat sections of rat liver; undiluted serum) acted as controls in relation to the method (see below).

(iii) 97 normal subjects (40 men, 57 women), healthy blood donors consecutively during one day appearing at the blood bank, Rigshospitalet, Copenhagen, aged 20–62 years (mean 33 years), were studied as controls to the MS patients. These controls all claimed to be comfortable on the day of blood sampling and a month previously, when routinely asked. In addition, two pooled standard sera were studied: (i) a serum from 75 healthy blood donors (sex and age unknown), (ii) a standard human serum (Behring 04 1008).

Immunocytochemistry

As substrates for the immunoenzyme cytochemical (IEC) test were used rat brains and rat and hog pituitaries.

Ten adult Wistar rats (5 males and 5 females, 150–200 g, age 3 months) were killed by decapitation after diethyl ether anesthetization. In order to effect adequate and rapid penetration of the fixative the roof and the rostral part of the skull were removed. After fixation in situ for 36 h at room temperature in Bouin's fluid containing 10% of a saturated aqueous solution of $HgCl_2$, the brains together with the pituitary gland were dissected free and embedded in paraffin after dehydration in a graded series of ethanol and clearing in methyl benzoate followed by benzene. Serial sagittal and transverse sections of the paraffin-embedded tissue were cut 6 μm and used as antigen substrate in the IEC tests. The hog pituitaries were obtained at a local abbatoir and fixed 20 min post mortem.

Distribution and morphological characteristics of the cell types specialized in production of a specific hormone in the rat anterior pituitary were evaluated with reference antisera: (i) anti-bovine somatotropin (anti-bSTH) and anti-ovine prolactin (anti-oPRL) [ovine-P-S9 and P10 were kindly donated by the National Institute of Arthritis, Metabolism, and Digestive Diseases (NIAMDD)], the corresponding antisera were produced in rabbits (B.L. Hansen and G.N. Hansen 1982); (ii) anti-(1-24)ACTH (Synthetic human (1-24)ACTH, Synacthen, Organon, The Netherlands) antiserum (a gift from Dr. L. Hummer, Glostrup Hospital, Copenhagen, Denmark) was prepared in guinea pigs (Hummer 1978); (iii) rabbit antisera to

the beta (β) subunits of human lutropin (anti-hLBHβ, NIAMDD batch #1), human follitropin (anti-hFSHβ, NIAMDD batch #1), human thyrotropin (anti-hTSHβ, NIAMDD batch #1), and rat PRL (anti-rPRL-S-8) were generously donated through the NIAMDD hormone distribution program. These antisera were used as primary sera in the unlabeled antibody–enzyme method (Sternberger 1979) with the peroxidase–antiperoxidase (PAP) complex (DAKO Immunoglobulins, Copenhagen, Denmark, code Z 113) diluted 1:80. As bridging antibodies were used swine anti-rabbit IgG (DAKO code Z 196) diluted 1:10.

The human sera were kept frozen at −21°C until they were used in a 1:100 standard dilution as primary sera on the rat pituitary and brain sections. This standard dilution was chosen after titration of the two pools of normal human sera to a level with negligible unspecific background staining. In addition, sera from SLE patients were examined by stepwise dilution from the concentrated stage in order to verify the standard ANA-test. As detective antiserum was used rabbit anti-human IgG conjugated with horseradish peroxidase (DAKO code P 214) diluted 1:100. As substrate for the peroxidase was used 0.0025% H_2O_2 together with 0.0125% 3,3′-diaminobenzidine-tetrahydrochloride (SIGMA D8126) as leucostain in 0.05M Tris–HCl pH 7.6. All sera were diluted in 0.05M Tris–HCl pH 7.2 containing 0.5M NaCl and 0.1% Triton X-100. To diminish unspecific binding, all tissue sections were coated with normal rabbit serum (DAKO code X902) diluted 1:30 in the same buffer containing 0.25% human serum albumin (Behring 455010A) 1 h prior to the 24-h application of primary sera. Method controls included substitution of normal swine IgG (DAKO code X901) diluted 1:10 for the anti-rabbit IgG and omission of primary serum from the dilution buffer. All positive human sera were tested repeatedly during a period of at least 3 months. Serum titers were established by IEC end-point dilutions.

Immunocytochemical controls

First level controls, examining the specificity of the positive immunoreaction, were in the case of the patients' sera limited to the first antibody layer being replaced by non-immune human sera. In the case of reference antibodies, these were absorbed in solution 24 h at 4°C with their proper antigen, before being applied to the sections. The procedural details were as described elsewhere (G.N. Hansen 1983). Second level controls (method specificity) were determined by successive omission of the bridging antibodies (swine anti-rabbit-IgG) and the detective antibody complex (PAP).

Other antibody examinations

The IEC-positive sera were examined for antibodies against smooth and striated muscles, gastric parietal cells (PCA), mitochondria, thyroid follicular and microsomal fraction, adrenals and parotis. Routine methods were used. In the muscle antibody test the sera were used at 1:20 dilution, all other tests were performed with undiluted sera.

Statistics

The Chi-square test was used for evaluating the IEC reactions on pituitary distal

lobe ± reaction on hypothalamic nuclei of MS sera vs sera from healthy subjects. This test was also used for evaluating the difference of IEC reactions between relapsing and progressive-type MS. The Wilcoxon two-sample rank test was used for testing the difference between mean titers of IEC reactive sera in the groups of MS vs healthy subjects.

Results

Immunocytochemical controls

First level controls of the reference antibodies showed that preabsorption with the homologous antigen inhibited the immunostaining for each of the 6 antisera used. Intensity of staining and number of stained pars distalis cells decreased as antiserum dilution increased. Regarding the patients' sera and the positive sera from healthy human subjects, a replacement of these by non-immune sera revealed negative reaction. Successive omission of antibody layers (second level controls) followed by the enzyme reaction, in all cases revealed negative reactions, except for erythrocytes which possess endogenous peroxidase activity.

Table 2 summarizes IEC reactions obtained when using the human sera as primary sera on the rat paraffin-embedded tissue sections.

TABLE 2

SEROLOGICAL DATA ON MS AND SLE PATIENTS COMPARED WITH NORMAL SUBJECTS

Data	MS patients	SLE patients	Normal subjects
No. of subjects	33 (8M, 25W)	10 (2M, 8W)	97 (40M, 57W)
Age (yr)	23–60 (mean 42)	24–76 (mean 41)	20–60 (mean 33)
Immunoreaction on [a]			
distal lobe tissue	9/33 (27.3%)	0/10 (0%)	9/97 (9.2%)
mean titer	1577		333
range	400–3200		100–800
neural lobe tissue	2/33 (6.1%)	0/10 (0%)	0/39 (0%)
mean titer	2700		
range	400–5000		
hypothalamic nuclei	2/33 (6.1%)	0/10 (0%)	0/97 (0%)
mean titer	2700		
range	400–5000		
Anti-nuclear factor [a]	0/33 (0%)	8/10 (80%)	2/97 (2.1%)

Tested by means of the indirect antibody-enzyme method on fixed, paraffin-embedded sections of rat brain and pituitary. Initial dilution of human sera 1 : 100.

MS patients (Table 1)

Nine of the 33 sera tested revealed finely granular cytoplasmic immunoreaction with discrete cells in the distal lobe of the rat pituitary gland (Figs. 1 and 2). The distribution of positive cells was the same for all these sera. The immunoreaction was pronounced but variable. Many cells, in between the reactive cells, remained unstained. In shape and distribution these cells corresponded to cells harboring peptides of the somatotropin family. This could be confirmed by IEC staining with rabbit reference antibodies (anti-bSTH, -rPRL, -(1-24)ACTH, -hLHβ, -hFSHβ, -hTSHβ) of sections adjacent to those treated with MS sera. The mean titer of the reactive MS sera was 1 577 (range 400–3 200). Additionally, sera from 2 of the 33 MS patients revealed reaction with the neural lobe (Fig. 3) tissue and with perikarya in different hypothalamic areas: the supraoptic nucleus (Fig. 4), the paraventricular nucleus, and the periventricular preoptic nucleus. Further, one of these two sera revealed reaction with a network of beaded nerve terminals connecting the hypothalamic nuclei with the neural lobe. The titers of the two sera were 400 and 5 000. Altogether 11 of the 33 MS sera (mean titer 1781) revealed binding to material either in the distal or the neural lobe of the rat pituitary (Table 1). In the present investigation, the immunocytochemical reactions were not related to clinical status or to medical treatments of the patients.

SLE patients

Of the 10 SLE patient sera tested, 8 demonstrated nuclear IEC in rat brain and pituitary tissues (Fig. 5). The range of titers was from 100 to 2 000. None of these sera showed reaction with cytoplasmic material in the distal lobe cells or with the neural lobe tissue.

Normal subjects

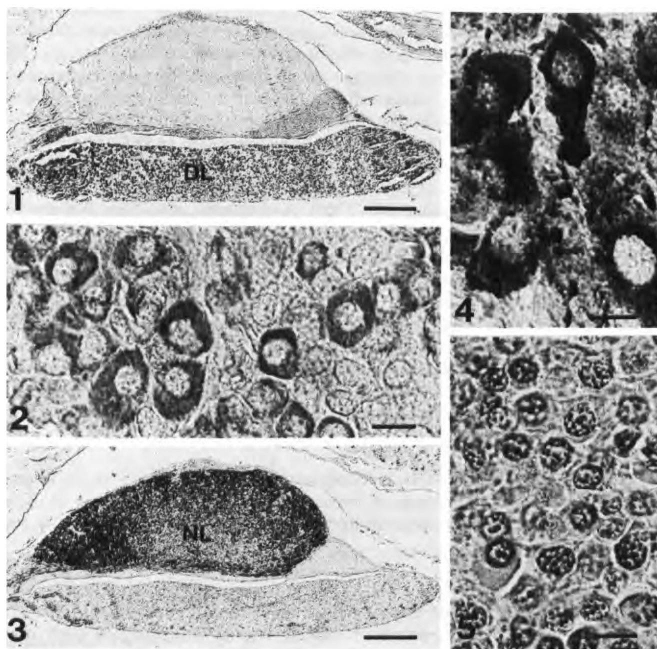
Sera from 9 of the 97 blood donors yielded immunoreactive deposits in discrete cells in the distal lobe. The pattern of staining was indistinguishable from that obtained after treatment with positive MS sera. The titers of these 9 immunoreactive donor sera were between 100 and 800 (mean 333). None of the 97 sera demonstrated IEC staining with hypothalamic perikarya, and none of 39 donors tested on the rat neural lobe revealed IEC staining with material in this tissue. Two of the sera showed anti-nuclear activity indistinguishable from the IEC reaction pattern revealed by the SLE sera. The two pooled standard sera were both negative as regards the IEC test on the rat tissue.

All positive results were confirmed on different days. Some sera were thawed 10 times during a period of 1 year without loss of IEC reactivity.

To obviate problems of species differences in peptide aminoacid sequences (rat vs human) sections of hog pituitaries were immunocytochemically treated in parallel with the rat tissue material. The incidence of immunoreaction on the hog pituitary sections corresponded to the above data on the rat pituitaries.

Prevalence of other antibodies in sera containing pituitary antibodies

One of the MS sera, neural lobe reactive (titer 5 000), revealed a medium PCA



Figs. 1 - 5. Sections (6 μm) of rat brain and pituitary, Bouin-sublimate fixed, paraffin-embedded, showing immunocytochemical reaction products after incubation with sera from either MS or SLE patients.

Fig. 1. Section treated with MS serum revealing pronounced immunoreaction in distal lobe (DL). ×39; scale bar 250 μm.

Fig. 2. Detail of section illustrated in Fig. 1 showing immunoreactive cells (dark gray) in distal lobe. ×840; scale bar 10 μm.

Fig. 3. Reaction products in neural lobe (NL) tissue after incubation with MS serum. ×39; scale bar 250 μm.

Fig. 4. Immunoreactivity in perikarya in anterior portion of supraoptic nucleus revealed after incubation with MS serum. ×840; scale bar 10 μm.

Fig. 5. Detail of distal lobe showing nuclear immunoreaction after incubation with SLE serum. ×840; scale bar 10 μm.

reaction. All other IEC-positive sera in the MS group as well as in the blood donor group showed no reaction with smooth and striated muscles, mitochrondria, thyroid microsomal or follicular fractions, adrenals or parotis.

Statistics

Patients with MS had a significantly higher frequency ($0.01 > P > 0.005$) of positive IEC reactions on the distal lobe ± hypothalamic nuclei compared to normal subjects (11/33 or 9/33 vs 9/97). The frequency of IEC reaction in the distal lobe did not differ significantly ($P > 0.1$) in the groups of relapsing and progressive type MS (5/24 vs 4/9). There was a significant difference ($P \leq 0.01$) between mean titers of distal lobe reactive sera in MS compared to normal group sera (1 577 vs 333).

Discussion

The development of immunocytochemical techniques (Sternberger 1979) has made it possible to use tissue sections with known antigens as substrate for the detection of autoantibodies against peptides in human sera. Thus, autoantibodies have previously been demonstrated, in serum from patients with diabetes mellitus and polyendocrinopathies, against endocrine cells in pancreatic islets and in the anterior pituitary (Botazzo et al. 1975; Botazzo and Doniach 1977; Botazzo et al. 1980; Mirakian et al. 1982). In these studies were used unfixed cryostat sections of human tissue and indirect immunofluorescence for the detection of human antibodies.

A recurring question in immunocytochemical techniques concerns the specificity of the antibodies used (Sternberger 1979; Larsson 1981). First level controls are of the utmost importance, and so far absorption represents the only way of detecting antibody specificity. In the present study, the specificity of the first level autoantibodies observed in the human sera was not yet established. On the basis of serial sections and comparison of the staining patterns produced with various reference antibodies, we suggest that most, if not all, of the reactive cells in the rat pars distalis may be identified as somatotrophs or prolactotrophs. This may indicate that the autoantibodies in MS sera possess specificities against antigenic determinants in rSTH and rPRL possibly identical with antigenic determinants in hSTH and hPRL. Although cross-reactivity between human and rat STH has not been demonstrated by RIA or agar diffusion studies (Hayashida 1970), immunocytochemical data from several laboratories suggest a significant immunological relationship between hSTH and rSTH. Thus, anti-hSTH has been used to localize somatotropic cells in the rat distal lobe (Nakane 1968; Nayak et al. 1969; Martin-Comin and Robyn 1976; Lechan et al. 1981). In addition, immunocytochemical data have confirmed the existence of immunological relationship between human, rat, and ovine PRL (L'Hermitte et al. 1972; Pasteels et al. 1972; Martin-Comin and Robyn 1976). However, there may be alternative explanations for these apparent reactions between MS sera and rSTH and rPRL. Thus, Witorsh (1980) has provided evidence for the presence of human placental lactogen-like immunoreactivity in somatotropic cells in

the rat distal lobe. Somatotrophs may also contain met-enkephalin (Weber et al. 1978), renin (Celio and Inagami 1980), and β-LPH-like (Lechan 1981) immunoreactivities. The distribution of the immunoreaction revealed by the one MS serum in the rat brain resembled that previously reported for PRL (Hansen et al. 1982), ACTH, and opioid peptides (see Lechan et al. 1981). The possibility for antibody specificities against peptides not yet discovered also exists.

As the MS sera reactive on rat pituitaries did also show reaction on hog pituitaries, the relatively low incidence of reactions cannot be a reflexion of species differences in peptide amino-acid sequences. Other explanations may however be offered: (i) some sera may reveal false negative reactions due to an antigen/antibody ratio of 2:1 if the antibodies yield stable immunocomplexes; (ii) the autoantibodies might not be present in serum during all stages of the disease; (iii) other peptides, not present in the antigen substrates, might be involved; (iv) other immunoglobulin classes might be involved.

Concerning the SLE sera it was found that the IEC procedure and the standard procedure used for estimation of ANA are comparable at a high serum concentration. Our findings, that SLE sera diluted 1:100 revealed 8 of 10 to be immunopositive, show that this source of variation is due to a dilution of the antibodies equivalent to the results generally obtained by titration. Further, it may be concluded that the SLE autoimmune condition does not reveal IgG antibodies reactive with pituitary peptides.

The incidence of immunoreaction in sera from healthy human subjects suggests that there are anti-distal lobe peptide antibodies in some human sera. This parallels the situation for anti-neural antibodies (Watts 1981) and for antibodies against oligodendrocytes (Kennedy and Lisak 1979; Traugott 1981). The reason for the presence of such antibodies in normal control subjects is unknown.

In contrast to the low titer antisera observed among the healthy subjects, high titer sera were observed among the MS patients. At present, an immediate causal relationship of these presumed anti-peptide autoantibodies to the pathology of MS cannot be seen. In our opinion, however 3 possible hypotheses concerning this relationship may be formulated: (i) these peptide antibodies represent unspecific cross-reactivity resulting from the liberation of cross-reactive antigens during the process of demyelination or from secondary production of autoantibodies; (ii) these peptide antibodies might be linked to the loss of suppressor T cells observed by some investigators during active MS (e.g., Reinherz 1980) through peptide modulation of immunobalance. This topic has become increasingly interesting during the last few years (Maclean and Reichlin 1981), but no precise connection between peptides and different lymphocyte subsets has as yet been elucidated; (iii) these peptide antibodies might be directly involved in the MS brain plaque development. This has to be viewed in the light of the increasing evidence that sequences of peptides formerly believed to possess 'hormonal' qualities only, turn out to be produced and distributed in the brain, with a presumably signal modulatory function (Krieger and Liotta 1979; Krieger et al. 1980; B.L. Hansen and G.N. Hansen 1982; B.L. Hansen et al. 1982; Palkovits 1982).

This concept implies receptors for peptides either on the glial cells or on the

related neurons. Thus a breakdown of glial cells by immunodestruction mechanisms, perhaps in combination with a temporarily compromised blood–brain barrier, may be effectuated by autoantibodies against peptides bound to their receptors either on the glial cells or on the neurons. In the latter case, the trophic interaction with the glial cells may be interrupted (Kriegler et al. 1981). Based on this hypothesis, it could be assumed that MS is a disease in analogy with myasthenia gravis and Graves' disease, in which autoantibodies have been demonstrated to receptors or their mediators.

In conclusion, we observed for the first time, a higher incidence and titer of antibodies against pituitary peptides in sera from MS patients than in sera from healthy control subjects. Whether these antibodies are involved in the development of some of the brain plaques in MS is unknown at present, but the findings demand further studies.

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