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Natural Catalytic Antibodies
in Norm and in HIV-Infected Patients

Georgy A. Nevinsky
Institute of Chemical Biology and Fundamental Medicine of SD of RAS
Russia

1. Introduction

Antibodies (Abs) have been first characterized as proteins which are produced by the immune system and have a sole function of binding other molecules, called antigens, with the goal of eliciting an immune response. In this classical conception, Abs act similarly to enzymes in specific binding other molecules. However, in contrast to enzymes they do not have the ability to catalyze chemical conversions of their bound partners. For the vast majority of Abs, this observation is correct. However, in a 1946 consideration of enzyme function, Linus Pauling first hypothesized that the active center of an enzyme is closely juxtaposed to a "strained configuration" of its substrate (that is, targeted against the structure of the transition state) rather than to the native conformation of the substrate molecule (Pauling, 1946). This idea led Jencks in 1969 to propose that Abs generated in an anti-hapten immune response against chemically stable analogs of the transition-state of a reaction of interest could potentially display an enzymatic activity (Jencks, 1969).

In 1985, a general method for generating catalytic monoclonal Abzs against transition state analogs, and a way to use those Abs to accelerate chemical reactions, was first described (Schochetman & Massey, 1985). One year later two groups were able to produce the first monoclonal Abs with catalytic properties, which were generated against hapten analogs of the transition states for *p*-nitrophenylphosphorylcholine (Pollack et al., 1986) or for monoaryl phosphonate esters (Tramontano et al., 1986a, 1986b). The artificial monoclonal anti-hapten catalytic Abs were termed abzymes (derived from **a**nti**b**ody en**zyme**).

The evolution of the technology of artificial Abzs during the last ~two decades has led not only to the rapid development of direct approaches for the generation of Abs with specified properties, but also to the creation of strategies to revise the targeting specificity of individual Abzs. Such modifications of antigen binding specificity can be achieved genetically *in vitro*, by application of the site-directed mutagenesis, or genetic selection or screening (using approaches such as phage display). Alternatively, modification can be induced directly on purified antibody, *via* selective chemical modification by direct introduction of catalytic groups into the Ab combining site. Some studies describing these approaches include (Keinan, 2005, and refs therein). The employment of the approaches have demonstrated that the substrate specificity (and/or the specific activity) of some artificial Abzs is comparable to or even higher than that of enzymes with the same catalytic activity (Barbas et al., 1997; Janda et al., 1997; Keinan, 2005, and refs therein).

Artificial Abzs against transition chemical states of different reactions have been studied intensively (Thayer et al., 1999). Mechanistic basis for the activity of such Abzs is becoming well understood (Janda et al., 1997; Thayer et al., 1999; Keinan, 2005, and refs therein). The field of artificial Abzs has been amply reviewed recently (Martin & Schultz, 1999; Suzuki, 1994; Keinan, 2005, and refs therein), for more detailed description of the relevant reactions.

During last two decades it has become clear that auto-Abs from the sera of patients with different autoimmune (AI) diseases can possess enzymic activities (Suzuki, 1994; Keinan, 2005; Nevinsky et al., 2002a, 2002b, 2003, 2005, 2010a, 2010b, and refs therein). The first example of a natural Abz was an IgG found in bronchial asthma patients, which hydrolyzes intestinal vasoactive peptide (VIP) (Paul et al., 1989), the second was an IgG with DNase activity in SLE (Shuster, et al., 1992), and the third was an IgG with RNase activity in SLE (Buneva et al., 1994). Later, different natural catalytic IgG and/or IgA, IgM hydrolyzing oligopeptides, proteins, DNA, RNA, nucleotides, and polysaccharides were detected in the sera of patients with several autoimmune (AI) and viral pathologies, and Abzs with these and other different activities were discovered in healthy human milk (for review see Nevinsky et al., 2002a, 2002b, 2003, 2005, 2010a, 2010b).

The phenomenon of catalysis by auto-Abzs is extremely interesting and potentially applicable in many different fields including new types of efficient catalysts, new generation of drugs, evaluation of the functional roles of Abzs in innate and adaptive immunity, and understanding of certain aspects of self-tolerance and of the destructive or positive responses in AI diseases. The field of monoclonal Abzs with immunotherapeutic potential has recently been reviewed (Wentworth et al., 1998; Tellier, 2002; Zhou et al., 2002; Nishi, 2003; Stockwin and Holmes, 2003; Hanson et al., 2005; Gabibov et al., 2006; Planque et al., 2008; Wójcik & Kieć-Kononowicz, 2008). Some general possibilities of present and future therapeutic Abs and Abzs application were discussed in (Stockwin & Holmes, 2003). Abs and Abzs can be used to neutralize pathogens, toxins and endogenous mediators of pathology. As cell-targeting reagents, Abs can be used to modulate cytoplasmic cascades or to tag specific cells for complement- or effector-mediated lysis. Abs can also be modified to deliver toxic or modulatory payloads (small molecules, radionuclides and enzymes) and engineered to bind multiple epitopes or even to have novel catalytic activity. The modular structure of Igs and the availability of Ab fragment libraries also make it possible to produce variable-domain therapeutics (Fab, single-chain and domain of Abs). Although exhibiting less favorable kinetics *in vivo*, these fragments are straightforward to express and easily penetrate tissues, making them especially useful as neutralizing or delivery agents. The number of approved Abzs is expected to increase in the near term, as the platform is adopted as a viable alternative to small molecule discovery (Stockwin & Holmes, 2003). The Abzs strategy can be employed for new methods of drug synthesis, as well as for in vivo therapies. Catalytic antibodies seem to be a promising tool for therapeutic purposes, because of their specificity and stereoselectivity. For instance, cocaine-hydrolyzing Abzs have been developed, and may provide a novel approach to the problems of drug addiction (Hanson et al., 2005). Possible application of Abzs for prodrug activation and their potential utility in clinical oncology was also discussed (Nishi, 2003). Abzs have two distinct advantages over canonical enzymes: first, they can be selected to perform reactions not catalyzed by endogenous enzymes, and second, they can be humanized to minimize their immunogenicity (Stockwin & Holmes, 2003).

2. Features of the immune status of patients with AIDS, bacterial, and autoimmune diseases

HIV-1 is the etiologic agent of an extremely dangerous human disease, AIDS (Fauci et al., 2008, and refs therein). The association of immune dysfunction in patients with HIV infection and AIDS and the development of AI diseases are very interesting. At this moment the spectrum of reported autoimmune phenomena in AIDS patients is increasing (for review see Zandman-Goddard & Shoenfeld, 2002). A special feature of ADs is high concentrations of auto-Abs (Abs to many different endogenous antigens) (Zouali, 2001; Pisetsky, 2001). The development of AI diseases is characterized by spontaneous generation of primary Abs to proteins, nucleic acids and their complexes, polysaccharides, nucleotides etc. (Earnshaw & Rothfield, 1985; Raptis & Menard, 1980). Later the secondary idiotypic and then antiidiotypic Abs to the primary ones are usually generated, etc. Immunization of animals with DNA or RNA and especially their complexes with proteins leads to the production of anti-DNA and anti-RNA Abs (Gottieb & Shwartz, 1972; Mitsuhashi et al., 1978).

During frank loss of immunocompetence, AI diseases that are predominantly T cell subtype CD8 driven predominate. There is evidence for B cell stimulation and many auto-Abs are reported in HIV patients. HIV-dependent activation of B lymphocytes leads to the production of auto-Abs not only to different viral proteins including HIV reverse transcriptase (RT) and integrase (IN), but also to human cell components, and various immune complexes including anti-cardiolipin, anti-beta2 GPI, anti-DNA, anti-small nuclear ribonucleoproteins, anti-thyroglobulin, anti-thyroid peroxidase, anti-myosin, and anti-erythropoietin and possibly other human cell and blood components (Fauci et al., 2008; Zandman-Goddard & Shoenfeld, 2002). The list of reported autoimmune diseases in HIV/AIDS includes SLE, anti-phospholipid syndrome, vasculitis, primary biliary cirrhosis, polymyosits, Graves' disease, and idiopathic thrombocytopenic purpura (Zandman-Goddard & Shoenfeld, 2002). The presence of AI phenomena and production of auto-Abs in chronic bacterial and viral infections including HIV could be related to molecular mimicry between microbial or viral and host antigens (Zandman-Goddard & Shoenfeld, 2002; Hentati et al., 1994; Ternynck et al., 1991), altered self, abnormal expression of immunoregulatory molecules, and the anti-idiotypic network (Barzilai et al., 2008).

Interestingly, some other viral infections similarly to ADs can also demonstrate AI reactions leading to the formation of Abs to various human and viral antigens. Compared with healthy donors, concentrations of DNA and anti-DNA Abs are higher not only in patients with SLE (36% of SLE patients), but also in multiple sclerosis (17-18%), primary Sjogren's syndrome (18%), Hashimoto's thyroiditis (23%), myasthenia gravis (6%), rheumatoid arthritis (7%), autoimmune hepatitis (Shoenfeld et al., 1988, 1989), and also in lymphoproliferative (Kozyr et al., 1998) and some viral diseases including viral hepatitis, AIDS (Gololobov et al., 1994), and TBE (Garmashova et al., 2004). In the sera of patients with several AI diseases, RNA and anti-RNA Abs were also detected (Blanco et al., 1991; Sato et al., 1994; Hirokawa et al., 2002; Ikeda et al., 2003).

AIDS, TBE, and hepatitis demonstrating strong reorganization of immune system have some similarities with typical AI diseases such as SLE (HT, and others) which is a systemic AI polyetiologic diffuse disease that is characterized by disorganization of conjunctive tissues with the paramount damage to skin and visceral capillaries (Hhachn, 1996). All known AI and viral diseases like AIDS, viral hepatitis, and TBE are characterized by significant disturb the immune status of the patients accompanied by humoral and cellular

AI reactions, with detectable tissue-specific and organ-nonspecific Abs (Bigazzi, 1983; Sugiama & Yamamota, 1996; Nevinsky, 2010b). At the same time, microbial and viral infections expose the human organism to different components from the parasite's cells and viral particles, including protein, DNA, RNA, lipids, and polysaccharides. The sera of mice infected with different microbe's bacterial pathogens contain a variety of Abs to the parasite's antigens and to human lipids, proteins, and nuclear components, including anti-DNA Abs (Ternynck et al., 1991; Unterkircher et al., 1993; Matsiota-Bernard et al., 1993, 1996; Hentati et al., 1994; Boekel et al., 1997; Wun et al., 2001). The origin of anti-DNA Abs in the infections remains speculative; some of them may arise inadvertently in the course of a normal immune response due to the induction by Abs that bear structures (mimotopes) mimicking DNA (Wun et al., 2001). The immunoregulatory effect of the infection seems to be related, at least partially, to the increase in a particular population of Abs, the polyreactive Abs (Matsiota-Bernard et al., 1996). It has been proposed that bacterial and viral infectious agents can act in some cases through the mechanism of molecular mimicry and stimulate development of different AI diseases. For instance, the agents responsible for molecular mimicry in multiple sclerosis include measles, hepatitis B, herpes simplex, influenza, papilloma, and Epstein-Bar viruses (Steinman, 2001). Thus, AI reactions in different AI, viral and bacterial infection diseases are very similar and may be strongly associated.

3. The origin of artificial and natural abzymes

Artificial Abzs can be obtained by immunization of animals with chemically stable analogs of transition states of chemical reactions (reviewed in Martin & Schultz, 1999; Nevinsky et al., 2000a; Tanaka, 2002; Tanaka and Barbas, 2002; Dias et al., 2002; Keinan, 2005). On the other hand, artificial antiidiotypic Abs can also possess catalytic activity (Barbas et al., 1997; Wentworth et al., 1998). Building on earlier observations on the existence of idiotypic determinants related to the antigen, Jerne proposed that the immune system is self-regulated by a network of idiotype–anti-idiotype interactions (Jerne, 1974). The simplified model of this network may be schematically presented as follows:
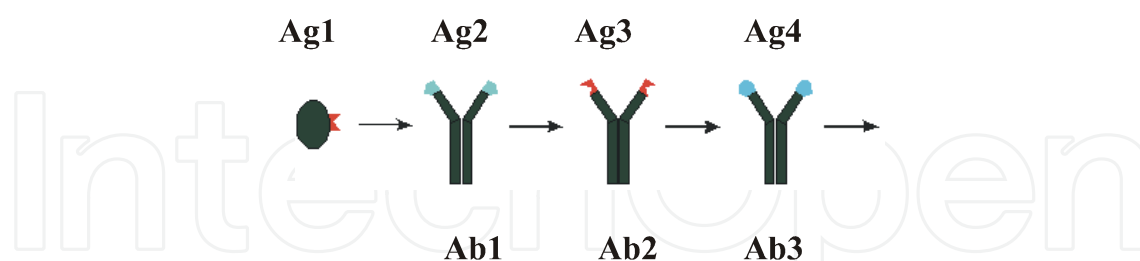
Antibodies 1 and 2 are termed idiotype and anti-idiotype, respectively, etc. There is convincing evidence that such idiotype–anti-idiotype networks are actually present in the body. The presence of blood serum Ab4 (in the notation shown in the scheme) has been confirmed in experimental animals (Jerne, 1974).

If the active site of an enzyme plays the role of antigen triggering this anti-idiotypic chain, it is logical to suggest that the secondary anti-idiotypic Ab2 may possess the structure, a part of which represents an "internal image" or "mould" of the active site of this enzyme, and, consequently, these Abs may possess some properties of this enzyme. This remarkable property of idiotypic mimicry has been exploited to raise monoclonal antiidiotypic Abzs with several different catalytic activities (reviewed in Keinan, 2005; Nevinsky et al., 2005).

The origin of natural Abzs in different AI, viral and bacterial diseases may be complex. Similarly to artificial Abzs against analogs of transition states of catalytic reactions, naturally occurring Abzs with DNase and RNase activities may be Abs raised directly against free DNA and RNA or these nucleic acids acting as haptens bound to different proteins and resembling transition states of catalytic reactions (Nevinsky et al., 2003, 2005, 2010a, 2010b, and refs therein). Immunization of rabbits with pure DNA and RNA generated Abs interacting with DNA and possessing weak DNase and RNase activities (Krasnorutskii et al., 2008a, 2008b). Many SLE anti-DNA Abs are directed against histone-DNA nucleosomal complexes appearing as a result of internucleosomal cleavage during apoptosis (Founel & Muller, 2002). Apoptotic cells are the primary source of antigens and immunogens in SLE, and certain features in recognition, processing, and/or presentation of apoptotic auto-antigens by antigen-presenting cells can trigger AI processes (Founel & Muller, 2002). Anti-DNA–protein and anti-RNA–protein complexes and other antinuclear antibodies were found in the sera of patients with multisystem connective tissue disease (Gottieb & Shwartz, 1972). Therefore, we have emulated such natural complexes using complexes of DNA and RNA with methylated bovine serum albumin (mBSA). Immunization of rabbits with complex of DNA and RNA with mBSA elicited production of 10-50-fold more active DNase and RNase IgGs, while pIgGs from animals immunized with mBSA were catalytically inactive (Krasnorutskii et al., 2008a, 2008b). Immunization of healthy rabbits with DNase I, DNase II, and pancreatic RNase A also produced anti-idiotypic IgGs with intrinsic DNase and RNase activities (Krasnorutskii et al., 2008c, 2008d, 2009). Thus, DNase and RNase Abzs in different AI diseases may be a cocktail of Abs against complexes of DNA and RNA with proteins and antiidiotypic Abzs to very different DNA- and RNA-hydrolyzing enzymes.

Healthy humans and patients with many diseases with insignificant AI reactions usually lack Abzs or develop Abzs with very low catalytic activities, often on a borderline of the sensitivity of detection methods (Nevinsky et al., 2002a, 2002b, 2003, 2005, 2010a, 2010b, and refs therein). Natural Abzs hydrolyzing DNA and RNA are described from the sera of patients with several AI (SLE; Shuster, et al., 1992; Buneva et al., 1994; Andrievskaya et al., 2000, 2002; Vlassov et al., 1998), Hashimoto's thyroiditis and polyarthritis (Vlasov et al., 1998), multiple sclerosis (MS) (Baranovskii et., 1998, 2001; Nevinsky et al., 2001), asthma (Galvita et al., 2007), and viral and bacterial diseases: viral hepatitis (Baranovskii et al., 1997; Vlasov et al., 1999), tick bone encephalitis (TBE; Parkhomenko et al., 2010), AIDS (Odintsova et al., 2006a), and several diseases caused by different bacterial infections (Parkhomenko et al., 2009). It was shown, that like in the case of AI-patients, IgGs with DNase activity from autoimmune mice are the earliest and statistically significant markers of pathology and these activities are detectable at the pre-disease stage, when there are no visible markers of SLE pathology or significant proteinuria, and anti-DNA titres are within the typical ranges of these indicators for healthy mice (Dubrovskaya et al., 2003; Andryushkova et al., 2007; Kuznetsova et al., 2007).

Using different approaches convincing evidence was provided that, similarly to Abzs from SLE and MS patients (Savel'ev et al., 2003; Ivanen et al., 2002, 2004), amylase activity is intrinsic to autoimmune mouse polyclonal IgGs (Andryushkova et al., 2006, 2007). It was shown that the relative activities of IgGs from MRL-lpr/lpr mice in the hydrolysis of DNA, ATP, and polysaccharides correlate very well with some visible (pink spots, baldness of the head and parts of the back, general health deterioration, etc.) and biochemical (proteinuria, Ab titers to native and denatured DNA) markers of AI pathologies during various stages of

mouse SLE (Andryushkova et al., 2006, 2007, 2009). Similarly to Abzs with DNase and RNase activities, catalytic Abs with polysaccharide-hydrolysing activity can be Abs directly against polysaccharides and their complexes with proteins and enzymes and second antiidiotypic Igs against different enzymes hydrolyzing polysaccharides (Andryushkova et al., 2006, 2007, 2009; Nevinsky et al., 2005, 2010a, 2010b).

IgGs and/or IgMs and IgAs hydrolyzing different peptides and proteins were also found in AI and other diseases: vasoactive intestinal peptide (VIP) in asthma (Paul et al., 1989), thyroglobulin in HT and rheumatoid arthritis (Li et al., 1995; Kalaga et al., 1995), prothrombin in multiple myeloma (Thiagarajan et al., 2000), protein factor VIII in haemophilia A (Lacroix-Desmazes et al., 1999), and myelin basic protein (MBP) in MS (Polosukhina et al., 2004, 2005, 2006, 2006; Legostaeva et al., 2010). Some healthy humans produce Abzs with low VIP- (Paul et al., 1989), and thyroglobulin-hydrolyzing activities (Kalaga et al., 1995), but usually healthy volunteers and patients with many diseases with insignificant autoimmune reactions lack Abzs with proteolytic activity (Nevinsky et al., 2002a, 2002b, 2003, 2005, 2010a, 2010b, and refs therein). Since immunization of AI mice results in a dramatically higher incidence of Abzs with a higher activity than in conventionally used normal mouse strains (Tawfik et al., 2002; Nishi, 2002), the formation of Abzs in AI and some viral diseases may be much more profuse. The question is why autoimmunization of AI patients and mice results in a dramatically higher incidence of catalytically inactive Abs and Abzs with enzyme properties as compared with healthy humans and animals.

MRL-lpr/lpr mice spontaneously developing a SLE-like disorder are a very promising model to study the mechanisms of natural Abzs generation and their role in the pathogenesis of pronounced AI disturbances. A mutation in the *lpr* gene of these mice leads to a deficit in functional Fas ligand and dysregulation of apoptosis in homozygotes (Watanabe-Fukunada et al., 1992; Nagata & Suda, 1995). As a result, the mice develop SLE-like phenotype, including accumulation of double-negative T cells (CD4- CD8- B220+ TCR+) in the peripheral lymphoid organs.

Recently we have carried out the first analysis of possible correlations between the relative activities (RAs) of mouse IgGs in the hydrolysis of DNA, ATP, and polysaccharides with several clinical and biochemical markers of AI pathologies (proteinuria, Ab titers to native and denatured DNA) at various stages of mouse SLE (Andryushkova et al., 2006, 2007, 2009). An ever-growing number of observations suggested that AI diseases may originate from defects in hematopoietic stem cells (Ikehara et al., 1990). Therefore, lymphocyte proliferation and apoptosis at different stages of the AI disorder development in MRL-lpr/lpr mice were also studied. It was shown that IgGs from the sera of 2-7 month-old control non-autoimmune (CBAxC57BL)F1 and BALB/c mice and 2-3 months-old MRL-lpr/lpr mice (conditionally healthy mice) are catalytically inactive (Andryushkova et al., 2006, 2007, 2009). During spontaneous development of deep SLE-like pathology a specific reorganization of immune system of these mice leads to conditions associated with a production of IgGs hydrolyzing DNA, ATP, and polysaccharides with low catalytic activities (conditionally pre-diseased mice) (Andryushkova et al., 2006, 2007, 2009). First significant changes in differentiation and proliferation of mice bone marrow hematopoietic stem cells (HSC; granulocytic-macrophagic colony-forming unit; erythroid burst-forming unit, and granulocytic-erythroid-megacaryocytic-macrophagic colony-forming unit) in pre-diseased in comparison with healthy mice are most likely only temporary, since a transition from the pre-diseased to diseased mice is associated not only with an increase in the RAs of different Abzs and

proteinuria, but also with a significant additional change in the profile of HSC differentiation. This change seems to be the most important factor in the irreversible switching of the mouse immune system to an AI mode, since the changes in cell proliferation and apoptosis in different organs occur mainly on transition from healthy to pre-diseased mice and the observed differences in these indices between pre-diseased and diseased mice are insignificant (Andryushkova et al., 2006, 2007, 2009). Immunization of healthy young AI mice leads to the highest increase in urine protein, titers of anti-DNA Abs as well as DNase, amylase and ATPase Abz activities, occurring in parallel with a significant decrease in apoptosis, especially in bone marrow, thymus and spleen. However, the profile of HSC differentiation in immunized mice is quite different from the pre-diseased and spontaneously diseased mice, but comparable with that for young healthy animals. It was shown, that in contrast to spontaneously diseased AI mice, immunization with DNA does not remarkably affect bone marrow stem cells; the increased levels of anti-DNA Abs and Abzs in immunized mice may be mainly provided by an activation of lymphocyte differentiation and proliferation in different organs, first of all in the spleen, with a concomitant decrease in apoptosis. A significant decrease in apoptosis in the immunized mice may be an important factor providing the increased number of specific lymphocytes producing auto-Abs and Abzs, which are normally eliminated. Very high urine protein concentration and visible markers of SLE demonstrated by the immunized mice may be a result of kidney and spleen dysfunction (Andryushkova et al., 2006, 2007, 2009). Overall, in contrast to immunization of healthy mice an appearance of Abzs and increase in their activity is associated with changes in differentiation and proliferation of mice bone marrow HSC. At the onset of AI diseases (pre-disease condition), Abs are usually contain catalytic Abzs produced by a single clone, or at least a relatively narrow repertoire of Abzs with relatively low relative activities. In the course of chronic AI pathology development, the repertoire of Abzs constantly widens and Abs with significantly higher RAs can be found. In addition, the number of Abzs with high RAs usually increases during exacerbation of AI pathologies (Nevinsky et al., 2003, 2005, 2010[a], 2010[b], and refs therein). It should be mentioned, that the detection of Abzs was shown to be the earliest and statistically significant indicator of development of different autoimmune diseases in humans (Nevinsky et al., 2003, 2005, 2010[a], 2010[b], and refs therein) and animals (Andryushkova et al., 2006, 2007, 2009).

4. Catalytic antibodies of HIV-infected patients

4.1 Purification of natural abzymes

Natural Abzs from sera of patients are usually polyclonal in origin and are products of different immuno-competent cells (Nevinsky et al., 2000[b], 2002[a], 2002[b], 2003, 2005, 2010[a], 2010[b], and refs therein). Natural Abz purification is one of the most complicated aspects of their study; it was discussed in detail in review (Nevinsky et al., 2000[b]).

In study Abzs from the sera of HIV-infected patients, electrophoretically and immunologically homogeneous AIDS Ab fraction (pIgG+pIgM+pIgA) was first purified by chromatography of the serum proteins on Protein A-Sepharose under conditions that remove non-specifically bound proteins (Odintsova et al., 2006[a], 2006[b]; Baranova et al., 2009, 2010). pIgMs were separated from pIgAs and pIgGs by FPLC gel filtration of the total Ab fraction on a Superdex 200 (Baranova et al., 2009, 2010). The homogeneity of the 150 kDa IgG was confirmed by SDS-PAGE with silver staining, which showed a single band under nonreducing conditions and two bands corresponding to the H and L chains after reduction

(Fig. 1). Since IgM has a very high molecular mass (~970 kDa), it cannot enter SDS-PAGE gels under nonreducing conditions (Fig. 1A, lane 2). Two bands corresponding to the H and L chains of pIgMs were evident after Ab reduction (Fig. 1B, lane 1). The absence of any protein bands in the gel corresponding to pIgMs under nonreducing conditions (Fig. 1A, lane 2) and the presence of only two bands corresponding to the heavy and light chains under reducing conditions (Fig. 1B, lane 1) demonstrates the absence of protein contaminations in the pIgM preparations.
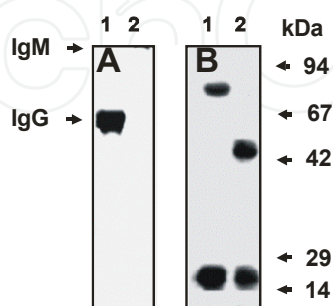
Fig. 1. SDS-PAGE in a nonreducing 4–15% gradient gel followed by silver staining of control pIgGs (lane 1) and pIgMs (lane 2) after affinity chromatography on protein A-Sepharose and FPLC gel filtration on a Superdex 200 column (A). SDS-PAGE of pIgGs (lane 2) and pIgMs (lane 1) in a reducing 12% gel (B).

4.2 Criteria to establish that catalytic activity is intrinsic to antibodies

The application of rigid criteria allowed the authors of the first article concerning natural Abzs (Paul et al., 1989) to conclude that VIP-hydrolyzing activity is an intrinsic property of Abs from the sera of patients with asthma. Later several additional criteria were proposed (for review see Nevinsky et al., 2000a, 2002a, 2002b, 2003, 2005).

It was shown that non-fractionated on affinity sorbents bearing immobilized DNA or specific protein substrates, pIgGs and pIgMs from HIV infected patients effectively hydrolyze DNA (Odintsova et. al., 2006a), HIV-1 RT, human casein, human serum albumin (HAS; Odintsova et. al., 2006b), and HIV-1 IN (Baranova et. al., 2009, 2010) but not many other tested proteins. We applied a set of strict criteria worked out previously (Paul et al., 1989, Nevinsky et al., 2000a, 2002a) for an analysis of DNase and proteolytic activity as an intrinsic property of AIDS IgGs and/or IgMs. The most important of these are: i) electrophoretic homogeneity of pIgGs and pIgMs (Fig. 1); ii) the complete absorption of AIDS IgGs and IgMs with the DNase or proteolytic activities by Sepharose bearing immobilized anti-light chain of human Abs leading to a disappearance of the activity from the solution and recover following its elution with an acidic buffer (pH 2.6); iii) FPLC gel filtration of IgGs using an acidic buffer (pH 2.6) did not lead to a disappearance of the activity, which tracked exactly with IgGs or IgMs. The fulfilment of these criteria was observed for Abzs with all activities mentioned above (Odintsova et. al., 2006a, 2006b; Baranova et. al., 2009, 2010).

To exclude possible artefacts due to hypothetical traces of contaminating enzymes, pIgGs were subjected to SDS-PAGE in a gel co-polymerized with calf thymus DNA, and their DNase activity was detected by incubating the gel in the standard reaction buffer (Fig. 2). Ethidium bromide staining of the gels after the electrophoresis and refolding of IgGs revealed sharp dark bands against a fluorescent background of DNA. Fig. 2B demonstrates

a typical example for AIDS IgGs (lane 1); there was no hydrolysis of DNA by control Abs from healthy donors (lane 2). Control human urine (lane 3) and bovine pancreatic DNase I (lane 4) also cleaved DNA, but produced bands in the position well below (33–36 kDa) the intact pIgGs (150 kDa). Since SDS dissociates all protein complexes, the detection of the DNase activity in the gel zone corresponding only to intact IgGs together with the absence of any other activity band or protein band (Fig. 2B), provides direct evidence that AIDS pIgGs hydrolyze DNA and are not contaminated by canonical DNases. In addition, after incubation of IgGs with DTT only light chains of AIDS IgGs demonstrated DNase activity (Fig. 2C).
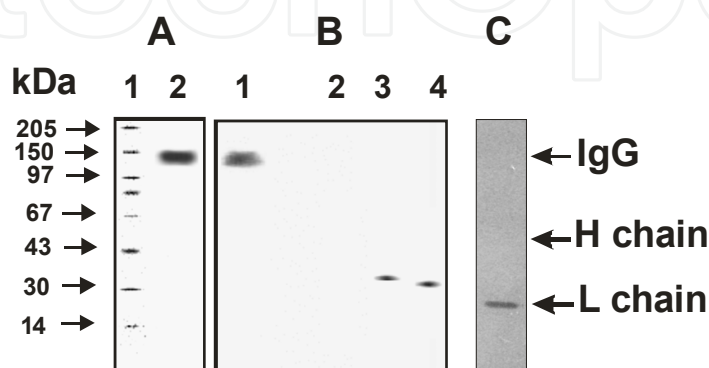

Fig. 2. *In situ* SDS-PAGE analysis of DNase activity of AIDS intact IgGs (lane 1), healthy humans (lane 2) (B); human urine (lane 3) and bovine pancreatic DNase I (lane 4, B) in nonreducing conditions. IgGs were analyzed in reducing conditions after Abs incubation with DTT (C). After electrophoresis, the gel containing 3 μg/ml thymus DNA (B and C) was incubated under special conditions for protein refolding and hydrolysis of nucleic acids; the nuclease activity was visualized by ethidium bromide staining of the gels (B and C). The longitudinal slices of the same gel were used for Coomassie R250 staining to reveal the positions of IgG bands (lane 2, A) and protein molecular mass markers (lane 1, A).

AIDS IgGs and IgMs were separated by SDS-PAGE respectively under nonreducing and reducing conditions and their proteolytic activity was detected after the extraction of proteins from excised gel slices (Baranova et. al., 2009, 2010). The detection of IN-hydrolyzing activity in the gel region corresponding only to IgG, together with the absence of any other bands of the activity or protein, provided direct evidence that IgG possesses IN-hydrolyzing activity. Similar results were obtained for AIDS IgGs hydrolyzing HIV RT, HSA, and human casein (Odintsova et. al., 2006b).

As mentioned above, pIgMs cannot enter the gel. The absence of IN-hydrolyzing activity from all gel zones corresponding to the intact pIgMs under nonreducing conditions (data not shown), together with hydrolysis of IN only with separated heavy and light chains of IgMs under reducing conditions and the absence of any other bands of the activity (Fig. 3) provides a direct evidence that IgM possesses IN-hydrolyzing activity.

It was shown (Odintsova et. al., 2006b), that in contrast to known different nonspecific proteases hydrolyzing many proteins, AIDS pIgGs non-fractionated by affinity chromatography on Sepharoses bearing specific immobilized proteins efficiently hydrolyze only: human casein > HIV-1 RT > HSA but not many other tested proteins. Later it was shown that AIDS IgGs and IgMs also hydrolyze HIV-1 integrase (for example, Fig. 4) (Baranova et. al., 2009, 2010).
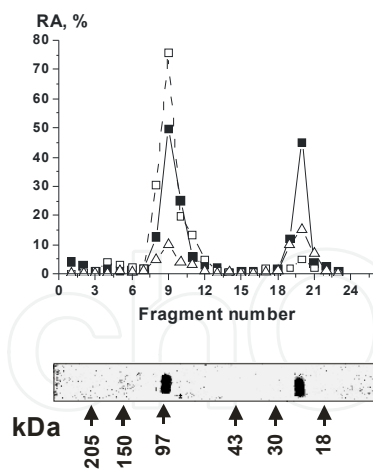
Fig. 3. SDS-PAGE analysis of IN-hydrolyzing activity of AIDS IgMs. After reducing SDS-PAGE of purified AIDS IgMs the gel was incubated under special conditions for renaturation of Abs. The relative IN-hydrolyzing activity (RA, %) was quantified using the extracts of fragments (2–3-mm each) of one longitudinal slice of the gel corresponding to 3 individual IgMs: (■), IgM1; (□), IgM2; (Δ), IgM3.

It was shown that immunogenic VIP (Paul et al., 1989), human MBP (Ponomarenko et al., 2006; Legostaseva at al., 2010), human milk casein (Odintsova et al., 2011), stimulate formation of Abzs which in contrast to canonical proteases efficiently hydrolyze only antigen-proteins, but not many other proteins tested. To analyze the "average" proteolytic activity of AIDS Abzs, two mixtures of equal amounts of electrophoretically homogeneous pIgGs (pIgG$_{mix}$) and pIgMs (pIgM$_{mix}$) with different relatively high and average activities from the sera of seven patients were prepared. After purification of anti-IN Abs on IN-Sepharose these Abzs hydrolyzed only IN and cannot hydrolyze other proteins including viral RT (Fig. 4). In addition, it was shown that AIDS pIgGs and pIgMs after their purification on sorbents bearing immobilized HIV RT (Fig. 4), human casein or HSA specifically hydrolyzed only cognate protein, but not many other proteins including HIV IN
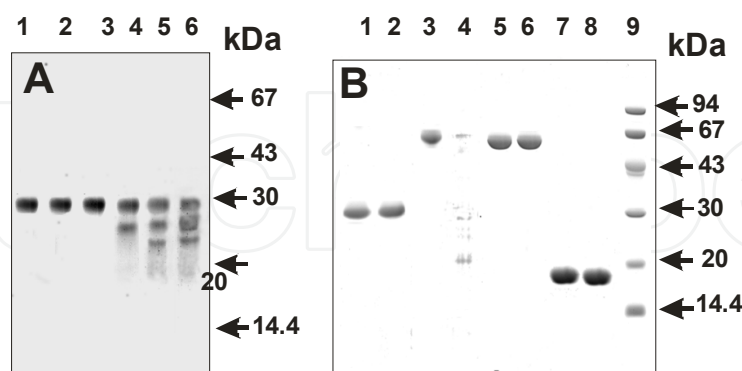

Fig. 4. SDS-PAGE analysis of protein hydrolysis by AIDS IgGs and IgMs. A, HIV-1 IN was incubated for alone (1) or in the presence of pIgGs from two healthy donors (2 and 3) and pIgGs from two different AIDS patients (4 and 5), or with AIDS IgGs purified on IN-Sepharose (6). B, AIDS IgMs purified on RT-Sepharose were incubated with different proteins without (odd numbers) and with Abs (even numbers): HIV-1 IN (1 and 2); p66 HIV RT (3 and 4); HSA (5 and 6); myelin basic protein (7 and 8). Lane 9 corresponds to mixture of standard protein markers with known molecular masses.

(Odintsova et al., 2006[b]). It means that within pools of AIDs pIgGs and pIgMs only specific anti-IN Abzs are able to hydrolyze intact globular molecules of viral integrase (Baranoba et al., 2009, 2010), while specific anti-HSA, anti-casein, and anti-RT IgGs hydrolyze their specific target proteins (Odintsova et. al., 2006[b]).

4.3 Comparison of the relative catalytic activity of Abs from different AIDS patients

Sera of ten healthy donors, 110 HIV-infected patients (18–40-years-old; men and women) including 65 at the stage of pre-AIDS and 45 at the stage of generalized lymphadenopathy (GL) according to the classification of the Center of Disease Control and Prevention were used to analyze the catalytic activities of IgGs and IgMs (Odintsova et al., 2006[a], 2006[b]; Baranova et al., 2009, 2010). Patients with pre-AIDS stage were characterized by a decrease in their body mass up to 10%, fungal, bacterial and viral lesions of skin and mucosal surfaces, shingles, repeating pharyngitis, sinusitis, otitis, and frequent acute respiratory infection.

Polyclonal IgGs from 10 healthy controls were inactive in DNA hydrolysis (Odintsova et al., 2006[a]). Similar results were obtained earlier for several groups of 10-20 healthy humans used as controls in the studies of DNase Abs from the sera of patients with AI diseases (Nevinsky et al., 2003, 2005, 2010[a], 2010[b] and refs therein).The type of plasmid supercoiled (sc) DNA hydrolysis by AIDS pIgGs did not depend on the Ab concentration and the rate of the hydrolysis linearly increased with the increase in IgG concentration and time of incubation (Odintsova et al., 2006[a]). Fig. 5 illustrates a cleavage of plasmid DNA (14 μg/ml) by Abs (0.3 mg/ml) from several AIDS patients after 4 h of incubation. One can see that in this period some Abs cause only single breaks in one strand of supercoiled DNA (lanes 1-3), whereas others cause multiple breaks and as a result the formation of linear DNA (lanes 4-6). The most active Abs hydrolyze DNA into short and medium length ODNs (lanes 7-10). It should be mentioned that Fig. 5 in principle illustrates a range of possible changes of the relative DNase activities for patients with not only AIDS but also with different AI diseases and viral pathologies previously analyzed (Nevinsky et al., 2000[a], 2003, 2005, 2010[a], 2010[b] and refs therein). When passing from one pathology to another only the values of a relative percent of patients with low, middle and high DNase activities is usually changed.
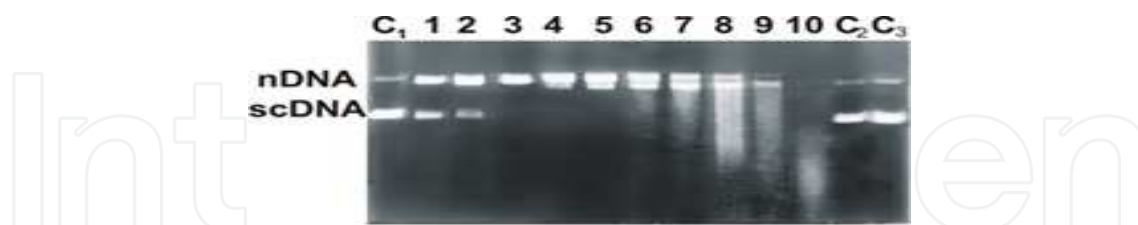

Fig. 5. DNase activities of catalytic AIDS IgGs from different patients in the cleavage of supercoiled (sc) and nicked (n) plasmid DNA (14 μg/ml). Lanes 1–10, IgGs (0.3 mg/ml; incubation for 4 h) from the sera of 10 different patients; C₁, scDNA incubated alone; C₂ and C₃, scDNA incubated with Ab from the sera of two healthy donors.

The efficiency of DNA cleavage was calculated from the increase of DNA in the band of scDNA (10-40 % of initial DNA hydrolysis); the measured RAs (%) for IgGs were normalized to standard conditions (0.1 mg/ml Abs, 2 h) and a complete transition of scDNA to its relaxed form was taken for 100%. While four AIDS IgG preparations were completely inactive, the remaining 106 IgG samples (96 %) demonstrated the RAs from 5 to 100% of scDNA hydrolysis (Table 1).

On the fist step we have analyzed the RAs of proteolytic activity of AIDS pIgGs in the hydrolysis of many different proteins including HIV RT, HSA, and human β-casein (Odintsova et al., 2006[b]). It was shown that among all proteins used, IgGs from different HIV-infected patients with detectable or high rate hydrolyze only HIV-1 reverse transcriptase (RT), human serum albumin (HSA), and human β-casein. Interestingly, the highest rate of the hydrolysis was observed for β-casein (Odintsova et al., 2006[b]). Therefore, the RA of 110 AIDS IgGs in the hydrolysis of casein was analyzed. The efficiency of casein cleavage was analysed by SDS-PAGE and calculated from the decrease in the band of non-hydrolyzed casein taking into account the control reaction incubated in the absence of IgGs.

Relative activity, % of scDNA hydrolysis	Number of IgG preparations with DNase activity**	Number of IgG preparations with casein-hydrolyzing activity***
0	4 (4 %)*	5 (5 %)
5 – 20	21 (19 %)	11 (10 %)
21 – 40	33 (30 %)	24 (21.4 %)
41 – 60	18 (16.4 %)	25 (22.7 %)
61 – 80	7 (6.4 %)	20 (18.2 %)
81 – 100	17 (15.5 %)	25 (22.7 %)
Number of preparations and stage of disease	Average DNase RA	Average protease RA
Generalized lymphadenopathy (45)	40.2 % ± 26.3 %	53.6 % ± 22.6 %
pre-AIDS (65)	44.7 % ± 21.3 %	51.5 % ± 25.9 %

*Percent of total number of patients (110) is given in parenthesis.
**A complete transition of 14 μg/ml scDNA to its relaxed form (0.1 mg/ml IgGs, 2 h) was taken for 100%.
***A complete hydrolysis of 0.1 mg/ml human β-casein (0.1 mg/ml IgGs, 2 h) was taken for 100%.

Table 1. The relative catalytic activities of AIDS IgGs in the hydrolysis of scDNA and β-casein in the case of total group and patients with different stages of diseases development.

Purified pIgGs from ten healthy donors were unable to catalyze casein hydrolysis, whereas 105 of 110 IgGs (95 %) demonstrated high or detectable casein-hydrolyzing activity (Table 1). With the development of the disease at the stage of pre-AIDS, IgGs from some patients demonstrated a high DNase activity (≥80 % of DNA hydrolysis) but the average RA was 44.7 % ± 21.3 % (Table 1). At the stage of generalized lymphadenopathy, IgGs from 4 of 45 patients (8.8 %) did not possess detectable DNase activity. However, the average RA value of DNase activity for this group of patients (40.2 % ± 26.3 %) was comparable with that for the pre-AIDS group. At the same time, the number of IgG preparations with very high activities (≥80 % of DNA hydrolysis) was significantly lower in the GL group. At the GL stage, 5 of 45 (11 %) Abs were completely inactive, while several IgGs demonstrated high RA in the hydrolysis of casein (up to 86.7%); the average RA for all 45 patients was 53.6 % ± 22.6 % (Table 1). All 65 IgGs from patients with pre-AIDS were catalytically active, but the average RA (51.5 % ± 25.9 %) was comparable with that for IgGs from patients with GL (Table 1). Each group of patients corresponding to GL and pre-AIDS stage was divided into

two subgroups with either rapid or slow progression of the disease. According to the recommendations of the Center of Disease Control and Prevention, the transition time from GL to pre-AIDS stage (≤ 2 and > 2 years, respectively) was used as the measure of the disease rate of progression. The number of GL patients demonstrating a detectable proteolytic activity was comparable in the case of rapid (41.7 %) and slow (47.8%) progression, while DNase activity was observed in 41.3 % of the patients with rapid progression and only in 29.5% of the patients with slow progression. In the pre-AIDS group, the number of IgGs with DNase and proteolytic activity was slightly higher in patients with rapid progression (55.8 and 58.1 %, respectively) than in patients with slow (50.7 and 51.8 %) progression of the disease.

On first glance, high activity of IgGs 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 Abs against casein, and five against human milk lactalbumin (Goldfarb, 2001). Thereby, the activation of β-casein synthesis in AIDS patients driven by not yet understood factor can not 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) (Grusby et al., 1990). 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 HIV virus life cycle, its replication, or development of AI reactions in AIDS patients.

At the same time, so far Abzs hydrolyzing HSA were found only in AIDS patients. A possible reason of production of HSA-hydrolyzing Abs in AIDS patients is also not known.

Later the RAs of AIDS IgGs (Baranova et al., 2009) and IgMs (Baranova et al., 2010) in the hydrolysis of IN were analyzed. Sera of 19 HIV-infected patients (18-40 yr old; men and women) including 13 at the stage of pre-AIDS and 6 at the stage of GL were used to study IN-hydrolyzing activity of IgGs, while 18 Ab preparations corresponding to pre-AIDS stage and 8 preparations to GL were used to analyze RAs of IgMs.

pIgGs and pIgMs from ten control healthy donors were unable to catalyze IN hydrolysis. Interestingly, 11 of 13 IgGs from patients with pre-AIDS (84.6 %) and 6 of 6 (100 %) with GL demonstrated detectable of high IN-hydrolyzing activity (Baranova et al., 2009). There was no statistically significant difference in the IgG RAs between the two groups of patients; average values of IgG IN-hydrolyzing RAs were 1.99 ± 1.68 for pre-AIDS and 3.4 ± 1.31 (µM IN/1h)/mg of Abs for GL patients. 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 (Baranova et al., 2010). 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 µM IN per hour per mg of Abs (range 0.3–7.3 µM IN per hour per mg of Abs) for pre-AIDS and 3.3 ± 2.6 µM IN per hour per mg of Abs (range 0–8.1 µM IN per hour per mg of Abs) for GL.

Overall, in the case of Abs with DNase, casein-, and IN-hydrolyzing activity we have found only a negligible difference in the RAs of Abs from HIV-infected patients at the GL and pre-AIDS stages. However, it is not surprising and agrees with the published data that a detection of Abzs is the earliest indicator of the development of many AI diseases in humans and animals (Andryushkova et al., 2006, 2007, 2009; Nevinsky et al., 2005, 2010a, 2010b, and refs therein). According to our data, various catalytic activities of Abzs are usually very easily detectable at the onset of AI diseases when the total concentrations of non-catalytic

Abs to specific auto-antigens have not yet increased significantly and correspond to their ranges for healthy donors. At the early stages of AI diseases, the repertoire of Abzs is usually relatively small but it greatly increases with the progress of the disease, leading to the generation of catalytically diverse abzymes with different activities and functions (Nevinsky *et al.*, 2003, 2005, 2010[a], 2010[b] and refs therein). In addition, AI reactions in the case of some viral diseases including AIDS patients are in some extent similar to AI diseases. At the same time, immunization of AI mice produces an unexpectedly high increase in the number of clones secreting various auto-Abs, including Abzs, in comparison with normal mice (Nishi, 2002; Tawfik et al., 2002).

HIV-1 RT- and IN-hydrolzying pIgGs and IgMs from HIV-infected patients were the first examples of catalytic Abzs produced in humans against viral proteins after a viral infection (Odintsova et al., 2006[b]; Baranova et al., 2009, 2010). In addition, it was shown for the first time that HIV infection stimulates autoimmune reactions leading to the formation of Abzs that hydrolyze at least two human proteins, HSA and casein. It is known that HIV infection stimulates the development of many AI diseases (Zandman-Goddard et al., 2002). One can suppose that in some other viral and bacterial infections may induce similar processes to some extent.

During many infections, the human organism is exposed to different bacterial components including protein, DNA, RNA, and polysaccharides (Ternynck et al., 1991; Unterkircher et al., 1993; Matsiota-Bernard et al., 1993, 1996; Hentati et al., 1994; Boekel et al., 1997; Wun et al., 2001). Because of their ability to bind a variety of exogenous antigens, including bacterial and viral ones, natural Abs play a major role in the primary line of defense against infections. Some results suggest that the synthesis of auto-Abs and Abs directed against bacterial antigens at least partially follow distinct pathways, but with the existing experimental data it is impossible to determine unambiguously whether these two Ab populations are produced by the same or distinct B-cell subpopulations (Matsiota-Bernard et al., 1993). Recently, DNase activity in the patients with diseases caused by several bacterial infections has been analyzed (Parkhomenko et al., 2008). The catalytic activities were significantly lower than in patients with different AI pathologies and increased in the following order: streptococcal infection (erysipelas) < urogenital chlamydiosis associated with arthritis (Reiter's disease) < meningococcal meningitis < shigellosis < suppurative surgical infections caused by *Staphylococcus aureus* < suppurative surgical infections caused by epidermal staphylococci < urogenital ureaplasmosis associated with reactive arthritis.

In addition, DNA-hydrolyzing IgGs was found in the sera of patients with hepatitis (Baranovskii et al., 1997) and tick bone encephalitis (Parkhomenko et al., 2010-11). Interestingly, TBE like HIV infection of humans stimulate formation of Abzs with several proteolytic activities (Parkhomenko T., personal communication). The RAs of IgGs in the hydrolysis of DNA increased in the following order: diabetes ≤ bacterial infections ≤ viral hepatitis < polyarthritis < Hashimoto's thyroiditis < AIDS ≤ MS < SLE (Nevinsky *et al.*, 2003, 2005, 2010[a], 2010[b] and refs therein).

Taking these observations together, we suggest that the specific activity of polyclonal Abs from the sera of patients with diseases caused by bacterial infections are usually lower than those for typical AI diseases and most probably they can differ in their biological functions. It was shown that the specific reorganization of immune system during the spontaneous development of a profound SLE-like pathology in MRL-lpr/lpr mice is associated with changes in the differentiation profile and the level of proliferation of bone marrow hematopoietic stem cells and with production of DNase, ATPase, and amylase Abzs

(Andryushkova et al., 2006, 2007, 2009). Immunization of healthy mice with DNA also leads to production of DNase Abzs; however, it is associated only with increased lymphocyte proliferation and suppression of apoptosis of lymphocytes in different organs (especially spleen), but not with a change in differentiation of the bone marrow cells. Immune processes after immunization of mammals with bacterial DNA, proteins, polysaccharides during many infectious diseases may be considered similar to those after immunization of healthy mice with DNA, different proteins and enzymes. According to theoretical analysis, the adaptive improvement of the catalytic turnover is limited by the rate of B cell receptor signal transduction, as rapid release of antigen fragments from catalytic B cell receptors aborts clonal selection, but production of catalysts can occur at increased levels under conditions of rapid B cell signaling in AI disease (Paul et al., 2006). In addition, the RAs of DNase Abs increased with the progress of the AI pathology, while the time course of immunization associated with some infections is usually not so long as compared with AI diseases, which can have chronic character. In contrast to AI diseases, treatment and recovery of patients with bacterial infections usually eliminates Abzs with various activities. In addition, Abzs may have protective functions in patients with bacterial infections. It was shown that the presence of IgG endowed with serine protease-like activity in the plasma of patients with sepsis strongly correlates with their survival (Lacroix-Desmazes et al., 2005).

In contrast to DNase abzymes, the polysaccharide-hydrolyzing Abs are usually present even in the sera of healthy humans and their activity remarkably increases in the sera of patients and animals with different AI diseases (Andryushkova et al., 2006; 2007) and especially with pathologies caused by viral infections (Buneva V.N., personal communication). Formation of specific Abs against DNA and other components of bacteria and some viruses during infections of healthy mammals suggests that the specific catalytic Abs can mostly hydrolyze these bacterial and viral components.

It is possible that co-action of Abzs with proteolytic and polysaccharide-hydrolyzing activities can at least partially degrade bacterial cell walls and viral particles and facilitate the entry of Abzs into the bacterial cells and hydrolysis of bacterial DNA, proteins and other components. This cooperative action of abzymes with different catalytic activities may have a protective effect against diseases caused by bacterial and viral infections. At the same time, in contrast to many viral and bacterial infections, HIV-infection stimulates AI reactions. Therefore, at the first stage of AIDS development catalytic Abzs against different viral components can protect humans, similarly to the situation in bacterial and viral infections that do not stimulate AI reactions. Later, due to molecular mimicry between viral and host antigens, viral antigens can affect hematopoietic stem cells and trigger the development of AI processes.

5. Extreme diversity of AIDS abzymes

5.1 Structural diversity of AIDS abzymes

DNase, RNase, ATPase, amylase, and protease Abzs may show very different contributions of variable domains of H- and L-chains to their active centers. Chromatographically separated light chains of IgGs from the sera of asthma patients were found to be active in the hydrolysis of VIP (Sun et al., 1994; 1997). The light chain of the VIP Abz was expressed in bacteria, purified, and found to possess an intrinsic catalytic activity (Tyutyulkova et al., 1996). The Abz-dependent hydrolysis of DNA and RNA by isolated light chains of IgGs from SLE, MS, asthma, and other AI patients, as well as from MRL-lpr/lpr mice, is more

efficient than by intact Abs (Dubrovskaya et al., 2003; Galvita et al., 2007; Andrievskaya et al., 2000, 2002; Baranovskii et al., 2001; Nevinsky et al., 2005, 2010[a], 2010[b]). A similar situation was observed for human milk IgGs and sIgAs with DNase and RNase activities (Kanyshkova et al., 1997; Nevinsky et al., 2000[a]). In addition, both H and L-chains of sIgAs had affinity to DNA-cellulose but only L-subunits hydrolyzed DNA and RNA (Nevinsky et al., 2000[c]). At the same time, it was demonstrated that the catalytic center of 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 (Kim et al., 2006).

It was shown that only separated light chains of AIDS IgGs hydrolyze DNA, while heavy chains is catalytically inactive (Odintsova et al., 2006[a]). On the contrary, both light and heavy chains of mouse IgGs after separation were active in the ATP hydrolysis (Andryushkova et al., 2009). Intact rat pIgGs and their separated H- and L-chains possess both peroxidase and oxidoreductase catalytic activity (Nevinsky et al., 2010[a]). The observed IN-hydrolyzing activity of AIDS pIgM L- and H-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, similarly to mouse monoclonal DNase (Kim et al., 2006), 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.

From the crystal structure of a catalytic Ab with esterase-like activity, it was concluded that the ligand *p*-nitrophenyl acetate interacts with amino acid residues of both light and heavy chains of Abzs and that both types of subunits are required for catalysis (Golinelli-Pimpaneau et al., 1994). Taken together, it is obvious that light and heavy chains of different Abzs including AIDS Abs can contribute to the active sites of Abzs in different ways.

The next question concerning the structural diversity of AIDS Abzs relates to the type of the proteolytic activity of their catalytic sites. Proteolytic IgGs from the sera of patients with asthma hydrolyzing VIP (Paul et al., 1989), Hashimoto thyroiditis and rheumatoid arthritis hydrolyzing thyroglobulin (Li eta l., 1995; Kalaga et al., 1995) are serine-like proteases, and their activity is most strongly reduced after incubation with specific serine protease inhibitors PMSF or AEBSF. It was shown that casein-hydrolyzing sIgAs from human milk (Odintsova et al., 2006; 2011) and hMBP-hydrolyzing IgGs and IgMs from the sera of patients with MS (Polosukhina et al., 2004, 2005, 2006) contain not only Ab subfractions with serine-like, but also specific subfractions with metal-dependent activity.

We have analyzed the type of proteolytic activity of AIDS pIgGs and pIgMs in the hydrolysis of IN and β-casein. It was shown, that in contrast to milk sIgAs (Odintsova et al., 2006; 2011) and similarly to several other proteolytic Abzs, only specific inhibitor of serine protease AEBSF significantly suppress AIDS pIgG-dependent hydrolysis of β-casein, while EDTA has no significant effect.

We have analyzed the type of IN-hydrolyzing activity of ten AIDS pIgGs; several typical examples are given in Fig. 6 (Baranova et al., 2009). Leupeptin, an inhibitor of many different proteases, demonstrated significant inhibition of proteolytic activity of only 2 of 10 individual AIDS IgGs (Table 2). A similar situation was observed for a specific inhibitor of acidic proteases, pepstatin A, which significantly inhibited IN-hydrolyzing activity of only two IgGs. Surprisingly, a significant inhibition of serine protease-like activity was also found only for 2 of 10 AIDS IgGs (Table 2). Proteolytic activity of five of ten AIDS IgGs was inhibited by 40-96 % after incubating the IgGs with EDTA. An incubation of IgGs with

iodoacetamide (a specific inhibitor of thiol proteases) usually has no remarkable effect on their proteolytic activity (Paul et al., 1989; Li et al., 1995; Kalaga et al., 1995; Odintsova et al., 2006; 2011; Polosukhina et al., 2004, 2005, 2006). Surprisingly, however, IN-hydrolyzing activity of all 10 IgGs was inhibited by iodoacetamide by 12-98 % (Baranova et al., 2009).
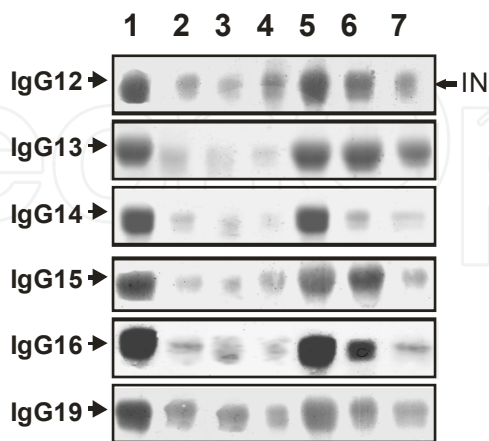

Fig. 6. SDS-PAGE analysis of a decrease in the intensity of protein band after its incubation with pIgGs from AIDS patients in different conditions. IN was incubated alone (1); in the presence of AIDS pIgGs from the sera of six different AIDS patients (IgG-12 – IgG19) and in the absence of other components (2) or in the presence of AEBSF (3), leupeptin (4), iodoacetamide (5), EDTA (6), and pepstatin A (lane 7). Arrows indicate the positions of molecular mass markers.

Similar surprising situation was observed for ten AIDS pIgMs with IN-hydrolyzing activity (Table 2) (Baranova et al., 2010). Leupeptin, significantly inhibited the proteolytic activity of only two of ten individual AIDS IgMs, and very weak inhibition was observed for one more preparation (Table 2). 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 2). 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 ten AIDS IgMs. Proteolytic activity of five of ten 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 relative activity of six preparations by 64–98% and inhibited three more preparations for ~8-10% (Table 2). As for AIDS IgGs, iodoacetamide inhibited the IN-hydrolyzing activity of all ten of ten Abs by 30–99% (Table 2). The inhibition of AIDS IgGs and IgMs with EDTA was comparable with that for IgGs from patients with MS (Polosukhina et al., 2004, 2005, 2006).

Iodoacetamide, a specific inhibitor of thiol proteases, usually does not significantly affect the activity of proteolytic Abzs (≤ 3–7% inhibition) (see above). 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 $65.7 \pm 20.6\%$) 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 $75.6 \pm 21.2\%$) in all ten Ab 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

Number of prep.	Inhibition, %*					
	AEBSF	Leupeptin	Pepstatin A	Iodoacetamide	0.1 M EDTA	Sum of effects**
IgG9	42±5	74±8	51±5	85±9	0	252
IgG10	0	70±7	0	83±9	96±9	249
IgG11	0	0	0	66±7	0	66
IgG12	0	0	0	66±5	44±5	110
IgG13	0	0	59±6	98±8	98±8	255
IgG14	0	0	0	87±7	3±1	90
IgG15	0	11±3	0	33±4	45±4	89
IgG16	0	0	0	78±8	40±3	118
IgG17	49±5	0	0	12±1.5	0	61
IgG19	0	0	0	49±5	2±1	51
IgM4	0	0	48 ± 5	97 ± 8	98 ± 2	243
IgM5	0	47 ± 5	36 ± 5	36 ± 5	80 ± 7	199
IgM7	8 ± 1	68 ± 7	0	95 ± 9	94 ± 8	265
IgM8	0	0	7 ± 2	99 ± 8	8 ± 2	114
IgM9	0	0	0	76 ± 6	0	76
IgM10	0	0	0	83 ± 7	93 ± 8	176
IgM11	0	0	0	94 ± 7	8 ± 3	102
IgM12	0	7 ± 1	0	55 ± 5	98 ± 8	153
IgM13	46 ± 5	0	88 ± 9	91 ± 10	64 ± 4	289
IgM23	80 ± 8	0	5 ± 2	30± 5	10 ± 2	125

**The decrease in the intensity of initial IN band estimated from SDS-PAGE electrophoresis data in the absence of inhibitor was taken for 100 %, for each preparation, a mean of 3 repeats are used.
 **Sum of the effects of different compounds on the proteolytic activity (leupeptin+Pepstatin+iodoacetamide + EDTA).

Table 2. Inhibition of proteolytic activity of individual IgGs and IgMs (from 10 AIDS patients) in the hydrolysis of HIV integrase by specific inhibitors of proteases of different types

resembling thiol, serine, acidic, and metal-dependent proteases, the ratio of which may be individual for every HIV-infected patient.

Interestingly, only IgM9 and three of IgGs (IgG11, IgG14, and IgG19) demonstrated significant inhibition by one inhibitor (iodoacetamide). At the same time other IgG and IgM preparations were sensitive to two or three inhibitors (Table 2). For example, IgM10 and IgM11 was 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 2). Surprisingly, three of ten 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 2). Very comparable situations were observed for AIDS IgGs and IgMs (Table 2).

In principle it is possible that the pools of IgGs and 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 three of ten 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 2). Three IgG preparations (IgG9, IgG10, and IgG13) demonstrated this sum from 249 to 255 % (Table 2). 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 2). However, since the proteolytic activity in seven of ten IgMs and five of ten IgGs was summarily suppressed by specific inhibitors of serine, acidic, metal-dependent, and thiol proteases by more than 100% (110–289%, Table 2), 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). Similar suggestion is reason in the case of several AIDS IgGs (Table 2) (Baranova et al., 2009, 2010).

5.2 pH optima diversity of AIDS abzymes

Theoretically, a mammalian immune system can produce up to 10^6 variants of Abs against one antigen. An extreme diversity of RNase and DNase IgG and/or IgM Abzs from the sera of patients with MS and SLE and autoimmune prone MRL-lpr/lpr mice was observed (Baranovskii *et al.*, 1998, 2001; Andrievskaya *et al.*, 2000, 2002; Kuznetsova *et al.*, 2007; Nevinsky *et al.*, 2003, 2005, 2010a, 2010b). It was shown that different patients (and animals) may have a relatively small or an extremely large pool of polyclonal nuclease Abzs containing different relative amounts of light chains of κ- and λ-types, demonstrating maximal activity at various optimal pHs, having a different net charge, activated or not by different metal ions, characterized by different substrate specificities. MS IgGs of all four subclasses (IgG1-IgG4) were catalytically active in the hydrolysis of hMBP (Legostaeva *et al.*, 2010) and DNA (Parkhomenko *et al.*, 2010).

We have analyzed the pH dependencies of the initial rates of DNA hydrolysis by AIDS catalytic IgGs (Odintsova et al., 2006a). Fig. 7A demonstrates three pH dependencies of different types which were revealed for catalytic pIgGs from the sera of 3 different patients. In contrast to all human DNases having one pronounced pH optimum in hydrolysis of DNA (Baranovskii et al., 2004), catalytic Abs usually show high DNase activity at a wide range of pH values between 5.5-9.0. Nevertheless, as one can see from Fig. 7A, one of the IgG preparations has a pronounced optimum at pH 8.0; second at pH 7.5, while third one demonstrates two marked pH optima at pH 7.5 and 8.5.

We have analyzed the pH dependencies of the initial rates of human casein hydrolysis by four individual AIDS IgGs. In contrast to all human proteases having one pronounced pH optimum (Horl et al., 1987; Rao et al., 1998), catalytic AIDS IgGs demonstrated high specific casein-hydrolyzing activity within a wide range of pH values (5.0–9.0) and the pH profile for each IgG was unique (Fig. 7B) (Odintsova et al., 2006).

It is well known that canonical mammalian, bacterial, and plant proteases, depending on their biological function, can have optimal pH values ranging from acidic (2.0) to neutral and alkaline (8-10) (Horl et al., 1987; Rao et al., 1998). We have measured the relative activity of AIDS IgGs and IgMs at pH from 3.0 to 10.5. In contrast to all human proteases, catalytic IgGs demonstrated high specific IN-hydrolyzing activity within a wide range of pH values (3.0–10).
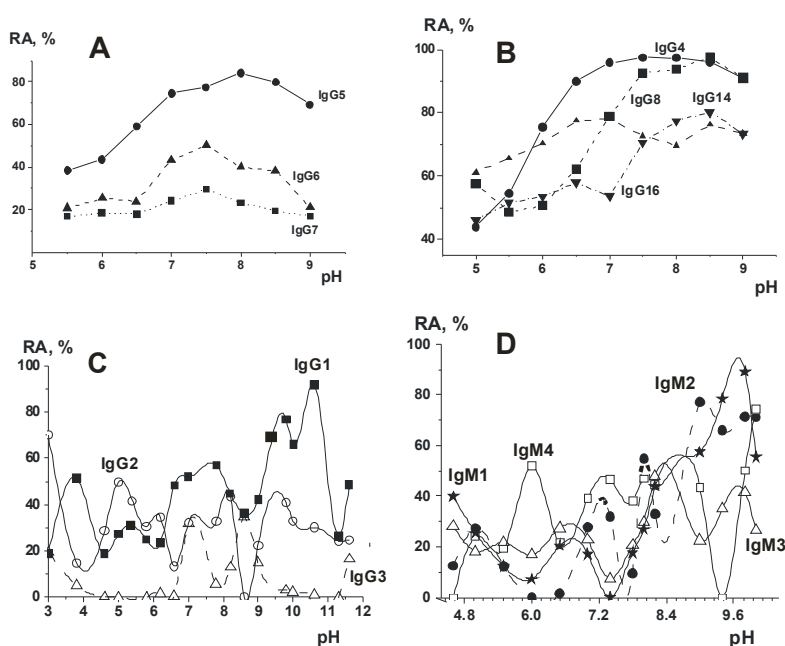
Fig. 7. pH dependences of the relative DNase (A), casein-hydrolyzing (B) activity of IgGs, and IN-hydrolyzing activity (RA) of individual pIgGs (C) and pIgMs (D) from the sera of several different AIDS patients (number of Ab correspond to number of patient). The average error in the initial rate determination at each pH from two experiments did not exceed 7-10 %.

The pH profile for each IgG and IgM was unique; each preparation demonstrated from three to seven brightly expressed optima at different pH from 3 to 11 (Fig. 7). Taking into account the effective hydrolysis of IN at pH 3.0, one cannot exclude that human immune system of AIDS patients could in principle produce IgGs and IgMs with a proteolytic activity similar to that of stomach acidic proteases. The above results clearly demonstrate that pIgGs and pIgMs from individual AIDS patients can consist of different sets of catalytic Ab subfractions demonstrating quite distinct enzymic properties in the hydrolysis of DNA, human casein, and integrase.

Overall, a pool of many auto-Abs may contain very different monoclonal Abzs with various pH optima. It should be mentioned that the RAs of Abzs from patients with different AI and viral diseases are usually compared at one fixed pH, in which all samples are more or less active. Changing the reaction pH, one can reveal not only the major fraction of Abzs in

different individuals analyzed, but also other subfractions of Abs, the activity of which may be comparable with or less than that of the major subfraction. In addition, the number of K_m and V_{max} values, characterizing interaction of different monoclonal of polyclonal Abzs with their specific substrates, can significantly increase when they measured at several pH values (Nevinsky, 2010b).

5.3 Affinity and relative catalytic activity diversity of AIDS abzymes

It was shown previously, that nuclease and protease Abzs from the sera of AI patients and animals are very heterogeneous in their affinity for cognate substrate and can be separated into many fractions by chromatography on affinity resins bearing immobilized substrate (Baranovskii et al., 2001; Andrievskaya et al., 2002; Semenov et al., 2004; Kuznetsova et al., 2007; Nevinsky et al., 1998, 2005, 2010a, 2010b). We have analyzed the affinity of AIDS pIgGs for human β-casein by chromatography on casein-Sepharose (Odintsova et al., 2006b). Interestingly, when IgGs were eluted from casein-Sepharose by a KCl gradient (0–3 M), the protein (and casein-hydrolyzing activity) was distributed all over the chromatography profile. A similar result was obtained at AIDS IgGs chromatography on RT- and HSA-Sepharoses (Odintsova et al., 2006b). The data indicate for extreme affinity heterogeneity of casein-, RT-, and HSA-hydrolyzing abzymes to cognate protein substrates (Odintsova et al., 2006).

We have subjected an equimolar mixture of pIgGs (and IgMs) from five AIDS patients to affinity chromatography on IN-Sepharose. Only 15±3 % of the total IgGs (Fig. 8A) and 17±3 % of the total IgMs (Fig. 8B) were bound to IN-Sepharose (Baranova et al., 2009, 2010).

As we have shown previously, the fraction of Abzs with different catalytic activities including in the serum of AI patients usually does not exceed 0.1-5 % of total Igs (Nevinsky et al., 2005, 2010a, 2010b). Therefore, it was surprising that IN-Sepharose can bind up to 15-17 % of the total pIgGs and pIgMs. At the same time, IN is known as a very hydrophobic protein which can interact nonspecifically with different hydrophobic compounds including other proteins. Taking this into account we could suppose that immobilized IN binds anti-IN pIgGs and pIgMs in a specific manner, and interacts with some other IgGs and IgMs non-specifically.
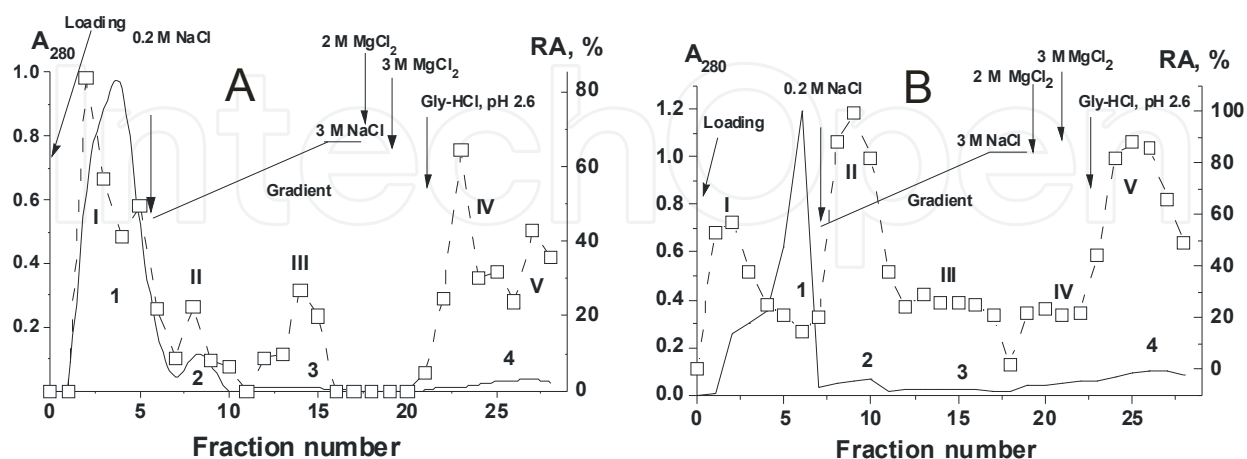

Fig. 8. Affinity chromatography of the mixture of five individual AIDS pIgGs (A) and pIgMs (B) on IN-Sepharose: (——), absorbance at 280 nm; the relative activity (RA) of IgGs and IgMs (5 μl of dialyzed eluate, total volume of the reaction mixture 10 μl) corresponding to complete hydrolysis of 0.3 mg/ml IN after 16 h of incubation was taken for 100%.

The pIgG and IgM fractions of the first peak (~83-85% of protein loaded on IN-Sepharose) with a very low affinity for IN possess high IN-hydrolyzing activity (peak 1, Fig. 8). As it was shown previously non-separated on affinity resins pIgGs and IgMs from AIDS patients contain small subfractions hydrolyzing specifically not only HIV IN (Baranova et al., 2009, 2010), but also HIV RT, HSA and human casein (Odintsova et al., 2006). However, the fractions of pIgGs and pIgMs having high affinity to IN-Sepharose and eluted from this sorbent with different concentrations of NaCl, $MgCl_2$ and acidic buffer (protein peaks 2-4, activity peaks II-V; Fig. 8) hydrolyzed only IN. Thus, IgGs and IgMs with IN-independent activities do not have affinity for IN-Sepharose, but some other Abs can be bound with IN non-specifically.

The total IN-hydrolyzing activities of pIgGs and pIgMs were distributed all over the chromatography profiles and in the case of both Abs five peaks of IN-hydrolyzing activity (I – V, Fig. 8) were brightly expressed. The data obtained are indicative of extreme heterogeneity of IN-hydrolyzing pIgGs and pIgMs in their affinity to IN.

When Abzs are highly heterogeneous, the dependence of V on the substrate concentration for non-fractionated Abs may appear inconsistent with simple Michaelis-Menten kinetics and may be described by a sum of several hyperbolic curves corresponding to different Ab subfractions. However, the contribution of some subfractions to the total curve may be small, or they may have comparable K_m and V_{max} (k_{cat}) values. As a rule, only when significant differences ($\geq$ 5-fold) exist between the K_m and V_{max} values for different Ab subfractions it is possible to determine these parameters characterizing individual subfractions of polyclonal Abz from the aggregated initial rate curves.

First, we have measured the K_m and V_{max} values in the reaction of IN hydrolysis using two individual preparations of pIgGs and pIgMs not fractionated on IN-Sepharose. The initial rate data obtained for these Abs at the increasing IN concentration were inconsistent with the Michaelis-Menten kinetics and the dependences corresponded to at least three or four hyperbolic curves with several segments reflecting different K_m values, which were approximately in the ranges of 5-10, 15-20, 30-50, and higher than 70-100 µM (Fig. 9). Similar situation was observed for the mixtures of equal amounts of electrophoretically homogeneous IgGs (pIgG$_{mix}$) and IgMs (pIgM$_{mix}$) from the sera of five AIDs patients.

For more detailed analysis of K_m and k_{cat} values characterizing different Ab fractions within total pool of Abzs (pIgG$_{mix}$ and pIgM$_{mix}$) we have analyzed several individual pIgG$_{mix}$ and pIgM$_{mix}$ fractions eluted from IN-Sepharose (Fig. 8). First, we have measured the K_m and V_{max} values in the reaction catalyzed by IgG$_{mix}$ (IgG$_{load}$) and IgM$_{mix}$ (IgM$_{load}$) corresponding to the second fraction eluted under loading of Abs on IN-Sepharose (Fig. 8). The dependencies of V/[S] (hyperbolic curves; Fig. 10A) and 1/V vs 1/[S] (Fig. 10B) demonstrated virtually normal Michaelis-Menten character for second fractions of pIgGs and pIgMs. The K_m and k_{cat} for IgG$_{load}$ (156± 40 µM; 0.3±0.1 min^{-1}) and IgM$_{load}$ (130± 30 µM; 2.0±0.4 min^{-1}) were determined. The V/[S] and 1/V vs 1/[S] dependences for individual fractions of pIgGs and pIgMs eluted from IN-Sepharose in gradient of NaCl concentration (IgG$_{salt}$ and IgM$_{salt}$) and by acidic buffer (IgG$_{acid}$ and IgM$_{acid}$) had also typical Michaelis-Menten character.

The affinity of pIgGs for IN (in terms of K_m values) increased with the increase of their affinity to IN-Sepharose; for IgG$_{salt}$ (K_m = 44±4.0 µM) corresponding to fraction 8 (eluted with the salt) and IgG$_{acid}$ (K_m = 14±1.0 µM) corresponding to fraction 24 (eluted with an acidic buffer) the affinity was 3.5– and 11-fold respectively higher than that for IgG$_{load}$ (156±40 µM). Similar situation was observed for the separated individual fractions of IgM$_{mix}$ (Fig. 8B); pIgM$_{solid}$ (K_m =43±4.0 µM; fraction 10 eluted with NaCl) and pIgM$_{acid}$ (K_m = 12.8±1.0 µM;
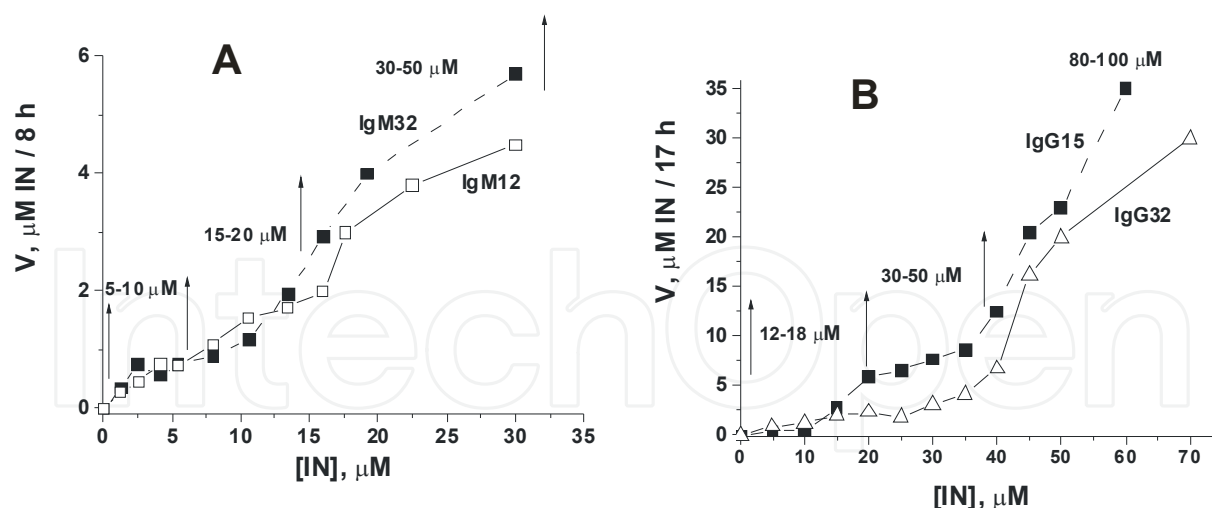
Fig. 9. The dependencies of the initial rates of IN hydrolysis upon the IN concentration in the reaction catalyzed by non-separated by affinity chromatography two individual pIgMs (A) and two pIgGs (B) from different patients in coordinates V *vs* [S]. IgM12, IgM32, IgG15, and IgG32 were used in different concentrations. Arrows show different hyperbolic fragments of complicated curves corresponding to the total dependencies.
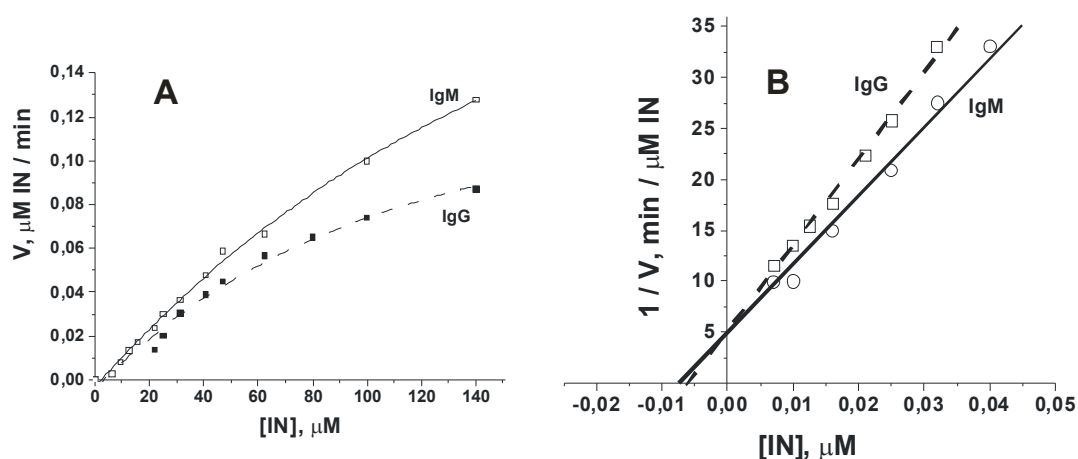

Fig. 10. The dependencies of the initial rates of IN hydrolysis upon the IN concentration in the reaction catalyzed by fractions of pIgG$_{mix}$ and pIgM$_{mix}$ purified on IN-Sepharose (Fig. 8; fractions number 2; IgG$_{load}$ and IgM$_{load}$) in coordinates V vs [S] (A). Determination of the K_m for IN and V_{max} values using the Lineweaver-Burk plot in the case of the same IgG$_{load}$ and IgM$_{load}$ (B).

fraction 24 eluted with an acidic buffer) demonstrated 3– and 10-fold respectively higher affinity than that for pIgM$_{load}$ fraction (130± 30 μM). The K_m and k_{cat} values corresponding to fractionated pIgGs and pIgMs agree with the relative contents and specific activities of the major Ab subfractions isolated by affinity chromatography (Fig. 8). On overall, the above data demonstrate extreme diversity of HIV IgGs and IgMs in their affinity to IN and in the relative k_{cat} values (Figs 8-10).

Affinity chromatography of DNase and RNase Abs from AI patients and animals (Baranovskii et al., 2001; Andrievskaya et al., 2002; Kuznetsova et al., 2007), healthy rabbits immunized with DNA, RNA, DNase I, DNase II, pancreatic RNase (Krasnorutskii et al.

2008[a], 2008[b], 2008[c], 2008[d], 2009) as well as AI Abs with proteolytic and other activities (Nevinsky et al, 1998; Semenov et al., 2004; Legostaeva et al., 2010) on resins bearing immobilized specific substrates using elution of Abs with different concentration of NaCl and an acidic buffer always leads to separation of Abs into many Abz subfractions with different affinity to immobilized substrate. In addition, the affinity of separated fractions for immobilized substrate increased gradually with the increase in eluting NaCl concentration, but the K_m (and V_{max}) values corresponding to each fraction eluted from affinity sorbent are individual for every patient analyzed. It means, that the apparent number of monoclonal Abzs with different catalytic properties within the polyclonal Abs pool may be significantly underestimated since it is impossible to separate Abzs with comparable affinities for a specific substrate or to distinguish monoclonal Abzs with similar kinetic parameters. Some minor monoclonal Abzs with a relatively high activity and even major Abzs with low activity may be hidden by major Abzs with high activity. As a consequence, the K_m (and V_{max}) values determined using Abzs not fractionated on affinity sorbents more often characterize the interaction of substrates with a major fraction of Abzs with the maximal content and highest relative enzymatic activity at condition used. At the same time, these characteristic are very useful for comparisons of Abs with different substrate specificities and from patients with different diseases. For example, it was shown that affinity of specific major fractions of AIDS IgGs non-fractionated on affinity resins to HSA ($K_m = (1.8 \pm 0.6) \times 10^{-8}$ M, $k_{cat} = (3.6 \pm 1.1) \times 10^{-5}$ min^{-1}) is 270-290-fold higher than to HIV RT ($K_m = (4.9 \pm 0.5) \times 10^{-6}$ M, $k_{cat} = (2.2 \pm 0.2) \times 10^{-3}$ min^{-1}) and to β-casein ($K_m = (5.3 \pm 0.5) \cdot 10^{-6}$ M, $k_{cat} = (2.0 \pm 0.2) \times 10^{-2}$ min^{-1}) (Odintsova et al., 2006). At the same time, the relative rate of β-casein hydrolysis was 9- and 550-fold higher than that for HIV RT and HSA, respectively. The K_m for casein (($7.3 \pm 1.2) \times 10^{-6}$ M; $k_{cat} = 0.75 \pm 0.05$ min^{-1}) estimated in the reaction catalyzed by polyclonal sIgAs from human milk (Odintsova et al., 2005) is comparable with that for IgGs from AIDS patients, while the k_{cat} is ~38-fold higher. This difference is most likely due to a higher content of anti-casein proteolytic Abzs in human milk in comparison with blood of AIDS patients.

The affinity of AIDS pIgGs hydrolyzing HIV RT, human casein, and HSA ($K_m = 0.018 – 5.3$ μM; see above) as well as IN-Sepharose-purified AIDS pIgGs and pIgMs for IN ($K_m = 12.8$-156 μM) in terms of K_m values is comparable with typical affinities ($K_m = 0.038$–7.3 μM) (Paul et al., 1989; Kalaga et al., 1995; Legostaeva et al., 2010; Nevinsky et al., 2005, 2010[a], 2010[b]) of Abzs hydrolyzing different proteins.

The K_m (and k_{cat}) values for plasmid scDNA in the reaction catalyzed by two individual non-fractionated AIDS IgG preparations were determined (Odintsova et al., 2006[a]). In the case of one of two preparations analyzed, the initial rate of DNA hydrolysis increases with increase in DNA concentration according to the Michaelis–Menten kinetics and only one pair of K_m (53 ± 9 nM) and k_{cat} (($2.1 \pm 0.1) \times 10^{-2}$ min^{-1}) was observed. For the second pIgG preparation two pairs of K_m (2.6 ± 0.1 and 4.4 ± 0.7 nM) and k_{cat} values (($6.7 \pm 0.1) \times 10^{-2}$ and $(29.6 \pm 5.0) \times 10^{-2}$ min^{-1}) were revealed. Thus, the affinity the scDNA substrate for AIDS IgGs varied (in terms of K_m values) in the range 2.6–53 nM, which correspond to typical K_d values for Ab–antigen interactions and is about 3–4 orders of magnitude higher than affinity of scDNA for DNase I ($K_M = 46$–58 μM) (Gololobov et al., 1995). These K_m values for scDNA are comparable with the K_m for plasmid DNA (43 nM) reported previously for IgG from SLE patients (Gololobov et al., 1995).

The catalysis mediated by artificial Abzs against reaction transition states is usually characterized by relatively low reaction rates: k_{cat} values are 10^2–10^6-fold lower than for canonical enzymes (Keinan, 2005). The known k_{cat} values for natural Abzs from AI patients

vary in the range of 0.001–40 min^{-1} (Gololobov et al., 1995; Kalaga et al., 1995; Nevinsky et al., 2005, 2010a, 20010b). There are only several exceptions. For example, the specific activity of RNase IgGs from AI patients was about 1-20% of that for RNase A and of six known human sera RNases, while poly(A) was hydrolyzed by Abzs 2-10-fold faster than by RNase A, one of the most active RNases known (Buneva et al., 1994; Baranovskii et al., 1997, 1998; Vlasov et al., 1998). At the same time, the specific activity of homogeneous Abzs of several SLE and MS patients was about 40-400% of that for RNase A (Baranovskii et al., 1998). In addition, the specific nucleotide-hydrolyzing activities of mouse polyclonal IgGs is ~1–4 orders of magnitude higher that those of known natural Abzs (Andryushkova et al., 2009). The k_{cat} values for AIDS pIgGs hydrolyzing scDNA ($(2.1-29.6)\times10^{-2}$ min^{-1}), human casein (2.0×10^{-2} min^{-1}), HSA (3.6×10^{-5} min^{-1}), and HIV RT (2.2×10^{-3} min^{-1}), as well IgGs ($0.3-2.9$ min^{-1}) and IgMs ($2.0-6.4$ min^{-1}) purified on IN-Sepharose (Table 2) in the IN hydrolysis were comparable with those for known Abzs (Odintsova et al., 2006a, 2006b; Baranova et al., 2009, 2010).

Currently there are no methods that can efficiently separate Abzs from catalytically inactive Abs against the same antigen. In addition, as it was shown above, IN-Sepharose interact not only with anti-IN IgMs and IgGs but bind non-specifically some other Abs. Even partial purification of IgGs and IgMs on IN-Sepharose (or other specific affinity resins) leads to significant increase in the k_{cat} value for IN and other substrates hydrolysis. Since the specific activities in all cases were calculated using the total concentration of purified pIgGs and pIgMs and affinity chromatography on IN-Sepharose (and other affinity sorbents) cannot separate catalytically active and inactive anti-IN Abs, the specific IN-hydrolyzing activities of the individual monoclonal subfractions in the pIgG and pIgM pools may be higher than those of non-fractionated or partially fractionated Abs. It should be mentioned that specific activities of some Abzs are often significantly lower than those for canonical enzymes with the same catalytic activities. However, it is impossible to dismiss the RAs of Abs from patients with AI and viral infection as biologically inessential since they are comparable with those for many canonical and very important enzymes, for example, restriction endonucleases and repair enzymes (Gololobov et al., 1995; Nevinsky et al., 2005, 2010, 2010). Thus, IN-hydrolyzing IgGs and IgMs from HIV-infected patients are very heterogeneous in their affinity to IN-Sepharose, demonstrate different K_m and V_{max} values and different subfractions of Abzs can hydrolyze various substrates at pH from 3 to 10. In addition, in contrast to other Abzs with proteolytic activity they can possess for different types of proteolytic activities: thiol-, metal-dependent, serine- and acidic-like.

6. Peculiarities of protein hydrolysis by AIDS abzymes and canonical proteases

6.1 Casein hydrolysis by AIDS abzymes

Casein hydrolyzing Abzs was found not only in the sera of HIV-infected patients (Odintsova et al., 2006b) but also in the milk of lactating women (Odintsova et al., 2005; 2011). At the first glance, no obvious immunizing factors could be found in clinically healthy pregnant and lactating women. However, pregnancy could "activate" or "trigger" autoimmune-like manifestations in clinically healthy women, and a sharp exacerbation of AI reactions can occur in some cases soon after childbirth (Amino *et al.*, 1982; Freeman *et al.*, 1986). Postnatal AI pathologies arise sometimes, including SLE, Hashimoto's thyroiditis, phospholipids syndrome, polymyositis, AI myocarditis, etc. (Amino *et al.*, 1982; Freeman *et al.*, 1986).
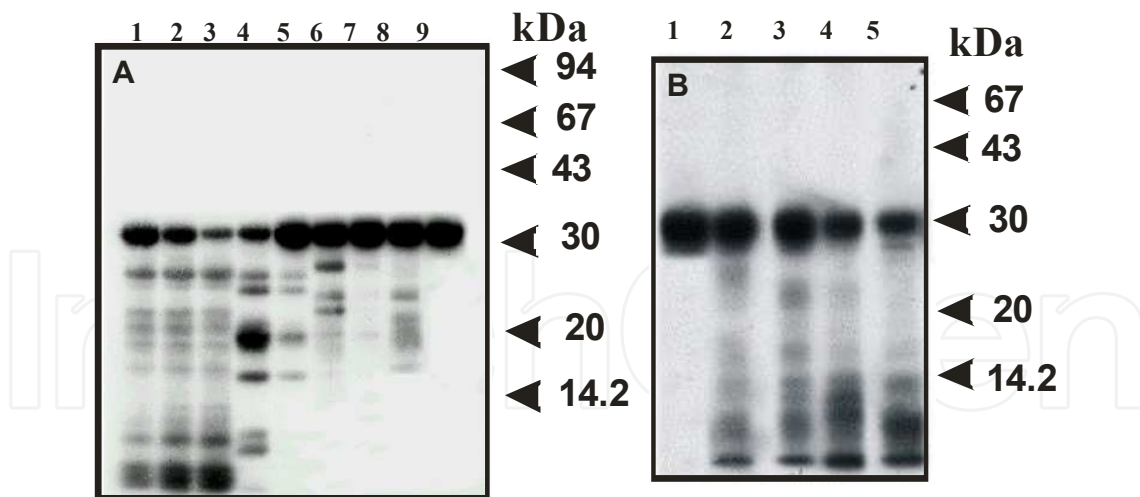
Fig. 11. SDS-PAGE analysis of products of [32P]casein hydrolysis by human milk sIgA and different proteases (A) or AIDS IgGs (B) (autoradiograph). A, Lanes 1, 2, and 3 correspond to [32P]β-casein incubated with sIgAs for 20, 40, and 60 min, respectively; lanes 4 and 5, incubation with trypsin for 10 and 15 min; lanes 6 and 7, incubation with proteinase K for 10 and 15 min; lane 8, incubation with chymotrypsin for 10 min; 9, casein incubated alone for 60 min. The reaction mixture (10 μl) for analysis of proteolytic activity of different proteases contained 6.2 μg/ml casein and sIgAs or enzymes at the following concentrations: 0.32 μg/ml trypsin, 0.064 μg/ml chymotrypsin, 0.1 μg/ml proteinase K, and 20 μg/ml sIgA. B, [32P]casein was incubated for 14 h in the absence of Abs (lane 1) and in the presence IgGs from the sera of different AIDS patients: lane 2, IgG-1 (14 h), lanes 3 and 4, IgG2 (7 and 14 h, respectively), lane 5, IgG-3 (14 h).

Parenteral or oral administration of various proteins to animals late in pregnancy leads to the production of the corresponding Abs at high levels (Fey *et al.*, 1973; Mestecky *et al.*, 1987). Thus, pregnant women may be effectively immunized by contacts with compounds of viruses and bacteria that are not immunogenic in other healthy humans. There may be also some degree of autoimmunization during pregnancy similar to that in AI patients (Nevinsky et al., 2005, 2010a, 2010b and refs therein).

It was shown that lactation is associated with the appearance of sIgA and IgG abzymes with DNase, RNase (Kanyshkova *et al.*, 1997; Nevinsky *et al.*, 2000a, 2000b), ATPase (Semenov *et al.*, 2004), amylolytic (Savel'ev *et al.*, 2001), protein- (Nevinsky *et al.*, 1998), lipid- (Gorbunov *et al.*, 2005) and polysaccharide (Karataeva *et al.*, 2006a, 2006b) kinase activities in human milk. The specific activities of milk Abzs are significantly higher than those of Abzs from the blood of healthy lactating women and patients with different AI pathologies (Nevinsky *et al.*, 2003, 2005, 2010a, 2010b and refs therein).

We have compared the hydrolysis of β-casein by canonical proteases, human milk sIgA (Fig. 11A) and three different individual AIDS IgGs (Fig. 11B) (Odintsova et al., 2006b; 2011). The patterns of β-casein hydrolysis by milk sIgA, AIDS IgG, trypsin, chymotrypsin, and proteinase K were quite different. In addition, there was observed remarkable difference in the hydrolysis of β-casein by three individual AIDS IgG-1, IgG-2, and IgG-3 (Fig 11B).

It should be mentioned that AIDS IgGs demonstrated only serine-like protease activity (Odintsova et al., 2006b), while milk IgAs additionally possess Me-dependent activity (Odintsova et al., 2011). Thus, possible ways of the production of Abzs with casein-hydrolyzing activity in healthy human mothers and in AIDS patients may be different.

6.2 Integrase hydrolysis by AIDS abzymes
6.2.1 Specific regularities of integrase interaction with DNA

HIV-1 integrase catalyzes insertion of a DNA copy of the viral genome into the host genome (Asante-Appiah & Skalka, 1999). Therefore IN, together with RT and protease, is the main important target of anti-HIV drugs.

Specific interactions between HIV IN and long terminal repeats are required for insertion of viral DNA into the host genome. The use of a method based on stepwise increase in ligand complexity allowed an estimation of the relative contributions of each nucleotide from oligonucleotides (ODNs) to the total affinity for IN (Bugreev et al., 2003). It was shown that IN interacts with ODNs through superposition of weak contacts with their bases and, more importantly, with the internucleotide phosphate groups. Formation of the IN complex with specific DNA cannot by itself account for the major contribution of enzyme specificity, which lies in the k_{cat} term; the rate of 3'-processin reaction is increased by more than 5 orders of magnitude upon transition from non-specific to specific oligonucleotides (Bugreev et al., 2003).

In solution, HIV-1 IN exists in a dynamic equilibrium of monomers, dimers, tetramers and high-order oligomers (Deprez et al., 2000). We have recently analyzed the activity of different purified oligomeric forms of recombinant IN obtained after stabilization by platinum crosslinking and shown that these forms do not share the same *in vitro* catalytic properties (Faure et al., 2005). While monomers were inactive for all specific IN activities, dimers were able to catalyze the 3'-processing and the insertion of only one LTR into a short target DNA. In contrast, a tetramer of IN catalyzed the full-site integration of the two viral LTR ends into a target DNA.

To characterize the influence of the determinants of DNA substrate specificity on the oligomerization status of IN, the small-angle X-ray scattering technique was used (Baranova et al., 2007). Under special conditions in the absence of ODNs IN existed only as monomers. IN preincubation with specific ODNs led mainly to formation of dimers, the relative amount of which correlated well with the increase in the enzyme activity. Under these conditions, tetramers were scarce. Nonspecific ODNs stimulated formation of catalytically inactive dimers and tetramers. Complexes of monomeric, dimeric and tetrameric forms of IN with specific and nonspecific ODNs had varying radii of gyration (R_g), suggesting that the specific sequence-dependent formation of IN tetramers occurs by dimerization of two dimers of different structure. From the data it was concluded that the DNA-induced oligomerization of HIV-1 IN is of extreme importance to provide substrate specificity and to increase the enzyme activity (Baranova et al., 2007).

6.2.2 Effect of DNA on the integrase hydrolysis by different proteases

It is known that a formation of multiple contacts between the same or various subunits of oligomeric enzymes is usually provided by multiple hydrophobic and electrostatic contacts and hydrogen bonds. A similar situation was observed for the dimeric forms of HIV-1 IN by X-ray crystallography (for review see Wlodawer 1999; Chiu & Davies, 2004). Analysis of effects of specific and nonspecific ODNs on the rate of IN proteolysis by chymotrypsin, trypsin, and proteinase K can provide useful information concerning a possible decrease in the accessibility of aromatic and positively charged amino acid residues after an enzyme binds its substrates, changes its conformation, or forms contacts between its subunits. It was interesting to compare the effect of different ODNs on the cleavage of IN by Abzs and

canonical proteases. The specific single-stranded (ss) 5'-GTGTGGAAAATCTCTAGCA (19-CA), ss 5'-GTGTGGAAAATCTCTAGCAGT (21-GT), ss 5'-ACTGCTAGAGATTTTCCACAC (21-COM, complementary to 21-GT and to 19-CA), double-stranded (ds) 21-GT (21-GT•21-COM) and ds 19-CA (19-CA•21-COM) corresponding to terminal repeats of viral DNA were used.

While nonspecific $d(pT)_n$ markedly decreased the IgG-dependent hydrolysis of IN, $d(pA)_n$ and $d(pA)_n$•$d(pT)_n$ demonstrated no detectable protective effect (Fig. 12) (Odintsova E., Baranova S., and Nevinsky G.A., personal communication).
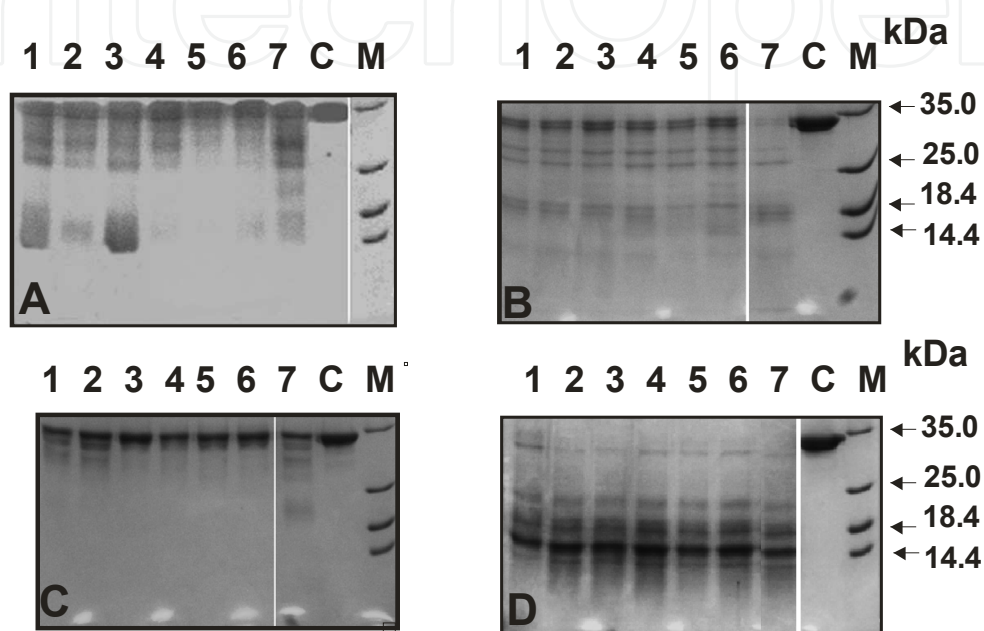

Fig. 12. SDS-PAGE analysis of HIV IN hydrolysis by IgGs and canonical proteases. Polyclonal IgGs from HIV-infected patients (A), chymotrypsin (B), trypsin (C), and proteinase K (D) were used for hydrolysis of IN after its preincubation in the absence and in the presence of various ODNs leading to the formation of different IN oligomeric forms. IN was pre-incubated for 1 h at 30°C in the absence (lane 7) or in the presence of saturating concentrations (0.2–1 mM) of ss $d(pA)_{24}$ (lane 1), ss $d(pT)_{24}$ (lane 2), ds $d(pA)_{24}$•$d(pT)_{24}$ (lane 3), ss 21-COM (lane 4), ss 21-GT (lane 5), ds 21-GT•21-COM (lane 6). Then, pre-incubated mixture was diluted 3-fold and one of three canonical proteases or pIgGs from HIV-infected patients was added. After 5–10 min incubation in the presence of trypsin (19 μM), chymotrypsin (1.6 μM), proteinase K (19 μM) and 6 h in the presence of pIgGs (0.17 μM) the reaction was stopped and the efficiency of IN hydrolysis was analysed by SDS-PAGE.

The best protection from the hydrolysis by IgGs was observed for ss and especially ds specific ODNs (Fig. 12). Overall, the protective effects of all specific and nonspecific ss and ds ODNs from hydrolysis of IN by chymotrypsin were comparable. Therefore, one can suggest that the formation of IN complex with specific and nonspecific ODNs led to a similar decrease in the accessibility of aromatic amino acid residues as a result of their shielding by ODNs and/or involvement of these residues to the formation of multiple contacts at the interfaces of IN oligomer subunits. In contrast to chymotrypsin, nonspecific ODNs strongly protects IN from hydrolysis by trypsin, which cleaves peptide chains mainly

at the carboxyl side of lysine and arginine residues (Fig. 12). Thus, these ODNs most probably stimulate formation of dimeric forms of IN with more Lys- and Arg-dependent electrostatic contacts between the monomers. A weak protective effect of specific and nonspecific ODNs was observed in the case of proteinase K, which is mostly sequence-independent. Thus, specific and nonspecific DNAs stimulate the formation of different IN oligomeric forms, in which aromatic and charged amino acid residues in different extent accessible for Abzs, chymotrypsin, and trypsin. The findings correlate with the results obtained by small-angle X-ray scattering, which show that all nonspecific and specific ODNs stimulate different changes in the structure of IN monomers and dimers free of DNA (Baranova et al., 2007).

MALDI-TOF analysis of the fragments formed after IN incubation with pIgG and pIgM purified on IN-Sepharose was carried out (Odintsova E., Baranova S., and Nevinsky G.A., personal communication). The cross-sections of longitudinal slices of the gel corresponding to the products with approximate mol. masses 29 ± 2 (P1), 22 ± 2 (P2), 16 ± 2 (P3), and 12 ± 2 (P4), as well as 30± 2 (P0) kDa were cut out as shown on Fig. 13 and the proteins were eluted from the gel. MALDI-TOF analysis has shown that P1–P4 fractions contain from four to eight major fragments with different molecular masses. The peptides found in the P1–P4 fractions were digested with trypsin under standard conditions for MALDI analysis, and the hydrolyzates were studied.
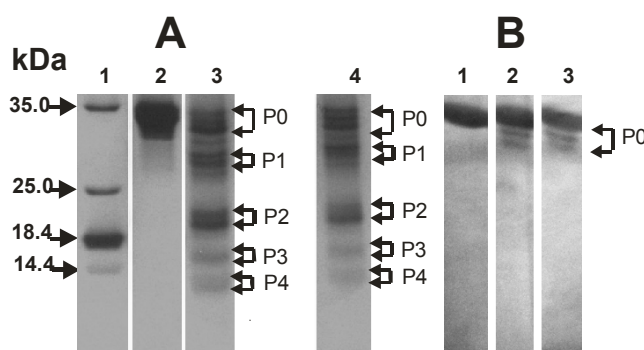

Fig. 13. SDS-PAGE analysis of IN hydrolysis by pIgG-1 (lane 3, A), IgG-6 (lane 2, B), pIgM-2 (lane 4, A) and pIgM-6 (lane 3, B) purified on IN-Sepharose after 9 h (A) and 2 h (B) of incubation in a nonreducing 12% gel followed by silver staining. Lanes 1 (B) and 2 (A), IN incubated in the absence of Abs. Lane 1 (A) protein molecular mass markers. Gel zones P1, P2, P3, and P4 (A) as well as P0 (B) were used for MALDI-TOF analysis (see the text).

Seven antigenic determinants (AGDs) have been reported for HIV IN corresponding to amino acid residues 5–22 (AGD1), 14–35 (AGD2) (Yi et al., 2000), 58–141 (AGD3), 141–172 (AGD4), 248–264 (AGD5) (Bizub-Bender et al., 1994), 208–228 (AGD6), and 251–271(AGD7) (Nilsen et al., 1996) (underlined in Fig. 14). Interestingly, 6–7 cleavage sites found by MALDI corresponded to the N-terminal stretch of residues 11–35, belonging to two overlapping antigenic determinants AGD1 and AGD2 (Fig. 14). Three clusters of cleavage sites were located within the long AGD3. A block of 12 closely spaced cleavage sites corresponded to the N-terminal part of AGD4. Only one cleavage site was located within AGD5 and four sites corresponded to AGD6 and AGD7. At the same time, some sites of IN cleavage, most notably a cluster of 16 sites between residues 175 and 202, did not corresponds to any IN AGD known at this moment.
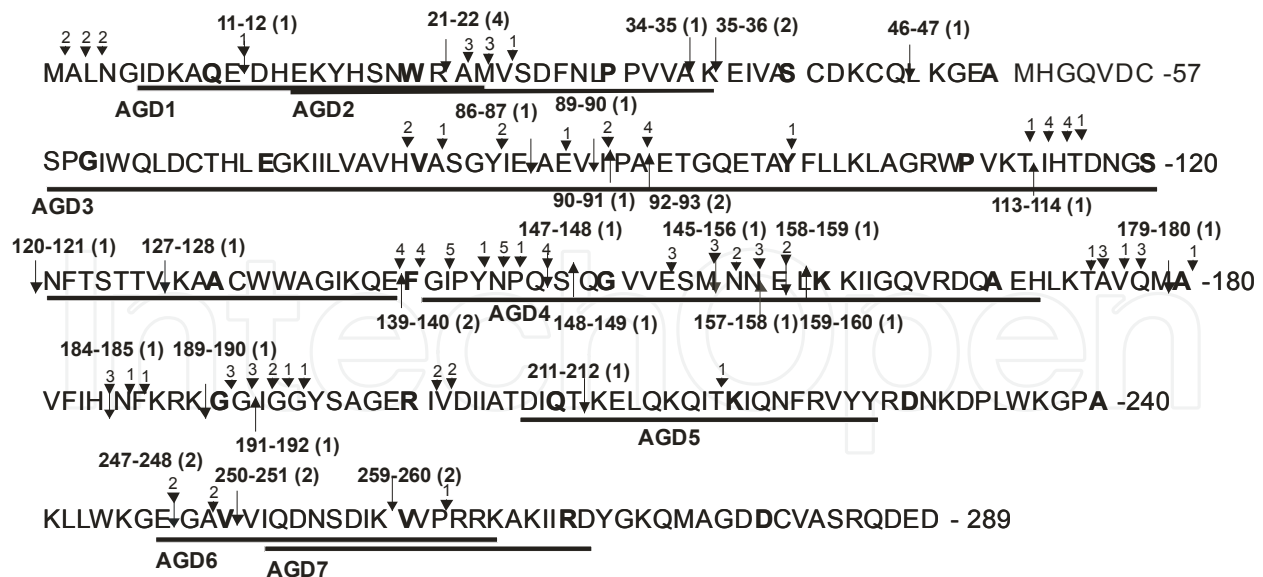
Fig. 14. All trypsin-independent points of IN cleavage determined from the MALDI-TOF analysis directly mol. masses of the P0–P4 fractions and after their cleavage by trypsin. Numbers next to the arrows show the position of the residue within the IN sequence; numbers under short arrows and in parentheses correspond to the frequency of these cleavage sites found using different approaches. Seven known antigenic determinants of IN are underlined in the figure.

Interestingly, a similar situation was observed for MS IgGs specifically hydrolyzing MBP; in addition to the sites of cleavage within four known AGDs of MBP, several sites were outside these determinants (Ponomarenko et al., 2006). Thus, the number of structurally different antigenic determinants in the case of IN may be great.

Interestingly, in contrast to the absence of hydrolysis of non-specific globular proteins by anti-IN Abs, they first cleave IN with the accumulation of long fragments corresponding mainly to known AGDs and then are capable of further degradation of these long intermediates, and the formation of very short products was observed after 72–100 h of IN incubation In with Abzs (Odintsova E., Baranova S., and Nevinsky G.A., personal communication). AIDS anti-IN pIgGs and IgMs hydrolyze specific 20-25-mer oligopeptides corresponding to the IN AGDs ~30–70-fold faster than nonspecific long 20-25-mer oligopeptides corresponding to AGDs of human myelin basic protein and HIV RT. In addition, AIDS anti-IN Abzs can hydrolyze very short 3-4-mer nonspecific oligopeptides 100–300-fold more slowly than specific ones. Therefore, the recognition and digestion of globular proteins and relatively short oligopeptides by the Abzs proceeds in different ways. Since catalytic centers of Abzs specifically hydrolyzing different proteins including IN are usually located on the light chains of Abs (Nevinsky et al., 2005, 2010a, 2010b, and refs therein), the observed hydrolysis of short oligopeptides can be a consequence of their interaction with light or heavy chains without significant contacts with alternative chains.

Interestingly, separated light chains of pIgGs, pIgMs, and pIgAs from the sera of patients with different AI and viral diseases usually significantly more active than intact Abs in the hydrolysis of DNA, RNA, oligosaccharides, and proteins (Nevinsky et al., 2005, 2010a, 2010b, and refs therein). This phenomenon may be a consequence of a higher affinity of intact Abs, as compared with separated light chains, for different substrates due to interaction of the

substrates with both light and heavy chains of Abzs. The separation of the light chains can lead to a decrease in the lifetime of the existence of the complex and, as a consequence, to an increase in the turnover number and V_{max} (k_{cat}) of the reaction catalyzed by L-chains. Taken together, the absence or very weak interaction of short substrates with heavy chains of AIDS Abzs in contrast with globular molecules proteins (and higher rate of the reaction) may be a main reason of a decrease of specificity of Abzs action in the case of short oligopeptides; one cannot exclude that light (or heavy) chains of some Abzs can effectively hydrolyzed short oligopeptides of any sequences.

We have shown that *in vitro* IgGs and IgMs hydrolyzing IN significantly decrease the 3'-processing and integration reaction catalyzed by IN (for example, Fig. 15) (Odintsova E., Baranova S., and Nevinsky G.A., personal communication).
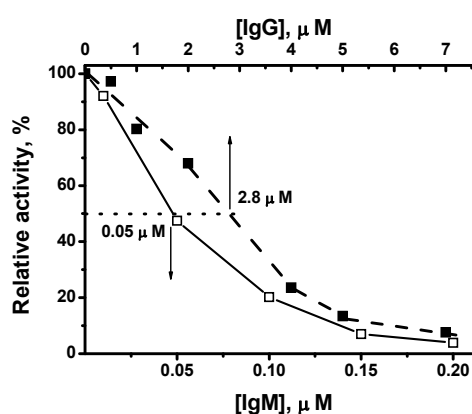

Fig. 15. Effect of IN-hydrolyzing IgGs and IgMs from AIDS patients on the rate of the 3'-processing reaction.

7. Conclusion

According to modern point of view, the immune response to the viral components is the most important factor providing slow transition of HIV infection to the stage of AIDS (Fauci et al., 2008). Since AIDS anti-IN anti-RT Abs can efficiently hydrolyze IN and RT (Odintsova et al., 2006b; Baranova et al., 2009, 2010), a positive role of abzymes in counteracting the infection cannot be excluded and these polyclonal and corresponding monoclonal Abzs with proteolytic activities are potentially interesting for designing new anti-HIV agents. In addition, detection of IN-hydrolyzing activity can be useful for diagnostic purposes and for assessment of the immune status in AIDS patients.

The field of monoclonal Abzs with immunotherapeutic potential has recently been reviewed (see "Introduction"). Abzs that cleave HIV envelope gp120 protein may find their use in the treatment of AIDS (Tellier, 2002; Stockwin & Holmes, 2003). pIgG degrading gp120 was also obtained taking advantage of the susceptibility of SJL mice to a peptide-induced AI disorder, experimental AI encephalomyelitis (Ponomarenko et al., 2006). Immunization of specific pathogen-free SJL mice with structural fragments of gp120 fused in-frame with the encephalitogenic MBP(85-101) peptide resulted in a pronounced disease-associated immune response against these antigens. This strategy can be generalized to create catalytic vaccines against viral pathogens (Ponomarenko et al., 2006). In addition, Abzs with different catalytic activities can be used for different purposes (see "Introduction").

In conclusion, a number of studies of Abzs show the extremely wide potential of the immune system in producing Abzs possessing very different enzymatic activities, which very often are not comparable with those of known enzymes, and natural Abs with specified and novel functions may have wide potential for biotechnology and medicine.

8. Acknowledgments

9. Abbreviations

Abs - antibodies

Abzs - abzymes - or catalytically active antibodies

AG - antigen

AI - autoimmune

AD - autoimmune disease

AIDS - human autoimmune deficit syndrome

BSA - bovine serum albumin

CC - correlation coefficient

HSCs - hematopoietic stem cells

sc - supercoiled

ss and ds - single- and double-stranded - respectively

CBA - (CBAxC57BL)F1 mice

HT - Hashimoto's thyroiditis

hMBP - human myelin basic protein

MFT - microsomal fraction of thyrocytes

MBP - myelin basic protein

MS - multiple sclerosis

nat-DNA and den-DNA - native and denatured DNA - respectively

MHO - maltoheptaose

pAbs and pIgGs - polyclonal Abs and IgGs - respectively

RF - rheumatoid factor

SLE- systemic lupus erythematosus - SDS-PAGE - SDS-polyacrylamide gel electrophoresis

TBE - tick-borne encephalitis

VIP - vasoactive intestinal peptide

RA - relative activity

CFU-GM - granulocytic-macrophagic colony-forming unit

BFU-E - erythroid burst-forming unit

CFU-GEMM - granulocytic-erythroid-megakaryocytic- macrophagic colony-forming unit

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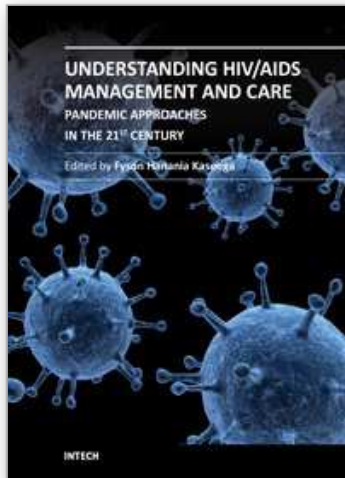
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