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HIV-1 integrase-hydrolyzing IgM antibodies from sera of HIV-infected patients

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Abstract

IgG abzymes (Abzs) with different catalytic activities are a distinctive feature of various autoimmune (AI) diseases. At the same time, data concerning IgMs with catalytic activities are very limited. Electrophoretically and immunologically homogeneous IgMs were isolated from the sera of acquired immunodeficiency syndrome (AIDS) patients by chromatography on several affinity sorbents. Several rigid criteria have been applied to show that the integrase (IN)-hydrolyzing activity is an intrinsic property of IgMs from HIV-infected patients but not from healthy donors. We present evidence showing that 22 of 24 (91.7%) IgMs purified from the sera of HIV-infected patients specifically hydrolyze only HIV IN but not many other tested proteins. Usually, proteolytic antibodies of AI patients are serine protease-like or metal dependent. Only 30% of IN-hydrolyzing IgMs were inhibited by specific inhibitors of serine proteases and 60% by inhibitors of metal-dependent proteases. Unusually, a significant reduction of the activity by specific inhibitors of acidic (in 20% of IgM preparations) and thiol proteases (in 100% of IgM preparations) was observed. Although HIV infection leads to formation of antibodies to many viral and human antigens, possible biological roles for most of them are unknown. Since anti-IN IgG can efficiently hydrolyze IN, a positive role of Abzs in counteracting the infection cannot be excluded. In addition, detection of IN-hydrolyzing activity can be useful for diagnostic purposes and for assessment of the immune status in AIDS patients.

Keywords: catalytic IgMs, HIV-infected patients, human blood antibodies, hy, drolysis of HIV integrase

Introduction

Autoantibodies and catalytically active antibodies are among the specific characteristics of various autoimmune (AI) diseases. Microbial and viral infections expose the organism to various foreign components, including proteins, DNA, RNA, lipids and polysaccharides. The sera of mice infected with *Salmonella typhimurium* (1, 2) or malaria (3, 4) and the sera of humans infected with *Trypanosoma cruzi* (5) or bacterial pathogens (6, 7) contain a variety of antibodies to the parasite's antigens and to human lipids, proteins and nuclear components, including anti-DNA antibodies. Different autoantibodies were found in patients infected with various hepatitis viruses (8, 9), cytomegalovirus (10), polyomavirus (11), Epstein–Barr virus (12) and echovirus (13). AI-like rheumatic manifestations are commonly encountered in patients with hepatitis (14). According to the modern point of view, the presence of AI phenomena in chronic bacterial and viral infections could be related to polyclonal B-cell activation and molecular mimicry between parasite and host antigens (12, 15–17), alteration of host antigens, abnormal expression of immunoregulatory molecules and activation of the anti-idiotypic network (15). To take one example, AI pathogenesis in multiple sclerosis may involve molecular mimicry by such agents as measles, hepatitis B, herpes simplex, influenza, papilloma and Epstein–Barr viruses (18).

HIV-1 causes an extremely dangerous human disease, acquired immunodeficiency syndrome (AIDS) [(19) and

references therein]. HIV-dependent activation of B lymphocytes leads to the production of antibodies to viral proteins, autoantibodies to human cell components and various immune complexes containing antibodies directed to cardiolipin, β_2-glycoprotein, DNA, small nuclear ribonucleoproteins, thyroglobulin, thyroid peroxidase, myosin and erythropoietin [(20) and references therein). The frequency of reported rheumatic manifestations in HIV-infected patients ranges from 1 to 60% (20). The list of reported AI diseases associated with HIV/AIDS includes systemic lupus erythematosus (SLE), antiphospholipid syndrome, vasculitis, primary biliary cirrhosis, polymyositis, Graves' disease and idiopathic thrombocytopenic purpura.

During the last two decades, it became clear that autoantibodies from the sera of patients with various AI diseases can possess enzymatic activities [abzymes (Abzs)] (21–26). Natural IgGs hydrolyzing DNA, RNA and polysaccharides have been described from the sera of patients with several AI diseases (22–27). IgGs hydrolyzing a number of peptides and proteins were also found in AI and other diseases, including antibodies degrading vasoactive intestinal peptide (VIP) in asthma (28), thyroglobulin in Hashimoto's thyroiditis and rheumatoid arthritis (29), prothrombin in multiple myeloma (30), protein factor VIII in hemophilia A (31), MBP in multiple sclerosis (32–35) and human casein in the milk of human mothers (36). Some healthy humans produce Abzs with low VIP-, thyroglobulin- (28, 29) and polysaccharide-hydrolyzing activities (27) but usually Abzs are lacking in healthy donors and patients with many diseases with insignificant AI reactions (23–26).

Similarly to artificial Abzs against analogs of transition states of catalytic reactions (21), naturally occurring Abzs may be antibodies raised directly against the enzyme substrates acting as haptens and mimicking transition states of catalytic reactions (23–26). For example, Abzs hydrolyzing VIP (28), thyroglobulin (29) and MBP (32–35) in the sera of patients with asthma, Hashimoto's thyroiditis and multiple sclerosis, respectively, are antibodies against these proteins; the same is true for casein-hydrolyzing human milk antibodies against casein (36). On the other hand, anti-idiotypic antibodies can be induced in AI diseases by a primary antigen and may show some of its features, including the catalytic activity (37, 38).

It is currently believed that Abzs may play a significant role in forming specific pathogenic patterns and clinical settings in various AI conditions through their broadened autoantibody properties (21–26). Anti-VIP Abzs can have an important effect in pathogenesis decreasing the concentration of VIP (39). The protease activity of target-specific Abzs can attack MBP of the myelin–proteolipid shell of axons and contribute to the pathogenesis of multiple sclerosis (32–35). DNase Abzs are cytotoxic causing nuclear DNA fragmentation and inducing apoptosis (40, 41). A decrease in the relative activities (RAs) of DNase Abzs from patients with Hashimoto's thyroiditis correlates with normalization of the concentration of thyroid hormones and an improvement of the patients' clinical status (42). Thyroglobulin-hydrolyzing IgGs of Hashimoto's thyroiditis patients (43) and proteolytic IgGs from patients with sepsis (44) may play a positive role in the recovery from these diseases.

HIV-1 integrase (IN) catalyzes insertion of a DNA copy of the viral genome into the host genome (45). Therefore, IN, together with reverse transcriptase (RT) and protease, is the main important target of anti-HIV drugs. The first Abzs observed in AIDS were polyclonal IgGs (pIgGs) hydrolyzing DNA (46). Discovered later, pIgGs from AIDS patients hydrolyzing RT (47) and IN (48) were the first examples of proteolytic IgG Abzs appearing in humans directly against these proteins due to the viral infection and AI reactions. However, in parallel with the formation of Abzs against viral proteins, the production of pIgGs hydrolyzing specifically human serum albumin and human casein was observed in HIV-infected patients (47).

Most of the described artificial and natural Abzs are pIgGs; however, secretory Igs A (sIgAs) with various catalytic activities were purified from the milk of human mothers and found to be more active than pIgGs [reviewed in (23–26)]. The examples of IgAs and IgMs with catalytic activities from the human sera are limited. Nevertheless, it was demonstrated that polyclonal IgMs (pIgMs) from the sera of SLE patients are more active in the hydrolysis of DNA and RNA than pIgGs from the same patients (49). Specific enzymic activities of pIgMs and polyclonal IgAs (pIgAs) specifically hydrolyzing MBP (34) and pIgMs with amylolytic activity (50) from sera of multiple sclerosis patients were also significantly higher than those of pIgGs.

The kinetic parameters of gp120 cleavage by IgMs from uninfected patients varied over a broad range depending on the patient, while pIgGs failed to cleave gp120 detectably (51). Abzs that cleave HIV envelope gp120 protein may find their use in the treatment of AIDS patients [reviewed in (52–54)].

Chemical reactivity of Ig micro and kappa/lambda subunits expressed on the surface of B cells and in secreted IgMs is found in the preimmune repertoire. Secreted IgMs display nucleophilic reactivity superior to IgG antibodies (55). IgMs catalyze the cleavage of model peptide substrates at the rates up to 344-fold greater than IgGs. Catalytic activities are observed in pIgMs from immunologically naive mice and humans without immunological disease, as well as in monoclonal IgM to unrelated antigens. Fab fragments of a monoclonal IgMs expressed catalytic activity, confirming the V domain location of the catalytic site (55,56).

The 2.6 Å resolution structure has been determined for the glycosylated Fab fragment of an IgM obtained from a subject with Waldenström's macroglobulinemia (57). The Fab fragment displayed the ability to cleave, by a nucleophilic mechanism, the amide bonds of a variety of serine protease substrates. An atypical serine, arginine and glutamate motif is located in the middle of the fragment antigen-binding site and displays an overall geometry that mimics the classical serine, histidine and aspartate catalytic triad of serine proteases (57).

IgMs purified from the sera of patients with Alzheimer disease and one monoclonal human IgM hydrolyze amyloid-β peptide (57). Increased amyloid-β peptide concentrations in the brain are thought to induce neurodegenerative effects. Therefore, peripheral administration of antibodies binding amyloid-β peptide has been suggested as a potential treatment for Alzheimer disease (57).

The phenomenon of Abzs can potentially be exploited to isolate efficient catalytic domains directed against clinically relevant AI, bacterial and viral epitopes. The data of Zhou *et al.* (52) indicate the existence of protease-like IgMs as innate immunity components with potential roles in B-cell development and antibody effector functions. Since IgMs are usually more active than IgG Abzs, they might be more perspective for therapeutic applications.

In this report, we use several methods to provide the first evidence that IgMs from HIV-infected patients can *in vitro* specifically hydrolyze HIV-1 IN.

Methods

Chemicals, donors and patients

All chemicals were from Sigma or GE Healthcare (New York, NY). Homogeneous HIV-1 IN was purified as in ref. (58). IN-Sepharose was prepared using BrCN-activated Sepharose according to the standard manufacturer's protocol.

Sera of 10 healthy donors and 24 patients (18- to 40-year-old men and women) including 16 at the stage of pre-AIDS and 8 at the stage of generalized lymphadenopathy (GL) according to the classification of the Center of Disease Control and Prevention were used to obtain proteolytic Abzs. The blood sampling protocol conformed to the local hospital human ethics committee guidelines.

Antibody purification

Electrophoretically and immunologically homogeneous pIgMs were obtained from healthy donors and AIDS patients by sequential affinity chromatography of the serum proteins on protein A–Sepharose and FPLC gel filtration on a Superdex 200 HR 10/30 column as in refs. (34, 47–50). The sera were prepared by addition of 4% sodium citrate to the blood samples (5 ml, 1/4 of the blood volume) and removal of cells by centrifugation (2000 r.p.m., 10 min). The solution was loaded on a protein A–Sepharose column (5 ml) equilibrated in Tris-buffered saline (TBS) buffer (0.15 M NaCl and 20 mM Tris–HCl, pH 7.5) and the column was washed with TBS to zero optical density. Proteins bound non-specifically were eluted with the same buffer supplemented with 1% Triton X-100 and 0.3 M NaCl. The total IgG + IgM + IgA fraction was eluted with 40 mM glycine–HCl (pH 2.6), the column fractions were collected to cooled tubes containing 50 μl of 0.5 M Tris–HCl (pH 9.0) and finally each fraction was additionally neutralized with this buffer and dialyzed against 10 mM Tris–HCl (pH 7.5) containing 0.1 M NaCl.

pIgMs were separated from pIgAs and pIgGs by FPLC gel filtration of the total antibody fraction on a Superdex 200 HR 10/30 column (GE Healthcare) equilibrated with 50 mM Tris–HCl (pH 7.5) containing 0.3 M NaCl as described previously (34, 47–50). Before gel filtration, the antibody samples were incubated in TBS containing 2.5 M $MgCl_2$ for 30 min at 20°C. Tris–HCl (pH 7.5) containing 3 M $MgCl_2$ (3 ml, 'salt cushion') was applied to the column before the samples. The antibodies were eluted with TBS. The type of antibodies (IgA, IgG or IgM) in the fractions during different chromatographies was determined by western blotting on a nitrocellulose membrane as described previously (34, 47–50). The protein corresponding to the central part of the pIgM peak was concentrated and used in the further purification and assay of catalytic activity.

In order to protect the antibody preparations from bacterial contamination, they were filtered through a Millex filter (pore size 0.2 μm). After 1 week of storage at 4°C for refolding, the antibodies were used in the activity assays as described below. To exclude possible artifacts due to hypothetical traces of contaminating enzymes, the pIgMs were analyzed using an in-gel assay (see below).

For gel filtration of antibodies after acid shock, pIgMs were incubated in 50 mM glycine–HCl (pH 2.6) containing 0.2 M NaCl for 20 min at 25°C. Separation of the IgMs under 'acid shock' conditions was done by FPLC gel filtration on a Superdex 200 HR 10/30 column using 10 mM glycine–HCl (pH 2.6) as described previously (34, 47–50). The column fractions were collected to cooled tubes containing 50 μl of 0.5 M Tris–HCl (pH 9.0) and finally each fraction was additionally neutralized with this buffer and dialyzed against 10 mM Tris–HCl (pH 7.5) containing 0.1 M NaCl and filtered through a 0.2-μm Millex syringe-driven filter. After 1 week of storage at 4°C for antibody refolding, the fractions were used in the standard analysis of protease activity.

The IgMs purified using protein A–Sepharose and FPLC gel filtration were chromatographed on IN-Sepharose (1 ml) equilibrated with 50 mM Tris–HCl (pH 7.5) containing 0.1 M NaCl, the column was washed with this buffer to zero optical density and pIgMs were eluted with 50 mM glycine–HCl (pH 2.6), neutralized and dialyzed against 50 mM Tris–HCl (pH 7.5) containing 0.1 M NaCl.

Chromatography of purified IgMs on Sepharose bearing mouse IgGs against light chains of human antibodies was performed similarly to the chromatography on Protein A–Sepharose.

In all cases, IgM concentration in the final solutions was measured using the Bradford assay with a BSA standard.

Antibody proteolytic activity assay

The reaction mixture (10–60 μl) containing 50 mM Tris–HCl (pH 7.5), 30 mM NaCl, 0.2–0.4 mg ml^{-1} IN (31 kDa) or one of the control proteins and 0.01–0.2 mg ml^{-1} pIgMs was incubated for 2–24 h at 37°C. To quantitatively estimate the protease activity, we have found a concentration for each IgM preparation corresponding to the reaction of the pseudo-first order [linear part of the dependence of the hydrolysis rate on the IgM concentration; [Abzs] << [S]. The progress of reaction was followed from accumulation of IN fragments within the linear regions of the time courses.

In some cases, IgMs (0.5–1 mg ml^{-1}) were preincubated for 30 min at 25°C with one of the specific inhibitors of different proteases: iodoacetamide (4 mM), pepstatin A (0.7 mM), leupeptin (50 μM), 4-(2-aminoethyl) benzenesulfonyl fluoride (AEBSF, 0.15 mM) and EDTA (0.1 M), and then, aliquots of these mixtures were added to the standard reaction mixture as described (32–34, 48). SDS–PAGE analysis of antibodies for homogeneity and for products of protein cleavage was performed in 5–18% gradient gels with 0.1% SDS and the polypeptides were revealed by silver or Coomassie Blue staining (32–34, 48). The gels were imaged by scanning and quantified using GelPro v3.1 software.

SDS–PAGE assay of proteolytic activity

SDS–PAGE analysis of antibodies was done in 4–15% gradient gels under non-reducing conditions in the presence of 0.1% SDS; for polypeptide separation, the electrophoresis was performed in a reducing 12.5% gel in the presence of 0.1% SDS and 10 mM dithiothreitol (DTT). The polypeptides were visualized by silver staining (34, 47–50).

For SDS–PAGE assay of proteolytic activity, IgGs (10–12 μg) were preincubated at 30°C for 30 min in 50 mM Tris–HCl (pH 7.5), 1% SDS and 10% glycerol (32–34, 48) in the presence or in the absence of 10 mM DTT. To restore the activity after reducing or non-reducing SDS–PAGE, SDS was removed by incubating the gel for 1 h at 22°C with 4 M urea, and the gel was washed 10 times with water. Then 3- to 4-mm cross sections of longitudinal slices of the gel were cut out and incubated with 50 μl of 20 mM Tris–HCl (pH 7.5), 5 mM $MgCl_2$ and 1 mM EDTA for 2 days at 4°C to allow protein refolding and elution from the gel. The eluates (10 μl; total reaction mixture volume 20 μl) were used in the activity assay as described above. Parallel longitudinal gel slices were used to detect the position of IgM and its different chains in the gel by silver staining.

Determination of the kinetic parameters

The K_m and V_{max} (k_{cat}) values were calculated from the kinetic data by least-squares non-linear fitting using Microcal Origin v5.0 software and presented as linear transformations using a Lineweaver–Burk plot (59). Errors in the values were within 15–30%.

Statistical analysis

The results are reported as the mean and the standard deviation of at least three independent experiments for each IgG preparation, averaged over at least eight different HIV-infected patients. Difference between groups of patients was analyzed using Student's *t*-test, $P < 0.05$ was considered statistically significant.

Results

Purification of antibodies

Protein A–Sepharose is mainly used for IgG purification. However, it can bind IgM and IgA with lower affinity (60, 61). In order to remove non-specific binding proteins, we used protein A–Sepharose chromatography under special conditions (see Methods) (32–34, 48). In order to destroy immunocomplexes, the solution was fractionated on an FPLC gel filtration column. The homogeneity of the pIgM was confirmed by SDS–PAGE with silver staining. Since IgM has a very high molecular mass (~970 kDa), it cannot enter SDS–PAGE gels under non-reducing conditions (Fig. 1A, lane 2). Two bands corresponding to the heavy and light chains of pIgMs were evident after antibody reduction (Fig. 1B, lane 2). The absence of any protein bands in the gel corresponding to pIgMs under non-reducing conditions (Fig. 1A) and the presence of only two bands corresponding to the heavy and light chains under reducing conditions (Fig. 1B) demonstrate the absence of protein contaminations in the pIgM preparations.

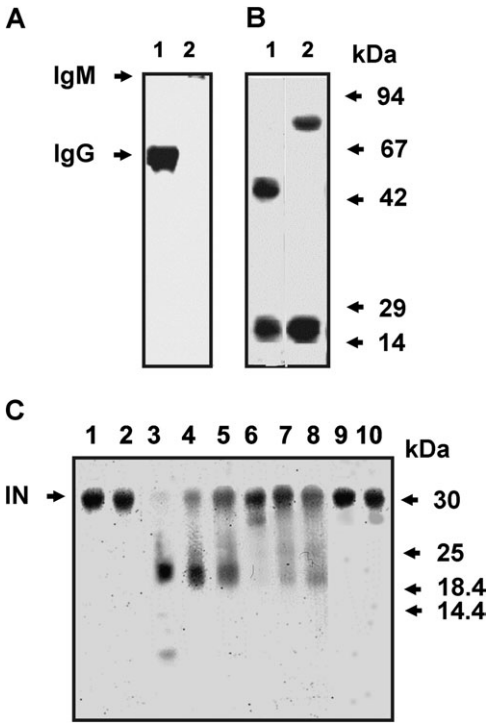

Fig. 1. SDS–PAGE in a non-reducing 4–15% gradient gel followed by silver staining of control pIgGs (lane 1) and pIgMs (lane 2) after FPLC gel filtration on a Superdex 200 column (A). SDS–PAGE of control pIgGs (lane 1) and pIgMs (lane 2) in a reducing 12% gel (B). Arrows indicate the positions of molecular mass markers. The hydrolysis of IN (0.4 mg ml^{-1}) by individual pIgMs from the sera of eight HIV-infected patients (lanes 3–10) after incubation for 10 h in the presence of 0.2–0.3 mg ml^{-1} IgMs (C). Lanes 1 and 2 correspond to IN incubated alone and in the presence of pIgMs (0.3 mg ml^{-1}) from a healthy donor, respectively.

First, we have analyzed the relative efficiency of hydrolysis of HIV IN (0.4 mg ml^{-1}) in the presence of 0.2–0.3 mg ml^{-1} IgM preparations from different patients (Fig. 1C). To estimate the IN-hydrolyzing activity quantitatively, we have found a special concentration for each IgM preparation (0.05–0.2 mg ml^{-1}) corresponding to the reaction of the pseudo-first order and used incubation and reaction depth within the linear regions of the time courses (5–24 h; 15–40% of IN hydrolysis; e.g. lanes 6 and 10 in Fig. 1C). The efficiency of IN cleavage was calculated from the decrease in the fraction of IN in the band of non-hydrolyzed protein taking into account the control reaction incubated in the absence of IgMs (lane 1, Fig. 1C).

Purified pIgMs from 10 control healthy donors were unable to catalyze IN hydrolysis (lane 2, Fig. 1C), whereas all 16 IgMs purified from patients with pre-AIDS (100%) and 6 of 8 IgMs (75%) from patients with GL demonstrated high or detectable IN-hydrolyzing activity (Fig. 1C). There was no statistically significant difference ($P = 0.71$) in the IgM RAs between the two groups of patients; the average values of IgM IN-hydrolyzing RAs were 3.8 ± 2.2 nM IN h^{-1} mg^{-1} antibodies (range 0.3–7.3 nM IN h^{-1} mg^{-1} antibodies) for pre-AIDS and 3.3 ± 2.6 nM IN h^{-1} mg^{-1} antibodies (range 0–8.1 nM IN h^{-1} mg^{-1} antibodies) for GL or 0.12 ± 0.07 and 0.1 ± 0.08 mg IN h^{-1} mg^{-1} antibodies, respectively.

Previously, we have analyzed the RAs of 19 individual IgGs from HIV-infected patients (49). There was no statistically significant difference in the IgG RAs between the two groups of patients; the average values of IgG IN-hydrolyzing RAs were 1.99 ± 1.68 μM IN h^{-1} mg^{-1} of antibodies for pre-AIDS and 3.4 ± 1.31 μM IN h^{-1} mg^{-1} of antibodies for GL patients. Twelve previously analyzed IgGs and 12 IgMs studied in this article were from the same HIV-infected patients. While only 1 of the 12 IgMs was inactive, two of the IgGs did not possess detectable activity. Overall, the coefficient of correlation between the RAs of IgGs and IgMs was 0.60 ($P < 0.05$).

Application of the strict criteria

We applied a set of strict criteria worked out previously (3–6, 8) for an analysis of IN-hydrolyzing activity as an intrinsic property of AIDS IgMs. The most important of these are (i) electrophoretic homogeneity of IgMs (Fig. 1), (ii) the complete absorption of AIDS IgMs with the IN-hydrolyzing activity by anti-L-antibody–Sepharose leading to a disappearance of the activity from the solution and recovery following its elution with an acidic buffer (pH 2.6; Fig. 2A) and (iii) FPLC gel filtration of IgMs using an acidic buffer (pH 2.6) did not lead to a disappearance of the activity, which tracked exactly with pIgMs (Fig. 2B).

To exclude possible artifacts due to hypothetical traces of contaminating proteases, the IgM was separated by SDS–PAGE under non-reducing and reducing conditions and its proteolytic activity was detected after the extraction of proteins from excised gel slices. As mentioned above, pIgMs cannot enter the gel. The absence of IN-hydrolyzing activity from all gel zones corresponding to the intact pIgMs under non-reducing conditions (data not shown), together with hydrolysis of IN only with separated heavy and light chains of IgMs under reducing conditions (Fig. 3) and the absence of any other bands of the activity (Fig. 3) or protein (Fig. 1A), provides a direct evidence that IgM possesses proteolytic activity.

Purification of IgMs on IN-Sepharose

We have subjected an equimolar mixture of pIgMs from five AIDS patients to affinity chromatography on IN-Sepharose. The IgM fraction bound to the affinity sorbent was eluted with 40 mM glycine–HCl (pH 2.6). Approximately 17 ± 3% of the total IgMs were bound to IN-Sepharose. As we have shown previously, the fraction of Abzs with different catalytic activities in the serum of AI patients usually does not exceed 0.1–5% of total Igs (23–26). Therefore, it was surprising that IN-Sepharose can bind up to 15 ± 3% of the total AIDS IgGs

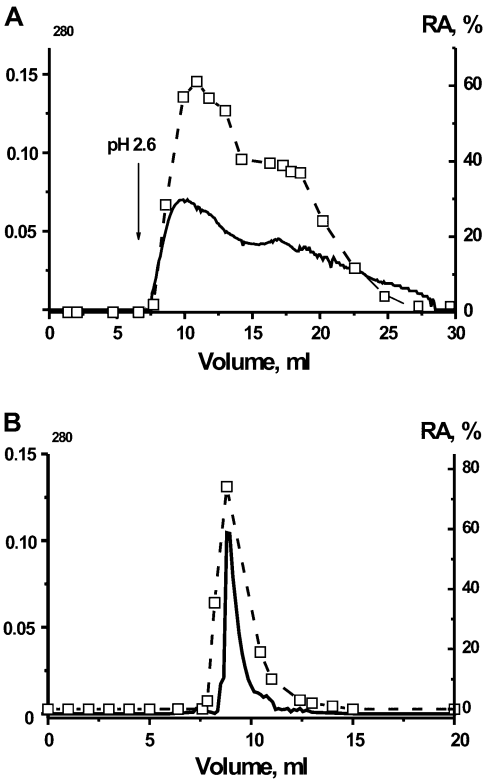

Fig. 2. Affinity chromatography of AIDS IgMs on anti-L-chain-Sepharose (A) and FPLC gel filtration of IgMs on a Superdex 200 column (B). After antibodies incubation in an acidic buffer (pH 2.6), they were loaded on the appropriate column: thin line, absorbance at 280 nm (A_{280}); the RA of IgMs (10 μl of eluate, total volume of the reaction mixture 20 μl) corresponding to a complete hydrolysis of 0.2 mg ml^{-1} IN after 20–24 h of incubation was taken for 100%. The average error in the initial rate determination from two experiments in each case did not exceed 7–10%.

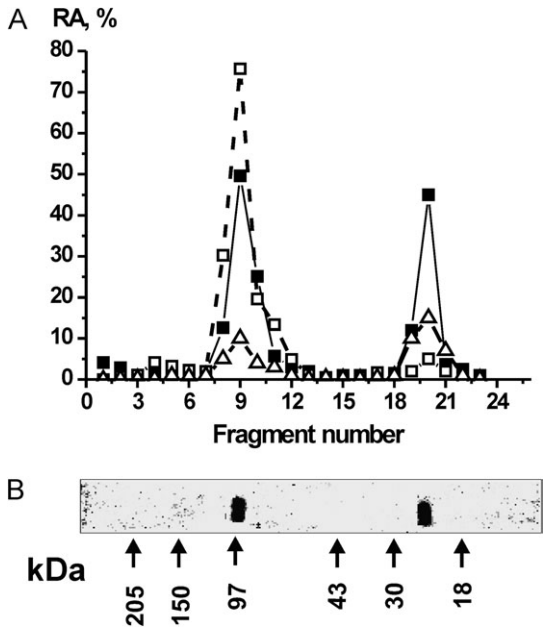

Fig. 3. SDS–PAGE analysis of IN-hydrolyzing activity of AIDS pIgMs. After reducing SDS–PAGE of purified IgMs, the gel was incubated under special conditions for antibodies refolding. The relative IN-hydrolyzing activity (RA, %) was quantified using the extracts of 26 fragments (2–3 mm each) of one longitudinal slice of the gel (A): filled squares, IgM7; open squares, IgM8; open triangles, IgM23. The RA of IgMs corresponding to a complete hydrolysis of 0.2 mg ml^{-1} IN after 24 h of incubation with 10 μl of extracts (total volume 20 μl) was taken for 100%. The average error in the initial rate determination from two experiments in each case did not exceed 7–10%. The position of heavy and light chains on the gel is shown (B). Arrows indicate the positions of molecular mass markers.

(48) and 17 ± 3% of the total IgMs. At the same time, IN is a very hydrophobic protein that can interact non-specifically with hydrophobic compounds, including proteins. Taking this into account, we suggest that immobilized IN might have bound anti-IN IgGs and IgMs specifically and some other IgGs and IgMs non-specifically (48). In spite of this possible binding of IN with other antibodies, the specific IgMs eluted from IN-Sepharose hydrolyzed only IN but not many other tested proteins (Fig. 4). Similar results were obtained previously for AIDS IgGs. Therefore, IgMs with IN-independent proteolytic activities do not have affinity for IN-Sepharose, while some other antibodies can be bound to it.

Analysis of the type of proteolytic activity

We have analyzed the type of IN-hydrolyzing activity of 10 AIDS pIgMs; one typical example is given in Fig. 5. The data

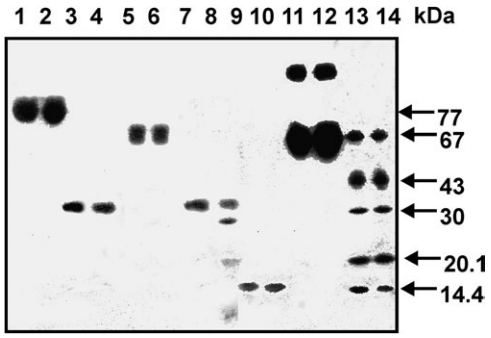

Fig. 4. SDS–PAGE analysis of hydrolysis of various protein substrates by pIgMs purified on IN-Sepharose. Proteins (0.2–0.4 mg ml^{-1}) were incubated for 16 h without (odd numbers) or with 0.1 mg ml^{-1} AIDS IgMs purified on IN-Sepharose (even numbers): human milk lactoferrin (lanes 1 and 2), human milk casein (lanes 3 and 4), p66 HIV-1 RT (lanes 5 and 6), HIV-1 IN (lanes 7 and 8; of the concentration of antibodies was 0.01 mg ml^{-1}), hen egg lysozyme (lanes 9 and 10), BSA (lanes 11 and 12) and a mixture of standard protein markers with known molecular masses (lanes 15 and 16). IgMs from AIDS patients purified on IN-Sepharose specifically hydrolyzed IN (lanes 7 and 8) but not other proteins (other lanes). Several proteins contained traces of protein contaminating proteins, which also were not hydrolyzed by AIDS IgMs (e.g. lanes 11 and 12). Arrows indicate the positions of molecular mass markers.

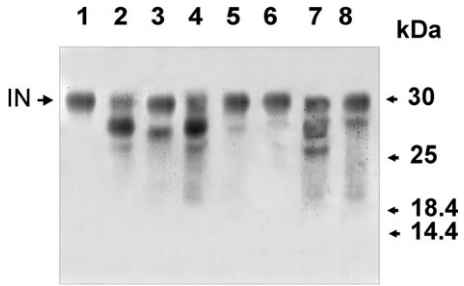

Fig. 5. SDS–PAGE analysis of a decrease in the intensity of IN band after its incubation with pIgMs from AIDS patients under different conditions. IN (0.3 mg ml^{-1}) was incubated alone for 9 h (lane 1), in the presence of pIgMs from one patient (0.1 mg ml^{-1}) but in the absence of other components (lane 2) or in the presence of AEBSF (lane 3), leupeptin (lane 4), pepstatin A (lane 5), iodoacetamide (lane 6), 0.01 M EDTA (lane 7) and 0.1 M EDTA (lane 8). For details, see Methods. Arrows indicate the positions of molecular mass markers.

for all nine IgMs from different donors are summarized in the Table 1. Leupeptin, an inhibitor of many proteases, significantly inhibited the proteolytic activity of only 2 of 10 individual AIDS IgMs, and very weak inhibition was observed for one more preparation (Table 1). Interestingly, leupeptin (lane 4) and EDTA (lanes 7 and 8) sometimes stimulated the hydrolysis of oligopeptides shorter than the initial IN (Fig. 5) with no significant effect on the cleavage of the initial IN. It may be a consequence of several types of proteolytic activities (acidic, thiol, serine protease-like and metalloprotease) associated with different molecules of pIgMs, with every specific inhibitor of IgM of one type possibly stimulating to some extent the hydrolysis of IN by antibodies with other activities (see below).

A specific inhibitor of acidic proteases, pepstatin A, significantly inhibited the IN-hydrolyzing activity of only three pIgMs and demonstrated weak inhibition of two preparations (Table 1). Surprisingly, a significant inhibition of serine protease-like activity by AEBSF was found only for two and weak suppression of the activity for another of 10 AIDS IgMs. Proteolytic activity of 5 of 10 AIDS IgMs was inhibited by 33–91% after incubating the IgMs with 0.01 M EDTA, while this chelating reagent at 0.1 M concentration decreased the RA of six preparations by 64–98% and inhibited three more preparations for ~8 to 10% (Table 1). An incubation of IgGs with iodoacetamide (a specific inhibitor of thiol proteases) usually has no effect on the proteolytic activity of Abzs from AI patients (28, 29, 32–35, 46). However, in our recent experiments, iodoacetamide inhibited the IN-hydrolyzing activity of all tested IgGs from HIV-infected patients by 12–98%. Similar result was obtained for IgMs; 10 of 10 antibodies were inhibited by iodoacetamide by 30–99% (Table 1).

Affinity of IgMs for IN

The dependency of the initial rate on the IN concentration in the reaction catalyzed by IgMs eluted from IN-Sepharose with an acidic buffer was consistent with Michaelis–Menten kinetics (e.g. Fig. 6). The K_m for IN (12.5 ± 2.0 μM) and k_{cat} (5.4 ± 1.1 min^{-1}) values were estimated.

Discussion

It has previously been shown that, in contrast to canonical proteases hydrolyzing many proteins, AIDS IgGs possess strict substrate specificity and efficiently hydrolyze only IN (48). In this article, similar result was obtained for AIDS IgMs; IN-hydrolyzing activity was shown to be an intrinsic property of IgMs derived from the sera of HIV-infected patients.

Many proteolytic Abzs are serine protease-like enzymes, and their activity is most strongly reduced after incubation with specific serine protease inhibitors phenylmethylsulphonylfluoride and/or AEBSF. These Abzs include IgGs hydrolyzing VIP, thyroglobulin, MBP or prothrombin from the sera of AI patients (28–30, 32, 33, 35); casein-hydrolyzing sIgAs from human milk (36); IgGs from HIV-infected patients (46); IgMs hydrolyzing MBP from multiple sclerosis patients (34) and IgMs hydrolyzing HIV gp120 from AIDS patients (51). In addition, it was shown that small fractions of pIgGs and pIgMs from patients with multiple sclerosis possess

Table 1. Inhibition of proteolytic activity of individual IgMs from nine AIDS patients by specific inhibitors of proteases of different types

Preparation ID	Inhibition, %[a]						
	AEBSF	Leupeptin	Pepstatin A	Iodoacetamide	10 mM EDTA	100 mM EDTA	Sum of effects[b]
IgM4	0	0	48 ± 5	97 ± 8	91 ± 8	98 ± 2	243
IgM5	0	47 ± 5	36 ± 5	36 ± 5	0	80 ± 7	199
IgM7	8 ± 1	68 ± 7	0	95 ± 9	86 ± 7	94 ± 8	265
IgM8	0	0	7 ± 2	99 ± 8	0	8 ± 2	114
IgM9	0	0	0	76 ± 6	0	0	76
IgM10	0	0	0	83 ± 7	64 ± 5	93 ± 8	176
IgM11	0	0	0	94 ± 7	0	8 ± 3	102
IgM12	0	7 ± 1	0	55 ± 5	66 ± 5	98 ± 8	153
IgM13	46 ± 5	0	88 ± 9	91 ± 10	33 ± 4	64 ± 4	289
IgM23	80 ± 8	0	5 ± 2	30 ± 5	0	10 ± 2	125

[a]The decrease in the intensity of the initial IN band estimated from SDS–PAGE electrophoresis data in the absence of inhibitor was taken for 100%; for each preparation, mean and standard deviation of three repeats are reported.
[b]Sum of the effects of different compounds on the proteolytic activity (leupeptin + pepstatin A + iodoacetamide + 100 mM EDTA).

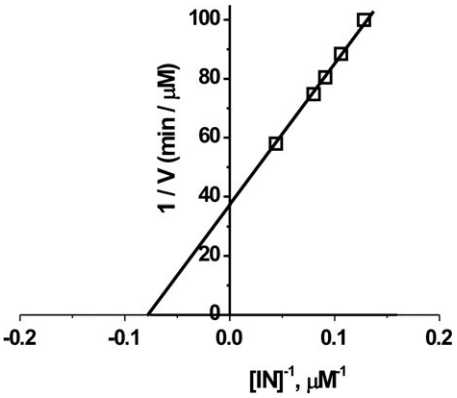

Fig. 6. The dependencies of the initial rates of IN hydrolysis on the IN concentration in the reaction catalyzed by pIgM (5 nM) purified on IN-Sepharose and determination of the K_m for IN and V_{max} values from the same data using the Lineweaver–Burk plot. Reactions were performed as described in Methods. The average error in the initial rate determination at each IN concentration from two independent experiments did not exceed 7–10%.

metal-dependent protease activity (33, 34). Some recently discovered Abzs possess not only serine protease-like and metalloprotease activities but also other types of protease activities. Likewise, IN-hydrolyzing pIgGs from 20% of the patients were inhibited by specific inhibitors of serine proteases, and the total pools of IgGs from 40% of patients contained antibody with metal-dependent protease activities but a significant suppression of the activity by specific inhibitors of acidic (20% of patients) and thiol (100% of patients) proteases was observed for the first time (48).

The effects of specific inhibitors of different proteases on the IN-hydrolyzing activity of AIDS IgMs varied from patient to patient (Table 1), but they were comparable with those for AIDS IgGs (48). A detectable or strong inhibition of serine protease-like activity was observed only for 3 of 10 preparations (8–80%). Six of 10 AIDS IgMs (60%) were significantly inhibited (⩾64%) by 0.1 M EDTA (Table 1), which was comparable with the properties of IgGs from patients with multiple sclerosis (33, 34) and with AIDS (48). A detectable or significant effect of pepstatin A (5–88% inhibition) on the proteolytic activity of five Abz preparations (50%) was revealed. Iodoacetamide, a specific inhibitor of thiol proteases, usually does not significantly affect the activity of proteolytic Abzs (⩽3–7% inhibition) (28–30, 32, 34–36, 46, 51). Therefore, it was surprising that the IN-hydrolyzing activity of AIDS IgGs was suppressed by iodoacetamide in all 100% preparations by 12–98% (average value 65.7 ± 20.6%) in a stark contrast with other known proteolytic Abzs. A similar result was observed for AIDS IgMs; iodoacetamide suppressed the IN-hydrolyzing activity by 30–99% (average value 75.6 ± 21.2%) in all 10 antibody preparations. Interestingly, there was no statistically significant difference in the inhibition of AIDS pIgGs and pIgMs by iodoacetamide ($P = 0.2$).

Our findings support the idea that the pools of pIgGs and IgMs of AIDS patients can contain IN-hydrolyzing Abzs of four types resembling thiol, serine, acidic and metal-dependent proteases, the ratio of which may be individual for every HIV-infected patient.

Interestingly, only one preparation (IgM9) demonstrated significant inhibition by one inhibitor (iodoacetamide), while IgM10 and IgM11 were strongly suppressed by iodoacetamide and EDTA; IgM23 was sensitive to AEBSF and iodoacetamide; IgM4 and IgM8 showed strong or at least some inhibition of the activity by three inhibitors (iodoacetamide, EDTA and pepstatin A), while IgM12 was sensitive to leupeptin instead of pepstatin A (Table 1). Surprisingly, 3 of 10 preparations (IgM5, IgM7 and IgM13) could be significantly inhibited by four different inhibitors. Of these, iodoacetamide and EDTA were common inhibitors for all three IgM preparations, while two other inhibitors were different: pepstatin A and leupeptin for IgM5, leupeptin and AEBSF for IgM7 and pepstatin A and AEBSF for IgM13 (Table 1).

In principle, it is possible that the pools of IgMs from AIDS patients may be cocktails of Abz molecules, with each molecule possessing only one of four alternative types of proteolytic activity: serine, acidic, thiol or metal dependent. Yet,

the effects of two, three and four inhibitors of different protease types did not always add to 100% inhibition. Only in 3 of 10 IgM preparations (IgM8, IgM9 and IgM11), this sum was less or comparable with 100% (76–114%), while for other seven IgMs, it varied from 125 to 289% (Table 1). Since IgM9 had only thiol protease-like activity and IgM8 and IgM11 could be significantly suppressed (94–99%) only with iodoacetamide but lost their activity only marginally in the presence of EDTA or pepstatin A (by 7–8%), it is most likely that in these patients, most of the Abz molecules possess only the thiol protease type of proteolytic activity (Table 1). However, since the proteolytic activity in 7 of 10 IgMs was summarily suppressed by specific inhibitors of serine, acidic, metal-dependent and thiol proteases by >100% (125–289%, Table 1), it is possible that the immune system of HIV-infected patients produces anti-IN Abzs with a combined structure of the active center, carrying amino acid residues typical of different proteases. For example, we suggest that the pools of IgM4, IgM7 and IgM13 (209–289% of the summarized inhibition) contain IgM molecules with extremely complicated active centers containing structural elements of thiol and metal-dependent proteases, which may be additionally combined with structural elements of the active centers of acidic proteases (IgM4), serine proteases (IgM7) or both (IgM13).

It is known that IN interacts with Mg^{2+} and Mn^{2+} ions and that these metals affect the enzyme's activity (46). Therefore, one cannot exclude that inhibition of IgMs by EDTA may be attributed to some structural changes due to a removal of metal ions from IN. At the same time, IgM preparations dialyzed against EDTA demonstrated nearly the same activity in the hydrolysis of IN preparations after their dialysis against EDTA or preincubation with an equimolar amount of $MgCl_2$ before adding to the reaction mixture. Thus, we can suggest that Me^{2+}-dependent structural changes in IN may be not highly relevant for the Abzs activity.

Figure 3 demonstrates that separated heavy and light chains of AIDS IgMs are active in the hydrolysis of IN. Interestingly, three of the preparations (IgG4, IgG7 and IgG12) having several easily detectable types of the proteolytic activities (Table 1) demonstrated comparable RAs of the separated light and heavy chains; Fig. 3 shows the data for IgG7. Heavy chains of IgG8 possessing only one high activity, that of the thiol protease type, were significantly more active than light chains, while light chains of IgG23 demonstrating a high serine protease-like and a lower metal-dependent activity were remarkably more active than the heavy chains (Fig. 3). Thus, most probably, there are different combinations of contribution of light and heavy chains of IgMs hydrolyzing IN to the total activity of antibodies from different HIV-infected patients. In this regard, it should be mentioned that the Abz-dependent hydrolysis of some proteins (28, 62), DNA and RNA by isolated light chains is more efficient than by intact antibodies (23–26). However, it was shown that the catalytic center of the recombinant variable fragment (scFv) of DNase IgGs from AI-prone MRL-lpr/lpr mice may be located at the interface between the light and heavy chains and that after separation both of these chains are able to hydrolyze DNA (63). In addition, isolated heavy and light chains of pIgGs from MRL-lpr/lpr mice hydrolyze different NTPs, NDPs, AMP and dAMP (64). The observed IN-hydrolyzing activity of AIDS pIgM heavy and light chains separated by SDS–PAGE (Fig. 3) may have different underlying causes. First, it is possible that these pIgMs contain a mixture of Abzs with only light or only heavy chains being catalytically active. However, similar to mouse monoclonal DNase (63) and nucleotide-hydrolyzing IgGs (64), catalytic centers of AIDS IgMs hydrolyzing IN may be located at the interface between the light and heavy chains, with both separated chains capable of hydrolysis of IN. One cannot exclude that active centers with different types of proteolytic activity may be localized on different heavy and light chains of IgMs.

The dependence of the initial rate on IN concentration in the reaction catalyzed by IgMs eluted from IN-Sepharose with an acidic buffer was consistent with Michaelis–Menten kinetics (Fig. 6). The K_m (12.5 ± 2.0 μM) and k_{cat} (5.4 ± 1.1 min^{-1}) values for IN hydrolysis were estimated. The affinity of IN-Sepharose-purified AIDS pIgMs for IN in terms of K_m values (K_m = 12.5 ± 2.0 μM; Fig. 6) is comparable with typical affinities [K_m = 0.038–7.3 μM (23–26, 28, 29, 36)] of Abzs for various proteins that they hydrolyze, including IN-hydrolyzing AIDS IgGs (K_m = 16 ± 6 μM) (48).

The catalysis mediated by artificial Abzs against reaction transition states is usually characterized by relatively low reaction rates: k_{cat} values are 10^2- to 10^6-fold lower than for canonical enzymes (21). The known k_{cat} values for natural Abzs from AI patients vary in the range of 0.001–40 min^{-1} (22–29, 36, 48–50, 65). The k_{cat} for AIDS pIgGs (an equimolar mixture of pIgGs from five AIDS patients) purified on IN-Sepharose (0.45 ± 0.15 min^{-1}) in the IN hydrolysis (48) was 12-fold lower than the k_{cat} for AIDS pIgMs purified by the same procedure (5.4 ± 1.1 min^{-1}). For the comparison of the RAs of pIgMs (~970 kDa) and pIgGs (~150 kDa) with different molecular masses, it is useful to estimate the apparent k_{cat} values at a fixed concentration of substrate as a ratio of the relative rates of the reaction and the total concentration of antibodies (V/[antibodies]). Using the relative average RA of 19 AIDS pIgGs [2.4 ± 1.5 nM IN h^{-1} mg^{-1} antibodies (48)] and average RA for 24 AIDS IgMs described above (3.6 ± 2.4 IN h^{-1} mg^{-1} antibodies), the relative apparent k_{cat} values for polyclonal antibody preparations can be calculated. Again, the average apparent k_{cat} value for pIgMs, (5.7 ± 3.8) × 10^{-2} min^{-1}, was ~9.5-fold higher than that for pIgGs, (6 ± 1.6) × 10^{-3} min^{-1}. These data are in agreement with the previously published results concerning a higher activity of catalytic IgMs and IgAs as compared with IgGs from the sera of patients with AI and viral diseases (34, 49–51).

Currently, no method can efficiently separate Abzs from catalytically inactive antibodies against the same protein. In addition, as shown above, IN-Sepharose interacts not only with anti-IN IgMs but also binds some other antibodies nonspecifically. Even partial purification of IgMs on IN-Sepharose led to a significant increase in the k_{cat} value for IN hydrolysis. Since the specific activities were calculated using the total concentration of purified pIgMs and affinity chromatography on IN-Sepharose cannot separate catalytically active and inactive anti-IN IgMs, the specific IN-hydrolyzing activities of the individual monoclonal subfractions in the pIgMs pool

may be higher than those of non-fractionated or partially fractionated pIgMs. At the same time, the apparent k_{cat} values for IN hydrolysis catalyzed by IgMs fractionated or not on IN-Sepharose were comparable with those described for artificial and natural Abzs (22–29, 36, 48–50, 58).

According to literature data, the immune response to the viral components is the most important factor slowing the transition of HIV infection to the stage of AIDS; antibodies to HIV RT and IN can protect humans from developing AIDS (19). In HIV-infected patients, the protease activity of anti-IN IgGs and especially IgMs can hydrolyze IN. We have shown that IgGs and IgMs hydrolyzing IN significantly suppress the 3′ processing and integration reaction catalyzed by IN *in vitro* (*Svetlana V. Baranova, Elena S. Odintsova, Valentina N. Buneva, Marina A. Kharitonova, Ludmila P. Sizyakina, Christina Calmels, Marie-Line Andreola, Vincent Parissi, Olga D. Zakharova and Georgy A. Nevinsky*, in preparation). Recently, it was shown that the serum of HIV-infected patients also contains Abzs hydrolyzing HIV RT (47). As antibodies posses not only binding activity but also can hydrolyze viral IN and RT, it is tempting to speculate that a cooperative hydrolysis of these proteins by Abzs with these catalytic activities may enhance the protective effect of antibodies against diseases caused by HIV infection.

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