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THE CHARACTERISTICS OF ANTIBODIES OF MICE IMMUNIZED BY HUMAN UNCONVENTIONAL MYOSIN 1C

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Specific antibodies produced against a protein of interest are invaluable tools for monitoring the protein structure, intracellular location and biological activity. Inoculation of murine lymphoma cells into the peritoneal cavity of immunized mice provides generation of ascitic fluid containing a significant amount of antibody with desired antigen specificity. Here we demonstrated that the intraperitoneal administration of murine lymphoma NK/Ly cells in mice immunized with 48 kDa isoform of human blood serum unconventional myosin 1c leads to generation of ascitic fluid that contained specific IgG-antibodies. These antibodies were capable of binding of the unconventional myosin 1c isolated from blood serum of patients with multiple sclerosis, rheumatoid arthritis and systemic lupus erythematosis, and could be used for diagnostics of several autoimmune diseases, the multiple sclerosis in particular.

K e y w o r d s: unconventional myosin 1c (48/Myo1c), murine NK/Ly lymphoma, IgG-antibodies.

Antibodies recognizing proteins of interest are valuable tools for monitoring the protein's structure, intracellular location and biological activity [1]. Usually, they are obtained by immunization of laboratory animals – rabbits or mice. However, using these animals can create some problems. For example, immunization of rabbits requires relatively big amount of antigen, while immunization of mice does not allow obtaining sufficient amount of antiserum needed for future investigation. In contrast to that, inoculation of murine lymphoma cells into peritoneal cavity of immunized mice provides a significant amount of ascitic fluid for obtaining an antibody with desired antigen specificity [2].

Recently, we have shown that TCA-extracted fraction prepared from blood serum of multiple sclerosis (MS) patients contains a 48 kDa protein that was identified by the MALDI TOF/TOF as N-terminal fragment of human unconventional myosin 1c (48/Myo1c) [3]. Screening of blood serum samples from different autoimmune patients for the presence of 48/Myo1c revealed its high level in MS and rheumatoid arthritis patients, relatively low level in systemic lupus erythematous (SLE) patients, and it was almost undetectable in blood serum of healthy human donors. These data allow suggesting that the level of p48 Myo1c in patient's blood serum is a potential marker for diagnosing autoimmune disease. A sufficient amount of monospecific antibodies for this protein has an important precondition for its further successful investigation. We consider that inoculation of murine lymphoma NL/Ly cells into the peritoneal cavity of immunized mice can provide a generation in the ascitic fluid of a significant amount of monospecific IgG-antibodies against blood serum and cellular isoform(s) of human unconventional Myo1c.

The main goal of our work was to obtain a sufficient amount of antibodies with affinity for blood serum 48 kDa form of unconventional myosin 1c and characterized it using Western–blot and dot-blot analysis.

Materials and Methods

Serum samples of peripheral blood of 28 MS patients (diagnosed according to the McDonald diagnostic criteria for MS) was donated by prof. Tetyana Nehrych (Danylo Halytsky Lviv National Medical University, Ukraine). These samples were collected under the approval of Bioethics Review Board of the Danylo Halytsky Lviv National Medical University in accordance with the regulations of the Ministry of Health of Ukraine. A documented consent was obtained from all patients under study, and the form

of this consent was approved by the Bio-Ethics Review Board.

Purification of 48/Myo1c protein from blood serum. According to the developed protocol [3] 1 ml of blood serum was diluted 2-fold with phosphate buffer saline (PBS), and 100% TCA was added to 10% final concentration. After 30-min incubation on ice, the solution was centrifuged for 15 min at 10 000 g. The supernatant containing TCA-soluble compounds was isolated and mixed with acetone in 1 : 6 ratio followed by incubation at -20 °C for 18 h. The precipitate was pelleted by centrifugation for 10 min at 10 000 g. The pellet was diluted in distilled water and protein concentration was measured at 280 nm using a NanoDrop ND1000 spectrophotometer (NanoDrop Technologies, USA). The solution was stored at -20 °C until use. To remove albumin from 48/Myo1c preparations, they were incubated with Cibacron Blue 3GA Agarose (Sigma, USA) according to the manufacturer's protocol.

Immunization of BALB/c mice. Mice immunization was performed using white laboratory BALB/c mice (8–12 weeks). All animals were maintained in a specific pathogen-free animal facilities with water and commercial food provided ad libitum. Animal treatment was approved by the Institution Bioethical Committee. Mice were injected intraperitoneally with a complete Friend's adjuvant (CFA, Difco Lab., USA) and 50 μg of 48/Myo1c protein (total volume 100 μl) [4]. Immunization with the same amount of protein in the incomplete Friend's adjuvant (IFA, Difco.Lab., USA) was repeated in 2 and 4 weeks. At the last injection, mice were immunized with protein (50 μg per mice) in PBS, and during 10 days mice were intraperitoneally administrated with 1 000 000 of NK/Ly lymphoma cells. After 10 days, ascites from bowl were taken for analysis. Blood was obtained from marginal ear veins and further analyzed.

SDS-electrophoresis and Western-blot analysis. SDS-electrophoresis of TCA-extracted blood serum proteins was performed in 12% PAG [5] and followed by protein staining on gel by Coomassie Brilliant Blue G dye. For Western-blot analysis, proteins were transferred from gel onto a nitrocellulose membrane, and the membranes were blocked (1 h at 23 °C) with 5% non-fat milk in the PBS containing 0.05% Tween-20. The blots were washed three times for 5 min each with the PBS supplemented with 0.01% Tween-20, and then probed with specific antibodies. To identify 55 and 25 kDa polypeptides in a pool of TCA-extracted proteins of blood serum, polyclonal anti-human IgG (whole molecule) rabbit HRP-conjugated antibody (Sigma-Aldrich, product number A8792) in 1:40,000 dilution was used (2 h, 24 °C). Immunoconjugates were detected by the enhanced chemiluminescence (ECL).

Dot-blot analysis. For dot-blot analysis 2 μl of Myo1c preparation (total concentration 0.5 mg/ml) were spotted on nitrocellulose stripe in 6 dilutions (1:1, 1:2, 1:4, 1:8, 1:16 and 1:36). The stripes were blocked (2 h at 23 °C) with 3% BSA in the PBS buffer [6]. The blots were washed with PBS-Tween-20, three times for 5 min each, and then probed with the specific Abs, isolated from blood serum and ascites of immunized mice, diluted in 1 : 200 ratio in the blocking buffer. To identify antibodies attached to Myo1c polypeptide, polyclonal anti-mouse rabbit antibody (AVIVA SYSTEM BIOLOGY, product number ARP56292) in 1 : 1,000 dilution was incubated for 2 h at room temperature. Detection was performed using 3,3′-diaminobenzidine diluted in TBS (pH 7.5).

Results and Discussion

Blood serum has been extensively explored as a source of bio-markers, as it may contain not only blood proteins *per se*, but also proteins originating from different tissues of the body [7, 8]. It is estimated that up to 10 000 proteins (and/or their fragments) may be present in blood serum, and most of them are there in very low concentrations [9]. Selection of a protein preparation, and especially enrichment procedures, may aid in successful search for the bio-markers. Recently, we used TCA-precipitation/extraction methods combined with MALDI TOF/TOF mass-spectrometry to identify earlier unknown 48/Myo1c in blood serum of MS patients [3]. We also detected 48/Myo1c in blood serum of patients with rheumatoid arthritis, Alzheimer disease (unpublished), and multiple myeloma. Low level of this protein was found in blood serum of healthy human donors, but it was not detected in blood serum of patients with diabetes 1, liver cirrhosis, thyroiditis, and recurrent miscarriage (unpublished). The source of 48/Myo1c in human blood serum, as well as its biologic activity, is to be studied.

The 48/Myo1c was isolated from blood serum of MS patients using TCA-precipitation, as described earlier [3]. To remove serum albumin, the TCA-soluble fraction of blood serum proteins was subjected to affinity chromatography on Cibacron Blue 3GA Agarose. The purity of 48/Myo1c was checked

by SDS-PAG electrophoresis (Fig. 1). The impurities in 48/Myo1c preparation were less than 10%, and it was used for mice immunization that was conducted according to the protocol earlier used for others antigens [4]. To improve the yields of mouse anti-48/Myo1c, we used the protocol of specific antibody accumulation in ascitic fluid obtained after intraperitoneal administration of mouse NK/Ly lymphoma cells in the immunized mice. In 10 days after cell administration, the ascitic fluid was taken. Antibodies of the ascitic fluid were precipitated with 50% ammonium sulfate and tested for antigenic specificity. The antibody preparations obtained from the ascitic fluid of two immunized mice (Fig. 2, *A*, lanes *3*, *4*) were characterized using dot-blot (Fig. 2, *B*, lanes *3*, *4*) and Western-blot (Fig 2, *C*, lanes *1*, *2*) analyses. It was found that both antibody preparations are capable of binding to 48/Myo1c antigens present in blood serum. Dot-blotting demonstrated that the amount of IgGs in the studied antibody preparations correlated with titer of their binding with 48/Myo1c antigen. It is suggested that IgGs is a main antibody isotype involved in immune response of immunized mice. To check whether mouse anti-48/Myo1c have an affinity for both blood serum and cellular unconventional myosin 1c, Western blotting was performed (Fig. 2, *C*). As a source of cellular myosin 1c, Triton X-100-extracted fraction of human T-leukemia Jurkat cells was used. It was found that antibody preparation isolated from the ascitic fluid of immunized mouse contains IgGs possessing an ability to bind blood serum 48/Myo1c (Fig. 2, *C*, lane *1*) belonging to different isoform of cellular unconventional myosin 1c. The obtained data allow using these antibodies for identification of 48/Myo1c in blood serum samples of patients with different disease. On Fig. 3 typical distributions of the level of this protein detected in TCA-extracted fractions of blood serum of patients with MS, rheumatoid arthritis and healthy human donors are shown. The identification of TCA-extracted of 48/Myo1c was done by using mass-spectrometry only for samples of MS patients [2]. Thus, we used this antibody to check the antigenic relation of isolated proteins. It was found that antibody obtained from the ascitic fluid of mice immunized with 48/Myo1c protein of blood serum of MS patients preferentially bound 48 kDa

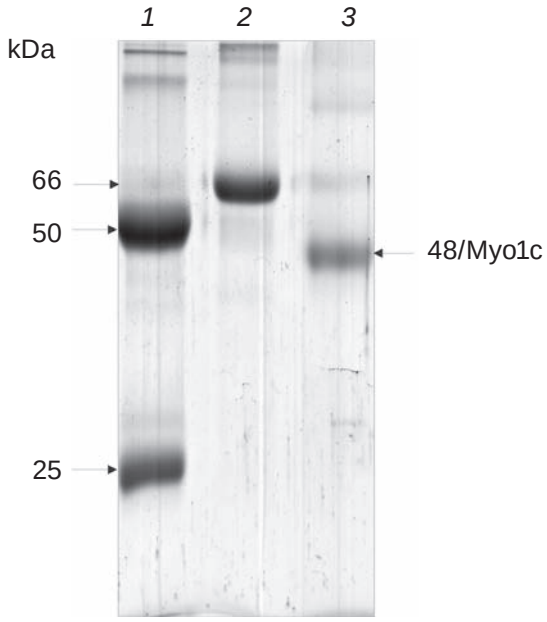

Fig. 1. Electrophoregram of SDS-electrophoresis in 12% PAG of proteins extracted with 10% TCA from blood serum of the multiple sclerosis patients. Lane 1 – polypeptides of heavy (50 kDa) and light (25 kDa) chains of IgG. Lane 2 – bovine blood serum albumin. Lane 3 – summarized 48/Myo1c preparation isolated from blood serum of 10 MS patients

polypeptide. It could be a confirmation that 48 kDa proteins extracted with 10% TCA from blood serum of patients with multiple sclerosis, rheumatoid arthritis, systemic lupus erythematosis and healthy human donors (NHD) all belong to 48 kDa form of human unconventional myosin 1c.

Myosin 1c is a member of the unconventional class I myosins of vertebrates, and it directly links plasma membrane with the microfilament cortical web [10]. This myosine has not been described in blood serum yet. It is implicated in such cell functions as cytoskeleton organization, cell motility and nuclear transcription. Myo1c is abundantly expressed in murine B lymphocytes, and it is preferentially located at the plasma membrane, especially in the peripheral processes such as microvilli [11, 12]. One can also suggest that 48/Myo1c is involved in neutrophil netosis at the cytoskeleton dynamics and membrane protein anchoring or sorting in B lymphocytes.

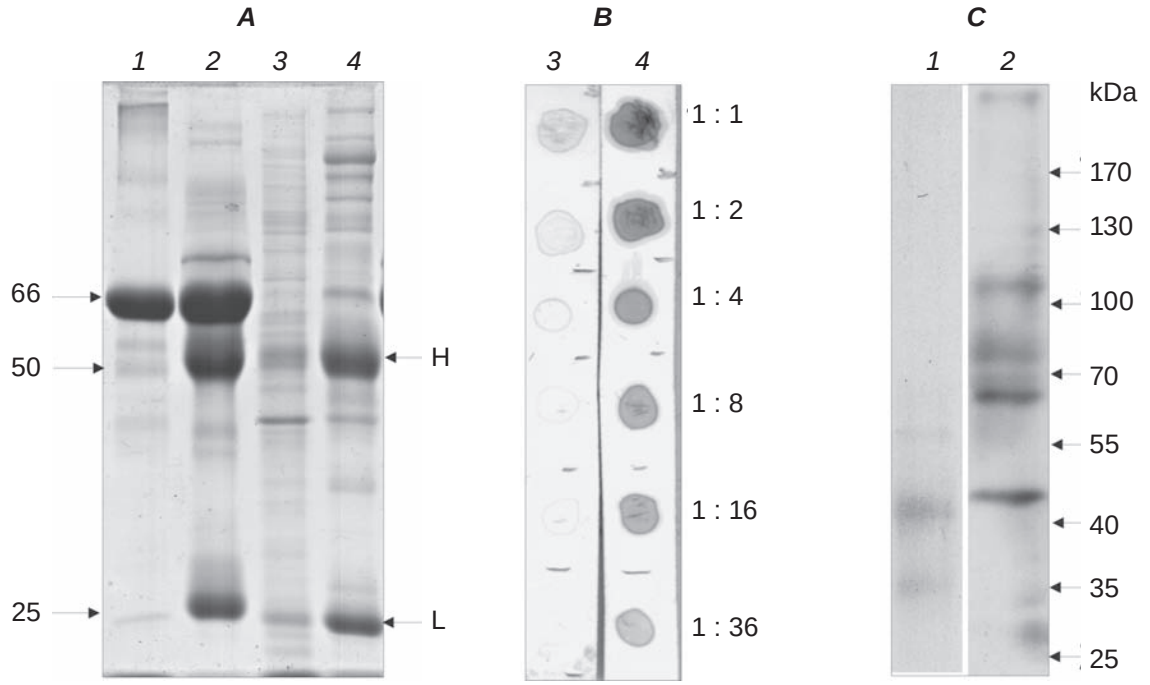

Fig. 2. Characteristics of anti-48/Myo1c antibodies isolated from the ascitic fluids of two immunized mice. A – electrophoregram of SDS-electrophoresis in 12% PAG of proteins isolated from mouse blood serum (lane 2), and ascitic fluids of the immunized mice (lanes 3, 4) by precipitation with 50% ammonium sulfate. Arrows on the left side indicate polypeptides belonging to blood serum albumin (66 kDa), heavy chains (50 kDa), and light chains (25 kDa) of IgGs. H-, L- the positions of heavy and light chains of IgG molecule. B – results of dot-blot analysis of the affinity of IgG-antibodies precipitated with blood serum of the immunized mice (lanes 3, 4) toward 48/Myo1c obtained from blood serum of MS patients. C – results of Western blotting of a 48/Myo1c preparation (lane 1) and Jurkat T-leukemia cells lysate using the IgG-antibodies precipitated with the ascitic fluid obtained from the immunized mouse (lanes 3, 4). On the right side, protein molecular mass standards are shown

In this manner, intraperitoneal administration of murine lymphoma NK/Ly cells in immunized mice allows obtaining 2 ml of ascite fluid with high titer of IgG-antibodies possessing specificity towards blood serum and cellular isoform of human unconventional myosin 1c.

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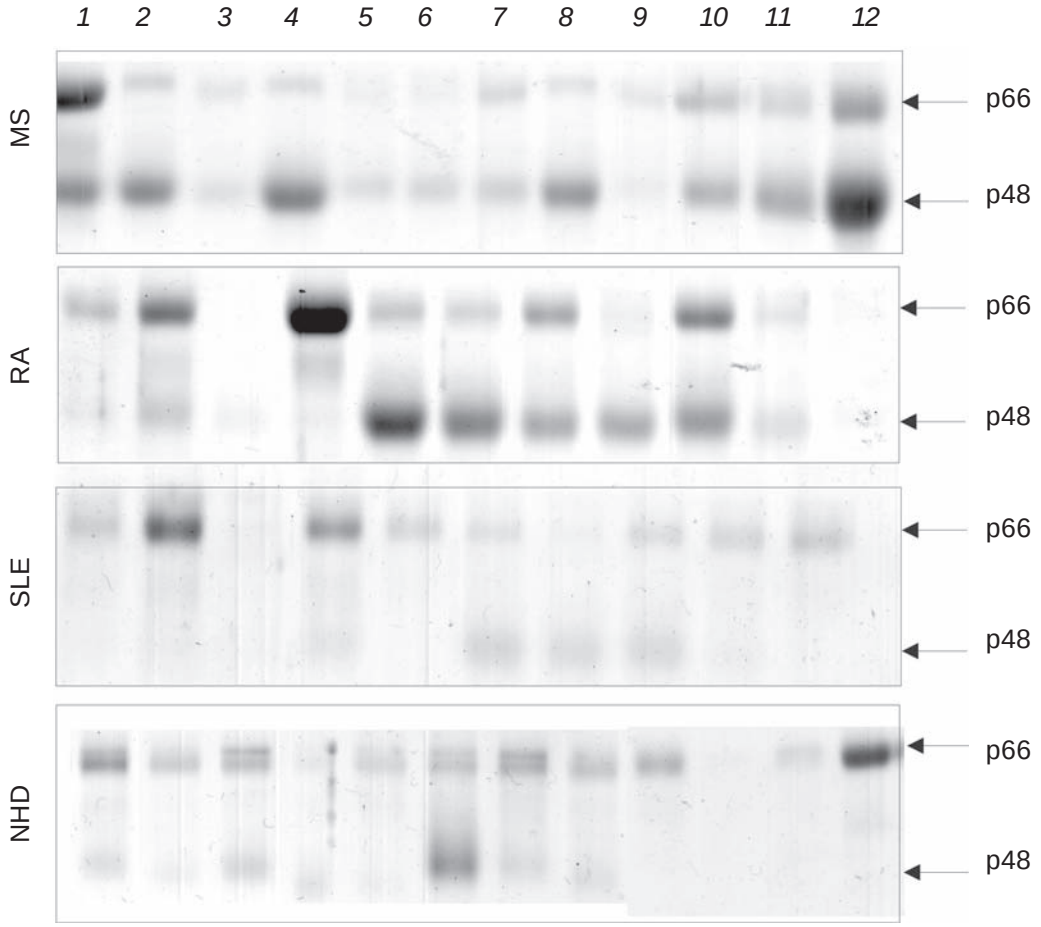

Fig. 3. Electrophoregram of SDS-electrophoresis in 12% PAG of proteins extracted with 10% TCA from blood serum of the multiple sclerosis patients. Lane 1 – polypeptides of heavy (50 kDa) and light (25 kDa) chains of IgG. Lane 2 – bovine blood serum albumin. Proteins were extracted with 10% TCA from blood serum of individual MS patients. Arrows on the left side point on the polypeptides belonging to blood serum albumin (66 kDa), heavy chains (50 kDa), and light chains (25 kDa) of IgGs. Arrows on the right side point on the polypeptides identified by the MALDI-TOF mass spectrometry as human blood serum albumin (HSA) and 48 kDa form of the unconventional myosin 1c (48/Myo1c)

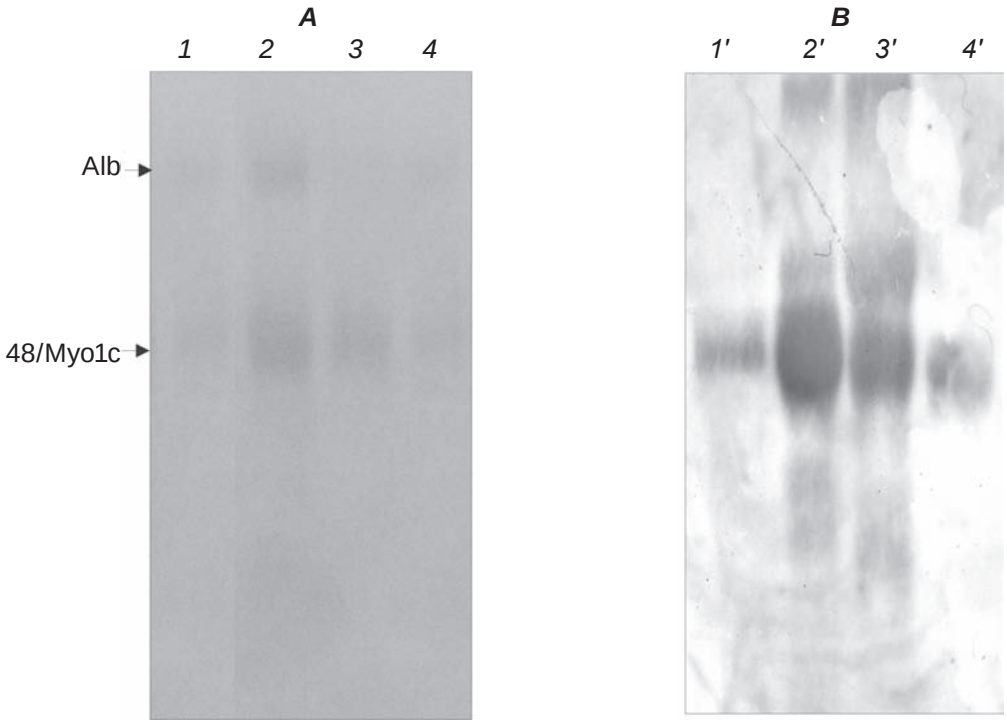

Fig. 4. Results of Western-blot analysis of TCA– extracted proteins isolated with blood serum of a healthy donor (lanes 1,1') and patients with MS, RA and SLE (lanes 2,2'; 3, 3'; 4, 4', respectively) using a polyclonal mice antibodies against 48/Myo1c. A – the membrane stained with Ponceau S. B – immunoconjugates detected by ECL. Arrows on the left side, indicate the polypeptides belonging to blood serum albumin (66 kDa) and short form of 48/Myo1c

ХАРАКТЕРИСТИКА АНТИТІЛ МИШЕЙ, ІМУНІЗОВАНИХ НЕКОНВЕНЦІЙНИМ МІОЗИНОМ ЛЮДИНИ

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Антитіла, що виробляються імунізацією протеїном, є безцінними інструментами для моніторингу його структури, внутрішньоклітинної локалізації та біологічної активності. Для імунізації мишей необхідна невелика кількість антигену, але низький вихід антитіл недостатній для їх подальшого застосування. Перевивка клітин лімфоми в черевну порожнину імунізованих мишей забезпечує генерацію асцитичної рідини, що містить значну кількість антитіл необхідної антигенної специфічності. У роботі показано, що внутрішньочеревинне введення клітин мишачої лімфоми NK/Ly мишам, імунізованим ізоформою неконвенційного міозину 1с 48 кДа (48/Myo1c), яка виділена із сироватки крові людини, призводила до утворення в асцитичній рідині специфічних IgG-антитіл. Встановлено, що одержані антитіла здатні зв'язувати 48/Myo1c, виділений із сироватки крові хворих розсіяним склерозом або ревматоїдним артритом, системним червоним вовчаком, і можуть бути використані для діагностики автоімунних захворювань, зокрема розсіяного склерозу.

Ключові слова: ізоформа міозину 1с (48/Myo1c), NK/Ly лімфома, IgG антитіла.

ХАРАКТЕРИСТИКА АНТИТЕЛ МЫШЕЙ, ИММУНИЗИРОВАННЫХ НЕКОНВЕНЦИОННЫМ МИОЗИНОМ ЧЕЛОВЕКА

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Антитела, вырабатываемые иммунизацией протеином, являются важнейшими инструментами для мониторинга его структуры, внутриклеточной локализации и биологической активности. Для иммунизации мышей необходимо небольшое количество антигена, но низкий выход антител недостаточен для их дальнейшего применения. Перевивка клеток лимфомы в брюшную полость иммунизированных мышей обеспечивает генерацию асцитической жидкости, содержащей значительное количество антител с требуемой антигенной специфичностью. В работе показано, что внутрибрюшинное введение клеток мышиной лимфомы NK/Ly мышам, иммунизированным изоформой неконвенционного миозина 1c 48 кДа (48/Myo1c), выделенной из сыворотки крови человека, приводило к образованию в асцитической жидкости специфических IgG-антител. Установлено, что полученные антитела обладают способностью связывать Myo1c, выделенный из сыворотки крови больных рассеянным склерозом, ревматоидным артритом и системной красной волчанкой и могут быть использованы для диагностики аутоиммунных заболеваний, в частности, рассеянного склероза.

Ключевые слова: изоформа миозина 1c (48/Myo1c), NK/Ly лимфома, IgG антитела.

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