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Hydrolysis of myelin basic protein by polyclonal catalytic IgGs from the sera of patients with multiple sclerosis

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Abstract

Various catalytic antibodies or abzymes have been detected recently in the sera of patients with several autoimmune pathologies, where their presence is most probably associated with autoimmunization. Recently we have shown that DNase, RNase, and polysaccharide-hydrolyzing activities are associated with IgGs from the sera of patients with multiple sclerosis (MS). Here we present evidence demonstrating that highly purified MS IgGs (but not Igs from the sera of healthy individuals) catalyze specifically hydrolysis of human myelin basic protein (hMBP). In contrast to many known proteases, IgGs do not hydrolyze many other different proteins. Specific inhibitors of acidic and thiol proteases have no remarkable effect on proteolytic activity of IgGs. However, specific inhibitor of serine (PMSF, AEBSF, and benzamidin) and metal-dependent (EDTA) proteases significantly inhibit activity of proteolytic abzymes. Interestingly, the ratio of serine-like and metal-dependent activities of MS IgGs varied very much from patient to patient. The findings speak in favor of the generation by the immune systems of individual MS patients of a variety of polyclonal anti-MBP IgGs with different catalytic properties.

Keywords: human blood - multiple sclerosis - catalytic IgG - hydrolysis of myelin basic protein

Introduction

Catalytically active artificial and natural antibodies (Abs) or abzymes (Abzs) have been studied intensively (see reviews [1-7]). The first example of a natural Abz was an IgG found in bronchial asthma patients which hydrolyzes intestinal vasoactive peptide (VIP) [8], the second was an IgG with DNase activity in SLE [9], and the third was an IgG with RNase activity in SLE [10].

Catalytic IgGs and/or IgMs hydrolyzing RNA and DNA [9-17], polysaccharides [18-19] or peptides and proteins [20-25] have been described in the sera of patients with autoimmune (AI) pathologies including SLE [9-13], Hashimoto's

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thyroiditis [14, 20], polyarthritis [14], rheumatoid arthritis [19, 21-22], lymphoproliferative diseases [16], polynephritis and malignant tumors [19, 20], severe hemophilia [23], multiple myeloma [24, 25], and with two viral diseases, viral hepatitis [13, 15], and acquired immunodeficiency syndrome [17].

We could not detect DNase or RNase activities in Abs from sera of 50 normal men and women or from patients with influenza, pneumonia, tuberculosis, tonsillitis, duodenal ulcer, and some types of cancer [15]. Some healthy patients demonstrated Abzs with low proteolytic [8] and polysaccharide-hydrolyzing activities [18-19].

During pregnancy and immediately after delivery (*i.e.* at the beginning of lactation), the woman organism is frequently characterized by an immune status similar to that of patients with autoimmune diseases [6-7]. Pregnancy could "activate" or "trigger" autoimmune-like manifestations in clinically healthy women, and a sharp exacerbation of autoimmune reactions can occur in some cases soon after childbirth [6-7]. Natural Abzs were also described in the milk of healthy human mothers including sIgAs and/or IgGs possessing DNase and RNase [26-28] or ATPase activities [29-30], and protein kinase activity [31-32] which are the first natural Abzs with synthetic activity. These Abzs show specific activities significantly higher than Abs found in the sera of AI patients. This is in agreement with the results of [33] that the specific activities of milk amylolytic Abzs were 5-50 times higher than those of Abzs in AI patients.

Multiple sclerosis (MS) is a chronic demyelinating disease of central nervous system. Its etiology remains unclear, and the most widely accepted theory of MS pathogenesis assigns the main role in the destruction of myelin-proteolipid shell of axons to inflammation related to autoimmune reactions [34-35]. Evidence supports activated CD4+ myelin-reactive T cells as major mediators of MS [35]. In addition, several recent findings imply an important role of autoreactive Abs against myelin autoantigens and B cells in the pathogenesis of MS (reviewed in [35-37]). An important dual role of autoreactive Abs is suggested: they may be harmful in lesion formation but also potentially beneficial in repair [36]. Occurrence of elevated Ab levels and oligoclonal IgG bands in the cerebrospinal fluid as well as clonal B cell accumulation in the cerebrospinal fluid and lesions of MS patients is one of the main lines of evidence [38].

According current point of view abzymes may play a significant role in utilizing broadened autoantibody properties in the formation of pathogenic patterns and clinical settings for different autoimmune conditions [3-7]. Recently we have shown that homogeneous IgGs from the sera and cerebrospinal fluid of MS patients are active in the hydrolysis of DNA and RNA [39-40]. IgGs and IgMs from the sera of patients with MS were found to possess amylolytic activity hydrolyzing maltooligosaccharides, glycogen, and several artificial substrates [41]. A target catalytic response against MBP in MS patients was recently declared [6-7, 42]. In addition, MBP-hydrolyzing activity was observed in sera of animal models: nonimmunized autoimmune SJL/J mice revealed immune responses to MBP, skeletal muscle myosin and cardiac myosin, and highly purified Abs from their serum show specific proteolytic attack against target antigens [42]. Thus, different catalytic auto-Abs may be an important part of autoimmune response in MS patients.

In this study we have present for the first time application of rigid criteria allowed us to conclude that MBP-hydrolyzing activity is an intrinsic property of IgGs from the sera of MS patients MS. The substrate specificity of IgGs from MS patients was analyzed.

Materials and methods

Chemicals, donors, and patients

hMBP was purified and kindly provided by the Department of Biotechnology, Research Center of Molecular Diagnostics and Therapy (Moscow), all other chemicals were from Sigma or Pharmacia. Highly purified pig MBP was obtained according to [43]. pMBP-Sepharose was obtained by immobilizing of 4 mg of pMBP per ml of BrCN-activated Sepharose according to the standard protocol of Pharmacia.

Sera of 10 healthy donors and of 25 patients (16-55 yr old; men and women) with clinically definite MS according to the Poser criteria [44] were used to search for abzymes.

ELISA of Anti-MBP autoantibodies

Anti-hMBP auto-Abs were measured by ELISA. An optimization of all component concentrations including buffers and time interval of all operations for achievement of maximal difference between control and experimental samples was carried out. Sodium carbonate buffer (50 μl**,** pH 9.6) containing 0.01 mg/ml hMBP was added to ELISA strips and incubated overnight at 22^{o}C. The assembled strips were washed with TBS buffer containing 0.01% NaN_3 and 0.05% Triton X-100 and two times with the same buffer without Triton X-100. The block of strips surface was performed for 2 h at 37^{o}C using TBS containing 0.2% egg albumin, 0.01% NaN_3. The strips were washed 10 times with water and then with TBS containing 0.01% NaN_3.

Human blood plasma was diluted 300-2000 times with TBS containing 0.2% egg albumin, 0.01% NaN_3 and 0.05% Triton X-100 and 100 μl of final solution was added to the strips and incubated for 2 h at 37^{o}C. After washing of the strips with water (10 times) and TBS, 100 μl TBS containing 0.2% egg albumin and 0.01% NaN_3 were added, incubation 2 h at 37^{o}C. The strips were washed 10 times with water and incubated with 100 μl TBS containing 1 μg/ml conjugate of monoclonal anti-human IgG with horseradish peroxidase for 30 min at 37^{o}C and washed again 10 times with water. After addition of 50 μl citric-phosphate buffer containing 3,3',5,5'-tetramethylbenzidine and H_2O_2 the strips were incubated for 15 min at room temperature and the reaction was stopped by addition of 50 μl of 50 % H_2SO_4.

The relative concentrations of anti-MBP Abs in samples analyzed were expressed as an optical density of the solution at 450 nm (units $À_{450}$; average of 3 measurements) corresponding to human plasma diluted 1:300.

Antibody Purification

Blood (1 ml) of healthy donors and MS patients was incubated for 1-2 h at 25^{o}C to achieve complete coagulation, carefully separated from the clot, and centrifuged. Proteins were precipitated with ammonium sulfate (50% saturation). The pellet was dissolved in 1.5 ml buffer A (150 mM NaCl, 50 mM Tris-HCl, pH 7.5) and loaded on a 1 ml Protein G-Sepharose column equilibrated in buffer A. Proteins adsorbed nonspecifically were eluted with this buffer containing 1% Triton X-100 and 0.3 M NaCl. Abs were eluted in 0.1 M glycine-HCl, pH 2.6. The column fractions were collected, neutralized, and dialyzed against 10 mM Tris-HCl, pH 7.5, and then their enzymatic activities were measured after 1-3 week of keeping at 4^{0}C.

In some cases electrophoretically homogeneous catalytic IgGs were chromatographed on Sepharose bearing immobilized pig MBP. The column (1 ml) was equilibrated with 20 mM Tris-HCl, pH 7.5, and protein was applied and the column washed with this buffer containing 1% Triton X-100 and 0.3 M NaCl. Abs were eluted in 0.1 M glycine-HCl, pH 2.6, neutralized with 1.5 M Tris-HCl, pH 9.0, and dialyzed as described above.

Chromatography of IgGs under "acid shock" conditions was carried out using FPLC gel-filtration on Superdex 200 HR 10/30 (Pharmacia) using 0.1 M glycine-HCl, pH 2.6, as described previously [26-33]. Fractions collected after the gel-filtration were dialyzed against 20 mM Tris-HCl, pH 8.0, and used in activity assays as described below.

SDS-PAGE analysis and gel assay of proteolytic activity

SDS-PAGE analysis of Abs for homogeneity in a nonreducing condition was performed in 5-16% gradient gels (0.1% SDS); for polypeptide spectrum was performed in a reducing 12% gel (in the presence of 0.1% SDS and 1% 2-mercaptoethanol) according to Laemmli as described previously [11-18]. The polypeptides were revealed by silver-staining [45] and by Western blotting to a nitrocellulose membrane [46].

Analysis of proteolytic activity of IgGs after SDS-PAGE was performed similarly to the analysis of amylolytic and nuclease activities of Abzs [18, 26, 33, 41]. To restore the MBP-hydrolyzing activity of IgGs after SDS-PAGE, SDS was removed by incubation of the gel for 1 h at 30^{o}C with 2 M urea and washed 10 times with H_2O. Then 3-4-mm cross sections of longitudinal slices of the gel were cut up and incubated with 50 μl of 20 mM Tris-HCl, pH 7.5, containing 5 mM $MgCl_2$ and 1 mM EDTA for 2 days at 4^{o}C to allow protein refolding and eluting from the gel. The solutions were removed from the gels by centrifugation and used for assay of hMBP hydrolysis as described below. Parallel control longitudinal lanes were used for detecting the position of IgG on the gel by silver staining.

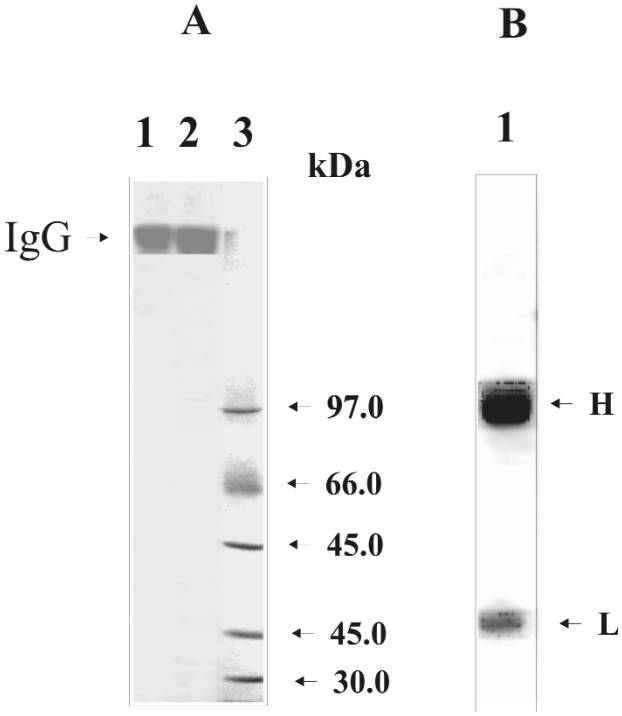

Fig. 1 (panel A) SDS-PAGE in a nonreducing 3-15 % gradient gel followed by silver staining of IgG after chromatography on Protein G- Sepharose (lane 1) and pMBP-Sepharose (lane 2). (panel B) SDS-PAGE of IgG in a reducing 12 % gel. Lane 3 and arrows indicate the positions of molecular mass markers.

Ab proteolytic activity assay

The reaction mixture (20 μl) for analysis of MBP-hydrolyzing activity of IgGs containing 5 mM Tris-HCl, pH 8.0, 0.006 % Triton X-100, 40-150 μg/ml human or pig MBP, and 10-50 μg/ml of Abs was incubated for 1-3 h at 37°C.

Substrate specificity of IgGs was analyzed using Abz preparations purified on MBP-Sepharose. Different human proteins (lactoferrin, albumin, thyroglobulin, casein, lactalbumin, lisozyme), egg lisozyme and albumin, as well as the mixture of some protein molecular mass markers (BSA, egg ovalbumin, bovine carbonic anhydrase and α-lactalbumin) were used for analysis of substrate specificity of MS IgGs.

In some cases reaction mixtures contain one of specific inhibitors of different proteases: iodineacetamide (7 mM), pepstatin A (0.7 mM), leupeptin (0.7 mM), PMSF (1 mM), AEBSF (1 mM), benzamidin (60 mM), and EDTA (7 mM).

In all cases the cleavage products were analyzed by Laemmli nonreducing SDS-PAGE in 12 % or 5-16 % gradient gels with following silver staining. The gels were imaged by scanning and quantified using GelPro v3.1 software.

Results and discussion

Serum anti-MBP Abs in MS patients have been reported before [42, 47-48], though not in all studies [49-51]. We have used ELISA to compare the relative levels of Abs against MBP in the sera of 25 patients with MS and 10 healthy donors. The concentrations of auto-Abs for healthy donors were not zero and have changed from 0.03 to 0.20 A_{450} units, in average 0.09 ± 0.04. A_{450} units. Relative indexes of anti-MBP Abs for 25 MS patients varied from 0.67 to 0.98 A_{450} units, in average 0.8 ± 0.1 A_{450} units. Thus, all MS

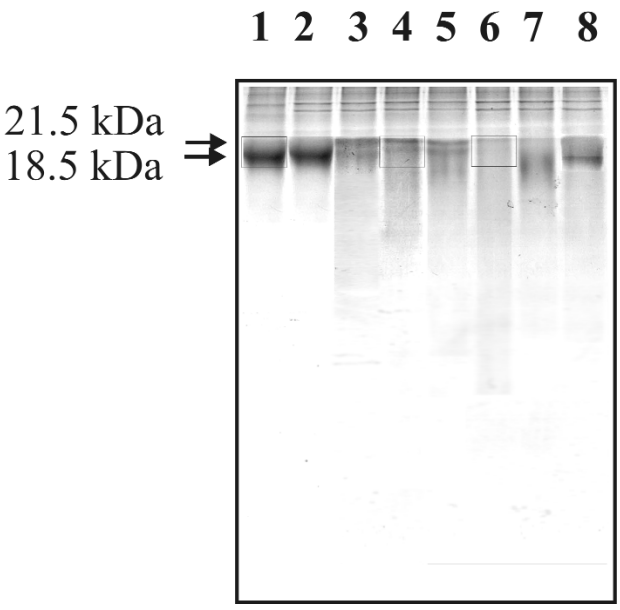

Fig. 2 SDS-PAGE analysis of hMBP hydrolysis by IgGs from healthy donors (lane 2) and six different MS patients (lanes 3- 8); lane 1 - hMBP incubated alone. For evaluation of the relative activity of Abzs the intensity of the main 21.5 and 18.5 kDa bands of hMBP incubated without IgGs was taken as 100 % (the protein region is shown on lane 1). The Abz relative activity was estimated from a decrease of protein intensity in the gel region corresponding to the main bands (for example, see lanes 4 and 6). Details of experiments are given in "Materials and methods".

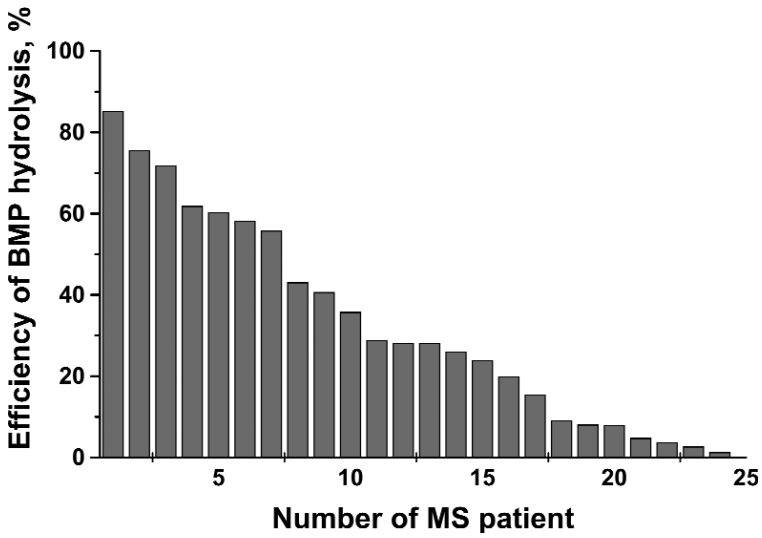

Fig. 3 The relative activity of IgGs from the sera of 25 different MS patients in the hydrolysis of hMBP. The relative activity of Abzs was estimated from a decrease of protein in the gel region corresponding to the main bands of hMBP as described in Fig 2. The reaction mixtures were incubated for 3 h. For other details see "Materials and methods".

patients analyzed by us demonstrated significantly higher levels of serum anti-MBP Abs than healthy individuals.

To search for abzymes in the serum of every healthy donor and MS patient, the IgG fraction was purified by chromatography on Protein-G Sepharose in conditions to remove non-specifically bound proteins. The homogeneity of the 150 kDa IgG was confirmed by SDS-PAGE with following silver staining, which showed a single band in nonreducing conditions and two bands corresponding to the H and L chains after reduction (Fig. 1).

We have analyzed hMBP- hydrolyzing activity of IgGs isolated from 25 MS patients and from 10 clinically healthy volunteers. It is known that due to alternative splicing of cDNA animal and human MBP can consist of several related forms of different molecular masses (21.5, 18.5, 17.5, and 14.0 kDa) [52]. In addition, different form of hMBP may be partially hydrolyzed in human cells and during protein purification [43]. Therefore, highly purified preparations of MBP are usually not homogeneous and according to SDS-PAGE they contain several protein bands, of which ~18.5 kDa is usually major [43]. As one can see from Fig. 2 (lane 1), MBP preparations used by us are contained two pronounced protein bands, but one (~18.5 kDa) of these bands was major. The incubation of hMBP with IgG from the sera of MS patients (Fig. 2, lanes 3-8) results in a obvious decrease of the intensity of silver-stained 21.5 and 18.5 kDa hMBP bands, in comparison with the intensity of these bands of hMBP incubated in the absence of Abs (Fig. 2, lane 1), what is considered as a result of hMBP hydrolysis by IgG fraction. The relative activities of IgGs from the sera of MS patients significantly varied from patient to patient. 7 of 10 preparations of IgG fraction from healthy donors did not show detectable hydrolysis hMBP (for example, lane 2, Fig. 2), while in the case of three donors a decrease of hMBP amount was estimated as 3-5 % which is on borderline of the sensitivity of the method used. Interestingly, 4 of 25 IgG preparations from MS patients demonstrated low proteolytic activity ($\leq$ 5 %), while 21 remaining preparations hydrolyzed hMBP for 10 - 86 % (Fig. 3). Thus, in contrast to healthy donors IgG preparations from ~84 % of investigated MS patients demonstrate detectable or significant activity in hydrolysis of hMBP.

Since Abs can form complexes with other proteins, and Ab-mediated catalysis is sometimes characterized by relatively low reaction rates, it is very important to prove that catalytic activities of Ig fractions was not due to admixture of enzymes. The application of rigid criteria allowed the authors of the first article concerning natural Abzs [8] to conclude that VIP-hydrolyzing activity is an intrinsic property of Abs from the sera of patients with asthma. One of these criteria is the presence of catalytic activity only in Abs of asthma patients and not in healthy donors. Our data on a significant hMBP proteolysis only by IgG

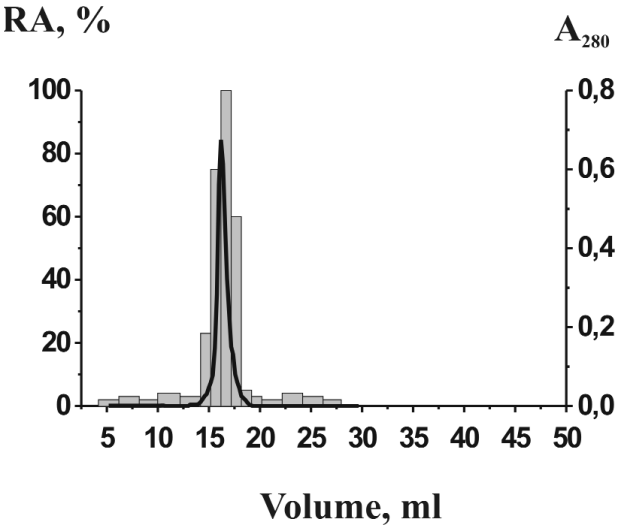

Fig. 4 FPLC gel-filtration of catalytic MS IgG on Superdex 200 HR 10/30 column after exposure to acidic conditions: (–) - absorption at 280 nm, columns show the relative activity (RA) of IgG in hydrolysis of hMBP. The maximal activity of one of the fractions was taken as 100 %.

preparations from MS patients in comparison with those from healthy donors satisfy this requirement.

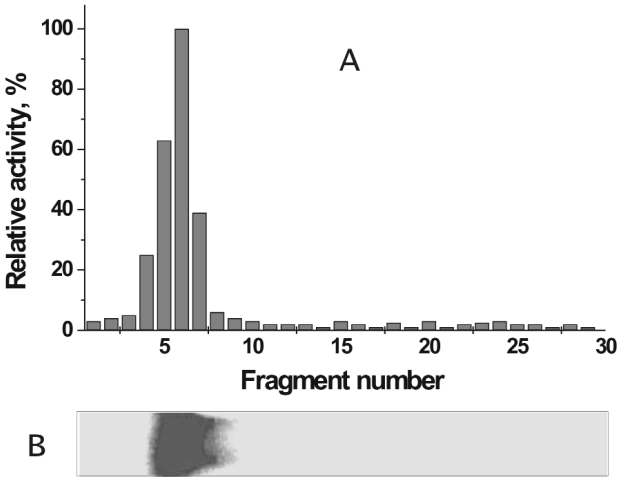

Fig. 5 SDS-PAGE analysis of hMBP-hydrolyzing activity of MS catalytic IgGs. After electrophoresis the gel was incubated using special conditions for renaturation of protein (*see* Methods). Then the relative MBP-hydrolyzing activity was revealed using extracts of 2-3 mm fragments of one longitudinal band of the gel (panel A). The second control longitudinal band of the same gel was stained with silver (panel B).

To prove that hMBP-hydrolyzing activity of IgG fraction isolated from the sera of MS patients is an intrinsic property of Abs, we have applied some of other rigid criteria. The most important of these are given below: electrophoretic homogeneity of IgGs (Fig. 1); b) complete adsorption of the hMBP-hydrolyzing activity by anti-IgG Sepharose and Protein A-Sepharose leading to a disappearance of catalytic activity from the solution and its elution from the adsorbent with buffer of low pH (data not shown); c) FPLC gel-filtration of IgGs under conditions of "acidic shock" (pH 2.6) did not lead to a disappearance of the activity, and the peak of proteolytic activity tracked exactly with 150 kDa IgGs (Fig. 4).

To exclude possible artifacts due to hypothetical traces of contaminating enzymes, the IgGs were separated also by SDS-PAGE and their hMBP-hydrolyzing activity was detected after extraction of proteins from the separated gel slices (Fig. 5). Since SDS dissociates any protein complexes, and the electrophoretic mobility of usually low molecular mass proteases cannot coincide with that of IgG, the detection of proteolytic activity in the gel region corresponding only to IgG, together with the absence of any other bands of the activity or protein (Fig. 5), provides direct evidence that IgG possesses hMBP-hydrolyzing activity.

We have shown in many articles [4-7, 11-12, 32-33] catalytic heterogeneity of polyclonal nuclease and polysaccharide-hydrolyzing Abzs from patients with different autoimmune diseases including MS; they can demonstrate maximal activity at various optimal pHs, are activated or not by metal ions, and are characterized by different substrate specificities. Here we analyzed a possible proteolytic diversity of polyclonal IgGs of MS patients. It was shown that incubation of IgGs with iodineacetamide (specific inhibitor of thiol proteases) and pepstatin A (specific inhibitor of acidic proteases) did not demonstrate remarkable effect on the IgG-dependent hydrolysis of hMBP. Leupeptin, the inhibitor of many different proteases demonstrated weak inhibition of proteolytic activity of IgGs. The maximal decrease of proteolytic activity of IgGs was observed after incubation of these Abs with specific inhibitors of serine proteases PMSF, AEBSF, and benzamidin, as well as after addition of EDTA to the reaction

mixture. It should be mentioned that the effects of specific inhibitors of serine and metal-dependent proteases on hMBP-hydrolyzing activity of IgG varied from patient to patient over the range of 30-80 % and 10-80 % inhibition, respectively. Fig. 6 demonstrates the effect of some inhibitors on the proteolytic activity of IgG preparation from the sera of one MS patient; there is a significant effect only for serine protease inhibitors benzamidin (lane 5), and AEBSF (lane 7). Taken together, our findings argue in favor of idea that polyclonal IgGs of MS patients contain mainly Abzs of two types: serine and metal-dependent proteases.

It is known that pig and human MBPs have 80-90% homology [53]. We have shown that IgGs from the sera of MS patients hydrolyze human and pig MBP with comparable efficiency. Pig MBP was used for preparing of MBP-Sepharose. We have purified the fractions of IgGs having affinity to MBP-Sepharose. In order to compare the substrate specificity of these abzymes, the hydrolysis of various proteins by IgGs from different MS patients was analyzed. In contrast to known different nonspecific serine proteases, these IgGs hydrolyze effectively only human and pig MBPs. Catalytic IgGs did not hydrolyze many different human proteins (lactoferrin, albumin, thyroglobulin, casein, lactalbumin, lysozyme), egg lisozyme and albumin, as well as bovine serum albumin, carbonic anhydrase and milk α-lactalbumin. Fig. 6B demonstrates the absence of IgG-dependent hydrolysis of some of these proteins.

Thus, it is obvious that MBP-hydrolyzing activity is an intrinsic property of IgGs derived from the sera of MS patients and does not due to admixture of any possible proteases. As shown above polyclonal IgGs can consist of different repertoires of hMBP-hydrolyzing subfractions and in some cases one of these subfractions may be predominant.

Abzymes have been studied primarily in the context of autoimmune diseases where their biological role remains unknown: do they have a positive or negative function ? [1-7]. In bronchial asthma it has been suggested that respiratory tract dysfunction may stem from the protease activity of auto-Abzs, resulting in a deficit of VIP, which plays a major role in the pathophysiology [54]. Anti-VIP Abzs are cytotoxic and mice immunized with anti-hormone IgGs from human sera develop asthma [54].

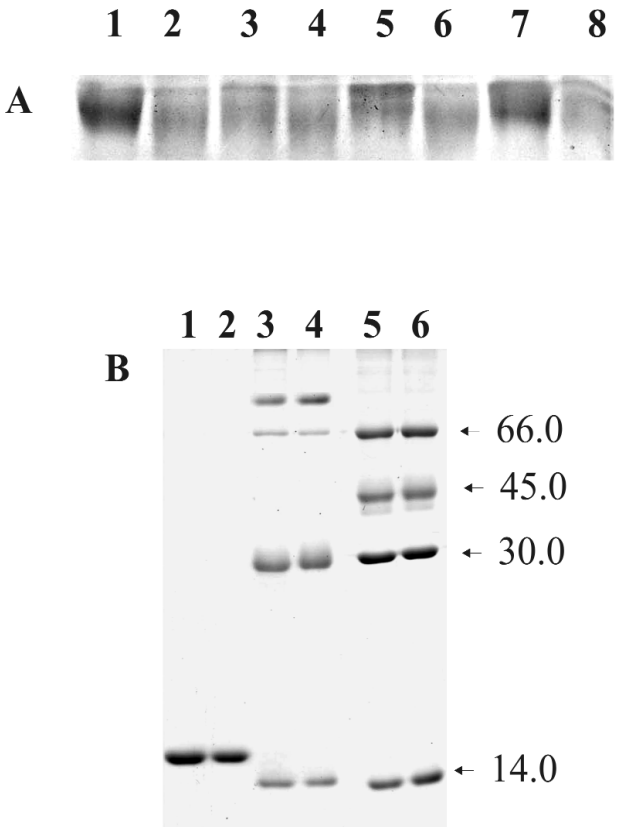

Fig. 6 (**A**) SDS-PAGE analysis of MBP hydrolysis by IgG preparation from the sera of one MS patient in different conditions: lane 1 - hMBP incubated alone; lanes 2-8 - hMBP incubated with IgG in the absence of other components (2) or in the presence of leupeptin (3), pepstatin A (lane 4), benzamidin (5), iodineacetamide (6), AEBSF (7) and EDTA (8). For details see "Materials and methods". (**B**) SDS-PAGE analysis of IgG-dependent hydrolysis of different proteins; odd and even numbers of lanes correspond to proteins incubated with and without IgG, respectively: 1 and 2 - egg lysozyme, 3 and 4 - mixture of human milk lactoferrin (~80 kDa), albumin (67 kDa), casein (~30 kDa), and lactalbumin (~14 kDa); 5 and 6 - mixture of some protein molecular mass markers - BSA (67 kDa), egg ovalbumin (43 kDa), bovine carbonic anhydrase (30 kDa), and bovine milk α-lactalbumin (14.4 kDa).

In MS, protease activity of anti-hBMP Abzs could attack hMBP of myelin-proteolipid shell of axons. Consequently, the Abzs may play an important role in MS pathogenesis. As was shown

before, DNase Abzs from MS patients are cytotoxic and induce cell death *via* apoptotic mechanism [7]. Taking in account that high-affinity anti-DNA Abs were a major component of the intrathecal IgG response in the patients with MS [55], it is reasonably to propose that anti-DNA Abs including DNA-hydrolyzing Abzs may promote important neuropathologic mechanisms in this chronic inflammatory disorder. Taken together, we suggest that hMBP- and DNA-hydrolyzing Abzs may cooperatively promote MS pathogenesis development.

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