Development of monoclonal antibodies against axenic amastigotes of *Leishmania infantum* strain in Iran: implication for diagnosis of Kala-azar

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A R T I C L E I N F O	A B S T R A C T
	Objective(s): Leishmaniasis is endemic in 88 countries. Amastigote forms of *Leishmania* are experts at exploiting host cell processes to establish infection. Monoclonal antibodies are key reagents used in the diagnosis of infectious and non-infectious diseases. The aim of this study was to produce monoclonal antibodies against axenic amastigotes of the *Leishmania infantum* strain in Iran. ***Materials and Methods:*** First, standard strains were cultured and axenic amastigote antigens of *L. infantum* were obtained. Since then, BALB/c smice were immunized and antibody titers were determined. For hybridoma cell formation, lymphocytes isolated from spleen of immunized mice and myeloma cells were fused at a ratio of 10 to 1 in the presence of polyethylene glycol, followed by limiting dilution for the isolation of monoclones. Subsequently, antibody isotypes were determined by using the isotyping kit. The best clone was injected intraperitoneally to pristane-primed mice for large scale production of monoclonal antibodies. The specificity of antibody was determined with Western blotting. ***Results:*** Approximately 25 positive monoclones were obtained, of which four hybrids producing anti-amastigotes *L. infantum* monoclonal antibodies with high optical density (OD), selected and designated as 8D2 FVI6, 8D2 FVI3, 6G2 FV4 and 6G2 FV3. Results from isotype determination showed the IgG2b sub-class in 6G2FV2 and 8D2FVI6 monoclones. ***Conclusion:*** This study produced monoclonal antibody against amastigotes of Iranian strain of *L. infantum* for the first time. These antibodies have reactivity against Iranian strain of *L. infantum* and can be used in the diagnosis of Kala-azar.

Introduction

Although in recent years identifying of *Leishmania* species using molecular methods such as PCR-RFLP and kDNA-PCR is performed in some research centers, 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 high level of polymorphisms in different species of *Leishmania*, these methods are not applicable in most of ordinary laboratories. It seems that mAbs usage is more appropriate to diagnose leishmaniases, specially it should be mentioned that the specificity of procedure using monoclonal antibodies are higher than other procedures (1).

Leishmaniasis is widely distributed around the world and is greatly important for humans as a leading cause of serious infectious diseases. Leishmaniasis is one of the most important contagious diseases caused by parasites of the genus *Leishmania*, a common parasite throughout the world and Iran. Although many efforts have been made to control the disease, leishmaniasis is still one of the health problems in the world (2-5).

Three forms of this disease have been identified in humans in which visceral leishmaniasis is the most threatening form; visceral leishmaniasis is endemic in 62 countries as well as in the Mediterranean region and Iran (6-10). Previous studies showed that the etiological cause of kala-azar in Iran (Ardabil, Fars, East Azarbaijan, North Khorasan, Qom and Bushehr) is the *L. infantum* strain (11-16).

From a morphological point of view, *Leishmania* can be categorized into two forms, amastigote and promastigote. The axenic amastigotes (AxAs) type is culturable *in vitro* and requires conditions like macrophage phagolysosome for growth (17-22). Amastigotes, which are produced in this condition, are designated as axenic. Culturing axenic amastigotes is performed for most of the *Leishmania* species and shows successful outcomes (23-25). According to the reports, since the infectivity of macrophage by AxAs forms, such as amastigote forms, are greater than promastigotes (26) it seems that this form of parasite will be ideal form for production of monoclonal antibodies. Data reported by an investigation shows that while, no expression

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of amastin gene observed with promastigote of *Leishmania*, amastin gene could be selectively expressed by AxAs of *L. tropica*. Also, specific expression of amastin has been confirmed by RT-PCR using amastigote and AxAs. Moreover, this study has shown that cpb gene was only expressed by amastigotes and AxAs and not promastigotes. Therefore because there are strongly similarities between amastigotes and AxAs, it seems that AxAs has ability to be useful on preparation of monoclonal for differentiation of parasites as well as in purification of antigens and drug screening (26, 27). In addition, as we know the antigens of amastigotes are the first one presented to immunological system of the host. Therefore, axenic amastigotes type of *L. infantum* was used in this study for preparation of monoclonal antibody.

The infection fate in leishmaniasis depends on two important factors, the immunologic status of the host plus species and the strain of parasites. *L. infantum* causes a lethal disease called Kala-azar (28-30). Health programs have failed to control this disease and there is no efficient preventive vaccine. Therefore, treatment is the only way to counter this disease. The first step in the treatment is diagnosis of the parasite in an appropriate time, and its distinction from other diseases. Although there are some useful practical methods for the diagnosis of leishmaniasis, the sensitivity is still a problem. These methods have different sensitivities, some of which show low sensitivity and specificity. More specific methods such as monoclonal antibodies (mAbs) to develop an enzyme-linked immunosorbent assay (ELISA) method may be more convenient in a common laboratory. These antibodies are used as efficient tools in diagnostic, treatment and research approaches to recognize microorganism antigens (31-34). Herein, in this study we produced mAbs against AxAs form of Iranian *L. infantum* in order to design an ELISA kit in the future.

Materials and Methods
Culture of L. infantum strains (promastigotes and amastigotes forms): Promastigote culture
The Iranian strain of *L. infantum* (MHOM/IR/04/IPI-UN10) isolated from a patient and reference strain of WHO (MHOM/TN/80/IPT1) was used in this study. At first, promastigotes of these strains were cultured in NNN (Novy-MacNeal-Nicolle) special media. Then, the parasites were transferred to RPMI-1640 medium (Gibco, Germany), supplemented with fetal bovine serum (FBS) 10% (Biosera, UK), 2 mM L-glutamine (Gibco, Germany) penicillin (100 U/ml) and streptomycin (100 µg/ml) (Merck, Germany). They were incubated in 24°C to reach appropriate concentration. After that, the cultured promastigotes were used to obtain amastigotes like forms parasites.

Culture of axenic amastigote
Late logarithmic phase cultures of *L. infantum* promastigotes were transferred to RPMI-1640 medium supplemented with 25% FBS and incubated at 37 °C (5% CO_2) for 16–24 hr. Thereafter, the cells were centrifuged (1200×g at room temperature for 10 min) and resuspended in the same medium supplemented

with (10 mM) succinate (Merck, Germany), titrated to pH 5.5 and incubated as above. Under this situation, promastigotes differentiated to amastigotes like form (AxAs) within 120 hr and amastigote forms without flagellate were produced (31-33).

Preparation of AxAs L. infantum antigens
Harvested amastigotes were counted ($4×10^9$ parasite cells) and their antigens were extracted using the freeze (five times at liquid nitrogen) and thaw at 37º C. For preparation of antigens, different dilutions were prepared in several vials. Amastigotes antigens were collected and stored at -70 °C until use (31, 33).

Immunization of mice
Four female BALB/c (6-8 weeks old) mice were injected intraperitoneally, and subcutaneously (at tail base) with 40 µg of soluble AxAs *L. infantum* antigens preparation in complete Freund adjuvant and 2 weeks later were boosted with the same amount of antigen in incomplete Freund adjuvant. Then the serum antibody titers of mice 30 days after injection were measured. When 1/1000 dilution of sera had positive reaction with antigen in ELISA, the mouse with highest OD in ELISA was selected for fusion. Three days before fusion, selected mouse was boosted with 40 µg antigen intravenous through the tail (34-36). All experiments in this study performed according to Ethic Committee of Tehran University of Iran.

Extraction and collection of spleen cells
Three days after final immunization, mouse was sacrificed and the spleen of mouse was isolated aseptically. Using a 20 ml syringe containing RPMI medium and with needle tip 25, spleen cells were collected. This procedure was repeated several times until all spleen cells were drained. On the suspension, 5 ml of ammonium chloride was added and then centrifuged (2000 × g 10 min) to lyse erythrocytes.

Cell fusion
Sp2/0-Ag14 cells (IBRC C10106) were used for fusion and cultured in special medium. Several cultures of these cells were prepared and their growth rate was assessed precisely, and cultures with live cells higher than 90% were selected for fusion. The cells were kept in exponential growth phase and retained at this phase for fusion. Isolated lymphocytes from spleen of immunized mice and myeloma cells were fused at a ratio of 10 to 1 in the presence of PEG (polyethylene glycol; MW 1450, Sigma) and then fused cells were transferred to the complete culture medium containing HAT (hypoxanthine-aminopterin-thymidine) medium (Sigma, USA), 2% , L-glutamine (2 mM), 20% FBS, 5% CO_2 1% penicillin (100 U/ml) with streptomycin (100 µg/ml) and were incubated at 37 °C. After 1 week, HAT medium were replaced with HT (hypoxanthine and thymidine) medium (Sigma, USA). Hybridoma cell presence and the colonies were identified using an invert microscope. Part of these cells were suspended in a special freezing medium and reserved in liquid nitrogen for future tests. Also, some parts of them were used for subsequent analyses (34, 35).

Table 1. Positive hybrids obtained from 4 fusions with highest OD

Fusion III		Fusion V		Fusion IV		Fusion VI	
Hybrids	OD	Hybrids	OD	Hybrids	OD	Hybrids	OD
1A4 F III	0.719	6G2 FV	0.819	1D6 FIV	1.465	5G6 FVI	1.163
4B4 F III	0.745	8E6 FV	0.801	4C9 FIV	0.998	5H10 FVI	1.107
4G1 F III	0.748	8F4 FV	0.850	6C4 FIV	0.999	6G2 FVI	1.192
8E4 F III	0.692	8G8 FV	0.858	7F6 FIV	0.994	8D2 FVI	1.515

(OD_{450}) Absorbance of samples at 450 nm

Table 2. Results of hybridoma proliferation for isolation of antibody producing monoclones using limiting dilution method

Hybrid(8D2 FVI)		Hybrid(6G2 FV)			
Monoclons	OD	Monoclons	OD	Monoclons	OD
8D2 FVI6	1.510	6G2 FV6	0.889	6G2 FV10	0.777
8D2 FVI7	0.99	6G2 FV1	0.890	6G2 FV8	0.895
8D2 FVI1	0.880	6G2 FV7	0.900	6G2 FV2	1.410
8D2 FVI9	0.769	6G2 FV3	1.112	6G2 FV4	0.989
8D2 FVI10	0.567	6G2 FV5	0.899		
8D2 FVI8	0.898	6G2 FV9	0.788		

(OD_{450}): Absorbance of samples at 450 nm

Cloning of hybridoma cells by limiting dilution assay

Positive clones were selected. Each colon was suspended in culture medium using limiting dilution and split into 96-well plates to reach a uniform suspension so that approximately one cell was placed in each well and incubated at 37 °C. They were cultured in complete culture medium plates with feeder layer and supplements such as oxalate, pyruvate, and insulin like growth factor (Merck, Germany). Consequently, mAb producing monocolones were isolated.

Production of ascitic fluids

Hybridoma cells with (the highest OD) producing mAbs were grown in RPMI-1640 (Gibco) supplemented with 10% FBS, harvested and washed twice in phosphate-buffered saline (PBS), (Sigma). Eight days after pristane injection, BALB/c mice were injected intra-peritoneally with $2×10^6$ hybridoma cells suspended in 0.5 ml PBS. Fluid was collected from the peritoneal cavity 10 days after the injection of the cells. Ascitic fluid was kept at 4 °C for 1 hr and centrifuged at (4000× g 20 min). Supernatant was collected and stored at -70 °C until use (34, 35).

Isotype determination

Two monoclones with higher OD were selected. The class and subclass of the mAbs were determined by ELISA with a mouse monoclonal sub isotyping kit containing rabbit anti-mouse IgG1, IgG2a, IgG2b and IgA (Sigma, USA).

SDS-PAGE and Western-immunoblotting

SDS-PAGE was performed according to the Laemmli method. Gels were stained with Coomassie Blue and later with silver staining. Proteins separated by SDS-PAGE were transferred to nitrocellulose sheets (Schleicher & Schuell, 0.45 μm pore size) by semi-dry blotting technique. Subsequently, the nitrocellulose sheet was incubated with 1% BSA in PBS buffer for 1 hr and then incubated with hybridoma culture supernatant at 4 °C overnight. The membrane was washed with PBS containing 0.1% BSA and 0.05% Tween 20, and second antibody (HRP-conjugated goat anti-mouse). After washing, the immune complex was detected by a color reaction using diaminobenzidine as enzyme substrate containing 0.03% $COCl_2$ for color enhancement.

Diagnosis of L. infantum isolates by AxA monoclones

The mAb 8D2 FVI6 was used for diagnosis of *L. infantum* promastigotes isolated from three Kala-Azar patients using ELISA tests. The isolates, which was a gift from Dr Mohabali, (School of Public Health, Tehran), were coated at 96 well plates after freez-thaw, and followed by ELISA test.

Results

AxAs L. infantum antigen

Antigens $7×10^9$ from AxAs *L. infantum* parasites were obtained, then optimal number of antigens for assessment of antibodies was determined using the ELISA method.

Comparison of antibody titer in immunized mice

In different fusion programme (12 times), five mice were used for immunization, the mouse with higher OD in ELISA was selected for fusion. For example in fusions 6 the mouse 3 with OD equal 2.43 were selected for fusion (Figure 1).

Positive hybridomas with highest OD

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 third fusion: Subclones of 1A4 F III, 4B4 F III, 4G1 F III and 8E4 F III, from forth fusion: Subclones of ID6 FVI, 4C9 FIV, 6C4 FIV and 7F6 FIV, from fifth fusion: subclones of 8G8 FV, 8F4 FV, 8E6 FV and 6G2 FV and from sixth fusion: Subclones of 5G6 FVI, 5H10 FVI, 6G2 FVI and 8D2 FV hybridomas were isolated. Some of these hybridomas were reserved in liquid nitrogen for future analysis. Results are presented in Table 1.
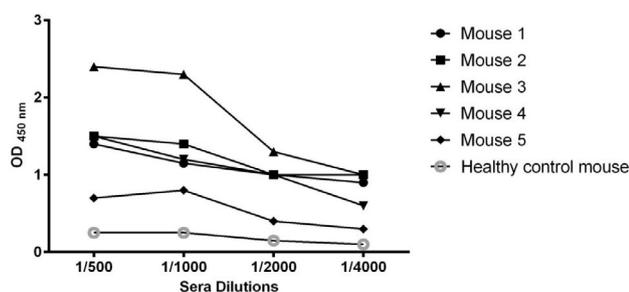
Figure 1. Comparison of antibody titer in immunized mice. Mouse numbers 1 to 5 are immunized mice by antigen (AXA *Leishmania infantum*). The highest OD is seen in mice 3

Monoclones obtained by limiting dilution method

In this study, about 1850 hybridomas were screened in which 84 showed positive hybridomas. Among them, 25 monoclones were positive and of these sets 12 monoclones had acceptable optical absorbance. Finally, 4 of them (8D2 FVI6, 8D2 FVI3, 6G2 FV4 and 6G2 FV3) showed absorbance higher than 1 in ELISA test. From these hybrids, anti-amastigotes *L. infantum* mAbs were obtained. Among above-mentioned hybridomas, positive ones namely 6G2FV(OD=1.365) and 8D2FVI(OD=1.415), which showed the highest amount of produced antibody against *L. infantum* amastigotes were selected and after preparing homogenous suspension were diluted by limiting dilution method. As a result, mAbs producing monoclones were isolated. Table 2 shows the results of hybridoma proliferation for isolation of antibody producing monoclones using limiting dilution method. Two cases of these monoclones (6G2 FV2, 8D2 FVI6) were selected for isotype determination.

Class and sub-classes of mAbs

Classification of mAbs (6G2 FV2, 8D2 FVI6) showed that the produced mAbs against AxAs *L. infantum* in cell fusion belong to IgG class and IgG2b sub-class. Table 3 shows class and sub-classes of mAbs identified. Two monoclones with higher OD (6G2 FV2, 8D2 FVI6) were used for determination of class and sub-classes. Both of them showed class IgG and IgG2b subclass.

The result of SDS-PAGE and Western blotting

To determine the specificities of mAb (8D2 FVI6), SDS-PAGE analysis was performed, followed by Western-blot analysis. As Figure 2 shows, Western blotting of monoclonal antibody (8D2 FVI6) confirms that this antibody has ability to recognize AxA *L. infantum* antigens.

Identification of L. infantum isolates

The mAb 8D2 FVI6 was used for identification of *L. infantum* promastigotes isolated from Kala-Azar patients using ELISA tests. As shown in Tabal 4, this mAb has ability to recognize also promastigote of different isolates of *L. infantum*.

After mAbs 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 are able to detect different species of *leishmania* (37).
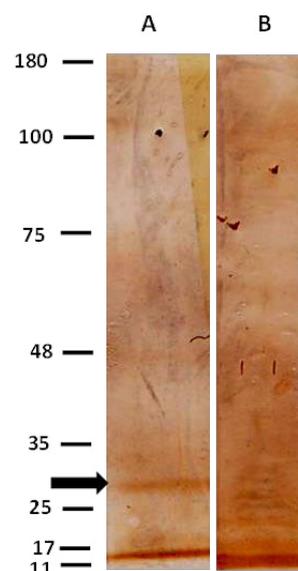

Figure 2. Western blotting of monoclonal antibody (8D2 FVI6). A sharp single band was seen in ⁓30 kDa (shown by arrow). A: AxA *Leishmania infantum* antigens loaded, B: Promastigotes of *Leishmania infantum* antigens loaded

In 1983, Greenblatt *et al.* developed a mAb that could act against *L. major* and showed reaction against other species of the parasite (38). During the past decades, specific mAb against *Leishmania amazonensi, mexicana and donovani* have been produced and applied in immunological diagnosis and taxonomic studies of *Leishmania* species (39-41). Regarding the limited number of studies carried out on preparation of monoclonal antibodies against antigens of *Leishmania* species, adequate information about the AxAs *L. infantum* antigens is not available. Also, there has been no investigation around the developing mAb against Iranian strain of *L. infantum*. Therefore, producing specific mAb against amastigote form of Iranian strain of *L. infantum* seems to be essential.

Since, the man and rodents are the main host for different species of *Leishmania*, and parasites are introduced themselves inside the macrophages as amastigote form, it seems that preparation of monoclonal antibodies against amastigote like parasite could be useful agent in process of leishmaniases diagnosis.

In this study, 25 positive monoclones were obtained and from which 12 monoclones showed acceptable titer of antibodies. Moreover, 4 of them (8D2 FVI6, 8D2 FVI3, 6G2 FV4 and 6G2 FV3) showed higher titer of antibody (OD >1) in ELISA test.

In the third fusion, from eight 96-well plates, only 6 positive hybrids were obtained. However, 4 hybrids (1A4 F III, 4G1 F III, 4B4 F III, and 7A6 F III) showed in primary study positive but in the reassessment, these 4 hybrids turned to negative with lower OD. It seems that this phenomenon happened because of chromosome instability in hybridoma cells (42).

According to Table 1, three fusions including fusion 4, 5 and 6 were ontained successfully. About 10% of each plate contained positive clones and this ratio seems to be acceptable. Clones with high absorbance in interaction with AxAs *L. infantum* were selected for proliferation

Table 3. Class and sub-classes of mAbs identified

mAbs	8D2 FVI6				6G2 FV2			
Class	Anti mous IgG			Ig	Anti mous IgG			Ig
sub-class	IgG1	IgG 2a	IgG 2b	IgA	IgG 1	IgG 2a	IgG 2b	IgA
OD	0.11	0.143	1.685	0.12	0.128	0.114	1.781	0.111
B	0.012	0.023	0.043	0.011	0.076	0.089	0.079	0.059

(OD$_{450}$): Absorbance of samples at 450 nm , B; Blank

Table 4. Identification of different isolates of Leishmania infantum (promastigotes) and AxAs of _Leishmania infantum_ by mAb prepared from 8D2 FVI6

Samples	_Leishmania infantum_ isolates (Promastigote)			AxAs _Leishmania infantum_	PC	NC	**B**
	S1	S2	S3				
OD	0.526	0.802	0.795	1.250	2.519	0.069	0.034

PC: Positive control (Immunized mouse serum); NC: Negative control (Normal mouse serum); B: Blank (BSA); Dilute of immunized mouse positive serum in wells:1/1000; (OD450) Absorbance of samples at 450 nm

using limiting dilution method and about 30% of wells were positive and monoclone. Hybrids with OD>1 (6G2 FV3, 6G2 FV4, 8D2 FVI3, 8D2 FVI6) were reserved in liquid nitrogen for future studies and probably kit development. 30% range is appropriate and coincides with $a=e^{-b}$ formula. This is Poisson'distribution and according to the Goding 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 is shown in them contain real mAbs (43, 44).

After limiting dilution performing and obtaining monoclones, class and sub-classes were identified. Clone 6G2FV2 was from IgG class and IgG2b sub-class (OD=1.685), and 8D2FVI6 clone was from IgG class and IgG2b sub-class (OD=1.781). The accuracy of the mAb (8D2 FVI6) was shown by Western blotting which shows that this antibody could recognize AxA _L. infantum_ antigen at about 30 kDa area. There is inconsistency between our results and data reported by Debrabant _et al._, who have shown the band of axenic amastigote of _L. donovani_ at about 40 kDa areas (45). The difference between two proteins may be attributed to the distinct strains has been used in analysis of both studies.

Diagnosis of _L. infantum_ isolates by mAb (8D2 FVI6) was indicated that the mAb aginst AxA has ability to recognize different isolates of _L. infantum_.

Taken together, these mAbs have capacity to be used in _Leishmania_ diagnostic kits and also for purification of antigen to be tested.

Conclusion

Hybridoma technologies can be used for the reliable development of mAbs and their subsequent production in different cells. In principle, the method has been widely applied in various fields such as diagnosis applications, disease monitoring and identifying prognostic markers (46-50). This study produced mAbs against amastigotes 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 flowcytometry tests for research and diagnosis (46-50). Taking into the account

that the main resistance mechanism against _leishmania_ parasite is cellular immunity, it is proposed that produced mAbs could be used to purify related antigens in axenic amastigotes and used to find a candidate molecule for vaccine studies. As well as identifying species using mAbs in areas where leishmaniasis is prevalent may be of great significance, however, it will be helpful for epidemiologic studies and important for treatment of travelers from non-endemic to endemic areas of leishmaniases including Iran. These antibodies may also provide help for the affinity purification of antigens or suitable targets for chemotherapeutic agents.

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