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Mitotic activity of anti-histone H1 sIgA-antibodies from milk of healthy mothers

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Aim. *Earlier, we have shown that antibody (AT) preparations obtained by precipitation with 50 % ammonium sulfate from milk of some healthy mothers possess pro-proliferative activity toward transformed and tumor cells in vitro (Kit et al., 2008). We hypothesized that this effect is associated with the presence of the anti-histone H1 sIgAs in AT preparations.* ***Methods***. *To check this hypothesis, we obtained electrophoretically homogeneous anti-histone H1 sIgAs from milk of healthy mothers by sequential chromatography on protein A-Agarose, protein G-Sepharose and histone H1-Sepharose respectively. These Ab were tested on a proliferative activity toward human T-leukemia Jurkat and human melanoma SK-MEL cells.* ***Results***. *It was found that anti-histone H1 sIgAs are able to stimulate proliferation of both tumor cell lines. Mitotic effect of these AB was confirmed with an increase of signal proteins involved in cell proliferation (c-Myc, MAP-and cdc2-protein kinases), detected by Western-blot analysis. We also studied the antigenic reactivity of anti-histone H1 sIgAs toward SK-MEL cell proteins. It was observed that these AB possessed an affinity for a number of melanoma cell proteins with molecular masses of 60, 55, 48 and 38 kDa.* ***Conclusions***. *It has been found that anti-histone H1 sIgA antibodies can stimulate proliferation of human T-leukemia Jurkat and human melanoma SK-MEL cells in vitro. The cross reactivity of these AB could serve as an explanation of their mitotic activity toward the target cells.*

Keywords: human milk, autoantibodies, anti-histone H1 sIgA, tumor cell, proliferation.

Introduction. Immune system of mammals ensures the protection of the organism from harmful environmental agents and is involved into the regulation of biological functions, defining its homeostasis [1]. An important role in maintaining homeostasis play antibodies (AT), directed to foreign and self-antigens (auto-Ab) [1, 2]. Auto-ATs were found in the organisms of patients with autoimmune and oncological diseases as well as in clinically healthy people [3]. In healthy people, auto-ATs are mainly presented by polyspecific low-affinity IgM or high-affinity IgGs (anti-idiotypic AT), involved into the regulation of immune response [3, 4]. Humans with autoimmune diseases were revealed to have high-affinity IgG auto-AT, which may participate in the development of autoimmune processes [5]. The determination of the level of these auto-ATs in human blood serum is a new approach, widely used in the diagnostics of various autoimmune diseases as well as in predicting the clinical course in patients [6].

Auto-ATs of various specificity were also found in human secretory fluids. A significant level of secretory immunoglobulin A (sIgA) with affinity to human actine, myosin, tubulin and spectrin was detected in saliva and colostrum of clinically healthy women [7]. These immunoglobulins were called polyspecific sIgA-antibodies (poly-sIgA).

Poly-sIgAs with broad antigenic specificity are believed to be produced by B1-lymphocytes and to ensure

the protection of mother and child from pathogenic microflora [8].

Another type of auto-AT, revealed in human secretions, is low-affinity anti-alpha-galactosyl AT (anti-Gal AT). These AB interact with carbohydrate residues Gal-α-1,3-Gal-β-1,4-GalcNAc-R (alpha-galactosyl epitopes) [9, 10]. They are synthesized in about 1 % B-lymphocytes in response to the effect of antigens of gastric bacteria. Anti-Gal ATs were found in human milk, saliva, vaginal smears and bile. They are capable of causing agglutination of rabbit erythrocytes (REA-AT) are binding bovine thyroglobulins, containing alpha-galactosyl epitopes [11].

Contrary to the secretory fluids where the sIgA level is high (3–5 mg/ml), the concentration of sIgA in blood serum of clinically healthy people is low (0.01–0.02 mg/ml). It was shown that the sIgA level in human blood serum essentially increases due to some chronic diseases of liver and gastrointestinal system, autoimmune, oncological and a number of infectious diseases of viral and bacterial origin. The functional activity of secretory immunoglobulins in blood plasma is poorly studied; although there are the data about their influence on immune cells [11–19].

Our previous studies have demonstrated that the preparations of antibodies, isolated from colostrum and milk of healthy mothers have different effect on T-leukemia Jurkat cells. Depending on donors, they are capable of either induce apoptosis of Jurkat T-cells or stimulate proliferation of these cells *in vitro* [20]. These data demonstrate that secretory antibodies may also influence the growth and viability of human tumor cells. Our further studies showed that [the] cytotoxic activity of secretory AT preparations towards tumor cells could be linked with anti-DNA sIgA-antibodies [21], though the mitogenic effect of secretory antibodies remained unstudied.

Previously we have found that blood serum of patients with multiple myeloma contains IgG-antibodies with affinity to histon H1 (anti-histon H1 sIgA-antibodies), capable of stimulating *in vitro* proliferation of T-leukemia CEM-T4 cells [22].

We assumed that the milk of some mothers might contain anti-histon H1 sIgA-antibodies possessing mitogenic activity similar to the anti-histon H1 IgG-antibodies from blood serum of patients with multiple myeloma.

The aim of this work is purification of electrophoretically homogeneous preparations of anti-histon H1 sIgA-antibodies from human milk and study of their effect on tumor cells *in vitro*.

Materials and Methods. Mother's milk was provided by the Lviv Regional Perinatal Center according to the cooperation agreement.

The isolation of antibodies with affinity to histon H1 (anti-histon H1 AT) from human milk. Anti-H1 sIgA was purified from milk of clinically healthy mothers by sequential chromatography according to the scheme, described in [23]. As the first step, the milk was centrifuged at 5,000 rpm with subsequent purification of the fraction of total AT (IgG and sIgA) on chromatography column with protein A-agarose ("Sigma", USA) according to [26]. Further IgG and sIgA-antibodies were separated on chromatography column with protein G-Sepharose. The fraction of sIgA (1–3 mg of protein) that did not bind the sorbent, was dialyzed against 20 mM tris-HCl-buffer, pH 7.5, containing 140 mM NaCl (tris-saline buffer, TSB), for 18 h, and applied to column with histon H1-Sepharose (1 ml), previously balanced by the same buffer. The column was washed with TBS and anti-histon H1 AT was eluted with 0.1 M glycine-HCl, pH 2.6. The eluate was neutralized with 1.5 M tris-HCl, pH 7.5, and dialyzed against TBS for 18 h. Protein concentration in the preparations of anti-histon H1 AT was measured at NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, USA) at 280 nm wavelength.

The effect of anti-histon H1 AT preparations of human milk on tumor cells in vitro. Human T-leukemia Jurkat cells, and SK-MEL human melanoma cells were used to study the effect of sIgA preparations. Cell lines were obtained from the collection of R. E. Kavetsky Institute of Experimental Pathology, Oncology and Radiology, NAS of Ukraine. The cells were cultivated in Carrel flasks in RPMI-1640 and DMEM mediums ("Sigma Chem. Co.", USA) with addition of 10 % bovine embryonic blood serum ("Sigma Chem. Co."), 50 μg/ml gentamicin ("Sigma Chem. Co.") until sub confluent state of the cells. Jurkat cells in the concentration of 1.5·106 cells/ml were seeded into 96-well cultural

plastic vial, and SK-MEL cells – into 24-well vials at starting concentration of 2.7·104 cells/ml.

After 2 h incubation to the suspension of cells was added the preparation of anti-H1 sIgA AT (final concentration of 0.04 mg/ml) and incubated for 24, 48 and 72 h. Dead cells were stained by 0.1 % trypan blue solution. The number of unstained living and stained dead cells was calculated in the hemocytometry chamber under light microscope Biolam R (LOMO, Russian Federation).

Western-blotting of cell lysates. To the preliminary washed in phosphate-buffer saline (PBS) cells was added lysing buffer (1 % triton X-100, 20 mM tris-HCl, pH 7.4 in the presence of the mixture of protease inhibitors (Complete^{TM}, "Roche", France)) in ratio 50 µl of lysing buffer per 1 million cells. Cells were performed for 30 min on ice and cell lysates were centrifuged for 15 min at 12,000 g. The supernatant was dissolved in 4-fold Laemmli buffer and heated at 900C for 5 min. Samples were kept at – 20°C until used. Proteins were separated by denaturing electrophoresis in 12 % polyacrylamide gel (PAAG) at 0.1 % SDS according to Laemmli [24]. Proteins from PAAG were transferred to nitrocellulose membrane ("Hybond", USA) with subsequent processing of obtained blots using specific antibodies. Membranes were blocked at room temperature for 1 h by 5 % solution of skimmed milk powder in PBS, containing 0.05 % twin-20. Then the membrane was incubated in the presence of monospecific anti-c-Myc rabbit antibodies, phospho-p44/42 MAP Kinase (Thr 202/Tyr204), cdc2 (Tyr15) ("Cell Signaling", USA) and β-actine ("Sigma") for 12 h at 4 °C.

Immunoreactive proteins on the membrane were revealed using conjugates of horseradish peroxidase and goat antibodies (Sigma), specific to rabbit IgG. For estimation of proteins amount on the membrane the β-actine-specific rabbit antibodies were used.

To determine the cross-reactivity of anti-histon H1 sIgA we used Western-blotting analysis with two alternative methods of detection. In one case the cell lysates were separated by SDS-electrophoresis in 12 % PAAG with following electroblotting proteins on nitrocellulose membrane. The membranes were blocked by 3 % albumin solution in PBS with 0.05 % twin-20 for 18 h at 40 °C, and then incubated with previously biotinylated anti-histon H1 sIgA-antibodies (60 µg/ml) in the blocking buffer. Immunoreactive proteins were detected after treatment of membranes with avidin-horseradish peroxidase solution (1:10,000). In the second case, the membranes after blocking were incubated with native anti-histon H1 sIgA-antibodies (60 µg/ml) dissolved in buffer for blocking. Immunoreactive proteins were detected after treatment of membranes by rabbit IgG antibodies, conjugated with horseradish peroxidase, mono specific towards heavy chains of human IgA ("Sigma-Aldrich", USA) in 1:6,000 dilution.

Statistical processing of investigation results. All experiments were repeated 3–5 times. Average values and standard deviations (M ± m) are presented in the work. Statistical analysis was performed by Student's criterion (t). The data at $p \leq 0.05$ were considered reliable. The charts and statistical processing of the data were performed using Origin 4.0 and Excel 97 software.

Results and Discussion. It was previously found that sIgA-antibodies possess the affinity to protein A-agarose, but contrary to IgG-antibodies, they are not capable of binding to protein G-Sepharose [25]. According to these data, anti-histon H1 sIgA was isolated from milk by sequential affinity chromatography, including: obtaining total fraction of milk AT by chromatography on the column with protein A-agarose (Fig. 1, path *1*), purification of sIgA antibodies from IgG by chromatography on the column with protein G-Sepharose (Fig. 1, path *2*); isolation of anti-histon H1 sIgA-antibodies by chromatography on the column with histon H1-Sepharose. Electrophoretic analysis of these purified proteins demonstrated their similarity to polypeptides corresponding to the secretory component (SC), heavy (H) and light (L) chains of sIgA [26].

The purified preparations of anti-histon H1 sIgA-antibodies were studied for their ability to affect the growth and survival of cells *in vitro*. Target cells were Jurkat T-cells (human leukemia) and SK-MEL (human melanoma). As seen from the data, presented in Fig. 2, *a*, growth-stimulating effect of anti-histon H1 sIgA on Jurkat line cells was observed at the 24th and 48th h with maximal growth (1.4-fold) at the 72 h of incubation. The similar effect was also observed for the antibody-treated SK-MEL melanoma cells (Fig. 2, *b*). Here the maximum of cell growth was detected at the 48th h of incubation.

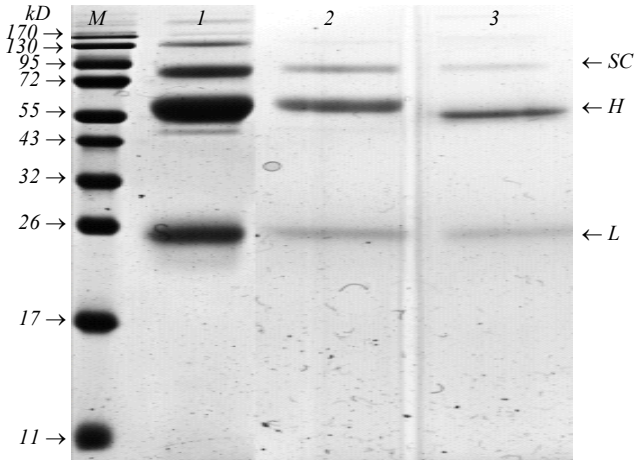

Fig.1. Electrophoresis in 12% PAAG in the presence of sodium dodecylsulfate of sIgA preparations, purified from human milk by consequential chromatography on columns with protein A-agarose (*1*), protein G-agarose (*2*) and histon H1-sepharose (*3*) *M* – markers of molecular mass of proteins. Arrows on the right hand indicate the position of sIgA polypeptides on the gel (*SC* – secretory component; *H* – heavy and *L* – light chains)

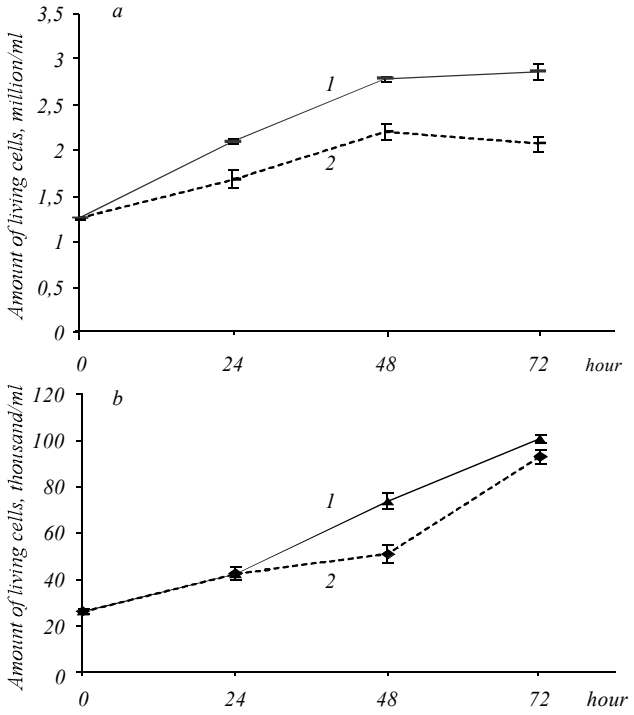

Fig.2. The effect of anti-histon H1 sIgA-antibodies on the growth of human T-leukemia Jurkat (*a*) and human melanoma SK-MEL cells (*b*)

These data demonstrate that anti-histon H1 sIgA-antibodies, isolated from milk of clinically healthy mothers, are capable of stimulating proliferation of both types of tumor cells. To prove this assumption we studied the level of some signaling proteins, directly involved in the regulation of cell proliferation. For this we used Western-blotting of proteins of Jurkat and SK-MEL cells, treated and untreated by anti-histon H1 sIgA-antibodies (Fig. 3). It was established that the level of MAP-kinase (p42/p44 Erk 1/2) (42, 44 kDa) in the cells, incubated with AT, was essentially increased comparing with the control cells. Additionally, in treated by anti-histon H1 sIgA-antibodies cells was revealed increased level of the transcriptional factor c-Myc (57 kDa) which is an intracellular molecular target for MAP-kinase, participates in the regulation of cellular growth and may initiate cell proliferation [27–29].

We also detected considerable increase of the phosphorylated form of cyclin-dependent kinase p-cdc2 (Tyr15) (34 kDa) in the Jurkat and SK-MEL cells in the presence of anti-histone H1 sIgA. These data suggest that the AT treated cells are in phase G1/S of the mitotic cycle [30].

Thus, we determined that the anti-histon H1 sIgA-antibodies, isolated from milk of healthy mothers, are capable of stimulating proliferation of both human T-leukemia Jurkat cells and human melanoma SK-MEL cells *in vitro* (Fig. 3, *a, b*).

Mechanism involved in the stimulation of cell proliferation by the anti-histon H1 sIgA-antibodies remains unknown. We assume that pro-proliferative effect of these secretory antibodies could be linked with their cross reactivity to positively charged proteins. We revealed also the antibodies with similar antigenic specificity and pro-proliferative activity in the blood serum of patients with multiple myeloma and systemic lupus erythematosus [22–31]. This property might provide the interaction of anti-histon H1 sIgA-antibodies with varied protein antigens of target cells.

To check this assumption, the SK-MEL cells were lysed in hypotonic buffer in the presence of 1 % triton X-100, then antigen specificity of the anti-histone H1 sIgA-antibodies towards lysate proteins was determined by Western-blotting using two independent ways. In one case, immune reactive proteins were detected using the previously biotinylated anti-histon H1 sIgA with their subsequent identification on the membrane by conjugate avidin-horseradish peroxidase (Fig. 4, path *1*). In the second case, for detection of the immune reactive proteins on the membrane we used

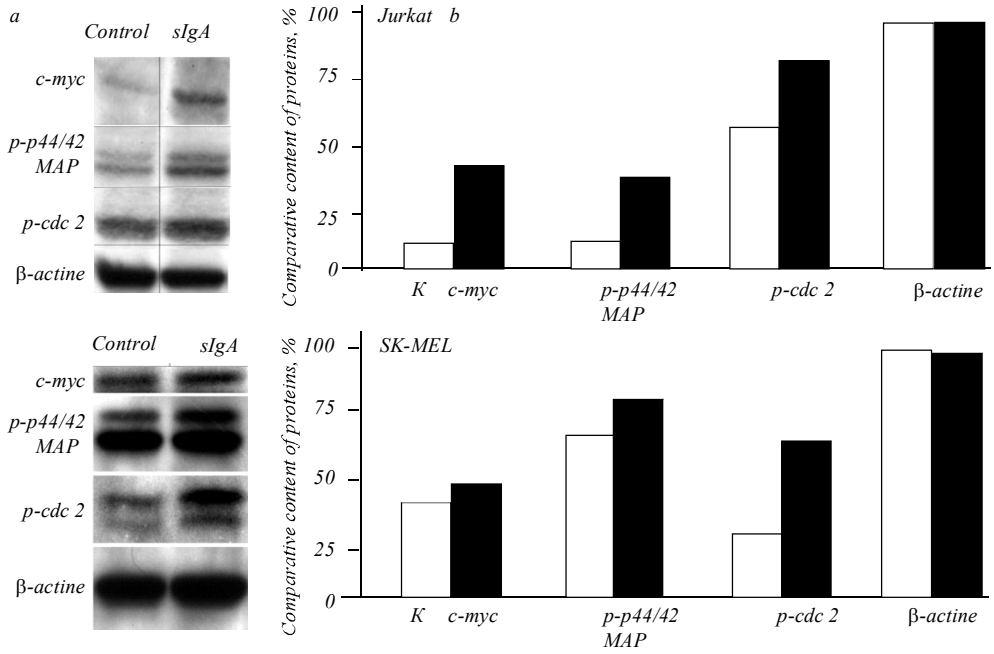

Fig.3. The level of some proteins, involved in the regulation of cell proliferation in Jurkat and SK-MEL cells after their incubation with the anti-histon H1 sIgA-antibodies: *a* – immunoblotting of proteins of cell lysates; *b* – comparative content of regulatory proteins in cells

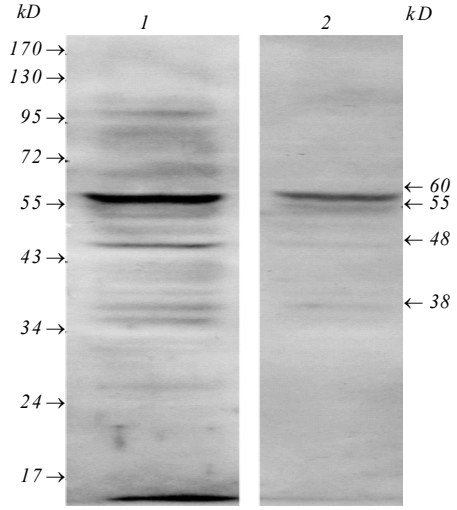

Fig.4. The affinity of anti-histon H1 sIgA-antibodies towards proteins of melanoma SK-MEL cells: *1* – membranes were treated with previously biotinylated anti-histon H1 sIgA-antibodies, avidin-horseradish peroxidase, were used for detection of antigens; *2* – membranes were treated with anti-histon H1 sIgA-antibodies, immune complexes were determined using rabbit IgG-antibodies, monospecific to heavy chains of human IgA, conjugated with horseradish peroxidase.

rabbit antibodies, monospecific to alpha-chains of human IgA (Fig. 4, path *2*). The data obtained demonstrated that the anti-histon H1 sIgA-antibodies possess an affinity to different proteins of melanoma cell preferentially binding with p60, p55, p48 and p38 kDa. Although the nature of these proteins is yet to be determined, one may suggest that these protein antigens include receptors of the plasmatic membrane, involved in the induction of cell proliferation (for instance, receptors of polypeptide growth factors [32]).

Since it is well-known that colostrum and milk of mothers contain a broad spectrum of biologically active compounds, possessing growth-stimulating as well as growth-inhibiting activity towards different types of cells [20, 21, 25], the data obtained do not allow us to claim that pro-proliferative activity is a function of only antibodies. The steroid hormones and growth factors, previously found in human milk, could also be engaged in this effect [33].

At present, the effect of anti-histon H1 sIgA-antibodies toward normal human blood lymphoid cells needs to be clarified. Our previous data demonstrate that these antibodies do not have considerable effect on the human blood lymphocytes *in vitro*. Meanwhile they are capable of stimulating the differentiation of isolated monocytes into macrophages in the presence of phorbol myristate acetate (unpublished results).

Conclusions. It was established that the sIgA-antibodies from healthy mothers' milk, isolated by chromatography on the column with histon H1-Sepharose (anti-histon H1-sIgA), are capable of stimulating the proliferation of human T-leukemia Jurkat and human melanoma SK-MEL cells in vitro. This effect may be a result of cross reactivity of these antibodies to membrane protein receptors of target cells.

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Мітотична активність анти-гістон H1 sIgA-антитіл молока клінічно здорових породіль

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Summary

Мета. *Раніше нами показано, що препарати антитіл (АТ), отримані з молока деяких клінічно здорових породіль осадженням 50 %-розчином сульфату амонію, володіють проліферативною активністю щодо трансформованих і пухлинних клітин in vitro (Кіт Ю. та ін., 2008). Ми припустили, що мітотична активність цих препаратів АТ може бути пов'язана з присутністю у них анти-гістон H1 sIgA-антитіл.* ***Методи***. *Для перевірки цієї гіпотези з молока клінічно здорових породіль методом постадійних хрома- тографій білків на білок А-агарозі, білок G-сефарозі і гістон H1-сефарозі одержано електрофоретично гомогенні анти-гістон H1 sIgA і досліджено їхній вплив на життєздатність Т-клітин лінії Jurkat лейкозу людини та клітин лінії SK-MEL меланоми людини.* ***Результати***. *Встановлено, що анти-гістон H1 sIgA стимулюють проліферацію клітин лінії Jurkat, а також клітин лінії SK-MEL in vitro. Мітотичний ефект цих АТ підтверджено імуноблотингом за зростанням у клітинах рівня деяких сигнальних білків, залучених до проліферації (c-Mус, MAP- і cdc2-протеїнкінази). Нами також досліджено антигенну реактив- ність анти-гістон H1 sIgA щодо білків лізатів клітин SK-MEL. Визначено, що ці АТ виявляють спорідне- ність до низки білків з молекулярною масою 60, 55, 48 і 38 кДа.* ***Висновки***. *Показано, що анти-гістон H1 sIgA-антитіла здатні стимулювати проліферацію Т-клітин ліній лейкозу Jurkat та меланоми SK-MEL людини in vitro. Мітотична дія цих АТ може бути пов'язана з їхньою перехресною імунореактивністю.*

Ключові слова: молоко породіль, автоантитіла, анти-гістон H1 sIgA, пухлинні клітини, проліферація.

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Митотическая активность анти-гистон H1 sIgA-антител молока клинически здоровых рожениц

Резюме

Цель. *Ранее нами показано, что препараты антител (АТ), полученные из молока некоторых клинически здоровых рожениц осаждением 50 %-м раствором сульфата аммония, обладают пролиферативной активностью в отношении трансформированных и опухолевых клеток in vitro (Кот Ю. др., 2008). Мы предположили, что митотическая активность этих препаратов может быть связана с присутствием у них анти-гистон H1 sIgA-антител.* ***Методы***. *Для проверки этой гипотезы из молока клинически здоровых рожениц методом постадийной хроматографии белков на белок А-агарозе, белок G-сефарозе и гистон H1-сефарозе получены электрофоретически гомогенные анти-гистон H1 sIgA и исследовано их влияние на жизнеспособность Т-клеток лейкоза человека линии Jurkat и клеток меланомы человека линии SK-MEL.* ***Результаты***. *Установлено, что анти-гистон H1 sIgA стимулируют пролиферацию клеток линии Jurkat, а также клеток линии SK-MEL in vitro. Митотический эффект этих АТ подтвержден иммуноблоттингом по возрастанию в клетках уровня некоторых сигнальных белков, вовлеченных в пролиферацию (c-Mус, MAP- и cdc2-протеинкиназы). Нами также исследована антигенная реактивность анти-гистон H1 sIgA к белкам лизатов клеток SK-MEL. Определено, что эти АТ проявляют сродство к ряду белков с молекулярной массой 60, 55, 48 и 38 кДа.* ***Выводы***. *Показано, что анти-гистон H1 sIgA-антитела способны стимулировать пролиферацию Т-клеток лейкоза линий Jurkat и меланомы SK-MEL человека in vitro. Митотическая активность этих АТ может быть связан с их перекрестной иммунореактивностью.*

Ключевые слова: молоко рожениц, аутоантитела, анти-гистон H1 sIgA, опухолевые клетки, пролиферация.

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