

BLOOD SERUM IMMUNOGLOBULINS OF PATIENTS WITH MULTIPLE MYELOMA ARE CAPABLE OF HYDROLYSING HISTONE H1

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Background: Recently we have shown that the immunoglobulins G from blood serum of some multiple sclerosis patients are capable of cleaving histone H1. **Aim:** To check whether histone H1-hydrolyzing abzymes could be detected not only in blood plasma of autoimmune patients, but also during cancer development, particularly during the onset of multiple myeloma. **Methods:** Immunoglobulins were isolated from blood serum of multiple myeloma patients (n = 11) by precipitation with 50% ammonium sulfate and tested for proteolytic activity toward linker and core calf thymus histones. Antibody preparations able to cleave histone H1 were subjected to affinity chromatography on histone H1-Sepharose with following analysis of chromatographic fractions' protease activity. To prove that antibody molecules are responsible for hydrolysis of histone H1, gel filtration at acidic pH with subsequent examination of protease activity of chromatographic fractions (pH-shock analysis) was used. **Results:** It was found that 3 of 11 antibody preparations are capable of hydrolyzing calf thymus histone H1 but not core histones. It was shown that histone H1-hydrolyzing activity of 2 proteolytically active antibody preparations is associated with IgGs that possess affinity towards histone H1. pH-shock analysis proved that protease activity towards histone H1 is intrinsic property of IgG molecules. **Conclusions:** We demonstrated the existence of previously unknown histone H1 hydrolyzing IgG abzymes in the serum of multiple myeloma patients. Possible biological role of hisH1-hydrolyzing antibodies in patients with multiple myeloma was discussed.

Key Words: antibodies, abzymes, multiple myeloma, blood serum, proteolytic activity.

There are increasing evidences that interaction of antibodies with antigens can lead to degradation of antigens. Antibodies (Ab) possessing catalytic activity have been named catalytically active antibodies or abzymes [1]. Abzymes were detected in human organism during a variety of pathological conditions, such as systemic lupus erythematosus (SLE), autoimmune thyroiditis, asthma, multiple sclerosis, etc [1, 2]. Peptides, proteins, nucleic acids, and oligosaccharides can serve as substrates for catalytically active antibodies in human and other mammals [3, 4]. Since abzyme's target molecules were identified as autoantigens (i. e., DNA, thyroglobulin, vasoactive intestinal peptide), the involvement of abzymes in pathology of autoimmune disorders has been clearly documented [1–4]. Catalytically active autoantibodies typically found in humans with autoimmune disorders, have also been detected in cancer patients. DNA-hydrolyzing activity of IgG auto-ABs from sera of patients with various types of lymphoproliferative diseases was described [5, 6]. Tests for abzymes presence in patients with hematological tumors and SLE revealed the linkage of anti-DNA Ab catalysis with mature B-cell tumors, and increased probability of DNA-abzymes formation on the background of autoimmune conditions [1]. These data suggest possible similarity between the mechanisms of abzyme formation at SLE and B-cell lymphomas. Peptide-hydrolyzing and DNA-hydrolyzing activities of Bence Jones proteins from blood serum of myeloma patients are also well studied [7, 8]. There are numerous data demonstrating that catalytic activity of anti-DNA IgGs and Bence Jones proteins is as-

sociated with their cytotoxic activity and correlates with the pathogenesis of the disease [9–13].

These data suggest that abzymes can play an important physiological role at autoimmune and oncological diseases. Therefore, their further studies are needed for better understanding of humoral immunity functions under normal and pathological conditions.

Recently, we have discovered a novel abzyme possessing histone H1 cleaving activity. It was demonstrated that IgGs isolated by affinity chromatography from blood serum of patients with multiple sclerosis and sIgAs isolated from colostrums of healthy woman possesses the ability to cleave histone H1 of calf thymus, but not cleave core histones, bovine serum albumin and chicken egg lysozyme [14]. Here we show that histone H1-hydrolyzing IgGs are present in blood serum of some patients with multiple myeloma.

MATERIALS AND METHODS

Patients. Peripheral blood serum samples of 11 patients (35–55-year old; men and women) diagnosed with multiple myeloma were analyzed. An informed consent was obtained from all patients as it was approved by the Review Board of the Institute of Blood Pathology and Transfusion Medicine AMS of Ukraine in accordance with the regulations of the Ministry of Health Protection of Ukraine.

Isolation of immunoglobulins (Igs). Igs were obtained from blood serum of patients with multiple myeloma by 3-fold precipitation of serum proteins by ammonium sulfate (50% saturation). The pellet was dissolved in 20 mM Tris-HCl buffer, pH 7.5, and dialyzed against the same buffer for 18 h. Igs concentration was determined with the use of NanoDrop ND 1000 spectrophotometer (NanoDrop Technologies, USA). Preparations of Igs were analyzed by electropho-

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Abbreviations used: Ab – antibody; auto-Ab – autoantibody; Igs – immunoglobulins; SLE – systemic lupus erythematosus.

resin in 12% PAGE in the presence of 0.1% SDS [15]. The gels were stained with Coomassie G 250.

Purification of antibodies with affinity to histone H1 (anti-hisH1 Ab). Anti-hisH1 Abs were isolated from two catalytically active preparations of Igs on a column with hisH1-Sepharose as described in [14]. That was done by placing 2–2.5 mg Igs onto a column with histone H1-Sepharose (1 ml) equilibrated with TBS (140 mM NaCl, 20 mM Tris-HCl, pH 7.5). The column was washed with TBS, and anti-hisH1 Abs were eluted with 1 M NaCl. Fractions of anti-hisH1 Abs were dialyzed against 100 mM NaCl, 20 mM Tris-HCl, pH 7.5, for 18 h and analyzed by electrophoresis in 12% SDS-PAGE.

Gel exclusion chromatography of Ab at conditions of dissociation of immune complexes (pH shock).

The preparation of anti-hisH1 Ab capable of hydrolyzing histone H1 was precipitated with 50% ammonium sulfate. The pellet was dissolved in 0.1 M glycine-HCl (pH 2.6) and separated by gel filtration on a Toyopearl TSK HW55 column (180 x 5 mm) in the same buffer. Chromatographic fractions (300 μ l) were collected, neutralized with 1.5 M Tris-HCl, pH 8.8, dialyzed against buffer A for 18 h, and analyzed by electrophoresis in a gradient (7–18.5%) PAGE in the presence of 0.1% SDS.

Histone H1 isolation. Total histones of calf thymus were kindly provided by Dr. M.D. Lutsik, and histone H1 was purified from that preparation as described in [19]. Total histones were diluted in distilled water and core histones were precipitated with 4% perchloric acid. After centrifugation, hisH1 was precipitated from supernatant by 30% TCA. Then histone H1 was additionally purified by chromatography on CM-Sephadex according to [16]. Histone H1 preparations were analyzed by SDS-PAGE (15%).

Proteolytic activity assays. We used two preparations containing calf thymus hisH1 and total histones fraction as substrate for proteolytic activity assays. The proteolytic reaction was performed in buffer (20 mM Tris-HCl, pH 7.5, and 0.1 M NaCl) in the presence of 0.3 mg/ml histone H1 and 0.05–0.3 mg/ml antibodies for 1 h at 37 °C. To analyze proteolytic activity of the chromatographic fractions, aliquots (30 μ l) were supplemented with 6 μ g hisH1, and mixtures were incubated for 3 h at 37 °C. The reaction was stopped by addition of 4-fold amount of denaturing buffer (0.26 M Tris-HCl, pH 6.8, 4% SDS, 8% 2-mercaptoethanol, 40% glycerol), and the reaction products were separated by electrophoresis in 12% PAGE in the presence of 0.1% SDS. The gels were stained with Coomassie G 250.

Cell proliferation study by MTS assay. The method was described in [17]. MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium was used in the assay (Promega Co., USA). Human leukemia T-cells of CEM line were obtained from Cell Collection at the Institute of Experimental Pathology, Oncology and Radiobiology, National Academy of Sciences of Ukraine (Kyiv, Ukraine). Cells were maintained in RPMI-1640 medium (Sigma Chemical Co., USA) supplemented with 10% fetal bovine serum (Sigma) and gentamycin (50 mg/ml, Sigma).

Cells were cultured in a humidified atmosphere at 37 °C and 5% CO₂. They were seeded into 96-well plate at starting concentration of 50 000 cells/ml, and 20 μ g/ml of Ab was added and incubated for 24 h, 48 h and 72 h. 10 μ l of MTS/PMS solution (2 mg/ml / 0.92 mg/ml, ratio 1 : 20) was added to each well and the plate was incubated for 1 h at 37 °C in a humidified atmosphere with 5% CO₂. Then the amount of soluble formazan produced by cell reduction of the MTS was measured according to the absorbance at 490 nm / 630 nm on the Microplate analyzer (BioTek, USA).

RESULTS

Analysis of proteolytic activity of Abs. Antibody preparations were isolated by precipitation with ammonium sulphate from blood serum of 11 patients with multiple myeloma. Electrophoretic analysis in the denaturing conditions showed that these preparations differ by ratio between various subclasses of immunoglobulins. Taking into account the molecular weights of human antibody's heavy chains [18], one can suggest that 5 of 11 Abs preparations are enriched with IgGs (Fig. 1, lines 1, 3, 5, 7, 9), 2 preparations mainly consist of IgM (Fig. 1, lines 4, 6) and 3 other preparation mainly contain IgA (Fig. 1, lines 2, 10, 11). To analyze proteolytic activity of Igs preparations, we used as substrate partly purified linker histone H1 that contained considerable amount of core histones (Fig. 2, line 12). Electrophoretic analysis showed that 3 of 11 Igs preparations obtained from blood serum of patients with multiple myeloma, effectively cleaved histone H1 but not core histones (Fig. 2, lines 5, 6, 10). This finding suggests that blood serum of multiple myeloma patients contains catalytically active antibodies hydrolyzing hisH1.

Validation of proteolytic activity of Abs. To prove that the ability of hydrolyzing hisH1 is an intrinsic property of antibody molecules following procedures were used [19].

Important condition for the detection of abzymes is the affinity of catalytically active antibodies toward their substrates. Since histone H1 served as a substrate for protease activity, two catalytically active preparations, enriched with IgG and IgM (Fig 2, lines 5, 6), were subjected to affinity chromatography on a column containing hisH1-Sepharose (Fig. 3, a). Four fractions containing proteins bound and unbound to histone H1, were obtained and analyzed by PAGE electrophoresis (Fig. 3, b). Electroporetic analysis showed that affinity isolated fractions of both Ab preparations mainly contain IgGs and small amount of others proteins of different molecular weight. (Fig. 3, b, lines 1, 2). It should be noted that IgM, in contrast to IgG, did not bind histone H1-sepharose (Fig. 3, b, line 4). The obtained data suggest that proteolytically active Ab preparations, isolated by precipitation with ammonium sulfate from blood serum of patients with multiple myeloma, contain IgG-class antibodies possessing affinity to histone H1. Ab fractions were analyzed for histone H1 hydrolyzing activity (Fig. 3, c). Analysis of proteolytic activity revealed that the fractions containing affinity purified IgGs effectively cleaved histone H1 (Fig. 3, c, lines 1, 2),

in comparison with fractions containing unbound Ab where proteolytic activity toward histone H1 was significantly lower (Fig. 3, c, lines 3, 4). Some proteolytic activity detected in unbound antibody fractions could be explained by specificity of affinity chromatography which does not allows a complete one-step isolation of proteolytically active abzymes from antibodies pool. On the other hand, it is not excluded that some antibodies with low histone H1-hydrolyzing activity does not bind to affinity column in the presence of 140 mM NaCl.

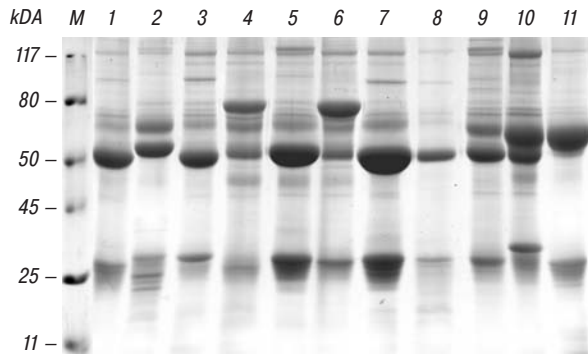


Fig. 1. SDS-electrophoresis of Ig, precipitated from blood serum of multiple myeloma patients by 50% sulfate ammonium. Lines 1–11 — preparations of Igs. M — molecular weight markers

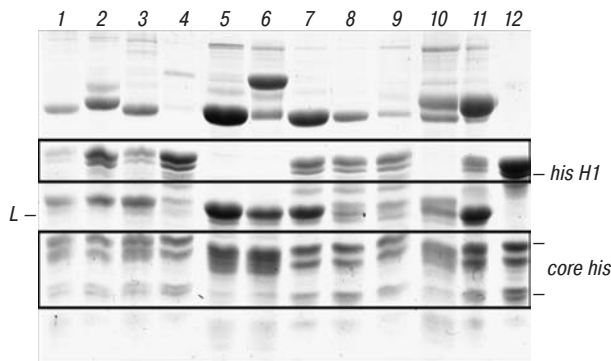


Fig. 2. Electrophoretic analysis of proteolytic activity of Igs, obtained from blood serum of patients with multiple myeloma (lines 1–11). Line 12 — histones before incubation with IgGs (control). Position of histone H1 and core histones on the gel are enclosed in boxes and shown by arrows. Positoin of immunoglobulines light chains (L) shown by arrow.

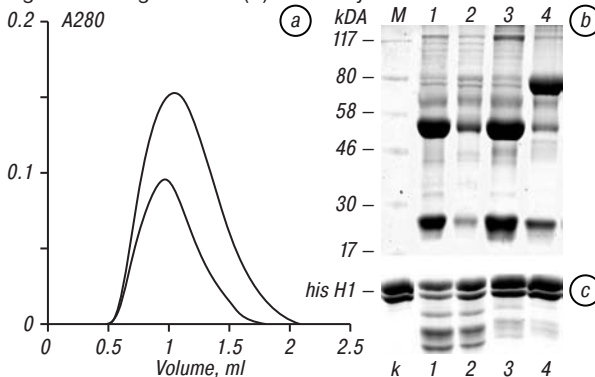


Fig. 3. Purification of Abs with affinity to histone H1 and analysis of their proteolytic activity. *a*, Affinity chromatography on histone H1-Sepharose of catalytically active Ig-preparations 5 and 6 (see Fig. 1, lines 5, 6). *b*, Electrophoretic analysis of binding proteins (lines 1, 2) and unbound proteins (lines 3, 4). M — molecular weight markers. *c*, Electrophoretic analysis of histone H1 hydrolysis in the presence of bound (1, 2) and unbound (3, 4) to histone H1-sepharose proteins. K — histone H1 in the absence of proteins

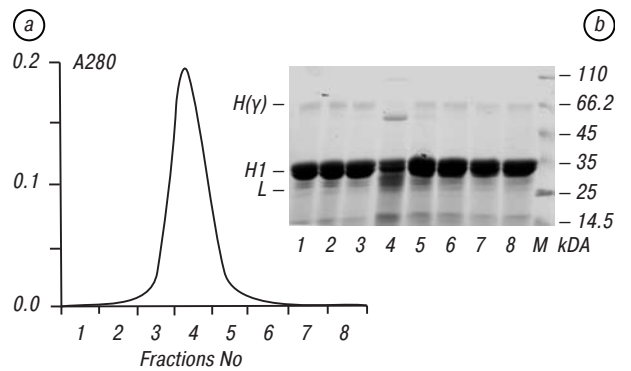


Fig. 4. Analysis of proteolytic activity of anti-histone H1 IgGs. *a*, Gel filtration of Ab in 0.1 M glycine-HCl buffer (pH 2.6). *b*, Electrophoretic analysis of products of histone H1 hydrolysis in the presence of chromatographic fractions. The arrows indicate positions of IgG polypeptides (H(γ) — heavy chain; L — light chain) and histone H1 on the gel. M — molecular weight markers

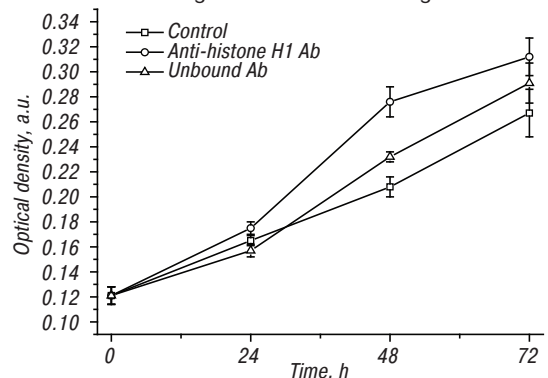


Fig. 5. Influence of anti-histone H1 AB on growth and survival of a human T-leukemia CEM cells

Thus, obtained data suggest that proteolysis of histone H1 in the presence of two Ab preparations (see Fig. 2, lines 5, 6) is caused by IgG capable of bind histone H1.

Presence of protease activity in the affinity purified antibody preparations does not guarantee their abzymatic properties. To prove that the ability to hydrolyze histone H1 is an intrinsic property of antibody molecules, another routine procedure — pH-shock analysis was used [19]. Catalytically active IgG preparations purified on histone H1-Sepharose (see Fig. 3, *a*), were combined and gel filtered at the conditions favorable for dissociation of immune complexes (Fig. 4, *a*) with subsequent analysis of chromatographic fractions for their ability to hydrolyze histone H1 (Fig. 4, *b*). The ability to hydrolyze hisH1 was detected only in the chromatographic fraction which contained IgGs (Fig 4, *b*, line 4). Absence of proteolytic activity in other chromatographic fractions indicates that hisH1-hydrolyzing activity of IgG belongs to immunoglobulins rather than to possible admixtures of proteases present in blood serum.

Anti-histone H1 Abs impact on cell proliferation.

Further, we compared the effect of catalytically active anti-histone H1 IgG (see Fig. 3, *b*, line 1) and catalytically inactive IgG (Fig. 3, *b*, line 3) on growth and survival of human T-leukemia cells *in vitro* (Fig. 5). An addition of anti-histone H1 IgG to human T-leukemia CEM cells significantly increased their time-dependent growth compared with action of IgG unbound to histone H1. This data demonstrated that anti-histone H1 IgGs isolated

from blood serum of patients with multiple myeloma can induce proliferation of lymphoid tumor cells.

DISCUSSION

Our data demonstrated that blood serum of 3 of 11 patients with multiple myeloma similarly to blood serum of some patients with multiple sclerosis [14], are capable of hydrolyzing calf thymus histone H1 but not cleave core histones. Undoubtedly, these results of the patients' screening are preliminary, and further studies are required.

Data obtained by gel filtration at acidic conditions, suggest that this proteolytic activity is an intrinsic property of IgGs. The affinity of catalytically active antibodies towards histone H1 allows us to classify them as anti-hisH1 autoantibodies [14, 20, 21]. Proteolytic activities of others immunoglobulines (IgM and IgA), revealed in antibody preparations, obtained from blood serum of patients with multiple myeloma, remains unknown and require additional investigation.

The role of proteolytically active IgGs remains unknown. Since histone H1 — hydrolyzing IgG of blood serum of patients with multiple myeloma belong to anti-histone H1 auto-Ab, one may speculate that their biological activity is similar to activity of anti-histone H1 Ab earlier revealed in blood serum of the patients with autoimmune diseases. It was shown that anti-histone H1 Ab might play an important role in the development of systemic lupus erythematosus [22, 23], and be considered as highly specific marker of disease progression [24].

According to its antigenic specificity, anti-histone H1 Ab could be also classified as antinuclear autoantibody [22–24]. Circulating antinuclear autoantibodies typically found at autoimmune conditions, have also been detected in cancer patients and in healthy elderly individuals [25]. It was found that antinuclear autoantibodies possess a complement-dependent and a complement independent toxicity towards different tumor cells [5, 6, 26]. There is a suggestion that antinuclear autoantibodies act as antineoplastic immune-surveillance agents [25].

An intriguing issue of anti-histone H1 Abs activity is their influence on the lymphoid cells. It has been found that anti-histone H1 Abs modulate tolerogenic status of dendritic cells, decrease the cytotoxicity of lymphokine-activated killer cells and human natural killer cells [27]. Those results suggest that immunosuppressive anti-histone H1 Abs decrease cellular immune reactivity in patients with multiple myeloma towards tumor cells, and in that way they might be involved in the disease development.

Our preliminary data show that catalytically active anti-hisH1 Ab can stimulate proliferation of myeloma cells. We suggest that pro-proliferative activity of anti-histone H1 IgGs is closely linked with biological function of histone H1. It is well known that histone H1 is present in the cell nucleus where it is involved in process of chromatin rearrangement [28, 29]. Recently, we have demonstrated that anti-hisH1 Ab isolated from blood serum of multiple sclerosis patients was capable to be internalized by human T-leukemia Jurkat cells *in vitro* [30]. We speculate that

proliferation of T-leukemia cells is caused by degradation of linker histone H1 after internalization of catalytically active anti-histone IgGs by the tumor cells and translocation of these Ab to the cell nucleus.

In conclusion, we have shown an existence of previously unknown histone H1 hydrolyzing IgG abzymes in the serum of some multiple myeloma patients. These abzymes were isolated and their stimulatory influence on human leukemia cells proliferation was demonstrated.

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