ISSN 0006-2979, Biochemistry (Moscow), 2006, Vol. 71, No. 3, pp. 251-261. © Pleiades Publishing, Inc., 2006.
Original Russian Text © E. S. Odintsova, M. A. Kharitonova, A. G. Baranovskii, L. P. Sizyakina, V. N. Buneva, G. A. Nevinsky, 2006, published in Biokhimiya, 2006, Vol. 71, No. 3, pp. 320-332.
Originally published in Biochemistry (Moscow) On-Line Papers in Press, as Manuscript BM05-155, December 18, 2005.

Proteolytic Activity of IgG Antibodies from Blood of Acquired Immunodeficiency Syndrome Patients

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Received June 29, 2005
Revision received October 4, 2005

Abstract—Proteolytic activity of polyclonal IgG antibodies (Abs) from the blood of AIDS patients was analyzed for the first time. These Abs were shown to display higher activity in hydrolysis of β-casein than in hydrolysis of human immunodeficiency virus (HIV)-1 reverse transcriptase (RT) or human serum albumin (HSA). Several abzymatic criteria were applied and it was shown that RT, HSA, and β-casein hydrolyzing activities are an intrinsic property of polyclonal Abs from AIDS patients. Casein-hydrolyzing Abs were detected in the blood serum for 95% of AIDS patients, and it was shown that they possess serine protease-like catalytic activity. The substrate specificities of polyclonal Ab proteases and typical human proteases are different. Depending on the patient, the IgGs exhibit various pH optima of proteolytic activity. The products of casein hydrolysis by Ab proteases were different from those in the case of trypsin, chymotrypsin, and proteinase K.

DOI: 10.1134/S0006297906030047

Key words: antibodies, natural abzymes, AIDS, proteolytic activity

Antibodies (Abs) against stable transition state analogs as well as natural immunoglobulins with catalytic activity are called abzymes and are well described in the literature (for review see [1-8]). During the last 15 years abzymes with different enzyme activities have been isolated from the blood of patients suffering from autoimmune diseases (AID) [4-8]. The first example of a natural abzyme was IgG isolated from the blood of bronchial asthma patients, and it is able to hydrolyze vasoactive intestinal peptide [9]. Later, IgGs with DNase [10] and RNase activities [11-13] were found in the blood of systemic lupus erythematosus (SLE) patients. IgG and/or IgM hydrolyzing DNA, RNA [11, 14-17], and polysaccharides [18, 19] isolated from the blood of patients suffering from various AIDs (SLE [11], Hashimoto's thyroiditis [20], polyarthritis [16], multiple sclerosis [21-23]), lymphoproliferative diseases [14], polyneuritis, malignant tumors [18, 19], as well as two viral diseases, viral hepatitis [17] and acquired immunodeficiency syndrome (AIDS) [15], were also described.

Other known IgGs from AID patients hydrolyze autoantigenic proteins: thyroglobulin (Hashimoto's thyroiditis and rheumatoid arthritis) [24, 25], prothrombin (multiple myeloma) [26], protein factor VIII (hemophilia) [27], and myelin basic protein (multiple sclerosis; IgG, IgM, and IgA antibodies) [28-30].

Milk of healthy maternal patients contains sIgA with nuclease [7, 31, 32], ATPase [33, 34], protein kinase [35, 36], and polysaccharide hydrolyzing [37] activities. According to a number of investigations, during pregnancy and especially after the beginning of lactation, the female organism has an immune status similar to that of AID patients ([38] and references therein). At the same time, according to our data, Abs from the blood of healthy donors, as well as patients with flu, pneumonia, tuberculosis, tonsillitis, duodenal ulcer, and some oncological diseases (uterine, mammary gland, and intestinal cancers) occurring without any significant disturbance of the immune status did not reveal noticeable catalytic activity [7, 38, 39].

AIDS is a viral disease resulting in the dysfunction of the immune system [40]. Expressed immune response toward viral components is the main factor determining

Abbreviations: Ab) antibody; AID) autoimmune diseases; AIDS) acquired immunodeficiency syndrome; DTT) dithiothreitol; HIV) human immunodeficiency virus; HSA) human serum albumin; SLE) systemic lupus erythematosus; RT) reverse transcriptase.

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slow progression of HIV infection to the AIDS stage, resulting in the depletion of T-helper cells and their functional incompetence. B-Lymphocyte activation during HIV infection leads to the appearance of the immune complexes and autoantibodies in serum. Structural similarity between the conservative regions of class I and II HLA molecules and viral glycoproteins gp120 and gp41 may cause generation of autoantibodies against HLA. Although systemic autoimmune reactions are rare during HIV-infections, sporadic autoimmune disorders (lymphopenia, neutropenia, anemia, and thrombocytopenia) are the basic clinical aspects of AIDS. The most common are autoantibodies against leukocytes, and less common against thrombocytes and neutrophils. Antinuclear Ab, Ab against cardiolipin, interleukin IL-2, CD4 and CD8 molecules, as well as some serum proteins, immunoglobulins and thyroglobulin [41], were also found in HIV-infected patients.

The aim of this work was the investigation of IgG proteolytic activity from the blood of AIDS patients.

MATERIALS AND METHODS

Isolation of antibodies. IgG antibodies were isolated from blood of 110 patients in the age between 18 and 40. According to the classification of the Center for Disease Control and Prevention, 45 of these patients were in generalized lymphadenopathy stage, 65 at pre-AIDS stage. Venus blood taken on empty stomach was mixed with 4% sodium citrate in a ratio of 4 : 1. The mixture was incubated for 3-5 h and centrifuged at 3000 rpm for 15 min. To precipitate the proteins, 1 ml of 50 mM Tris-HCl, pH 7.5, containing 150 mM NaCl and ammonium sulfate were added to an equal volume of blood until 50% saturation. After incubation for 1 h at 5°C, the precipitate was separated by centrifugation (12,000 rpm, 10 min), resuspended in 1 ml of 50 mM Tris-HCl buffer, pH 7.5, containing 150 mM NaCl, and then dialyzed against the same buffer for 12 h at 4°C.

Dialyzed fraction of total blood protein was centrifuged and the supernatant applied on a Protein A-Sepharose column (1 ml) equilibrated with buffer A (50 mM Tris-HCl, pH 7.5, 150 mM NaCl). Unbound proteins were removed by washing the column with the same buffer until the complete absence of absorption at 280 nm. Nonspecifically bound proteins and lipids were eluted using buffer A containing 1% Triton X-100, and then the column was washed with 50 mM Tris-HCl, pH 7.5, containing 0.3 M NaCl. Total Ab fraction containing IgG, IgA, and IgM was eluted with 0.1 M glycine-HCl, pH 2.6, and neutralized with 1.5 M Tris-HCl, pH 8.0, immediately after elution from the column. The Abs were dialyzed against buffer A.

IgG, IgA, and IgM were separated by high performance gel filtration on Superdex 200 HR 10/30 column (100 × 300 mm) (on a Sprint Biocad chromatography workstation; Pharmacia, Sweden) equilibrated with 20 mM Tris-HCl, pH 7.5, containing 0.3 M KCl. Prior to FPLC gel filtration, complexes were dissociated by mixing 250 μl of Ab solution (~1 mg/ml) with 83 μl of 3 M $MgCl_2$ and 166 μl of 3 M NaCl, incubated for 30 min, and then centrifuged (10,000 rpm, 10 min). Before loading the sample, 2 ml of the buffer containing 0.5 M $MgCl_2$ and 1 M NaCl was applied to the column, followed by application of Ab solution. Elution was carried out using 20 mM Tris-HCl, pH 7.5 (0.2 ml/min). Incubation of antibodies with high salt concentration resulted in efficient protein separation.

Additional Ab purification was performed by affinity chromatography on a column with casein-Sepharose (1 ml, 5 mg casein per ml resin). Abs (1.5 mg) were applied to the column equilibrated with 50 mM Tris-HCl, pH 7.5, or the same buffer containing 0.1 M KCl, and immunoglobulins were eluted by a KCl gradient from 0 to 1 M in the same buffer.

Isolation of casein from human milk. Partially purified casein was obtained from the milk of maternity patients according to the following procedure. Milk (0.5 liter) was centrifuged at 8000 rpm for 40 min, and the supernatant was collected and dialyzed against 300 mM NaOAc, pH 3-4, overnight at 6°C [35]. After that, the pellet was removed by centrifugation (10,000*g* for 40 min), washed with 0.3 M NaOAc, pH 3-4, and then centrifuged (7000*g* for 15 min). The pellet was resuspended in 50 mM NaOH containing 1.5 M NaCl. Highly purified casein preparations were obtained by high performance gel filtration on a Superdex-75 column equilibrated with the same buffer. Casein fractions were concentrated using a Centricon YM-10 (1000*g* at 4°C) until the final concentration of 1-2 mg/ml.

Electrophoretic and immunological analysis of proteins. Electrophoretic analysis of proteins was performed according to the method of Laemmli [42] in 4-15% gradient or 12.5% polyacrylamide gel. The proteins were incubated in 50 mM Tris-HCl, pH 6.8, containing 2% SDS, 10% glycerol, and 0.025% bromophenol blue for 1 min at 100°C and then applied to the gel. Electrophoresis was run for 3-4 h at current 15-20 mA. Proteins were stained by $AgNO_3$ [43] or Coomassie R-250 [44].

After electrophoresis, the Abs were transferred onto nitrocellulose membrane (immunoblotting) and visualized using a conjugate of specific Abs with horseradish peroxidase according to [45]. Control nitrocellulose membrane was rinsed with water three times and proteins were stained with 20 ml of fresh solution containing 550 mg of sodium citrate, 190 mg $FeSO_4$, and 40 mg $AgNO_3$. The sensitivity of this method is 50-100 ng/mm^2.

Determination of proteolytic and DNase activity of antibodies. The reaction mixture (20 μl) contained 20 mM Tris-HCl, pH 7.5, 0.1 mg/ml casein (or other

protein at the concentration of 0.1-0.3 mg/ml), and 0.05-0.2 mg/ml Abs. The following compounds were used as alternative substrates: α-lactalbumin, albumin, lactoferrin, lysozyme (human proteins), as well as bovine casein, α-lactalbumin, HIV-1 reverse transcriptase (RT), and a number of other proteins (see below). The pH optimum for reaction of casein hydrolysis was determined using the following buffers (20 mM): MES-NaOH (pH 4.5, 5.0, 5.5, 6.0, 6.5, 7.0) and Tris-HCl (pH 7.5, 8.0, 8.5, 9.0).

Classic protease inhibitors were used in some experiments: 0.01-0.1 M EDTA, 1.5 mM PMSF, 4 mM iodoacetamide, 1 μM pepstatin A, 50 μM leupeptin, and 100-150 μM AEBSF. The Abs were incubated with the given inhibitor concentrations for 30 min at room temperature and added to the reaction mixture.

All reaction mixtures were incubated for 1-16 h at 35°C, and the reaction products were analyzed by SDS-PAGE. The extent of the reactions was estimated by decrease in protein substrate band intensity compared to the control (incubation of protein in the absence of Ab) using GelPro Analyzer 9.11 software.

To optimize hydrolysis conditions (especially in the case of low efficiency cleavage of protein substrates) the time-dependent product formation was analyzed. Statistical reliability for protein hydrolysis in our experiments was ±4-5%. In the case of low percentage of hydrolysis (4-10%), the efficiency of substrate cleavage was estimated by the ratio of protein bands corresponding to initial substrate and products formed. Moreover, for more precise determination of the decrease in band intensity for initial substrate, the incubation time for low efficiency hydrolysis was increased up to 48-60 h. The obtained percentage of hydrolysis during 48-60 h was normalized to the standard incubation time (16 h). Both approaches to determination of hydrolysis efficiency in the case of low efficiency hydrolysis yielded the same data within the experimental error.

DNA hydrolyzing activity of Abs was determined as described previously [11-13] using electrophoresis of plasmid DNA in 1% agarose gel followed by product staining with ethidium bromide and gel photography.

Casein hydrolysis by classic proteases. The reaction mixture (10-20 μl) contained 20 mM Tris-HCl, pH 7.5, 6.2 μg/ml casein, and one of the classic proteases in a carefully chosen optimal concentration: 0.32 μg/ml trypsin, 0.064 μg/ml chymotrypsin, or 0.1 μg/ml proteinase K. The reaction mixture was incubated for 10-15 min at 30°C, after which the products were analyzed by SDS-PAGE in 12.5% polyacrylamide gel followed by silver staining.

Determination of casein-hydrolyzing activity of Abs after electrophoresis. After protein separation by SDS-PAGE in 4-15% gradient polyacrylamide gel, the control lane was cut out and stained with Coomassie R-250 solution. Experimental lanes were washed with 4 M urea solution for 1 h and then rinsed with water (10 times for 5-7 min) to remove SDS. Comparison of gel color before and after this treatment indicated that there was no protein elution during the process of gel washing with water. Then, the lanes were fragmented in pieces of 2-3 mm length. To elute the proteins from the gel as well for their subsequent renaturation, the fragments were disintegrated into small pieces and incubated in 20 mM Tris-HCl, pH 7.5, containing 5 mM $MgCl_2$ and 1 mM EDTA during 24-48 h at 4°C. After long incubation and periodic shaking of small gel pieces, efficient protein elution was observed. The gel pieces were removed by centrifugation (20,000*g* for 10 min), and the supernatant was used for determination of catalytic activity as described above.

Materials and reagents. HIV-1 RT was obtained as described in [46]. The following chemicals were used in this work: acrylamide, N,N′-methylene-bis-acrylamide, glycine, EDTA, Triton X-100, human lysozyme (Merck, Sweden); bromophenol blue, BrCN-Sepharose, Sephadex G-25 (Pharmacia, Sweden); TEMED, SDS, ethidium bromide, human α-lactalbumin and serum albumin (HSA), bovine casein (Sigma, USA); dithiothreitol (DTT), agarose (Serva, Germany); Tris (Helicon, Russia); Superdex-200 and Superdex-75 (Pharmacia Biotech, Sweden); QIAGEN (QUIAGEN Inc., USA). Other chemicals were of analytical grade.

RESULTS AND DISCUSSION

IgGs were isolated by affinity chromatography of blood proteins from healthy donors and AIDS patients on a Protein A-Sepharose column under conditions reducing the formation of nonspecific complexes. As we have shown previously [47-54] and confirmed in this work by SDS-PAGE data, Ab preparations do not contain contamination from any other protein after chromatography on the Protein A-Sepharose column. IgG, IgM, and IgA were separated by high performance gel filtration chromatography. The obtained IgG preparations were electrophoretically and immunologically homogeneous as shown by silver staining and immunoblotting of proteins after SDS-PAGE (Fig. 1). After all purification steps, Abs were catalytically active in hydrolysis of β-casein (mainly) and RT and HSA (to a lesser extent). We have shown that the activities of these polyclonal Ab are the intrinsic property of IgG (see below), and that Ab preparations obtained as described above can be used for the estimation of their relative proteolytic activity without additional purification.

Since casein-hydrolyzing activity of 10 Ab preparations analyzed in the beginning of this work was the highest compared to other activities of these preparations (RT and albumin hydrolysis), we undertook the comparative study of this activity using Ab preparations from the blood of 110 AIDS patients and 20 healthy donors. It was demonstrated that Abs from the blood of the 20 healthy

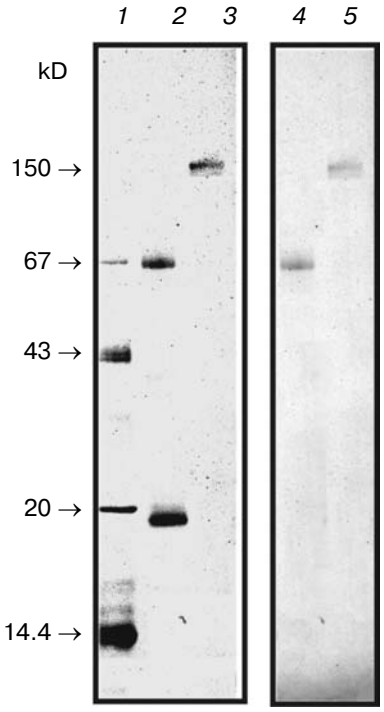

Fig. 1. SDS-PAGE analysis of homogeneity of IgG preparations from the blood of AIDS patients (4-15% gradient polyacrylamide gel): *2*, *3*) IgG after and before the incubation with 10 mM DTT, respectively (proteins stained with silver). Immunoblotting analysis of IgG using a conjugate of horseradish peroxidase with mouse Ab against human IgG heavy chain: *4*, *5*) Ab after and before treatment with 10 mM DTT, respectively; *1*) protein molecular weight markers.

donors did not possess the assayed casein-hydrolyzing activity. At the same time, casein-hydrolyzing activity was reliably detected in 105 (95%) out of 110 blood samples from the AIDS patients. However, the relative extent of Ab-dependent casein hydrolysis varied dramatically between the samples. After the incubation of casein with IgG for 16 h, the decrease in protein amount in the initial casein band did not exceed 5-20% for 10% of the IgG preparations; 22.7% of the IgG preparations resulted in 41-60% casein hydrolysis; 18.2% – in 61-80% hydrolysis, and the highest activity (81-100%) was displayed by 22.7% of the Ab preparations.

It is noteworthy that under given conditions some preparations of IgG abzymes from the patients in generalized lymphadenopathy stage displayed especially high proteolytic activity, up to 86.7%, but on average it was lower, within 53.6 ± 22.6%. As disease progressed to the pre-AIDS stage, the percentage of patients with reliably detectable IgG abzyme activity was higher (~100%). The average value of relative casein-hydrolyzing activity was practically unchanged (51.5 ± 25.9%), but the number of patients with the highest values of catalytic activity was lower.

Validation of antibody catalytic activity. The validation of catalytic activity of antibodies is usually based on examination of a large number of strong criteria, which are analyzed in detail in a monograph [6]. One of these criteria is the homogeneity of Ab preparations according to electrophoretic data, where protein bands are stained with silver (Fig. 1). Moreover, it was shown that IgGs are quantitatively adsorbed on anti-L-IgG-Sepharose and then eluted by acidic buffer (pH 2.6); the profiles of protease activity in the case of all three protein substrates (RT, HSA, and casein) correspond to the Ab profile (Fig. 2a).

It should be noted that among the criteria used at the present time there are those that undoubtedly indicate

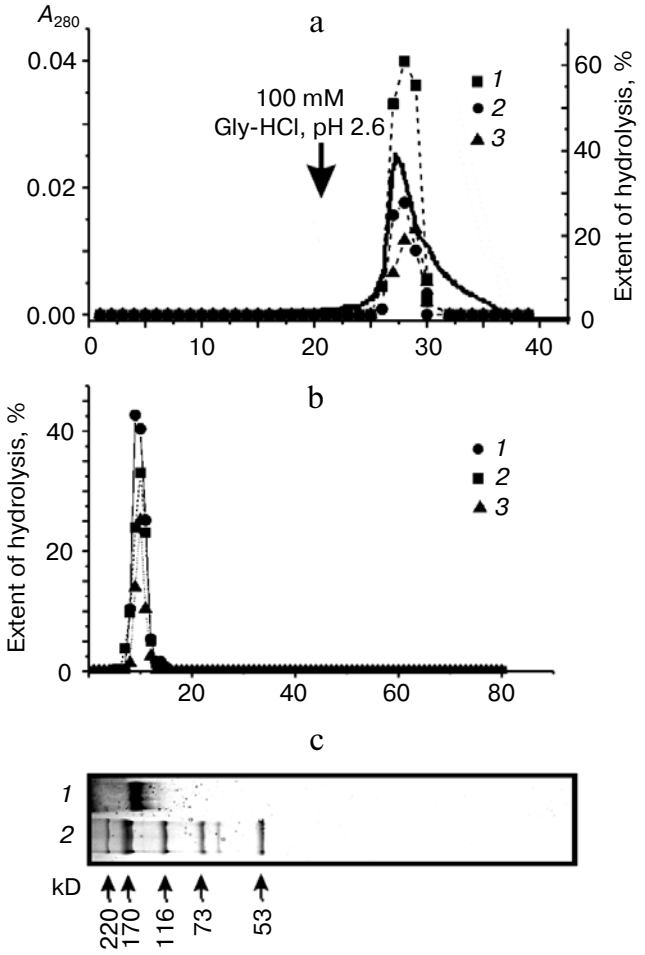

Fig. 2. a) Affinity chromatography of IgG preparation from the blood of AIDS patient on anti-IgG-Sepharose (relative efficiency of hydrolysis is shown, %): *1*) β-casein; *2*) RT; *3*) HSA. b) Analysis of catalytic activity of serum IgG from the blood of AIDS patients after SDS-PAGE. The gel lane was cut into fragments of 2-3 mm and the relative extent of hydrolysis (%) of the analyzed protein was determined (using the eluates corresponding to these fragments, and taking into account the initial protein amount incubated in the absence of eluate (100%)): *1*) β-casein; *2*) RT; *3*) HSA. c) The location of IgG protein band after electrophoresis (*1*) was determined by gel staining with Coomassie R-250; *2*) protein molecular weight markers.

that the activity belongs directly to Abs, not to any impurities. One of these criteria is the existence of antibody catalytic activity after protein separation by SDS-PAGE [4, 6, 7, 31, 32, 39, 55]. As we have shown previously, if this strongest criterion is exhibited, the others (less strong) are also exhibited [4, 6, 7].

As seen from data in Fig. 2b, the protein band possessing casein-, RT-, and HSA-hydrolyzing activities corresponds to the IgG from blood of AIDS patients. It is known that SDS-PAGE is one of the most rigorous techniques to break all types of noncovalent complexes. The absence of protease activity and other protein bands after SDS-PAGE indicate that all proteolytic activities are the intrinsic property of IgG and are not due to the contamination with serum proteases.

To investigate the substrate specificity of polyclonal IgGs from the blood of AIDS patients, various individual milk and serum proteins from humans, animals, as well as HIV RT were used as potential protein substrates. Ten abzyme preparations from different patients were used for analysis of substrate specificity. The same IgG preparations efficiently hydrolyzed RT, HSA, and β-caseins from human and bovine milk. Figure 3 illustrates the hydrolysis of some proteins by one of the 10 IgG preparations under conditions when both RT and HSA were almost completely (~100%) cleaved into short oligopeptides, but cattle β-casein (normally represented by two protein bands) and human β-casein are cleaved to a minor extent. At the same time, none of the following proteins, i.e., α-lactalbumin, lactoferrin, lysozyme, as well as bovine α-lactalbumin, were hydrolyzed by IgG preparations with appreciable rate during 16-48 h (see, for instance, Fig. 3). In addition, detectable hydrolysis was not observed for the proteins used as standard molecular weight markers for SDS-PAGE: α-macroglobulin, β-galactosidase, transferring, glutamine dehydrogenase, phosphorylase B, ovalbumin, and carboanhydrase.

It should be noted that the relative hydrolysis rates for the three most efficiently hydrolyzed protein substrates varied from substrate to substrate, but most of the analyzed IgG preparations hydrolyzed β-casein with higher rate than RT or HSA. The data obtained for one of the IgG preparations and given below illustrate the most characteristic situation in the terms of ratio between the relative activities of most IgG preparations in hydrolysis of various protein substrates: human casein (~50%), RT (~34%), and HSA (~36%).

It is known that different human proteases use any protein as a potential substrate. Recently we have shown that sIgA antibodies from the milk of healthy maternity patients specifically hydrolyze only human and bovine milk casein, but do not hydrolyze HSA, HIV RT, or other proteins [56]. Thus, substrate specificity of abzymes from the blood of AIDS patients is different from that of classical proteases and for milk sIgA abzymes.

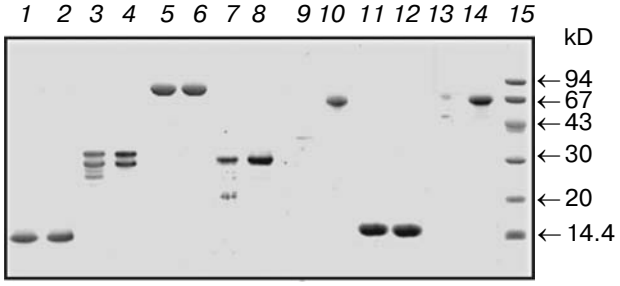

Fig. 3. SDS-PAGE analysis of IgG-dependent hydrolysis of various proteins: *1*, *2*) α-lactalbumin; *3*, *4*) bovine milk casein; *5*, *6*) human lactoferrin; *7*, *8*) human milk casein; *9*, *10*) HIV-1 RT; *11*, *12*) human lysozyme; *13*, *14*) HSA (lanes with odd numbers, in the presence of Ab; lanes with even numbers, in the absence of Ab); *15*) protein molecular weight markers.

It should be noted that the absence of reliably detected hydrolysis of some control proteins (see above) cannot, obviously, be an unambiguous indication of that abzymes capable of hydrolyzing these proteins are not produced in the AIDS patients. It cannot be excluded that the increase in number of the analyzed Abs, as well as enrichment of polyclonal antibody fraction by affinity chromatography on the sorbents with immobilized proteins, may lead to the generation of small abzyme subfractions capable of efficient hydrolysis of these proteins. Nevertheless, in the analysis of polyclonal IgG from the AIDS patients, the highest activity is displayed by abzymes directed towards specific hydrolysis of β-casein, RT, and HSA.

Analysis of protein affinity to casein. Natural abzymes are polyclonal Abs, representing the set of monoclonal Abs against various antigens, which sometimes can be separated into individual subfractions by affinity chromatography according to their affinity to the corresponding substrate [31, 32, 34, 35, 39, 47, 57]. Depending on the patient, between 30 ± 3% and 46 ± 4% of whole Ab pool was bound during affinity chromatography of homogeneous IgG preparations on a column equilibrated with 50 mM Tris-HCl buffer, both in the case of two healthy donors and five AIDS patients. This indicates significant nonspecific interactions of casein with various monoclonal IgG in the absence of salt. Taking this into account, we performed chromatography on a column equilibrated with buffer containing 0.1 M KCl. Affinity chromatography data for one of the IgG preparations (equimolar mixture of Abs from five AIDS patients) on casein-Sepharose (Fig. 4) indicates that ~5% of homogenous IgG preparation has an increased affinity towards immobilized casein. In the case of equimolar mixture of Abs from five healthy donors, reliably detectable amount of Ab interacting with the sorbent was not found (data not shown).

It is interesting that Ab fractions both interacting and not interacting with immobilized casein displayed activi-

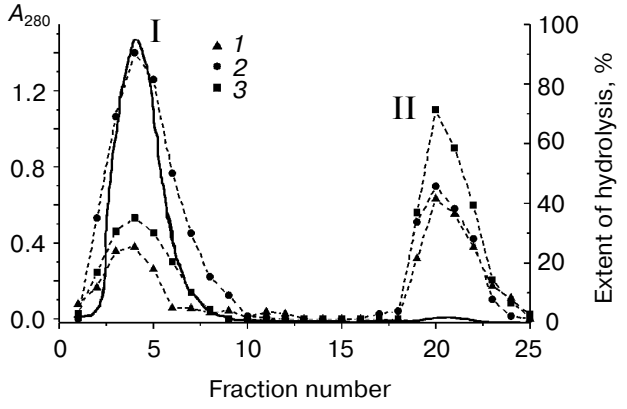

Fig. 4. Affinity chromatography profile for IgG preparation (equimolar mixture of IgG from five different patients) from the serum of AIDS patients on casein-Sepharose. Solid line, absorbance at 280 nm. Relative activity of Ab-dependent hydrolysis: *1*) RT; *2*) plasmid DNA; *3*) β-casein. Extent of hydrolysis is expressed as percentage of protein or DNA amount in initial band (100%) corresponding to the substrate incubated in the presence of Ab. I and II, two peaks of IgG.

ty in casein hydrolysis (Fig. 4), which indicates heterogeneity of the natural abzyme pool in its affinity to casein. This is in agreement with our data about extreme heterogeneity of abzymes with DNase [27], RNase [11, 12], ATPase [34], amylolytic [21, 37], and other activities [58, 59] by their affinity towards the substrates. During affinity chromatography on the sorbents with immobilized substrates, these abzymes can be resolved into several peaks, including Ab subfractions that are eluted upon application on the column due to its low affinity. Moreover, in the AID patients, depending on the individual and disease, narrow or rather broad repertoire of polyclonal abzymes can be produced. These abzymes can contain light chains of both κ and λ types, display maximal activity at different pH, have different total charge, or be characterized by different affinity towards the substrates, different dependence of activity on mono- and bivalent metal ions, and so on [4, 16, 23, 34, 47-50, 52, 53]. Such diversity in Ab properties may determine their differential behavior during affinity chromatography on the sorbents with immobilized substrates.

An interesting fact is that both the first and the second Ig peaks (I and II on Fig. 4) contain small Ab subfractions with catalytic activity. The I and II Ab peaks in the polyclonal IgG preparation shown in Fig. 4 correspond to 62 and 38% of its total activity in the hydrolysis of casein. Heterogeneity of abzymes by their affinity to casein-Sepharose can result from a number of reasons. First of all, from a theoretical point of view, the immune system of higher organisms can produce up to 10^6 various antibody molecules in response to the same antigen [65]. Moreover, the products of β-casein hydrolysis from human and bovine milk influence multiple biological functions of an organism, including antimicrobial, probiotic, and immunoregulatory functions, as well as metabolism [60-63]. This indicates the possibility of interactions between β-casein and a great number of various components in the cell and human blood. For instance, it is known that some Abs are polyreactive and can interact with different ligands with various affinity, including nucleic acids and proteins [31, 32, 34, 35, 39, 47, 57, 64, 65]. As follows from a number of investigations, the usual amount of abzymes with certain specificity in the blood of AID patients does not exceed 1-5% of the total IgG amount in the blood (for review see [5-7]), which, in general, is in agreement with the data on IgG sorption on a column with immobilized casein in the presence of salt. Nevertheless, it could not be excluded that a part of the Ab adsorbed on casein-Sepharose interacts with casein nonspecifically, or due to their polyreactivity. To test this assumption, the analysis of Ab activity in DNA and RT hydrolysis was performed (Fig. 4). As seen from Fig. 4, the major abzyme fraction with DNase activity (~71%) was eluted upon application of IgG on the column. Nevertheless, Abs in the second peak (~28%) also exhibited DNase activity (Fig. 4). Abzyme subfractions that were active in RT hydrolysis were distributed between the first and the second peak in the ratio of 38 and 62% (Fig. 4). These data can be an indication of that fact that β-casein is somehow able to interact with Abs having affinity to DNA, HIV RT, and, possibly, to other human blood proteins. It cannot be excluded that such interaction is realized due to the polyspecificity of Abs produced in the AIDS patients. In general, these data provide evidence that Ab fraction with affinity to casein-Sepharose is, most probably, not a specific Ab against this protein.

Determination of the type of Ab proteolytic activity. As it is known, higher eukaryotic organisms contain serine, thiol, and acidic proteases as well as metalloproteases. All abzymes with proteolytic activity [24-27] discovered so far are serine-type proteases. The first example of IgG antibodies with metalloprotease activity was recently found in the blood of multiple sclerosis patients. The activity of these antibodies is suppressed by EDTA and stimulated by several metal ions: Ni^{2+}, Cu^{2+}, Fe^{2+}, Mn^{2+}, Mg^{2+} [28-30].

To determine the type of casein-hydrolyzing activity of the IgG from blood of AIDS patients, the effect of the following specific protease inhibitors on Ab activity has been analyzed: EDTA (metalloproteases), iodoacetamide (thiol proteases), AEBSF (serine proteases). The following peptide inhibitors were also used in this work: pepstatin A (competitive inhibitor of acidic proteases) and leupeptin (selective irreversible inhibitor of trypsin-like and cysteine proteases). SDS-PAGE analysis of the products of IgG-dependent hydrolysis (Fig. 5) has shown that iodoacetamide, pepstatin A, and EDTA do not result in appreciable suppression of the activity of the studied Abs. During the reaction time (16 h), β-casein was cleaved

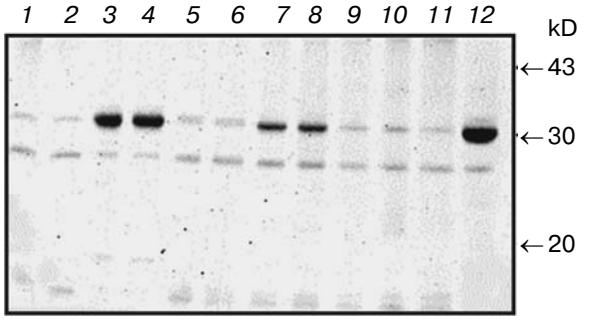

Fig. 5. SDS-PAGE analysis of β-casein hydrolysis products in 12.5% polyacrylamide gel before and after the treatment of Ab from serum of AIDS patients with protease inhibitors: *1*, *2*) EDTA; *3*, *4*) AEBSF; *5*, *6*) iodoacetamide; *7*, *8*) leupeptin; *9*, *10*) pepstatin A (the lanes with odd and even numbers correspond to Ab preparations from two different patients); *11*) Ab and casein in the absence of inhibitor; *12*) β-casein incubated in the absence of Ab. Location of protein molecular weight markers is shown on the right.

into short peptides. According to depletion of protein material in the initial casein band, leupeptin inhibited the casein hydrolysis reaction by ~30%. Addition of AEBSF to the reaction mixture (Fig. 5, lanes *3* and *4*) resulted in 90% inhibition of casein hydrolysis by Abs from the blood of AIDS patients. These data indicate that casein-hydrolyzing IgGs from the blood of AIDS patients as well as the majority of previously discovered proteolytic abzymes [24-27] are serine-type proteases.

Determination of pH optimum of casein hydrolysis. It is known that all classic proteases have one distinct pH optimum. At the same time, polyclonal Abs can contain a very large number of various monoclonal abzymes, which hydrolyze DNA [27], RNA [11, 12], ATP [34], and polysaccharides [21, 37] at different pH values. As a consequence, depending on the patient and the nature of AID, as well as on the stage of pathological development, polyclonal Abs can exhibit between one and several distinct pH optima. The abzyme repertoire in some AID patients is so broad that they are capable of hydrolyzing both DNA and RNA with approximately equal rates within the pH range from 5.5 to 9.5 [11, 12, 23].

We have investigated pH dependencies of casein-hydrolyzing Ab activity from the blood of AIDS patients using four IgG preparations with different levels of activity. As seen from Fig. 6, the four IgG preparations demonstrate absolutely different pH dependencies, and there is no single and unique pH optimum for any of them. Nevertheless, high rates of hydrolysis are observed for all four IgG preparations in the pH range from 8.0 to 9.0, and for three of them there is a tendency of increased hydrolysis rates at pH 6.0-7.5. These data indicate that heterogeneity of the IgG pool in the blood of AIDS patients varies from sample to sample.

Analysis of products of IgG-dependent casein hydrolysis. The products of casein hydrolysis by IgG from the blood of AIDS patients and various proteases were investigated. Figure 7 shows the data on hydrolysis of initial ^{32}P-labeled casein (lane *9*) by the control sIgA preparation from milk (lanes *1-3*), trypsin (lanes *4* and *5*), proteinase K (lanes *6* and *7*), and chymotrypsin (lane *8*). In the case of sIgA abzymes from human milk the formation of ~11 ^{32}P-labeled polypeptides with apparent molecular weights of 27.4, 25.4, 22.5, 21.5, 20.1, 18.8, 13.9, 12.9, 12.0, 10.7, and 9.8 kD was detected (lanes *1-3*). It is interesting to note that casein hydrolysis products with molecular weights from 28 to 12 kD in the case of trypsin (27.1, 24.8, 19.2, 15.9, 12.4, and 11.7 kD), chymotrypsin (24.0, 26.7, 16.8, and 15.1 kD), and proteinase K (24.0, 26.7, 16.8, and 15.1 kD) do not match those of sIgA abzymes from milk (Fig. 7a). Moreover, sIgA-dependent hydrolysis of β-casein results in accumulation of ^{32}P-labeled polypeptides with molecular weights of 9.8 and 10.7 kD (lanes *1-3*), whereas in the case of proteases the accumulation of these products is not observed (lanes *4-8*).

An especially interesting phenomenon takes place in the case of IgG antibodies from the blood of AIDS patients. Casein hydrolysis patterns in the case of three different Ab preparations are completely dissimilar (Fig. 7b). Based on the large number of experiments on casein (silver staining) and ^{32}P-labeled casein hydrolysis by these IgG preparations, apparent molecular weights of resulting polypeptides were calculated. In the first case the formation of seven major polypeptides with apparent molecular weights of 22.4, 20.5, 16.3, 14.4, 13.0, 8.7, and 7.6 kD (lane *2*) was observed, in the second case six polypeptides

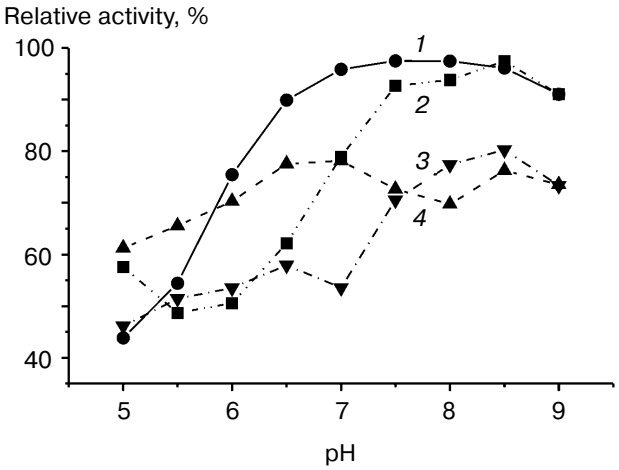

Fig. 6. Dependencies of relative catalytic activity of IgG preparations from the serum of four AIDS patients (*1-4*) in casein hydrolysis on the pH of reaction mixture. Extent of the reaction is expressed as a percentage ratio to the protein amount in the initial β-casein band (100%) incubated in the absence of Ab.

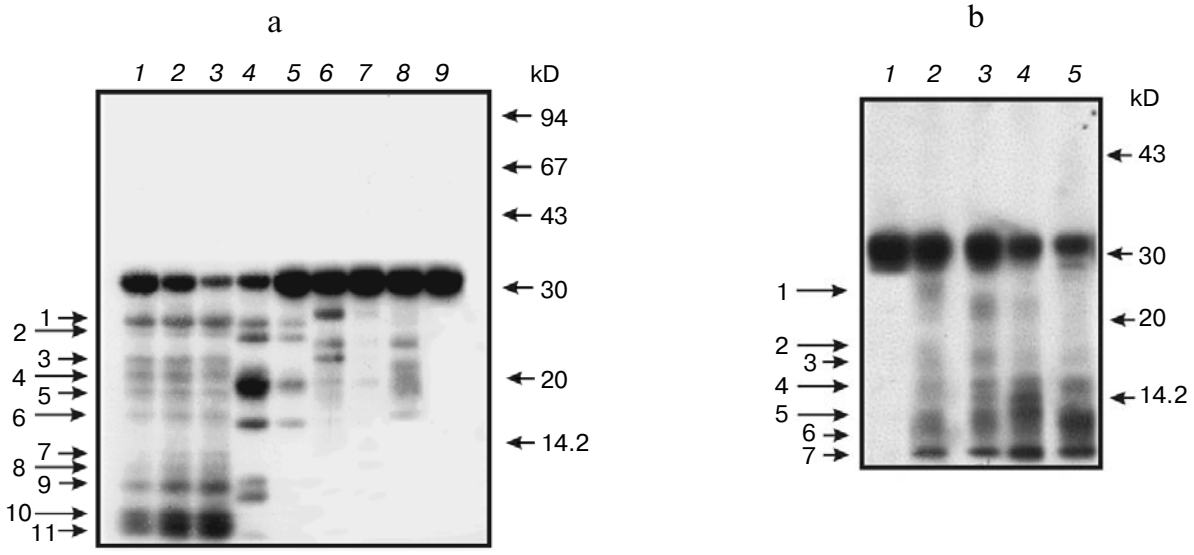

Fig. 7. SDS-PAGE analysis (radioautograph) of β-casein hydrolysis products by several proteases and sIgA from the milk of healthy maternity patients (a), as well as IgG from the blood of AIDS patients (b). a) Lanes: *1-3*) ^{32}P-labeled β-casein was incubated with sIgA during 20, 40, and 60 min, respectively; *4*, *5*) incubation with trypsin for 10 and 15 min; *6*, *7*) incubation with proteinase K for 10 and 15 min, respectively; *8*) incubation with chymotrypsin for 10 min; *9*) incubation of β-casein for 60 min in the absence of enzymes. The location of 11 major products of casein hydrolysis by sIgA from human milk is shown on the left. b) Lanes: *1*) incubation of β-casein in the absence of Ab; *2*) incubation with IgG from the first AIDS patient for 14 h; *3*, *4*) incubation with IgG from the second patient for 7 and 14 h, respectively; *5*) incubation with IgG from the third patient for 14 h. Arrows on the left show the location of seven major products of casein hydrolysis by the first IgG preparation (*2*). Apparent molecular weights of the main hydrolysis products in the case of all Ab preparations (see the text) were calculated according to the data of several experiments with the use of ^{32}P-labeled and non-labeled casein preparations in the case of each Ab preparation.

with molecular weights of 25.7, 19.7, 18.3, 14.5, 12.5, and 7.6 kD (lanes *3* and *4*), and in the third case four major polypeptides: 15.5, 13.2, 10.3, and 7.6 kD (lane *5*). These products of Ab-dependent hydrolysis do not completely match those for proteases, as well as for control sIgA preparations from human milk (for comparison see Figs. 7a and 7b).

It should be noted that all investigated sIgA preparations from human milk demonstrated similar patterns of β-casein hydrolysis [56]. At the same time, studies of IgG from the blood of AIDS patients demonstrated three different types of patterns for casein hydrolysis, which were dissimilar to that of Abs from human milk. So, in the case of preparations 2 and 3 (lanes *4* and *5*), the band corresponding to molecular weight of 22.4 kD was completely absent. In the case of preparation 3, no major polypeptide bands with molecular weights from 22.4 to 16.3 kD were found, even at low level of casein hydrolysis (data not shown). The common product for all the Ab preparations was a polypeptide with molecular weight of 7.6 kD. However, electrophoretic mobility of this polypeptide is very high, and corresponds to the mobility of a large number of different short polypeptides under experimental electrophoretic conditions. Taking this into account, it is most probable that this zone of the gel (7.6 kD) contains polypeptides that are different in molecular weight but are not effectively separated.

The characteristic feature of casein hydrolysis by IgG from the blood of AIDS patients (Fig. 7b) compared to hydrolysis by Abs from human milk (Fig. 7a) is smeared polypeptide bands. This may be associated with the fact that polyclonal sIgA from milk of each donor contain (besides the major abzyme fractions) minor Ab subfractions, which cleave casein at other amino acid sites than the major fractions, thus resulting in the emergence of other polypeptide bands with similar molecular weights, and, as a consequence, in a certain band smearing. It is noteworthy that casein hydrolysis patterns for five investigated IgG preparations from other AIDS patients were comparable with the three mentioned above, or resulted from a certain kind of superposition of those. These data indicate the possibility of substantial differences in the production of casein-hydrolyzing abzymes in healthy maternity patients and AIDS patients.

It should be mentioned that the determination of exact molecular weights of the products of Ab- and protease-dependent casein hydrolysis (and, as a consequence, amino acid sequences of the protein cleavage sites) is difficult at the present time. This is due to the primary structure of β-casein from human milk, which contains 212 amino acid residues and should have a molecular weight of ~24 kD [34]. However, for unknown reason, casein electrophoretic mobility on SDS-PAGE corresponds to apparent molecular weight of 33.6 kD.

Considering this, it does not appear to be possible to establish the relationship between the apparent and actual molecular weights of β-casein and its hydrolysis products.

Thus, we have demonstrated that IgG antibodies from the blood of AIDS patients display the highest activity in hydrolysis of HSA, β-casein from human and bovine milk as well as HIV RT. It is interesting that the repertoire of these abzymes (and also in the case of other investigated enzyme activities displayed by abzymes from AID patients [5, 7, 11-13, 16, 17, 20, 23, 38, 39, 47-54]) depends dramatically on the individual patients. Obviously, it should not be surprising, as it is known that the human immune system is capable of producing up to ~10^6 antibody variants against the same antigen [65], which can be realized at various extents and according to different pathways in the case of each particular patient. Affinity chromatography data regarding homogeneous IgG preparations on casein-Sepharose provide evidence about relatively low Ab affinity towards casein and possible casein interactions not only with casein-hydrolyzing Ab, but also with Ab possessing DNase and RT-hydrolyzing activities (Fig. 4). As it is known, polyreactive Abs produced by B-1 cells are normally present in the body [66]. During a number of viral infections (vesicular stomatitis, hepatitis B, measles, polyvirus infection), the initial stages of the immune response are T-independent, and, probably, occur with the involvement of B-1 cells. About 50% of total serum IgM and IgA, as well as some amount of serum IgG are synthesized by these very cells. Abs produced by B-1 cells react with single strand DNA, cytoskeleton proteins, bromelated erythrocytes, as well as with bacterial poly- and liposaccharides [66-68]. It should be noted that the titer of such Ab increases dramatically during several AID (SLE, rheumatoid arthritis) [69, 70]. Reaction against bacterial antigens defines the protective role of IgM, produced by B-1 cells. As low affinity Ab against several high molecular weight polyanions are able to cross react with various antigens, nonspecific binding of different abzymes observed upon affinity chromatography of Ab on casein-Sepharose may be a consequence of polyreactive nature of Ab from AIDS patients.

The distinctive feature of previously described abzymes from the blood of AID patients is their unusual (different from the classical enzymes) specificity towards the substrate. Thus, RNA-hydrolyzing antibodies upon AID hydrolyze ribooligoadenylates with high efficiency [47], which makes them substantially different from RNase A and human RNases [10, 47]. Different nature of DNase activity of DNase I and DNase II on one hand, and IgG and IgM abzymes from AID patients on the other hand is demonstrated in a number of investigations [71, 72]. Specificity of serine protease-type abzymes from the blood of bronchial asthma patients is different from the specificity of classical serine proteases [9, 73, 74]. Polyclonal abzymes from the blood of one of two asthma patients cleaved six peptide bonds, localized in the region between 14-22 amino acid residues of vasoactive neuropeptide, whereas Ab from the second donor cleaved one more peptide bond between 7-8 amino acid residues of this oligopeptide. These abzymes were specific towards the substrate and did not cleave other proteins and oligopeptides. IgM antibodies of AIDS patients cleaved HIV gp120 protein of virus coat also at several sites [75]. Approaches to the induction of abzymes with proteolytic activity in autoimmune animals using several proteins as well as antiidiotypic antibodies are reviewed by Gabibov et al. [76].

Abzymes from the blood of AIDS patients hydrolyze RT and HSA, as well as human and bovine caseins, whereas sIgA from the milk of maternity patients cleave casein only. This indicated that the immune response of AIDS patients to the blood proteins can be substantially expanded. Nevertheless, high activity of IgG from the blood of AIDS patients in hydrolysis of β-casein (which is not a typical component of human blood) is unexpected. However, it was recently shown by 2D electrophoresis that six of nine sera from AIDS patients contained antibodies against casein, and five against human milk lactalbumin [77]. Thereby, the activation of β-casein synthesis in AIDS patients driven by not yet understood factor cannot be excluded. It is interesting that mRNA corresponding to the gene encoding for β-casein is produced in mouse T-killer cells (also for unknown reason) [78]. In this way, it can not be excluded that genes, encoding for β-casein, as well as this protein itself, can play a special (but not yet known) role in the virus life cycle, its replication, or development of autoimmune reactions in AIDS patients.

This work was supported by the Program of Fundamental Research of the Presidium of Russian Academy of Sciences (Molecular and Cellular Biology, No. 10.5, and Fundamental Sciences for Medicine, No. 11.9), as well as by RFBR (grant No. 04-04-48211) and BFBR (grant No. 04-04-81017).

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