

# Cell Surface Glycans at SLE – Changes During Cells Death, Utilization for Disease Detection and Molecular Mechanism Underlying Their Modification

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## 1. Introduction

Autoimmune diseases develop when the immune system starts producing antibodies and T cells that are targeting components of the body. Such state occurs when the ability to recognition of self is disturbed and the immune cells attack healthy cells. The autoimmune diseases are frequently accompanied by self-destruction which is realized via apoptosis. There are two signaling pathways how the immune cells induce apoptosis in the target cells: 1) receptor-mediated; 2) receptor-independent. In the first mechanism, so called “death receptors” that are located on plasma membrane of the target cell are involved, while their “death ligands” are either located on the surface of the immune cell, or released by these cells and acting in free form. The corresponding ligand-receptor interactions cause activation of “death receptors” which use special “death domains” for contacting with specific intracellular signaling proteins and formation of death-inducing signaling complex (DISC). It is considered that this complex is capable of interacting with procaspase 8 and activating this initiator caspase. It is also probable that from this moment (activation of caspase 8), cascade of the apoptotic events gains irreversible character (Ashkenazi & Dixit, 1998). The receptor-independent mechanisms of apoptosis induction in which the immune cells take part, differ significantly (Vermijlen et al., 2001). These mechanisms are based on the ability of cytotoxic T cells to induce formation of special pores in plasma membrane of the target cells. Through these pores, calcium cations and protein granzyme B penetrates the target cells where they directly activate apoptotic enzymes – the caspases.

Fas-receptor that belongs to a family of tumor necrosis factor (TNF) receptors (Orlinick & Chao, 1998) is a typical “death receptor”. It is known that TNF is produced by activated macrophages and T cells as a host response to infection (Tartaglia & Goeddel, 1992). Its interaction with specific plasma membrane receptors induces production of transcription factors NF- $\kappa$ B and AP-1 which take part in the activation of specific genes whose products are involved in the inflammation and immunomodulation (Tartaglia & Goeddel, 1992).

After association with the adaptor protein TRADD, TNF receptor can interact with the procaspase 8, and this occurs during the TNF-induced apoptosis.

Apo2L or TRAIL is another TNF-like ligand that can induce apoptosis in various cell lines including tumor ones (Pradhan, Krahlting, Williamson, & Schlegel, 1997). Since the population of mature T cells gains sensitivity to the TRAIL-induced apoptosis after their stimulation with the interleukin 2, it is considered that TRAIL takes part in elimination of the peripheral T lymphocytes.

The receptor-independent apoptosis is the main mechanism by which the cytotoxic lymphocytes destroy virus-infected cells, as well as tumor cells (Ploegh, 1998). This mechanism is based on exocytosis of special dense granules which interact with plasma membrane of the target cells. These granules contain cytolytic substances which can polymerize in the presence of calcium cations and form macromolecular channels in the plasma membrane of the target cells. These channels are used for penetration of the granzyme B – serine protease that is capable of activating various caspases, for example the procaspase 3 (Goping et al., 2003). An elevated expression of the antiapoptotic mitochondrial protein Bcl-2 blocks such activation of the caspase 3, while the granzyme B blocks functioning of the Bcl-2. Thus, granzyme B is critical agent in the induction of apoptosis caused by the cytotoxic T cells.

### 1.1 SLE

Systemic Lupus Erythematosus (SLE) is a chronic, usually life-long, potentially fatal autoimmune disease characterized by unpredictable exacerbations and remissions with variable clinical manifestations. In SLE patients, there is a high probability for clinical involvement of the joints, skin, kidney, brain, lung, heart, serosa and gastrointestinal tract. Women and minorities are disproportionately affected, and Lupus SLE is most common in women of child-bearing age. A recent study identified a prevalence in the United States of 500 per 100,000 (1:200) in women (Belmont, 2010). SLE is a multifactorial disease involving genetic, environmental and hormonal factors. Its precise pathogenesis is unclear. There is growing evidence in favor of clearance deficiency of apoptotic cells as a core mechanism in SLE pathogenesis.

### 1.2 Clearance and SLE

Defective clearance of apoptotic cells causes secondary necrosis with a release of intracellular content and inflammatory mediators. This occurrence is considered as an intrinsic defect that can cause permanent presence of cellular debris responsible for the initiation of systemic autoimmunity in such diseases as SLE (for details see the review (Munoz, Lauber, Schiller, Manfredi, & Herrmann, 2010)). Macrophages respond and present self-antigens to T and B cells. Pathogenic autoantibodies are the primary cause of tissue damage in patients with lupus. The production of these antibodies arises by means of complex mechanisms involving every key facet of the immune system. Thus, restoring organism's ability to remove dying cells and impaired macromolecules (improving clearance efficiency) can serve as a perspective approach to treatment of autoimmune disorders and achieving clinical remission.

### 1.3 Ways to improve clearance

The apoptotic markers located in plasma membrane of the cell are very important, since they allow detecting apoptosis without violation of cell integrity. At present, phosphatidyl

serine externalization is the most widely used apoptotic marker on PM (Fadok et al., 1992). It is detected by the annexin V binding test (Reutelingsperger & Christiaan Peter, 1998). Recently, we found that apoptosis is accompanied by not only the loss of plasma membrane asymmetry caused by phosphatidyl serine externalization, but also by changes in cell surface glycoconjugates described by us (R. Bilyy & Stoika, 2007; R. O. Bilyy, Antonyuk, & Stoika, 2004; R. O. Bilyy & Stoika, 2003). Similar results were obtained by the group headed by Prof. Martin Herrmann (Heyder et al., 2003). Further findings of our (R. Bilyy et al., 2005; R. O. Bilyy et al., 2004) and other groups (Batisse et al., 2004; Franz et al., 2006; Franz et al., 2007) allowed us to consider that an increase in the exposure levels of  $\alpha$ -D-mannose- and  $\beta$ -D-galactose-rich GPs of the PM is a characteristic feature of the apoptotic cells. Their expression is substantially increased after apoptosis induction. Two independent mechanisms can lead to the appearance of altered surface glycoepitopes. One mechanism is the activation of surface sialidases resulting in exposure of desialylated (galactose-rich) surface glycoepitopes.

These glyconeoeitopes have been proposed for both the detection of apoptotic cells (R. O. Bilyy et al., 2004) and their isolation from the mixed populations (Stoika, Bilyy, & Antoniuk, 2006). Moreover, these glycoepitops can be directly involved in clearance of the apoptotic cells by the macrophages, serving as an “eat-me” signals of the apoptotic cells, as we have shown in (Meesmann et al., 2010). Our finding explains the previously known fact of surface glycopattern contribution to the clearance of dying and aged cells (Savill, Fadok, Henson, & Haslett, 1993). We effectively used changed apoptotic cell glycopattern for detection of dying cells in blood samples at the autoimmune disorders (R. Bilyy et al., 2009). We have proved that artificial desialylation of apoptotic and viable cells enhances their clearance by macrophages. This was confirmed in both cell lines and isolated human PMN and monocytes differentiated to macrophages (Meesmann et al., 2010).

Detection of both annexin V (van den Eijnde et al., 1997) and fluorescent conjugates of lectins (Heyder et al., 2003) usually requires using complex equipment like flow cytometer and/or fluorescent microscope. Evaluation of phosphatidyl serine externalization should be conducted as soon as possible after blood isolation, and cannot be done after the majority of available fixation procedure, since it would result in false-positive results; while the GPs are not affected by cell fixation or staining procedure. We have focused at the development of a test aimed to detect cell surface glycoconjugate changes during apoptotic cell death. We utilized the multivalency of lectin molecules for inducing agglutination of apoptotic cells, resulting from their altered surface GP content, and developed a specific test for apoptosis measurement (R. Bilyy & Stoika, 2007; Stoika et al., 2006).

## 2. Lymphocyte desialylation at SLE

Previously, we (R. Bilyy et al., 2009) demonstrated a significant increase in apoptosis incidence in the peripheral blood lymphocytes of RA patients comparing with lymphocytes of clinically healthy donors. That increase was detected by both flow cytometry and lectin-induced agglutination testing of apoptosis. We concluded that apoptosis-related changes in glycoconjugates of plasma membrane of the peripheral blood lymphocytes in RA patients can be used as a reliable and simple tool for apoptosis measurement during this and, probably, other autoimmune disorders. Detection of glycoconjugates via specific lectin binding is compatible with other available fixation/staining procedures, and can be recommended as an additional indicator in the multi-parameter automated detection systems.

Here we estimated changes in cell surface GP expression, namely changes in  $\beta$ -D-containing glycans, in the peripheral blood lymphocytes of SLE patients and clinically healthy donors, and compared these changes with the level of apoptotic cells detected by the alternative methods. Detection of  $\beta$ -D-containing glycans was performed by using VAA lectin staining, since this lectin binds to the surface of both early and late apoptotic cells, as can be seen at the confocal image on Fig. 1.

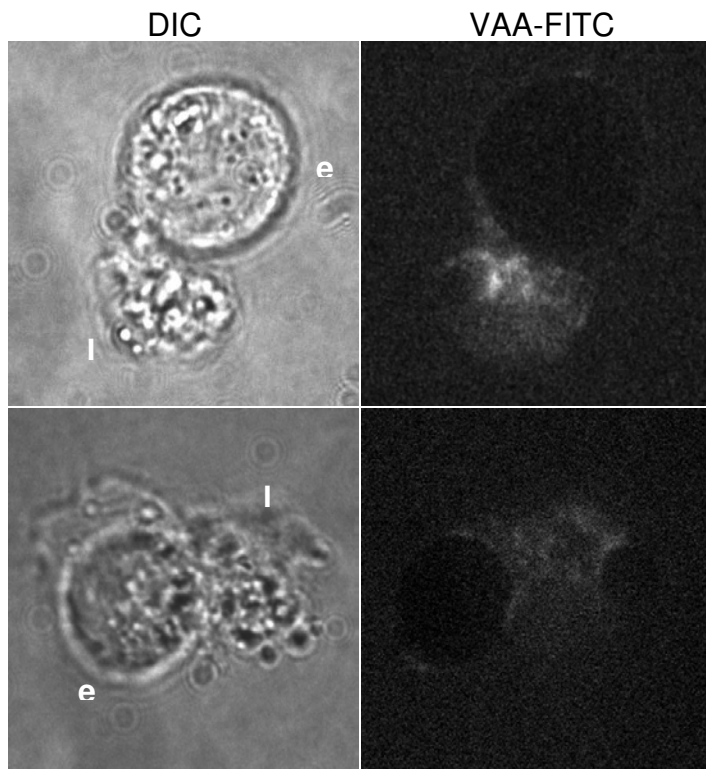
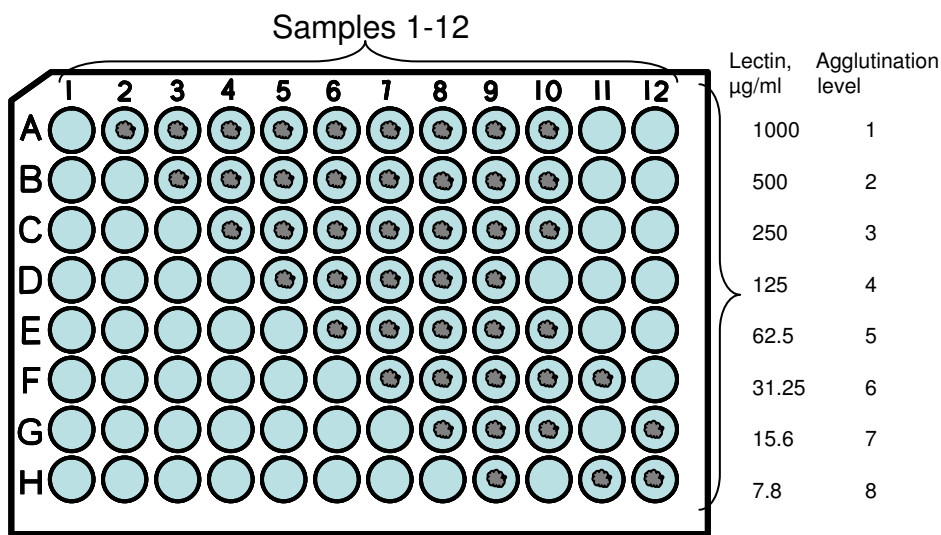


Fig. 1. Confocal microscopy of Jurkat T-cells at early (e) and late (l) stages of apoptosis progression, staining with VAA lectin. Lectin binds with cell surface, both cells are PI-negative.

Study of the peripheral blood lymphocytes of healthy donors revealed that their populations contained  $0.707 \pm 0.121$  % of cells with noticeable pre-G1 peak in cell cycle, with a range of 1.95% (minimal value - 0.14% and maximal value - 2.09), while the SLE patients were characterized by a markedly increased number of apoptotic cells (if judged by the appearance of G1 peak) -  $4.47 \pm 0.50$  %, with a wide range of 10.72% (minimal value - 0.86% and maximal value 11.62 %) (significance of the difference between two groups was  $P < 0.001$ ).

The lectin-induced agglutination test is based on the evaluation of minimal concentration of  $\beta$ -D-galactose specific *Viscum album* lectin (VAA) used for cell agglutination. The principle of lectin-induced agglutination test is described in Fig. 2. Previously, it was proved that the

level of these GPs was increased at apoptosis, and the concentration of lectin used for agglutination is in a reverse dependence upon the amount of cell surface GPs – the higher amount of the apoptotic like GPs is present on the cell surface - the less amount of lectin is needed for agglutination of these cells. Agglutination of lymphocytes of clinically healthy donors (0.32% and 0.12% of apoptotic cells), and of SLE patients (4.91% and 2.37% of apoptotic cells), as well as flow cytometry data on pre-G1 cell content are presented in Fig. 2.



Interpretation:												
0	1	2	3	4	5	6	7	8	7 <sup>2</sup>	- <sup>3</sup>	- <sup>4</sup>	Agglutination level
2000 <sup>1</sup>	1000	500	250	125	62,5	31,3	15,6	7,8	15,6	-	-	Lectin concentration

Fig. 2. Principle of lectin-stimulated agglutination test. Agglutination level corresponds to minimal lectin concentration, needed for cell agglutination. Notes: 1 - our data indicate that lectin concentration, 2000  $\mu\text{g/ml}$  agglutinates almost all intact cells. 2,3,4 - this conditions indicates possible errors in sample preparation and needs to be re-tested.

In the group of healthy donors, the mean lectin concentration needed to agglutinate lymphocytes was  $1,500 \pm 121.27 \mu\text{g/ml}$ , while in the group of SLE patients, this indicator equaled  $306.19 \pm 128.17$  (significance of the difference between two groups was  $P < 0.001$ ) (Table 1). Thus, the ratio between the lectin concentrations in two studied groups constituted almost 4 times. This could be caused by two reasons: 1. increased basal (overall) lectin binding by cells in population; 2. increased number of cells that specifically bind the lectin. To clarify these mechanisms, smears of peripheral blood lymphocytes were subjected to lectin-cytochemical analysis based on using VAA lectin with subsequent microphotography and densitometric study. It revealed that basal staining in control group was  $0.153 \pm 0.013 \text{ a.u.}$ , while in the SLE patients it was  $0.144 \pm 0.01 \text{ a.u.}$  There was no significant differences between two cell populations, while the number of cells that were intensively stained in both populations was significantly different (see Table 1). Thus, we suggested that difference in agglutination between lymphocytes of two groups is due to an increased percentage of cells exposing galactose-rich glycoconjugates on their surface.

	Healthy donors, n=18	SLE patients, n=23	p
% of apoptotic cells <sup>1</sup>	0.707 ± 0.121 %	4.471 ± 0.502 %	p<0.001
Agglutination <sup>2</sup>	1,500 ± 121.27 µg/ml	306.19±128.17 µg/ml	p<0.001
Basal VAA staining <sup>3</sup>	0.153 ± 0.013 a.u.	0.144 ± 0.010 a.u.	0.061
% of VAA stained cells	4.783 ± 0.936 %	8.27 ± 1.30 %	p<0.05

1 - judged by content of pre-G1 cells, measured by flow cytometry;

2 - measured by lectin-induced agglutination;

3 - measured by lectin-cytochemical analysis.

Table 1. Number of apoptotic cells and changes in plasma membrane glycoconjugates of lymphocytes in clinically healthy donors and SLE patients.

Correlation analysis of the amount of apoptotic cells detected by flow cytometry, and of minimal lectin concentration, needed for cell agglutination detected by lectin-stimulated agglutination, revealed a strong negative correlation between these two parameters ( $R=-0.764$ ,  $P<0.001$ , see Fig.4). As previously established, the agglutinating lectin concentration is reversely proportional to the amount of apoptotic cells. Thus, the amount of apoptotic cells established by both methods - pre-G1 cell detection by flow cytometry and the lectin-induced agglutination - is well correlated. It should be noted that lectin-induced agglutination is much easier and cheaper in performing.

The correlation study between the amount of apoptotic cells detected by the Annexin V-FITC labeling and by testing based on using mannose-specific lectin from *Narcissus pseudonarcissus* (both detected by flow cytometry) was performed, and strong correlation between both parameters ( $R=0.725$ ) was demonstrated (Heyder et al., 2003). Thus, specific changes in cell surface glycoconjugate pattern can be effectively used for detection of apoptotic cells at SLE and, probably, other at other autoimmune disorders.

The study of peripheral blood lymphocytes of 23 SLE patients and that of 18 clinically healthy donors revealed a significantly increased incidence of apoptosis in the SLE patients. That was detected by both flow cytometry and lectin-induced agglutination testing. High correlation between these results obtained by using two different methods suggests that apoptosis-related changes in plasma membrane glycoconjugates of the peripheral blood lymphocytes at SLE can be used as a reliable and easy tool for apoptosis measurement during autoimmune disorders. Detection of glycoconjugates via specific lectin binding is compatible with other available fixation/staining procedures. It can be recommended as an additional indicator in the multi-parameter automated detection systems.

Thus, the obtained data demonstrated that SLE was accompanied by an appearance of apoptotic cells possessing desialylated glycoepitopes (rich in terminal  $\beta$ -D-containing glycans). Taking into account the above described clauses that desialylated glycans are important for cell clearance and that SLE potentially results from insufficient cell clearance, an intriguing question appears- are there any desialylating agents in blood of SLE patients.

### 3. Desialylating abzymes at SLE

Mammalian sialidases (related enzymes including bacterial and viral are also referred as neuraminidases) are glycosidases responsible for the removal of sialic acids from the glycoproteins and glycolipids. They have been implicated to participate in many biological processes, particularly in lysosomal catabolism (Miyagi, Wada, Yamaguchi, Hata, &

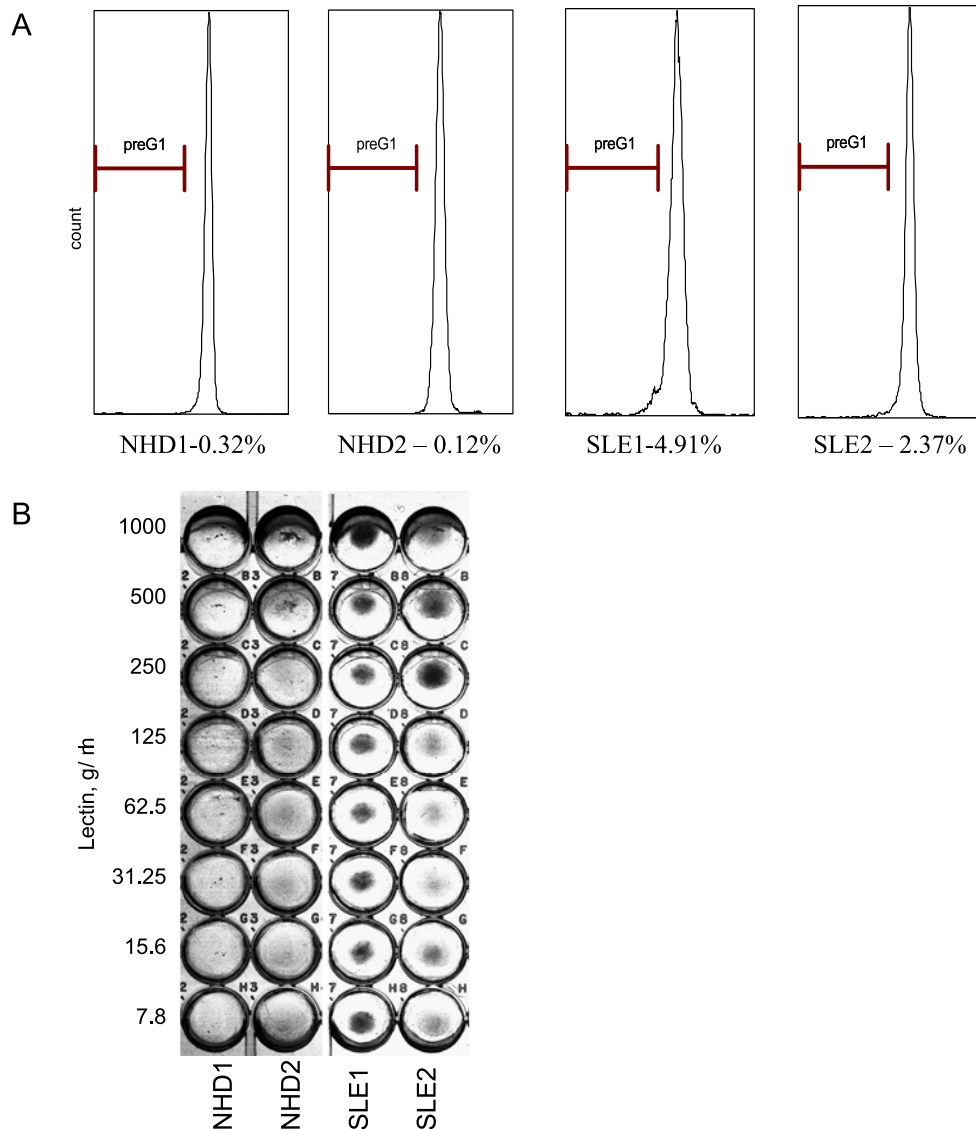


Fig. 3. Detection of apoptotic cells by measuring amount of pre-G1 cells using PI staining by flow cytometry (A) and minimal concentration needed for lectin-induced agglutination (B) of peripheral blood lymphocytes of two normal healthy donors and two SLE patients.

Shiozaki, 2008; Monti, Preti, Venerando, & Borsani, 2002). Altered sialylation of glycoproteins and glycolipids is observed as a ubiquitous phenotype in cancer. It leads to an appearance of tumor-associated antigens, aberrant adhesion and disturbance of transmembrane signalling (Miyagi, Wada, & Yamaguchi, 2008; Miyagi, Wada, Yamaguchi, & Hata, 2004). Aberrant sialylation is closely associated with the malignant phenotype of

cancer cells, including metastatic potential and invasiveness (Miyagi, Wada, & Yamaguchi, 2008; Miyagi et al., 2004; Miyagi, Wada, Yamaguchi, Shiozaki, et al., 2008). However, its biological significance and molecular mechanisms have not been fully elucidated.

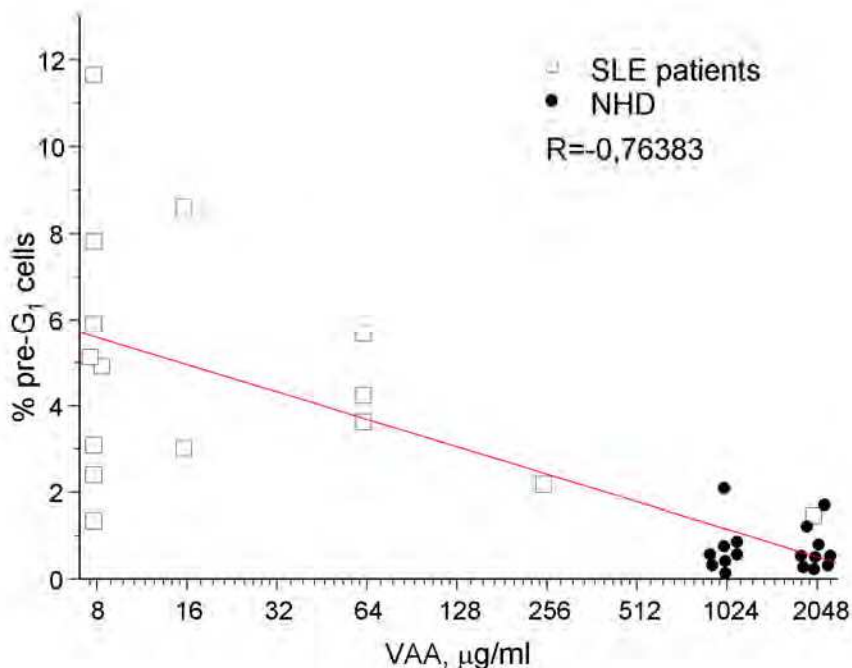


Fig. 4. Correlation analysis between specific lectin concentrations needed for lymphocyte agglutination and a percentage of the apoptotic cells. Lectin concentration needed for agglutination is reversely proportional to the amount of apoptotic cells.

Neuraminidases are abundant in prokaryotes and viruses, while only 4 sialidases are known in human (Miyagi, Wada, Yamaguchi, Shiozaki, et al., 2008). The last described one, Neu4, was reported only in 2003 (Comelli, Amado, Lustig, & Paulson, 2003). Neu 1 is a lysosomal sialidase, and Neu2 is localized in lysosomes and involved in digestion of N-glycans, and Neu3, known as ganglioside sialidase, is localized in plasma membrane and involved in ganglioside metabolism (Monti et al., 2000). Neu4 is localized in lysosomes (Seyrantepe et al., 2004) and can be translocated to mitochondria (Yamaguchi et al., 2005) and endoplasmic reticulum (Bigi et al.). However, none of known sialidases is active in the body fluids (blood or lymph). There is no evidence that plasma membrane sialidase Neu3 (or any other sialidase) can be shed from cell surface into the blood flow (Miyagi, Wada, Yamaguchi, Shiozaki, et al., 2008). While detecting increased neuraminidase activity on the surface of apoptotic cells (R. Bilyy, Tomin, & Stoika, 2010), we failed to detect any sialidase activity in culture media that could result from enzyme secretion/release during cell death.

We have focused our attention at the catalytic antibodies. These antibodies, now named as "abzymes", were first obtained in 1986 (Pollack, Jacobs, & Schultz, 1986; Tramontano, Janda, & Lerner, 1986), the first example of natural abzymes was IgG found in bronchial asthma



patients, cleaving intestinal vasoactive peptide (Paul et al., 1989). Abzyme's properties were discussed in more detail in recent reviews (Belogurov, Kozyr, Ponomarenko, & Gabibov, 2009; Georgy A. Nevinsky & Buneva, 2005; Planque et al., 2008; Taguchi et al., 2008). Abzymes were detected in human organism at a variety of autoimmune and non-autoimmune pathologies (Gabibov, Ponomarenko, Tretyak, Paltsev, & Suchkov, 2006; G. A. Nevinsky & Buneva, 2003), and various peptides, proteins, nucleic acids and oligosaccharides can serve as substrates for the catalytically active antibodies in human and other mammals (Hanson, Nishiyama, & Paul, 2005; Lacroix-Desmazes et al., 2006). The involvement of abzymes in pathogenesis of autoimmune disorders has been documented (Gabibov et al., 2006; Hanson et al., 2005; Lacroix-Desmazes et al., 2006; G. A. Nevinsky & Buneva, 2003). Catalytically active antibodies are typically found in patients with autoimmune disorders, however, they have also been detected in cancer patients. DNA-hydrolyzing activity of IgG auto-Ab from blood serum of patients with various types of lymphoproliferative diseases was described (Kozyr et al., 1998; Kozyr et al., 1996). Testing of the abzymes in patients with hematological tumors and SLE revealed a linkage of anti-DNA

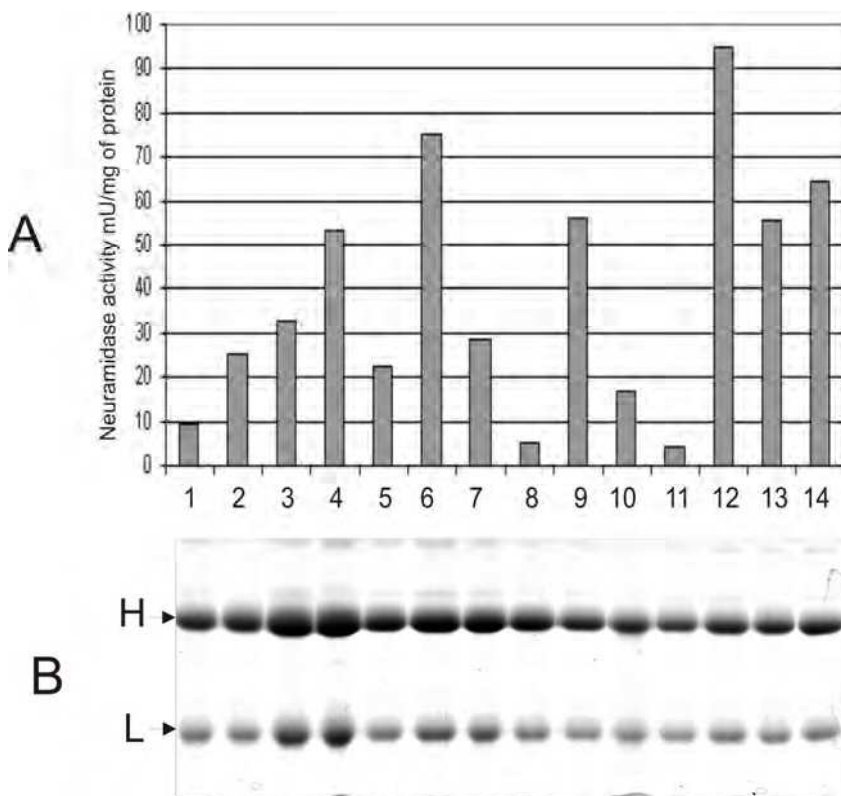


Fig. 5. Comparative analysis of sialidase activity of IgGs obtained by chromatography on Protein G - Sepharose from blood serum of SLE patients (A) and SDS-PAGE electrophoresis of IgG-preparations (B), n = 14. The position on the gel of heavy (H) and light (L) chains of IgGs molecules is indicated.

Ab catalysis with mature B-cell tumors, and an increased probability of DNA-abzymes formation at the autoimmune conditions (Gabibov et al., 2006). These data suggest a similarity between the mechanisms of abzyme formation at SLE and B-cell lymphomas. Peptide-hydrolyzing and DNA-hydrolyzing activities of Bence Jones proteins isolated from blood serum of myeloma patients are well studied (Paul et al., 1995; Sun, Gao, Kirnarskiy, Rees, & Paul, 1997). There are numerous data demonstrating that the catalytic activity of anti-DNA IgGs and Bence Jones proteins are associated with their cytotoxic activity and correlate with the disease pathogenesis (Gabibov, Kozyr, & Kolesnikov, 2000; Kozyr et al., 2002; Matsuura, Ohara, Munakata, Hifumi, & Uda, 2006; Sashchenko et al., 2001; Sinohara & Matsuura, 2000). Recently, we demonstrated that anti-histone H1 IgGs isolated from blood serum of multiple sclerosis patients, were capable of hydrolyzing histone H1 (Kit, Strykovych, Richter, & Stoika, 2008). IgGs with similar proteolytic activity were also found in blood serum of patients with SLE (Magorivska et al., 2010) and multiple myeloma (Magorivska et al., 2009). Recently, we have shown that in the blood serum of some multiple myeloma patients there are immunoglobulins IgG possessing sialidase activity (R. Bilyy, Tomin, Mahorivska, et al., 2010). These data suggest an important role of abzymes at the autoimmune and oncological diseases. However, further studies are needed for better understanding of humoral immunity functions under normal and pathological conditions. Here we demonstrated for the first time that blood serum of SLE patients contains catalytically active IgGs possessing sialidase activity. Biological consequences of such phenomenon are discussed.

The reason for studying neuraminidase activity of Ab in SLE patients is based on data showing that Ig preparations obtained by precipitation with 50% saturated ammonium sulphate from blood serum of 14 SLE patients possessed a significant capability of hydrolyzing neuramidase substrate 4-MUNA (Fig. 5), while Ig preparations of 12 healthy

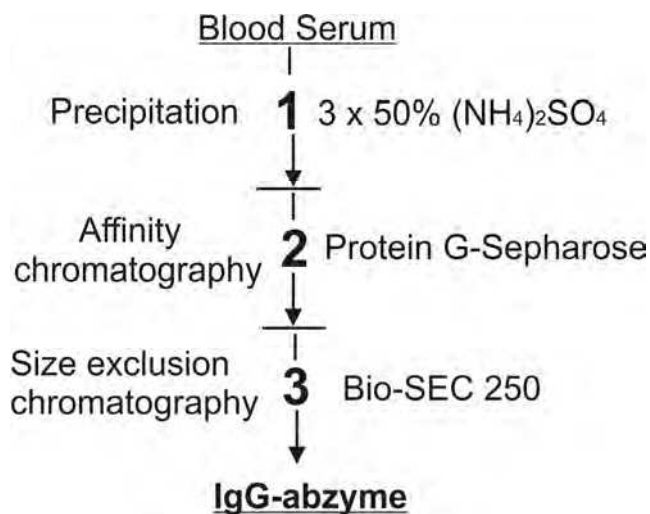


Fig. 6. Purification of IgG-abzymes from blood serum of SLE patients. Step 1 - Three-fold Ab precipitation with ammonium sulfate. Step 2 - IgG isolation by affinity chromatography on protein G-Sepharose column. Step 3 - HPLC size exclusion chromatography at pH 2.6, favoring dissociation of the immune complexes on Bio-Sec 250 column.

donors, obtained in the similar manner, were devoid of significant level of sialidase activity. Thus, we suggested that at least a part of this catalytic activity could be linked to abzymes present in the Ig preparations. To verify this suggestion, the catalytically active Ig preparations obtained with ammonium sulphate precipitation were further purified by the chromatography on protein G-sepharose column (Fig. 6) and additionally purified by HPLC SEC at neutral and acidic conditions (Fig.7). Besides, we obtained (Fab)<sub>2</sub>-fragments of this

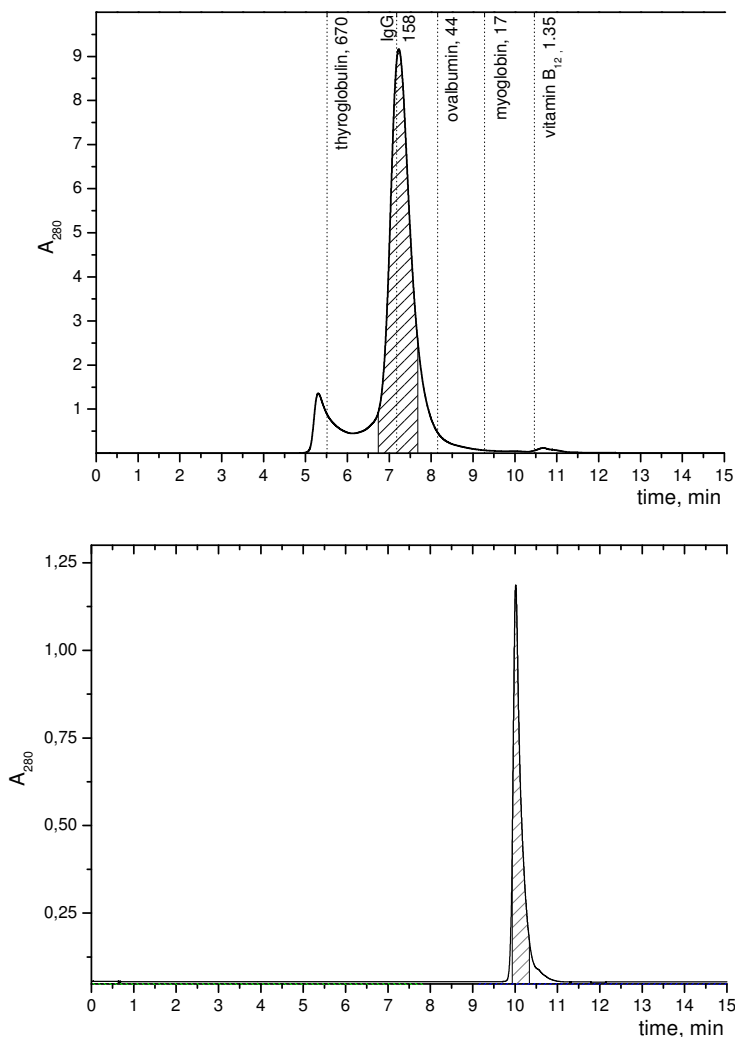
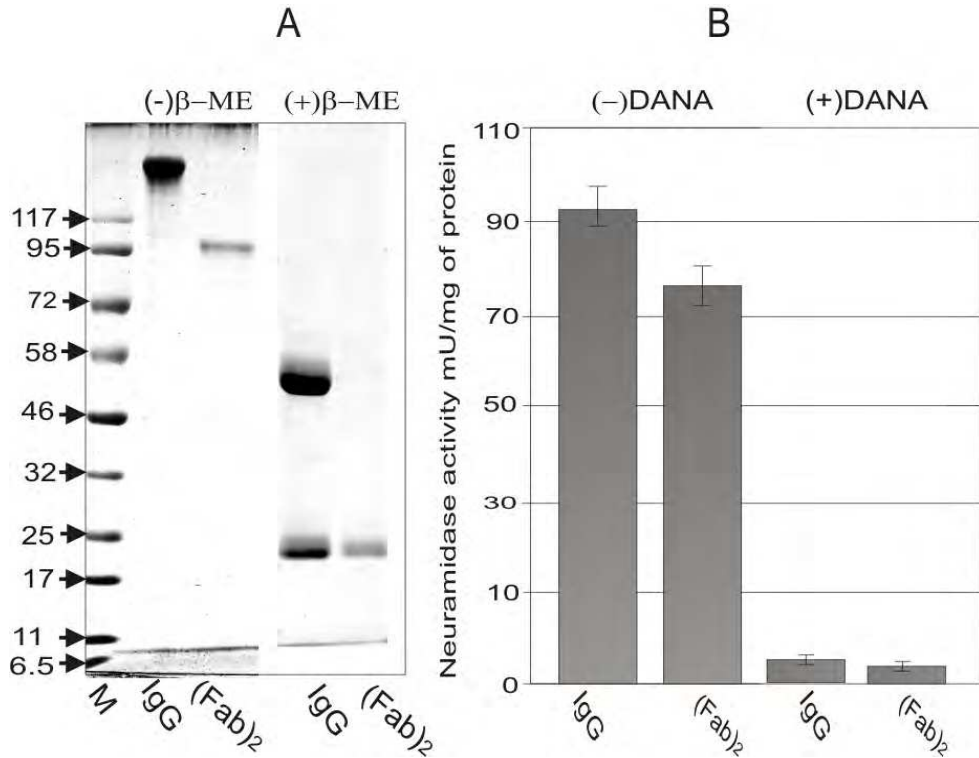


Fig. 7. Typical HPLC size exclusion chromatography on Bio-Sec 250 column (PBS, pH 6.8) elution profile of IgG preparation after purification by affinity chromatography on protein G-Sepharose (top) and additional size exclusion chromatography of this IgG sample at pH 2.6 (glycine\*HCl), favoring dissociation of the immune complexes on Bio-Sec 250 column (bottom). Peaks indicated by shading were collected and used for further analysis.

IgG, and studied their sialidase activity. It was found that both IgG preparation and its (Fab)<sub>2</sub> fragments possessed sialidase activity towards 4-MUNA, but not galactosidase activity towards 4-MU-Gal (Fig. 8). Sialidase activity towards 4-MUNA was not inhibited in the presence of 10 mM 4-MU ( $p < 0.05$ ).



A - Homogeneity determination of IgGs and their (Fab)<sub>2</sub> by SDS electrophoresis in gradient PAGE (5-16%) in the absence (-) or presence (+) of beta-mercaptoethanol (in non-reducing and reducing conditions, respectively). M: protein molecular mass markers (kDa).

B - Sialidase activity of IgGs and their (Fab)<sub>2</sub> in the absence (-) or presence (+) of specific sialidase inhibitor DANA.

Fig. 8. Evidences that sialidase activity of IgG preparations purified by the affinity chromatography on protein G-sepharose from blood serum of SLE patient is an intrinsic properties of antibodies.

To prove that sialidase activity of IgG fractions isolated from the SLE patients is an intrinsic property of the abzymes and is not caused by the co-purified enzymes/impurities, we applied the same criteria to the purity of catalytic Ab which have been proposed earlier (G. A. Nevinsky & Buneva, 2003; Paul et al., 1989). To rule out possible enzymatic contamination tightly bound to IgG molecule, we performed HPLC-SEC chromatography at the acidic conditions (pH 2.6), that are known to guarantee dissociation of antibody-antigen complexes (Hanson et al., 2005; G. A. Nevinsky & Buneva, 2003) (Fig. 7). It was confirmed by the SDS-PAGE electrophoresis and Western-blot analysis using anti(human)-IgG Ab that

the main chromatographic peak is an electrophoretically homogeneous IgG. Its sialidase activity was tested and shown to be attributable to IgG fraction. HPLC purification resulted in the retention of ~50% of original sialidase activity of protein-G purified IgG sample. Sialidase activity was significantly decreased when the reaction was performed in the presence of pan-neuraminidase inhibitor DANA that excludes a possibility of non-specific hydrolysis reaction. The mechanism of DANA action is connected with its resemblance of the unhydrolyzable transition-state analogue formed during sialic acid cleavage which is irreversibly bound by active centers of most neuraminidases (Chavas et al., 2005).

It is known, that the pH optimum of different sialidases is in range of pH 4–6.5. We have shown that isolated IgG is active under the physiological pH of blood serum. By using buffer systems in the pH range 3–9, we found that studied IgG samples revealed maximum speed reaction at pH range of 4.5–6.0, nevertheless at pH 7.4 all samples retained from 27 to 52% of their maximal activity, measured at NaCl concentration equal to that of blood serum. This suggests its potential enzymatic effectiveness in blood serum.

In order to determine the speed of catalytic reaction of both IgG and corresponding (Fab)<sub>2</sub>-fragments, we have calculated kinetic parameters ( $K_m$ ,  $V_{max}$ ,  $k_{cat}$ ) for sialidase reaction at 0.1–100  $\mu\text{M}$  concentrations of the substrate. Computer analysis demonstrated that the observed reaction belongs to a single substrate type, described by classical Michaelis-Menten equation. The calculated data for different studies sialyl abzymes were:  $K_m=44.4\div 1600 \mu\text{M}$  and  $k_{cat}=0.045\div 23.1 \text{ min}^{-1}$  (Fig. 9). The catalysis mediated by an artificial abzymes is usually characterized by relatively low reaction rates:  $k_{cat}$  values are  $10^2$ – $10^6$ -fold lower than for the canonical enzymes (Georgy A. Nevinsky & Buneva, 2005). The known  $k_{cat}$  values for natural abzymes vary in the range of 0.001–40  $\text{min}^{-1}$ . The  $k_{cat}$  values detected for MUNA hydrolysis (0.045–23.1 depending on sample) are comparable with the typical  $k_{cat}$  values established for others abzymes. To validate the kinetics assay, we have used *C. perfringens* neuraminidase as standard for kinetic parameters measurement. According to the obtained results, *C. perfringens*  $K_m$  equals to 89.2  $\mu\text{M}$  for 4-MUNA, which is in a good accordance with the literature data of 120  $\mu\text{M}$  (Li et al., 1994)(Inoue & Kitajima, 2006), while  $V_{max}$  was detected to be 2856  $\mu\text{mol}/\text{min}/\text{mg}$ . The obtained  $K_m$  value of IgG were of similar range, while  $V_{max}$  of IgG was significantly lower ( $k_{cat}$  significantly higher) than that of *C. perfringens* neuraminidase.

Principal question concerning a role of the discovered abzymes possessing sialidase activity is whether they can use as potential desialylation substrates also glycoproteins and glycolipids that are present in human's blood plasma and cells. Earlier, we have shown that sialyl-abzymes from multiple myeloma patients act towards human RBC by desialylating them and promoting agglutination with PNA lectins (R. Bilyy, Tomin, Mahorivska, et al., 2010). It is known that the peanut agglutinin (PNA) agglutinates human RBC after their sialidase treatment resulting in the exposure of sub-terminal Gal-residues (Nakano, Fontes Piazza, & Avila-Campos, 2006). Here, by incubating IgG preparation from SLE sera with RBC of NHD (blood group A(0)) and using subsequent agglutination test with different PNA lectin concentrations, we demonstrated an ability of IgG-abzyme obtained from blood serum of SLEs patient desialylate human RBCs directly. Agglutination was observed at PNA concentration 7  $\mu\text{g}/\text{ml}$ , while when the IgG preparation from NHD was used, agglutination was achieved at 250  $\mu\text{g}/\text{ml}$ ; PBS served here as a negative control (no agglutination at 1,000  $\mu\text{g}/\text{ml}$ , and *Clostridium perfringens* neuraminidase (10 mU) served as a positive control, agglutination at 7  $\mu\text{g}/\text{ml}$  of PNA. Thus treatment with sialyl abzymes from

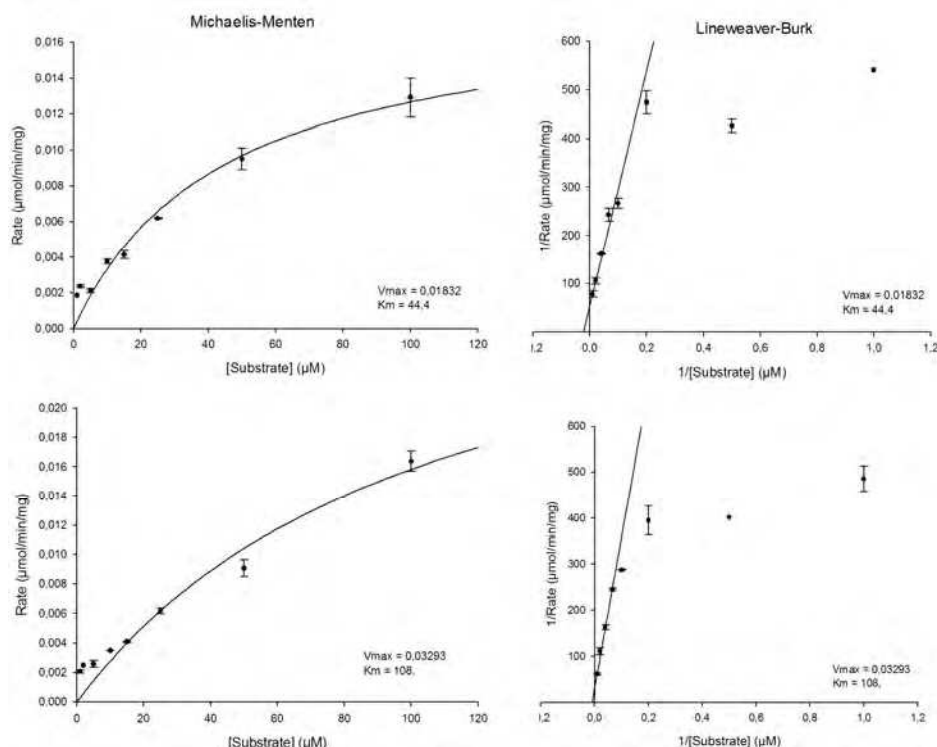


Fig. 9. Kinetic parameters (Michaelis-Menten and Lineweaver-Burk plots) of sialidase reaction catalyzed by the IgG (A) or its (Fab)<sub>2</sub>-fragments (B). The incubation time for all samples was 180 min.

SLE patient have increased the amount of desialylated glycoepitopes for 250 μg / 7 μg =35 times. We also used as substrates for sialyl abzymes: a) gangliodes of mouse brain and b) total surface glycans on eukaryotic (human T-leukemia Jurkat) cells. Ganglioside fraction was isolated from mouse brain and was incubated with sialil-abzyme and neuraminidase. Both sialil-abzyme and neuraminidase caused desialylation of GM3 and increase in the content of free sialic (neuraminic) acid (Fig.10). Treatment of human leukemia Jurkat cells with sialil-abzyme and neuraminidase caused a decrease in the level of α2,6-sialil-reach surface glycoconjugates (if judged by binding of FITC-labeled SNA lectin analyzed by flow cytometry) (Fig 11). Moreover, treatment of Jurkat cells with sialil-abzyme and neuraminidase also caused an increase in the level of desialylated glycoepitopes, if judged by binding of PNA lectin (biotinylated, treated with streptavidin-FITC and analyzed by flow cytometry) (Fig. 11).

Thus, isolated sialyl abzymes were desialylating both human RBC, gangliosides and total surface glycoepitopes of the eukaryotic cells and were active under pH and ion content values of human blood serum.

We have demonstrated previously unknown catalytic activity in the IgG antibodies of SLE patients – an intrinsic sialidase activity. Such activity was present in the IgG of blood serum

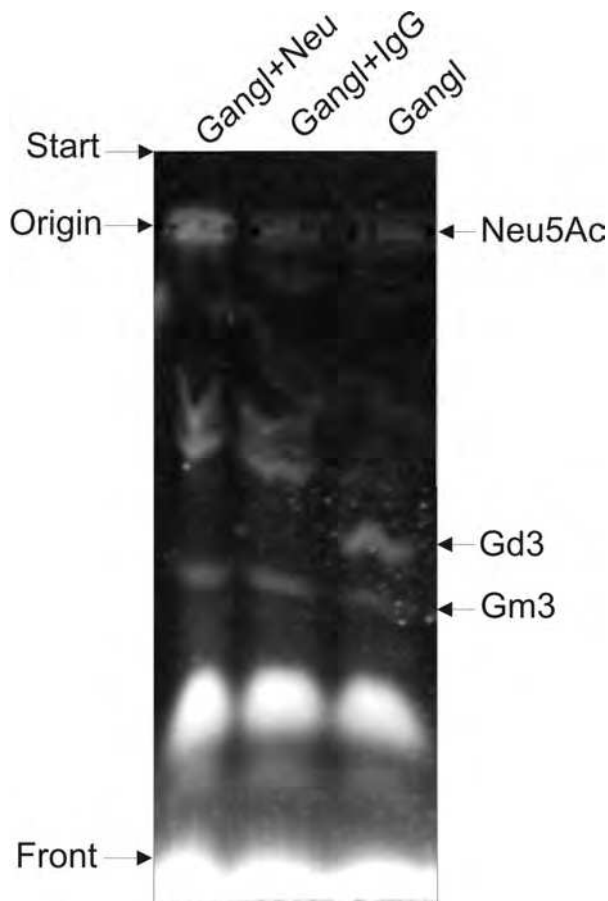
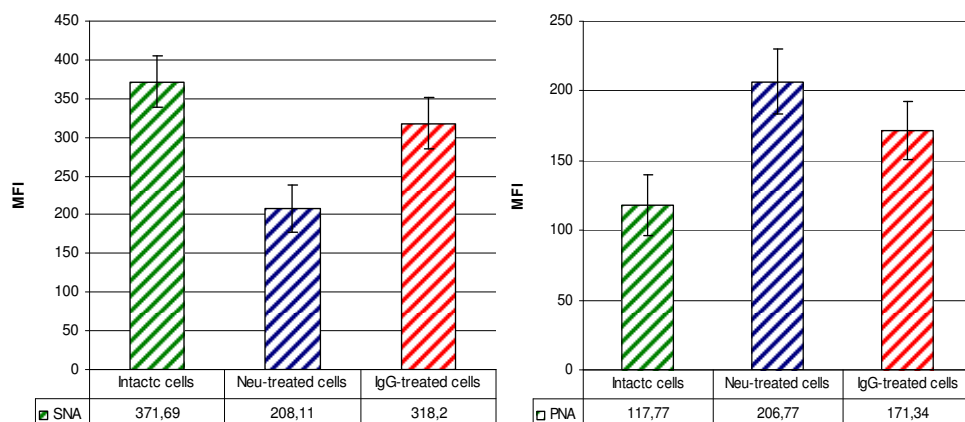


Fig. 10. Desialation of gangliosids by sialidase active IgGs obtained from blood serum of SLE patients possessing highest sialidase activity. Gangl+Neu – gangliosides incubated with neuramidase from *C. perfringens*; Gangl+IgG – gangliosides incubated with sialidase active IgGs; Gangl – gangliosides without incubation (control). The positions of free neuraminic acid (Neu5Ac), gangliosides GM3 and GD3 are shown by arrows on the right hand.

of SLE patients and absent in the IgG of NHD. Sialidase activity was detected under different conditions which exclude a possibility of contamination or artefacts. It was blocked by typical sialidase inhibitor (DANA), expressed under physiological pH, and corresponded to classical Michaelis-Menten kinetics. Since DANA is an unhydrolyzable transitional state analogue of hydrolysis reaction, one can assume that the mechanism of action of IgG with sialidase activity is similar to that of sialidase enzyme. Moreover, the described IgGs possessing sialidase activity were capable of direct desialylation of human RBCs, gangliosides and T-lymphocytes. The reason for appearance of discovered sialidase activity is not fully understood. One of the possible suggestions is their anti-idiotypic antibody as the "internal image" of an active site of endo- or exogenic sialidases, as known for other abzymes (Friboulet, Izadyar, Avalle, Roseto, & Thomas, 1994).



Jurkat cells were stained with FITC-labeled SNA (left), or biotinylated PNA lectin (right), stained with streptavidin-FITC. Cells were either treated with Neuraminidase, 10mU or sialil-abzyme, 10 uM for 3h at 37°C. Data represent normalized MFI of lectin binding. SNA lectin binds terminal  $\alpha$ 2,6-sialic acid residues, while PNA lectin binds desialylated glycoepitopes (Antonyuk, 2005).

Fig. 11. Analysis of lectin binding to human Jurkat T-cells.

The level of IgG molecule's sialylation was reported to be critical in defining their pro- or anti-inflammatory properties (Kaneko, Nimmerjahn, & Ravetch, 2006). Anti-inflammatory activity of immunoglobulins was tightly connected with the presence of  $\alpha$ 2,6-sialylated Asn<sup>297</sup> in the IgG molecule (Anthony, Nimmerjahn, et al., 2008). Macrophages receptors responsible for binding sialylated IgG and modulating its anti-inflammatory action were also described (Anthony, Wermeling, Karlsson, & Ravetch, 2008). Agalactosylated and desialylated IgG antibodies' action *in vivo* depends on binding of cellular Fc receptors (Nimmerjahn, Anthony, & Ravetch, 2007). Specific glycoforms, if present in populations of immunoglobulin molecules, are connected with disease-associated alterations and can serve as diagnostic biomarkers at rheumatoid arthritis and other diseases (Arnold, Wormald, Sim, Rudd, & Dwek, 2007). Blood serum level of desialylated form of IgG (IgG-G0) isolated from patients with rheumatoid arthritis, are more than 2 standard deviations above those levels in the age-matched healthy control (R. B. Parekh et al., 1985). They correlate with the disease activity and fall during remission periods (Rook et al., 1991). High levels of desialylated IgG-G0 are also characteristic for other disorders: Crohn's disease, SLE complicated by Sjogren's syndrome, and tuberculosis (Bond et al., 1997; R. Parekh et al., 1989; R. B. Parekh et al., 1985). The enzyme EndoS from *Streptococcus pyogenes* that cleaves IgG glycan between two GlcNAc residues, was used for "making autoantibodies safe" (Scanlan, Burton, & Dwek, 2008). The action of EndoS truncated IgG glycans and IgG molecules lost the ability to initiate activating signals through C1q, Fc $\gamma$ Rs and MBL, while the ability to interact with inhibitory Fc $\gamma$ RIIB was preserved (Collin, Shannon, & Bjorck, 2008).

#### 4. Summary

In previous studies, we have shown that cell surface glycopattern is changed during apoptosis. This, in part, results from activation of surface sialidases, with desialylated glycoproteins being characteristic markers of apoptosis (R. Bilyy & Stoika, 2007). Such



feature was successfully utilized for lymphocyte screening in the autoimmune patients (R. Bilyy et al., 2009). It is widely accepted that altered glycoepitopes (desialylated) are important surface markers for clearance of apoptotic cells (R. Bilyy & Stoika, 2007). We have shown (Meesmann et al., 2010) that desialylation of cell surface epitopes in viable cells, caused by sialidase, acts as an “eat-me” signal for macrophages and is needed for elimination of late apoptotic cells along with phosphatidylserine exposure, needed for elimination of early apoptotic cells. Apoptotic cells possess an elevated neuraminidase activity on their surface, however, we failed to detect any sialidase activity in culture media that could result from enzyme secretion/release during cell death. As we have shown, SLE – a disease resulting from insufficient cell clearance (Munoz et al., 2010) - is accompanied by the appearance of desialylated lymphocytes in blood stream. At the same time, some of SLE patients possessed abzymes with sialidase activity in their blood. The exact role of sialyl abzymes at SLE is currently unclear, as well as their relation to apoptotic cell desialylation and clearance. The abzymes possessing sialidase activity can change surface sialylation level and, thus, alter the glycocalyx of SLE patients’ cells and promote their clearance. They can also influence the immune response by acting towards blood serum IgG molecules.

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## 6. Abbreviations

GP - glycoprotein, PI - propidium iodide, PM - plasma membrane, RA - rheumatoid arthritis, Ab - antibodies, DANA - 2,3-dehydro-2-deoxy-N-acetylneuraminic acid, SLE - multiple myeloma, 4-MUNA - 2’-(4-Methylumbelliferyl)- $\alpha$ -D-N-acetylneuraminic acid, 4-MU-Gal - 4-Methylumbelliferyl- $\beta$ -D-galactopyranoside, NHD - normal healthy donors, RBC - red blood cells, SLE - systemic lupus erythematosus.

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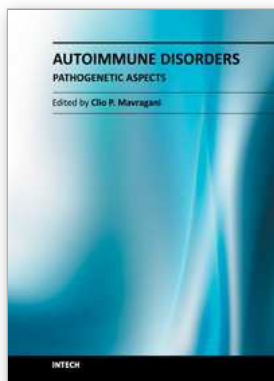
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