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Anti-idiotypic Monoclonal Antibodies Specific for Immunoglobulins Against the Lipopolysaccharide Antigen of *Brucella*

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Abstract

We have determined biological properties of the hybrid strain producing anti-idiotypic antibodies (AIAT) against immunoglobulins specific for the lipopolysaccharide of *Brucella* antigen after deep freezing during the *in vitro* and *in vivo* cultivation. From the cultural and ascitic medium, we got monoclonal preparations of anti-idiotypic antibodies by the method of ammonium sulfate salting and chromatography. In the reaction of immunoblotting and enzyme immunoassay, we have determined specific interaction of AIAT with brucellosis-positive sera of rabbits and cattle. This fact suggests that they may be used as an analog of the original antigen.

Keywords

Monoclonal anti-idiotypic antibodies; Hybrid cells; Electrophoresis; Chromatography; Immunoblotting; Enzyme immunoassay

Introduction

Structural diversity of antigen binding sites of antibodies is proved by their idiotypic diversity. Specific antibodies have repeated (cross-reacting, or general) idiotypes and so-called private idiotypes. Private idiotypes are unique markers of antibodies. Active site of antibodies for some idiotypes reproduces three-dimensional structure of the antigen against which the antibodies having particular idio type are directed; such active sites are designated as internal image of the antigen. Anti-idiotypic antibodies (AIAT) play an important role in the regulation of cellular and humoral immune responses [1-3].

Recent studies have shown the promise of studying the properties of AIAT. For example, Rerosa Federico, Favoino Elvira, Antonietta have reported that murine anti-idiotypic monoclonal antibodies 16D7 are able to recognize HP2/6 paratope of monoclonal antibodies (moAbs) against synthetic human CD4 molecules. The study of the ability of individual H and L chains to inhibit the binding of moAbs to CD4 antigen or moAb2 has shown that only the L chain 16D7 of moAb can specifically inhibit these interactions [4].

Scientists have reported about the processing of the vaccine on the basis of AIAT against plague and AIAT against the Marek's disease virus [5,6].

Gnedenko *et al.* have developed an Immunosorbent Assay with (Fab)₂ fragment of AIAT for the diagnosis of paroxysmal schizop hrenia [7].

Many researchers note the effectiveness of AIAT for the study of the mechanisms applied to regulate the immune response to vaccines and diagnostic products for various diseases. AIAT is the issue of the particular scientific interest that is proved by the fact that the production of recombinant preparations of AIAT has been increased in recent years. For example, scientists from China have constructed a gene of the single-stranded variable fragment of monoclonal AIAT against *Vibrio alginolyticus*. Recombinant AIAT are also reported by other authors [8-32].

Materials and Methods**Materials**

We have used BALB/c mice, a hybrid strain 2G9 – an agent producing monoclonal AIAT against immunoglobulins specific

for the lipopolysaccharide antigen of *Brucella*. We have also used lipopolysaccharide antigen of *Brucella*, single Brucellosis antigen, commercial polyclonal antiserum against brucellosis of rabbits and cattle, antispecies immunoglobulins labeled with horseradish peroxidase, commercial microcentrifuge chromatographic columns made by Thermo Scientific (USA). We used special sterile plastic dishes for cell culture in order to culture hybridomas: Petri dishes of 96-well flat bottom plates, pipettes, Petri dishes, mattresses with the growth area of 25 cm² (Nunc, Denmark).

Methods**Preparation of the culture medium**

We used commercial medium RPMI-1640 (Sigma, USA) for cell cultures cultivation [33-35]. We prepared complete growth medium for culturing the hybrid cells by adding sterile fetal bovine serum (Sigma, USA) inactivated by heating at 56°C for 30 min to incomplete medium in the amount of 10% of the volume. We have also used a serum-free culture medium EX-CELL (Sigma, USA).

Purified monoclonal antibodies were stored at –70°C without preservative agents or at 4°C with 0.1% sodium azide. Concentration of antibodies in ascitic fluid was determined by the method of M. Bradford.

Electrophoresis

Electrophoresis was performed in a 12% PAGE in the presence of sodium dodecyl sulfate (SDS) by the method of Laemmli *et al.* at the apparatus intended for vertical electrophoresis.

Chromatography

We added 400 µl of 25 mM Tris•HCl buffer, pH 8.0 in commercial microfuge chromatographic column “Thermo Scientific” (USA). After

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centrifugation for 5 min at 5,000 rpm the flow buffer was discarded. The procedure was repeated 2 times. Then we added 400 μ l of the sample at the column and centrifuged it at 5,000 rpm for 5 min. Then we discarded the flow buffer. The procedure was repeated twice. The column was washed by adding 400 μ l of 25 mM Tris•HCl buffer, pH 8.0 to the membrane, followed by centrifugation at 5,000 rpm for 5 min. The procedure was repeated twice. Protein was eluted with 25 mM Tris•HCl buffer, pH 8.0, containing 0.5 or 1M NaCl.

Immunochemical properties of monoclonal AIAT were determined by the method of immunoblotting and indirect enzyme-linked immunosorbent assay (ELISA).

Immunoblotting

Test AIAT were separated in 12% PAGE in the presence of SDS, followed by transfer of the separated proteins by the blotting method from the gel onto a nitrocellulose membrane. After the transfer the membrane was put in the refrigerator overnight in a 1% solution of bovine serum albumin (BSA) and incubated for 1 h at 37°C. After washing, the nitrocellulose membrane was placed in the solution of commercial polyclonal antisera for rabbits and cattle against *Brucella* for 1 h at 37°C. Washing was repeated, followed by transferring of antispecies antibodies labeled with horseradish peroxidase into the solution for 1 h at 37°C. The reaction was shown by chloronaphthol.

Results and Discussion

In order to determine biological properties of 2G9 and 2D9 strains after long cryopreservation, we carried out cell thawing and culturing.

Cell-containing ampoules were placed in a water bath at 37°C. After thawing, the cells were washed by centrifugation in incomplete growth medium and plated on the layer of macrophages in 25 ml mattress. In order to determine the quality of the medium and forming a nutritive layer, macrophages were isolated from inbred mice and cultured for 1-3 days before thawing the cells. Growth qualities of the medium were determined by culturing myeloma cells. Spreading of macrophages and formation of the nutritive layer was observed for 1-2 days (Figure 1).

Culturing was performed in the media supplemented with “nutritive layer” of macrophages and in macrophage-free medium. In both cases cells had a good attachment to the bottom of the mattress during the day. Cells grew well in the medium with nutritive layer. Monolayer was accumulated for 3-4 days (Figure 2). In macrophage-free medium there was a slight necrocytosis in the first 1-2 days of culturing. After washing out necrotic cells the growth of hybridomas

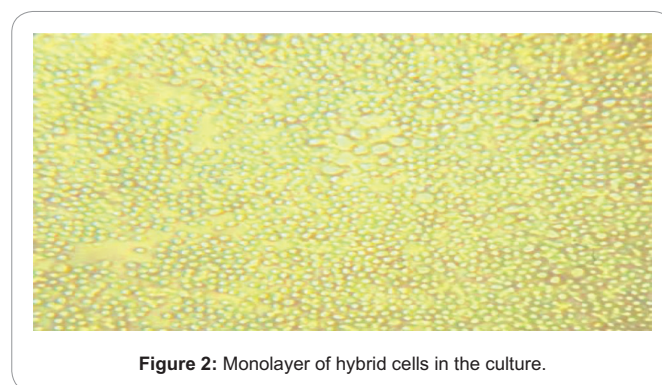


Figure 2: Monolayer of hybrid cells in the culture.

was restored. Our results indicated that after thawing hybrid cells may be cultured in macrophage-free medium.

Microscopy of cells in the growth dynamics *in vitro* showed that during freezing hybrid cells did not undergo any morphological changes and were presented by rounded cells with a good attachment to the substrate similar to the original myeloma cells by the size (Figure 2).

After controlling biological properties of hybrid cells we determined the presence of active AIAT in the culture fluid (CF). For this purpose, we have studied the interaction of CF with polyclonal antisera against brucellosis. The immunochemical activity of the used polyclonal antisera was pretested in the reaction with lipopolysaccharide antigen of *Brucella* and brucellosis single antigen in indirect ELISA test. The activity of antisera of rabbits in the reaction with antigens was higher than bovine serum. Accordingly, their titers were 1:12800 or higher, and the samples of bovine serum were titrated to the dilution of 1:400-1:800.

Wells of polystyrene plates were sensitized with antisera – 5 μ g of the protein per one well. After blocking and washing, CF samples of the studied strains were added. Subsequent procedures were performed in accordance with the standard procedure (Table 1).

ELISA results showed that the hybrid cells that were in the state of cryoconservation for a long time were able to retain their biological properties. The study of activity of AIAT produced by them with the help of ELISA test revealed that AIAT produced by the 2D9, 2G9 hybridoma cells preserved their activity. The cells of these strains were recloned. Immunochemical activity of the antibodies produced by the subclones did not differ from the values of the initial hybridomas. In order to obtain preparative amount of AIAT, cells were cultured in RPMI-1640 medium, then transferred to the serum-free medium EX-CELL. Cells were transferred gradually reducing the amount of fetal bovine serum down to 5%, 2.5%, and 0%. Hybrid cells grew well on the medium RPMI-1640 and medium EX-CELL. Concentration of the protein CF was 15-30 μ g/ml. After precipitation, purification, and concentration of the CF, we obtained 2 ml of the preparation of monoclonal AIAT with a protein concentration of 500 μ g/ml.

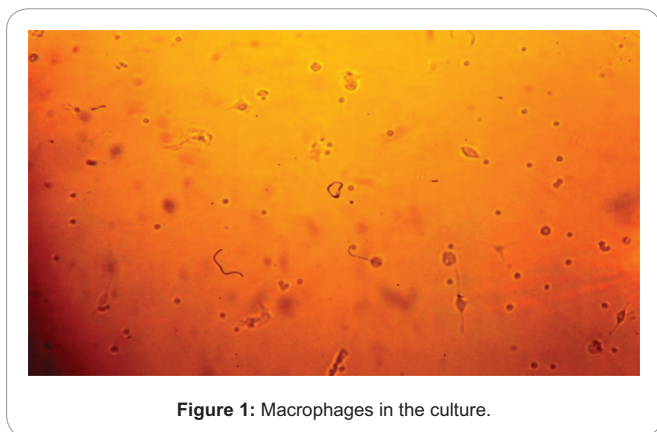


Figure 1: Macrophages in the culture.

Hybridoma strains	Values of extinction		
	2G9	0.579	0.789
2D9	0.307	0.355	0.590
X-63 – negative control	0.032	0.029	0.020

Table 1: Results of initial testing of CF hybridomas producing AIAT in ELISA with polyclonal serum for rabbits.

Dilution of anti-idiotypic antibodies	Optical density of AIAT reacted with			
	Rabbit serum against <i>B. Melitensis</i>		Positive bovine serum	
	2D9	2G9	2D9	2G9
1:100	0.475	0.514	0.393	0.481
1:200	0.281	0.301	0.239	0.297
1:400	0.236	0.242	0.203	0.214
1:800	0.217	0.207	0.187	0.190
1:1600	0.195	0.198	0.175	0.177

The values of the negative control: 0.089, 0.018, 0.022, 0.026.

Table 2: Immunochemical activity of AIAT reacting with polyclonal antisera.

In order to accumulate preparative amount of AIAT, the cells in the CF with most active AIAT were injected to BALB/c mice. Linear mice were preliminarily injected with 2, 6, 10, 14 tetramethyl pentadecane 0.5 ml per head. The preparation containing 1×10^6 cells in 0.5 ml of phosphate buffered saline were administered to mice intraperitoneally.

Ascites tumor has developed within 15-20 days. An amount of ascitic fluid from each mouse was small – 1-2 ml. AIAT were isolated from ascitic fluid by the double salting out of ammonium sulfate. Desalting was carried out on commercial chromatographic microcentrifuge column. The purity of immunoglobulin preparations was checked by electrophoresis.

Concentration of anti-idiotypic monoclonal antibodies in the purified preparation was determined by the method of Bradford. As a result, we got 7 ml of 2D9 ascites strain and 3.5 ml of 2G9 ascites strain with a final protein concentration of 1-2 mg/ml. Preliminary analysis of purified monoclonal AIAT from the ascetic fluid was carried out using indirect ELISA test. Polyclonal antisera were coated on the plate by 5 μ g per well, and AIAT were titrated with 1:100 dilution (Table 2).

We used commercial negative bovine serum against brucellosis as a negative control.

In the second variant, we used plates sensitized with 5 μ m of AIAT per well as an antigen. Polyclonal antisera were also titrated to 1:800-1:1600.

Immunochemical properties of antidiotypic antibodies were also determined by immunoblotting. In accordance with the data of Tables 1 and 2, AIAT produced by 2G9 strain were more active. For this reason, we performed immunoblotting with these antibodies. For this purpose we carried out electrophoresed in 12% PAGE. Upon the completion of electrophoresis, the control part of the gel was stained and the rest part was transferred to a nitrocellulose membrane by the standard technique (Figure 3).

After transferring the proteins the membrane was left overnight for blocking with BSA. The next day it was washed in accordance with the procedure and incubated with polyclonal antisera: positive rabbit serum bacteria against *Brucella abortus* and *Brucella melitensis*, bovine positive and negative serum against brucellosis.

After washing and development of preparation with chloronaphthol, the strips appeared in AIAT reaction with positive antisera. The fourth stripe reflects no AIAT reaction with negative serum. 2D9 and 2G9 hybrid strains during the long-term storage in deep freeze preserved their biological properties as producers of monoclonal AIAT – standard protein preparations. It is monoclonal AIAT that are used by the most researchers [4,5,7,18,19,23,28,30].

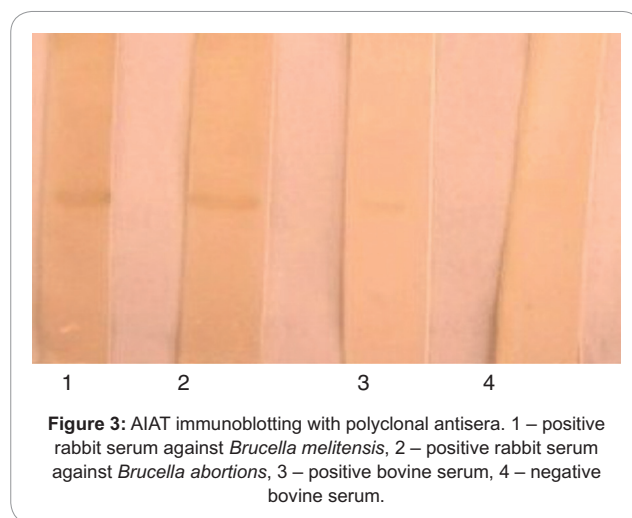


Figure 3: AIAT immunoblotting with polyclonal antisera. 1 – positive rabbit serum against *Brucella melitensis*, 2 – positive rabbit serum against *Brucella abortus*, 3 – positive bovine serum, 4 – negative bovine serum.

Analysis of AIAT immunochemical activity in ELISA test allows us to conclude on the presence of At2 β antibodies in the study population. These antibodies can be regarded as a prototype of lipopolysaccharide antigen of *Brucella*. Primary screening of AIAT was carried out by a number of authors in appropriate manner [30].

Immunoblotting demonstrated that the specific interaction with polyclonal rabbit and bovine antisera against *Brucella* occurs near light chains, which is consistent with the latest scientific data. For example, Rerosa, Federico *et al.* have reported that only the L chains of the received monoclonal AIAT are able to inhibit the binding of moAbs to CD4 antigen [4].

Thus, the results of the studies indicate the potential use of AIAT as biologically safe basis for the development of diagnostic and protective preparations.

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