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Preparation of Monoclonal Antibodies With Hybridoma Techniques Against Promastigote of *Leishmania infantum* Antigens in Diagnosis of Visceral Leishmaniasis

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Abstract

Background: Since the discovery of hybridoma cells, the uses of monoclonal antibodies (mAbs) are in vogue. Such antibodies with single isotype have high specificity. The developments in the field of cell culture and technology have led to the production of improved qualities of mAbs. In general, mAbs are important reagents used in biomedical research, as well as in targeted drug delivery systems.

Objective: The aim of this study was to apply different strategies to produce mAbs against proamastigote *Leishmania infantum* strain in Iran.

Materials and Methods: At first, standard strains were cultured and antigens of *L. infantum* were obtained. Afterward, BALB/c mice were immunized and antibody titers were determined. For hybridoma cell formation, isolated lymphocyte cells from spleen of immunized mice and myeloma cells were fused at the ratio of 10:1 in the presence of polyethylene glycol and followed by limiting dilution method for the isolation of monoclones.

Results: More than 20 positive monoclones were hybridoma, from which 3 clones had optical density over 1.5. We named these clones as 5D2 FVI6, 3G2 FV7, and 3G2 FV5 which were selected for limiting dilution. From these hybrids, anti-promastigotes *L. infantum* mAbs were obtained. The results of isotype determination showed IgG2b sub-class (and not IgG1, IgG2a and IgA) in 5D2 FV and 3G2 FVI monoclones.

Conclusion: This study produced mAbs against promastigotes of Iranian strain of *L. infantum* for the first time. These antibodies have reactivity against Iranian strain of promastigotes *L. infantum* and can be used in the diagnosis of visceral leishmaniasis.

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Background

Leishmaniasis is widely distributed around the world and is highly important for human as a serious infectious disease. It is one of the important contagious diseases caused by the parasite of the genus *Leishmania* which is common throughout the world including Iran. Although many efforts have been made to control it, leishmaniasis is still one of the health problems of the world and the region.[1-5] Three forms of this disease have been identified in humans. Visceral leishmaniasis (kala-azar) is the most threatening form which is endemic in 62 countries as well as the Mediterranean region and Iran (Table 1).[6-10] In some countries of the world, visceral leishmaniasis spreads rapidly. Previous studies have shown that the etiological cause of visceral leishmaniasis in Iran (Ardabil, Fars, East Azerbaijan, North Khorasan, Qom and Bushehr) is *Leishmania infantum* strain.[11-16]

From a morphological viewpoint, *Leishmania* can be categorized into two groups: amastigote and promastigote. Axenic amastigote (AxA) type is cultured in vitro and requires macrophage phagolysosome-like conditions to grow.[17-22] Amastigotes which are produced in this condition are named as axenic. Culturing axenic amastigotes is performed for most of *Leishmania* species, showing successful outcomes.[23-25] Therefore, in this study promastigote type of *L. infantum* was used.

The infection rate of leishmaniasis depends on 2 important factors: the immunologic status of the host plus species and strain of parasites. *L. infantum* causes the lethal disease of visceral leishmaniasis or kala-azar.[26-30] Health programs have failed to control this disease and there is not any efficient preventive vaccine yet. Therefore, the only way to counter this disease is to treat it. The first step in its treatment is diagnosis of it in appropriate time and distinction of the host from other diseases. Although there are some useful practical methods for diagnosis

Table 1. Number of Cases of Visceral Leishmaniasis in Different Countries From 2005 to 2016

Country	2016	2015	2014	2013	2012	2011	2010	2009	2008	2007	2006	2005
Bangladesh	255	544	650	1103	1902	2874	3800	4293	4840	4932	9379	6892
Brazil	3336	3289	3453	3253	2770	3894	3716	3693	3852	3604	3651	3597
China	ND	514	292	120	218	293	402	539	529	382	294	335
Colombia	37	21	31	13	9	11	34	54	33	54	44	66
Ethiopia	1593	1990	2705	1732	2381	2032	1936	1083	1356	1579	2375	2585
India	6249	8500	9241	13851	20572	33155	28382	24213	33598	44533	39173	32803
Kenya	692	894	880	181	457	406	ND	85	258	35	195	150
Paraguay	64	92	118	107	76	114	114	82	54	70	66	21
Somali	781	1031	1043	673	394	290	ND	507	583	ND	ND	ND
Sudan	3810	2829	3415	2389	5153	7418	6957	4880	3310	2788	1827	3713

ND: No data
Source: The World Health Organization (WHO), 2016.

of leishmaniasis, the sensitivity is still a problem. These methods have different sensitivities and in some of them, sensitivity and specificity are low. More specific methods such as monoclonal antibodies (mAbs) in an ELISA kit may be more convenient to use in a common laboratory. These antibodies are used as efficient tools in diagnosis, treatment and research approaches to recognize microorganism antigens. Taking all abovementioned issues into consideration, the aim of this study was to design applicable techniques to produce mAbs against promastigote Iranian type of *L. infantum*.

Materials and Methods

Culture of *Leishmania Infantum* Strains

Standard strain of *L. infantum* (MHOM/IR/04/IPI-UN10) was isolated from an Iranian patient, and reference strain (RS) of WHO (MHOM/TN/80/IPT1) was used in this study. These strains were taken from the Department of Immunology, Pasteur Institute of Iran.

At first, promastigotes of these strains were cultured in NNN (Novy-Ma.cNeal-Nicolle) special media. Then, the samples were transferred to liquid culture medium RPMI1640 (Gibco) containing fetal bovine serum (FBS) 10%, L-glutamine (2 mM) 1%, penicillin (100 u/mL) and streptomycin (100 µg/mL) 1%. They were incubated at 24˚C to reach appropriate concentrations.[31-33]

Harvested promastigotes were counted and their antigens were extracted using freeze-thaw method. For Optimization of antigens, different dilutions were prepared and coating was performed in several vials with five repetitions for each of them. Afterward, positive serum dilutions (1:1000) were obtained from immunized mouse (OD=1.12) and used in ELISA test.[31-33]

Immunization of Mice

Four female BALB/c (6-8 weeks old) mice were subjected to intra-peritoneal, subcutaneous, and subscapular injection of 40 µg of Soluble *L. infantum* antigens prepared in complete Freund adjuvant, and 2 weeks later, they were boosted with the same amount of antigen in incomplete Freund adjuvant. When 1:1000 dilution of sera had a positive reaction with antigen in ELISA, the mouse with the highest OD in ELISA was selected for fusion. Three days before fusion, the selected mouse was boosted with 40 µg of antigen into the tail.[34-36]

Cell Fusion

Leishmania infantum promastigotes were cultured at 25°C in RPMI1640 containing 10% fetal calf serum (FCS) and antibiotics. Freeze-thawed promastigote (40 µg) together with Freund complete adjuvant were intra-peritoneally and subcutaneously injected into the subscapular area in 6-week-old female mice. Three weeks later, the same dose of antigen was injected intra-peritoneally together with incomplete Freund adjuvant. When high titer of antibody was produced, 40 µg of antigen in saline was injected into the tail vain and 3 days later the mice were killed and lymphocytes from their spleen were fused with myeloma Sp2/0-Ag14 cells (IBRC C10106) in ratio of 10:1 by polyethylene glycol. Positive hybrids in HAT(Hypoxanthine-aminopterin-thymidine) medium were identified with ELISA.[34,35]

Limiting Dilution for Detection of Monoclones

Positive clones which produced special antibodies were selected. Each clone was suspended in culture medium using limiting dilution technique and divided into 96 platters to reach a uniform suspension in a way that 1 or 0.5 cells were placed in each well and incubated at 37˚C. They were cultured on complete culture medium plates with feeder layer and complements such as OPI growth factor. Consequently, mAbs produced by monoclones were isolated.

In the continuation of our experiment, we produced ascitic fluids. In addition, isotype determination was done by means of Sigma isotyping kit. All statistical analyses were performed using SPSS software version 16.0.

Results

Optimization of the Optimal Amount of Promastigotes *Leishmania infantum* Antigens

Obtained antigens from *L. infantum* parasites were optimized using ELISA method. According to Table 2, the average OD in Da (0.5×10^6) and the average Db (1×10^6) in comparison with positive control was relatively low ($P>0.05$). Dilution Dc (1.5×10^6) was selected not only for subsequent tests but also for assaying the amount of antibody. Moreover, *t* test showed that for Dd (2×10^6) and Dc (1.5×10^6), *P* value was 0.47 which was insignificant. Therefore, Dc dilution (1.5×10^6) could be considered as an appropriate concentration for future tests.

When 1:1000 dilution of sera had a positive reaction with antigen in ELISA, the mouse with the highest OD in ELISA (mouse 3 in Figure 1) was selected for fusion.

After pre-screening of hybridomas, further analysis was performed by cloning and sub-cloning using limiting dilution. These sub-clones were investigated by ELISA, from fourth fusion: 4G5 FV, 5D2 FV, 2G8 FV and 8E6 FV, from fifth fusion: 5D6 FIV, 3C9 FIV, 3C4 FIV and 7F6 FIV and from sixth fusion: 2G6FVI, 4H3FVI, 3G2FVI and 6D2FVI hybridomas were isolated. Some of these hybridomas were reserved in liquid nitrogen for future analysis. Results are presented in Table 3.

Among above-mentioned hybridomas, positive ones 5D2 FV (OD = 1.66) and 3G2 FVI (OD = 1.592), which showed the highest amount of produced antibody against *L. infantum* amastigotes, were selected, and after preparing homogenous suspension, they were diluted by limiting dilution method. As a result, mAbs-producing monoclones were isolated. Table 4 shows the results of hybridoma proliferation for the isolation of antibody-producing monoclones using limiting dilution method. Two cases of these monoclones were selected for Isotype determination.

Classification of mAbs (5D2 FVI6, 3G2 FV7) showed that the produced mAbs against *L. infantum* in cell fusion belonged to the IgG class and IgG2b sub-class. Table 5 shows class and sub-classes of mAbs identified in sample absorbance at 450 nm (OD_{450}).

Table 2. Optimized *Leishmania infantum* Antigens

Different Dilutions of *Leishmania infantum* Antigens							
0.5×10^6		1×10^6		1.5×10^6		2×10^6	
D_a	OD	D_b	OD	Dc	OD	Dd	OD
D_{a1}	0.273	D_{b1}	0.567	Dc_1	0.643	Dd_1	0.564
D_{a2}	0.389	D_{b2}	0.460	Dc_2	0.616	Dd_2	0.626
D_{a3}	0.388	D_{b3}	0.360	Dc_3	0.609	Dd_3	0.721
D_{a4}	0.485	D_{b4}	0.463	Dc_4	0.594	Dd_4	0.518
D_{a5}	0.377	D_{b5}	0.364	Dc_5	0.599	Dd_5	0.645
PC	0.887	PC	0.899	NC	0.054	B	0.022

PC, positive control (immunized mouse serum); NC, negative control (normal mouse serum); B: Blank (BSA), Da: (Dilution 0.5×10^6); Db, (Dilution 1×10^6); Dc, (Dilution 1.5×10^6) , Dd : (Dilution 2×10^6); Dilution repeats (Da1- Da5 , Db1- Db5, Dc1- Dc5 , Dd1- Dd5). Dilution of immunized mouse's positive serum in wells: 1:1000 (OD450) Absorbance of samples at 450 nm.

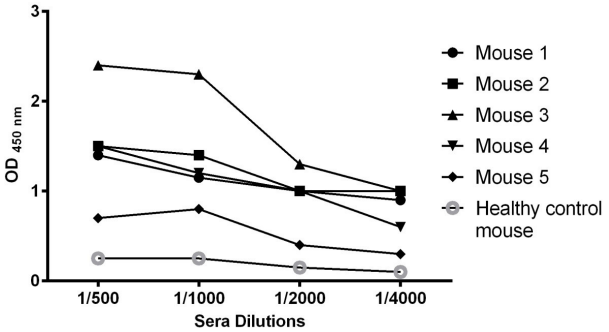

Figure 1. Comparison of Antibody Titer in Immunized Mice. Mouse numbers 1 to 5 are immunized mice by antigen (*L. infantum*). The highest OD is seen in mouse 3.

Discussion

Although in recent years identification of *Leishmania* species using molecular methods such as PCR-RFLP and kDNA-PCR is performed in some research centers,

Table 3. Positive Hybrids Obtained From 3 Fusions

Fusions With the Highest OD					
Fusion V		Fusion IV		Fusion VI	
Hybrids	OD_{450}	Hybrids	OD_{450}	Hybrids	OD_{450}
4G5 FV	1.11	5D6 FIV	1.065	2G6 FVI	1.163
8E6 FV	0.801	3C9 FIV	0.998	4H3 FVI	1.06
5D2 FV	1.66	3C4 FIV	0.769	3G2 FVI	1.592
2G8 FV	0.858	7F6 FIV	0.984	6D2 FVI	0.776

OD: optical density, FV: Fourth Fusion, FIV: Fifth Fusion, FVI: Sixth Fusion.
(OD450) Absorbance of samples at 450 nm
Fusion: Lymphocyte cells from spleen of immunized mice and myeloma cells were fused

Table 4. Results of Hybridoma Proliferation for Isolation of Antibody-Producing Monoclones Using Limiting Dilution Method

Hybrid (5D2 FVI)		Hybrid (3G2 FV)	
Monoclones	OD	Monoclones	OD
5D2 FVI6	1.610	3G2 FV6	0.989
5D2 FVI2	0.910	3G2 FV1	0.899
5D2 FVI4	1.080	3G2 FV7	1.670
5D2 FVI6	0.969	3G2 FV3	1.212
5D2 FVI2	1.267	3G2 FV5	1.599
5D2 FVI8	0.899	3G2 FV9	0.988

OD: optical density, FV: Fourth Fusion, FIV: Fifth Fusion, FVI: Sixth Fusion.
(OD450) Absorbance of samples at 450 nm
Fusion: Lymphocyte cells from spleen of immunized mice and myeloma cells were fused

Table 5. Class and Sub-classes of mAbs Identified

mAbs	5D2 FVI6				3G2 FV7			
Class	Anti-mouse IgG			Ig	Anti-mouse IgG			Ig
Sub-class	IgG1	IgG 2a	IgG 2b	IgA	IgG 1	IgG 2a	IgG 2b	IgA
OD	0.16	0.173	1.785	0.14	0.158	0.214	1.681	0.131
B	0.019	0.013	0.073	0.031	0.036	0.039	0.049	0.069

(OD450): Absorbance of samples at 450 nm
B: Blank (BSA); OD: optical density; mAbs: monoclonal antibodies; IgG, A: Immunoglobulin G, A; IgG 2a, 2b: Immunoglobulin G 2a, 2b; FIV: Fifth Fusion; FVI: Sixth Fusion.

these methods could not meet the needs of common laboratories and health programs because they are expensive and require special equipment. In addition, because of the high level of polymorphisms in different species of *Leishmania*, these methods are not applicable in most ordinary laboratories. It seems that the use of mAbs is more appropriate to diagnose leishmaniasis and recognize biochemical and immunopathological properties of the parasite.

After mAb discovery by Kohler and Milestein in 1975, there has been astonishing and fast progress in hybridoma technology and mAb application.[36] In 1982, De Ibarra et al produced mAbs, which were able to detect different species of *Leishmania*.[37] In 1983, Greenblatt et al developed a mAb that could act against *Leishmania major* and could also show reaction against other species of the parasite.[38] In addition, in 2009 Nejad Moghaddam and Abolhassani succeeded in preparing a monoclonal antibody against a common 57-kDa antigen of leishmania species.[39] Furthermore, Jaffe et al produced mAbs against *Leishmania donovani* for immunodiagnosis.[40]

During the past decades, a specific mAb was produced against *Leishmania amazonensi, Mexicana* and *Leishmania donovani* and applied in immunological diagnosis and taxonomic studies of *leishmania* species.[41-43] Regarding the limited number of studies on the antigens of *Leishmania* species, adequate information about the *L. infantum* antigens is not available. Moreover, there has not been any study on mAb development against Iranian strain of *L. infantum*. Therefore, producing a specific mAb against amastigote form of Iranian strain of *L. infantum* seems to be essential.

In this study, in the third fusion, from eight 96-well plates, only 16 positive hybrids were obtained, 4 of which (2A4 F III, 7G3 F III, 5B4 F III: negative, 5A3 F III: positive) with optical absorbance near to cut-off point of ELISA were considered suspicious. In the reassessment, these 4 hybrids turned to negative and showed optical absorbance below the ELIZA cut-off. The only positive hybrid in this fusion (5A3FIII; OD=0.761) lost its positivity in subsequent analysis. It seems that this phenomenon happened because of chromosome instability in hybridoma cells.[44]

According to Table 3, fourth, fifth and sixth cell fusions were successful and several clones were obtained. About 10% of each plate contained positive clones and this ratio was acceptable. Clones with high absorbance in interaction with *L. infantum* antigens were selected for proliferation through limiting dilution method and about 30% of wells were positive. Hybrids with OD>1 (2G2 FV3, 4G2 FV2, 5D3 FVI5, 7D2 FVI2) were reserved in liquid nitrogen for future studies and vaccine development. A range of 30% is appropriate and coincides with $a = e^{-b}$ formula. This is Poisson' distribution and according to Guding's interpretation, if $b = 1$, then "a" will be equal to 37. Therefore, if one cell is added to each well, it is probable that in minimum there are no cells in 37% of wells. Therefore, wells that cell proliferation was seen in them contained real mAbs.[45,46]

After performing limiting dilution and obtaining monoclones, class and sub-classes were identified. Clone 5D2 FVI6 was from IgG class and IgG2b sub-class (OD = 1.60), and 3G2 FV7 clone was from IgG class and IgG2b sub-class (OD = 1.670). These results were consistent with previous reports.[47] The benefits of using mAbs are: high sensitivity, specificity, low cost, and easy portability. Detection of *leishmania* products may constitute an important achievement to improve the clinical management of suspected leishmaniasis cases.

Conclusion

In this study, a monoclonal antibody was produced against proamastigotes of Iranian strain of *L. infantum* for the first time. It seems that these antibodies have appropriate reactivity against Iranian strain of *L. infantum* and could be used in ELISA, immunofluorescence, and flow cytometry tests for research and diagnosis. Considering that the main resistance mechanism against *Leishmania* parasite is cellular immunity, it is proposed that produced mAbs can be used to purify related antigens in *L. infantum* and to find a candidate molecule for vaccine studies.

Ethical Approval

The experiment was approved by State Ethics Committee, University of Mohaghegh Ardabili, Ardabil, Iran. Additionally, the advices of European Council Directive (86/609/EC) of November.24, 1986, were fully considered in the experimental procedures.

Conflict of Interest Disclosures

The authors declare that they have no conflict of interests.

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