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Obtaining and investigating immunochemical properties of monoclonal antibodies against rCTLA-4 protein

Abstract. Monoclonal antibodies are used to block control points of the tumor development of many oncological pathologies. One of the critical control points of tumor development of several oncological pathologies is the receptor for cytotoxic T-lymphocyte-associated protein 4 (CTLA-4). Monoclonal antibodies against the CTLA-4 receptor are laboratory-derived humanized antibodies. An essential step in the humanization of antibodies is the production of murine hybrid cells producing monoclonal antibodies. This article describes studies of mice monoclonal antibodies against a recombinant human CTLA-4 receptor (rCTLA-4) expressed in *Escherichia coli*. To obtain strains of hybrid cells producing monoclonal antibodies were used methods of hybridoma technology. As a result, hybrid cells producing monoclonal antibodies to CTLA-4 were obtained. Strains of hybrid cells have high productive activity *in vitro* and *in vivo*. Monoclonal antibodies react with rCTLA-4 protein, belong to the class of IgG1, and have a high binding constant. They efficiently bind to the rCTLA-4 receptor and block the interaction of rCTLA-4 with the commercial recombinant human B7-1 Fc and rhesus monkey PD-1 hFc proteins. These monoclonal antibodies to rCTLA-4 can be used to obtain recombinant humanized monoclonal antibodies to the human CTLA-4 receptor.

Keywords: Monoclonal antibodies, CTLA-4 receptor, oncology, recombinant protein.

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Introduction

Currently, monoclonal antibodies (mAbs) against the cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) are of great importance for immunotherapy aimed at blocking immunological control points of tumor development. Programmed cell death receptor 1 (PD-1), ligands for PD-1 and 2 (PD-L1, PD-L2), and antigen 4 associated with cytotoxic T lymphocytes (CTLA-4) are of great interest. Interest in CTLA-4 is attributed to the receptor being a homolog of the CD28 receptor and having a higher affinity for the B7 receptor. The interaction of CD28 and B7 receptors leads to increased T cell proliferation and cytokine production. CTLA-4 is essentially a competitor to the CD28 receptor, thereby interfering with the generation of the CD28:B7 signal that stimulates immunity. In addition, the binding of CTLA-4 and B7 can produce inhibitory signals that counteract the stimulatory signals from the binding of CD28:B7 and TCR: MHC [1]. PD-L1 is the central mediator of the evasion of cancer cells from immunity and is the only biomarker that allows the prediction of the effectiveness of the blockade of immunological checkpoints [2]. In addition, PD-L1, like CTLA-4, can bind to the B7.1 (CD80) receptor on dendritic cells and prevent the development of antitumor immunity [3].

In this regard, mAbs against PD1 are effective tools in treating melanoma [4,5,6,7]. Since 2011, mAbs against CTLA-4 (Ipilyumab) has taken a stable position in the pharmaceutical market of the world. The mechanism of action of Ipilimumab is based on the inhibition of CTLA-4 and an increase in the period of antitumor immunity. In 2014, two more drugs based on mAbs Pembrolizumab, Nivolumab, and Ipilimumab became commercially available. Studies have shown that the use of mAbs in 20% of patients with skin melanoma increased their immune response against malignancies by

several years. The use of Nivolumab or Pembrolizumab in combination with Ipilimumab was significantly more effective in the treatment of melanoma [8,9,10,11]. In the first phase of clinical trials, the effect of pembrolizumab on patients with melanoma and lung cancer was studied. Pembrolizumab showed no dose-dependent toxic effects and showed stimulatory antitumor activity against melanoma and lung cancer [12,13].

The use of mAbs for cancer immunotherapy control points is an essential study for the Republic of Kazakhstan in connection with an increase in malignant melanoma among the population. Here we report a study of murine mAbs against a recombinant CTLA-4 receptor expressed in *Escherichia coli*.

Material and methods

Animals, cells, and antigens

A total of 20 BALB/c mice (6-8-week old) and 150 outbred mice and X-63 myeloma cell lines were used. Anti-Mouse IgG1 and IgG2a Subclass Specific Antibodies (Jackson Immuno Research Inc), anti-Mouse IgG (whole molecule)-Peroxidase antibody produced in goat (Sigma-Aldrich, A4416) and 3,3',5,5'-Tetramethylbenzidine (TMB) Liquid Substrate System for enzyme-linked immunosorbent assay (ELISA) (Sigma-Aldrich, T0440) and recombinant human B7-1Fc protein were used in this study.

Production of mAbs

On the first day, BALB/c mice were injected intraperitoneally with 400 mg of rCTLA-4 antigen with 0.1 mL of incomplete Freund's adjuvant (Gibco, USA) in phosphate-buffered saline (PBS), pH 7.2-7.4. The following immunization was performed on days 7, 11, 12, 13, and 14 with 100 µg of antigens. The titer was evaluated using an enzyme-linked immunosorbent assay (ELISA) in a series of two dilutions of the serum of each animal, starting at 1:200. Cell hybridization was carried out according to the method of Oi and Herzenberg [14]. Cloning of hybrid cultured cells was performed by the limiting dilution method described by Coding [15].

To obtain a preparative amount of antibodies, hybrid cells were cultured in flasks with a volume of 150 cm³ for 8 days at 37°C. After cultivation, the culture medium was collected and separated from the hybrid cells by centrifugation at 150 g for 10 minutes. MAbs were purified from ascites fluid by salting out with ammonium sulfate to 50% saturation. The formed precipitate was centrifuged at 3000 g for 30 min at 4°C. The antibody pellet was resuspended in a minimal volume of PBS (pH7.2) and dialyzed against PBS during the day. Antibodies from the resulting solution were purified using HiTrap Protein A HP (GE Healthcare Life Sciences).

The binding affinity of mAbs

The method of Beatty et al. (1987) determined the binding affinity of mAbs. The recombinant protein was immobilized in three rows of a 96-well plate with the optimal concentration (10µg/mL) and three rows of wells with a protein concentration two times lower than the optimal concentration (5 µg/mL) in 0.05 M bicarbonate buffer, pH of 9.6 and incubated at 4°C for 12 h. After washing the plate and blocking with 1% bovine serum albumin (BSA), double dilutions of mAbs were introduced into the wells, starting from 10µg/mL, and incubated at 37°C for 1 h. After washing the plate, an antispecific conjugate was added at a dilution of 1:1000 and incubated at 37°C for 1 h. To develop the reaction, a TMB substrate was used, followed by the addition of a stop reagent. The optical density was measured at a wavelength of 450 nm. Based on the obtained data, graphs were constructed and the binding constant was determined.

The value of K_{aff} was calculated using equation (1).

$$K_{aff} = \frac{1}{4Ab' - 2Ab} \quad (1)$$

Where

Ab' – concentration of antibodies at 50% optical density of the reaction with rPD-1 in concentration 10 µg/mL;

Ab - concentration of antibodies at 50% optical density of the reaction with rPD-1 in concentration 5 µg/L.

ELISA

Solutions of rCTLA-4 (5µg/mL) in bicarbonate buffer (pH 9.6) were immobilized at 4 °C for 16 h. The plate was washed with 0.15 M PBS with Tween 20 (PBS-Tw20) (pH 7.4). After washing the plate, 1% BSA was added and incubated for 1 h at 37°C. Then, serial dilutions of mAbs beginning with 1:100 were introduced into wells. The plates were incubated as above and Anti-Mouse IgG (whole molecule)-Peroxidase antibodies produced in goat (Sigma-Aldrich) diluted 1:40000 were added to the wells. The incubation was performed as previously. To develop the reaction, a substrate of 3,3',5,5'-tetramethylbenzidine (TMB) was added and incubated at RT. The reaction was then stopped by the addition of 2M sulfuric acid. The intensity of the reaction product was measured at 450 nm.

Western blot

The recombinant protein was analyzed on a 12% SDS-PAGE by Laemmli [16]. Immunochemical properties of the rCTLA-4 were analyzed in Western blot by Towbin [17] method. The rCTLA-4 was transferred to a nitrocellulose membrane which was incubated in a 1% BSA at 4°C, 16 h. Then the membrane was incubated with 1:1000 mAbs for 1.5 h at RT. After a washing step with PBS-Tw20 the membrane was incubated with anti species peroxidase-conjugate for 1 h at 37°C. After the washing procedure protein detection was visualized by the adding 4-chloro-naphthol substrate.

Results

Obtaining hybridoma cells producing mAb to the rCTLA-4 protein

In the enzyme immunoassay, the titer of antibodies against rCTLA-4 in the serum of immunized mice was 1:25600. B-lymphocytes of immunized mice were used to obtain strains of hybrid cells producing mAbs. As a result of hybridization, out of 384 seeded wells, 100 showed the growth of clones of hybrid cells, which is 26% of clone formation. Antibody-producing hybrid cells, we cloned three times. After the third cloning, from 96 obtained clones, 81 subclones produced antibodies to rCTLA-4, which is 85% of positive clones. After cloning, the hybridoma cells did not change their productive and cultural properties for 16 passages in vitro condition (observation time). The productivity of hybrid strains was determined using ELISA for 8 days. Hybrid cells were seeded in 8 wells of a 24-well plate in an amount of 2×10 cells per well. From day 1 to day 8, the culture medium was selected from one well per day to determine the antibody-producing activity of hybridomas by ELISA. To determine the hybridoma productivity, a comparison of the reaction intensity in the tested row of wells with positive control, in which the concentration of murine antibodies is known, was used. The results of the analysis of the productivity of hybrid cells are shown in Fig. 1.

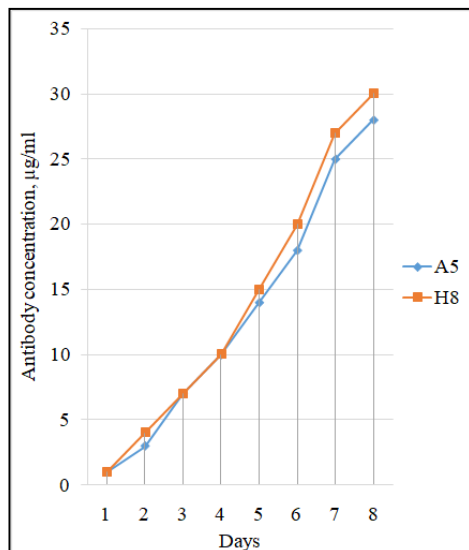


Figure 1. Productivity curve of hybrid cell strains producing mAbs to rCTLA-4 protein in vitro

Figure 1 shows that the concentration of antibodies in the culture fluid on the 8th day of cultivation reaches 17 - 30 µg/mL. To obtain a preparative amount of mAbs, hybridoma cells were cultivated in RPMI1640 medium within 6 days. The antibody concentration obtained was 4 mg/ml, from the total volume of cultural fluid was 150 ml.

Immunochemical properties of monoclonal anti-PD-1 antibodies

Indirect ELISA was used to determine the constant affinity (Kaff) of the mAbs. Serial dilution of mAbs was loaded onto rows on a microtiter plate with rCTLA-4 at two concentrations- 5 and 10 µg/mL. The dilution results from mAbs of four hybridoma cell culture pools in two concentrations of rCTLA-4 coating are shown in Fig. 2.

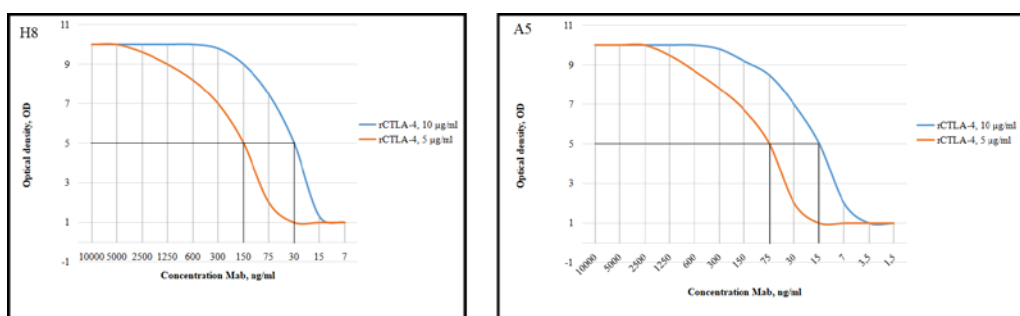


Figure 2. Experimental ELISA curve for anti-rCTLA-4 mAbs at two concentrations of protein

The calculated antibody concentrations (ng/mL) at OD-50 were H8 - 150 and 30; A5 - 75 and 15. According to formula (1), the affinity constant of monoclonal antibodies was calculated. This formula determines the level of interaction of the mAbs with the antigen and is based only on the total concentration of antibodies at 50% optical density of the reaction for 96 well plates immobilized with two concentrations of antigen, 5 and 10 µg/mL. The results of determining the binding constant are shown in table 1. To determine the subclass of mAbs in the ELISA used anti-mouse IgG1 and IgG2a subclass-specific antibodies (Jackson Immuno Research Inc).

Table 1

Main characteristics of monoclonal antibodies to rCTLA-4

Mab name	Subclass of antibodies	Productivity of hybridomas in vitro, $\mu\text{g/mL}$	Antibody sensitivity, ng/mL	Constant affinity
H8	IgG1	30	30	$3 \times 10^8 \text{M}^{-1}$
A5	IgG1	28	15	$5 \times 10^8 \text{M}^{-1}$

As can be seen from Table 1 the constant affinity of mAbs ranged from $3 \times 10^8 \text{M}^{-1}$ to $5 \times 10^8 \text{M}^{-1}$, which indicates the sufficient power binding of mAbs to this protein. The results of assessing the subclass of mAbs show that the mAbs to the rCTLA-4 protein synthesized by hybridoma belongs to the class G1 antibody.

Specificity and inhibited ability of monoclonal anti-rCTLA-4 antibodies

The main biological characteristics of mAbs are specificity, sensitivity, and affinity. The specificity demonstrates the ability of mAbs to react only with the certain protein to obtain antibody selectively. mAbs specificity was determined by Western blotting with heterogeneous proteins. The Western blot analysis showed a specific reaction of mAbs only with rCTLA-4 protein, the molecular weight of which is 20 kDa (Fig.3).

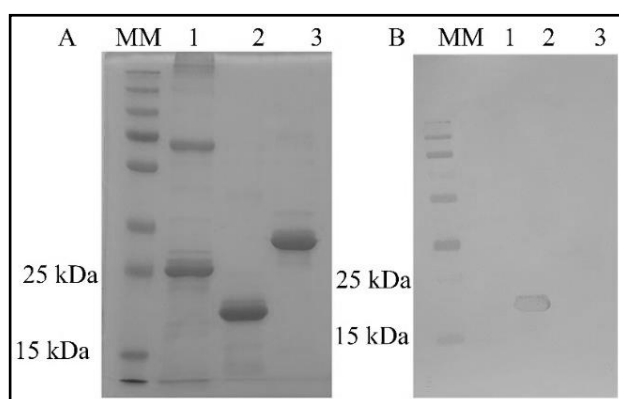


Figure 3. Electrophoresis (A) and Western blot (B) determine the specificity of mAbs to rCTLA-4 protein. Line 1 – rN protein SARS-Cov2; Line 2 – rCTLA protein; Line 3 - rPD-L1 rptein

The ability of mAbs to inhibit the reaction of rCTLA-4 with recombinant human B7-1Fc protein was researched by competitive ELISA assay. The solution of mAbs and rB7-1Fc protein was added to wells immobilized with rCTLA-4 a concentration of 10000 to 0,7 ng/ml . The solution of mAbs and rB7-1Fc was prepared in a ratio of 1:1. ELISA showed a decrease in the optical density of the reaction mAb+rB7-1Fc compared to rB7-1Fc (Fig.4).

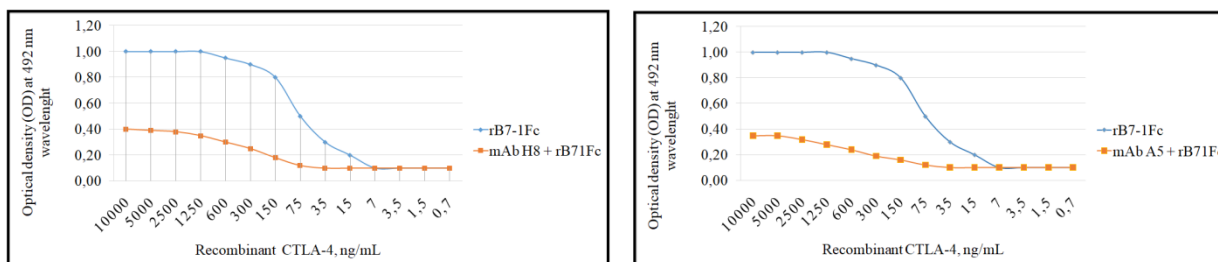


Figure 4. Experimental curves of competitive ELISA for rB7-1Fc protein and solution mAbs+rB7-1Fc protein

Discussion

mAbs against PD-1, PD-L1, and CTLA-4 have been successfully used as a treatment for various tumors and occupy a stable position in the pharmaceutical market of the world. The most widely used are three humanized mAbs: ipilimumab, pembrolizumab, and nivolumab [18]. mAbs can be used for the treatment of different types of malignant tumors. Japanese scientists have seen a positive effect in the treatment of diseases such as NSCLC, renal cell carcinoma, and melanoma in 18, 27, and 28% of patients, respectively. In these patients, also was observed the formation of long-term antitumor immunity. mAbs restrained the proliferation of some malignant tumors for 20-30 months [19].

In this work, five hybridoma cells producing mAbs to rCTLA-4 receptor were obtained. To get the strains of hybrid cells, we used the rCTLA-4 receptor obtained from *E. coli*. Analysis of the resulting murine mAbs showed a specific reaction with the extracellular fragment of the CTLA-4 receptor. mAbs had a sufficient affinity ranging from $3 \times 10^8 M^{-1}$ to $5 \times 10^8 M^{-1}$, specificity, and sensitivity to rCTLA-4. In our study, the resulting mAbs provide their unique specificity for CTLA-4 due to the interaction, which is likely to help in the development of therapeutic antibodies.

Wang et al. (2019), conducted studies on the effect of PD-1 receptor glycosylation on binding to mAbs. For these purposes, the authors obtained murine mAbs for recombinant human PD-1 (amino acid residues 21-167) obtained from *Escherichia coli* [20]. Mouse mAbs specifically bind to human PD-1 protein and inhibit the interaction of PD-1 and ligands. The mAbs obtained by the author had a binding constant of $3,5 \times 10^7 M^{-1}$. Then, the authors received humanized antibodies following the humanization of frame sequences of antibodies without changing affinity and specificity. Structural analysis showed that the specificity of mAbs is associated both with interaction with loops and with the glycan, parts of PD-1. The results showed that the N-glycosylation of PD-1 does not affect the binding strength of antibodies [20].

Conclusion

As a result of the work were investigated mAbs to a fragment of the extracellular domain of CTLA-4. The hybridoma cells have high productivity in vitro and in vivo. mAbs react with rCTLA-4 protein, belong to the class of IgG1, and have a high binding constant. Obtained mAbs efficiently blocked the reaction of rCTLA-4 with recombinant human B7-1Fc. Characterization of mAbs rCTLA-4 allows you to use them to obtain recombinant humanized mAbs to human CTLA-4 receptors.

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Conflict of interest. On behalf of all authors, the corresponding author states that there is no conflict of interest.

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rCTLA-4 ақуызна моноклоналды антиденелердің иммунохимиялық қасиеттерін зерттеу және алу

Аңдатпа. Моноклоналды антиденелер көптеген онкологиялық патологияларда ісік дамуының бақылау нүктелерін блоктау үшін қолданылады. Бірқатар онкологиялық патологиялар кезінде ісік дамуының маңызды бақылау нүктелерінің бірі цитотоксикалық Т-лимфоциттермен байланысқан ақуыз 4 (CTLA-4) рецепторлары болып табылады. CTLA-4 рецепторына қарсы моноклоналды антиденелер зертханалық шыққан ізгілендірілген антиденелер болып табылады. Антиденелерді гуманизациялаудың маңызды қадамы ол моноклональды антиденелерді шығаратын тышқан гибридті жасушаларын өндіру болып табылады. Бұл мақалада *Escherichia coli* экспрессияланған адамның рекомбинантты CTLA-4 рецепторына (rCTLA-4) қарсы тінтуірдің моноклоналды антиденелерінің зерттеулері сипатталған. Моноклоналды антиденелерді шығаратын гибридті жасуша штаммдарын алу үшін гибридтомдық технология әдістері қолданылды. Нәтижесінде CTLA-4-ге моноклоналды антиденелерді өндіретін гибридті жасушалар алынды. Гибридті жасуша штаммдары *in vitro* және *in vivo* жоғары өнімді белсенділікке ие. Моноклоналды антиденелер rCTLA-4 ақуызымен әрекеттеседі, IgG1 класына жатады және жоғары байланысу константасына ие. Олар rCTLA-4 рецепторымен тиімді байланысады және rCTLA-4-тің коммерциялық рекомбинантты Fc B7-1 адам ақуыздары мен резус макака PD-1 hFc әрекеттесуін блоқтайды. Бұл анти-rCTLA-4 моноклоналды антиденелер адамның CTLA-4 рецепторына рекомбинантты гуманизацияланған моноклоналды антиденелерді жасау үшін пайдаланылуы мүмкін.

Түйін сөздер: моноклоналды антиденелер, CTLA-4 рецепторы, онкология, рекомбинантты ақуыз.

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Получение и исследование иммунохимических свойств моноклональных антител к белку rCTLA-4

Аннотация. Моноклональные антитела используются для блокировки контрольных точек опухолевого развития многих онкологических патологий. Одной из критических контрольных точек опухолевого развития ряда онкологических патологий является рецептор цитотоксического Т-лимфоцитарно-ассоциированного белка 4 (CTLA-4). Моноклональные антитела против рецептора CTLA-4 представляют собой гуманизированные антитела лабораторного происхождения. Важным этапом гуманизации антител является получение мышинных гибридных клеток, продуцирующих моноклональные антитела. В этой статье описаны исследования мышинных моноклональных антител против рекомбинантного рецептора CTLA-4 человека (rCTLA-4), экспрессированного в *Escherichia coli*. Для получения штаммов гибридных клеток, продуцирующих моноклональные антитела, были использованы методы гибридомной технологии. В результате были получены гибридные клетки, продуцирующие моноклональные антитела к CTLA-4. Штаммы гибридных клеток обладают высокой продуктивной активностью *in vitro* и *in vivo*. Моноклональные антитела реагируют с белком rCTLA-4, относятся к классу IgG1 и имеют высокую константу связывания. Они эффективно связываются с рецептором rCTLA-4 и блокируют взаимодействие rCTLA-4 с коммерческими рекомбинантными белками Fc B7-1 человека и PD-1 hFc макака резус. Эти моноклональные антитела к rCTLA-4 можно использовать для получения рекомбинантных гуманизированных моноклональных антител к человеческому рецептору CTLA-4.

Ключевые слова: моноклональные антитела, рецептор CTLA-4, онкология, рекомбинантный белок.

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