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 promastigote 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 SD2 FV6, 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 SD2 FV and 3G2 FV1 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.

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. 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). 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.

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. Amastigotes which are produced in this condition are named as axenic. Culturing axenic amastigotes is performed for most of Leishmania species, showing successful outcomes. 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. 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...
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-MacNeal-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.

#### Immunization of Mice

Four female BALB/c (6-8 weeks old) mice were subjected to intra-peritoneal, subcutaneous, and subcapular 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.

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**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   | 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  | 120  |
| Paraguay     | 64   | 92   | 118  | 107  | 76   | 114  | 114  | 82   | 54   | 70   | 66   |
| Somalia      | 781  | 1031 | 1043 | 673  | 394  | 290  | ND   | 507  | 583  | ND   | ND   |
| Sudan        | 3810 | 2829 | 3415 | 2389 | 3153 | 7418 | 6957 | 4880 | 3310 | 2788 | 1827 | 3713 |

ND: No data
Source: The World Health Organization (WHO), 2016.
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 monoclonies were isolated. Table 4 shows the results of hybridoma proliferation for the isolation of antibody-producing monoclonies using limiting dilution method. Two cases of these monoclonies 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}).

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, different mAbs against L. infantum species can be produced by this method.

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 |
|---------------------------------------------------|------------|----------|------------|----------|
| Dc | OD | Dd | OD | Dc | OD | Dd | OD |
| Da | 0.273 | Da | 0.460 | Da | 0.643 | Da | 0.564 |
| Ds | 0.388 | Ds | 0.564 | Ds | 0.616 | Ds | 0.564 |
| Dc | 0.485 | Dc | 0.609 | Dc | 0.571 | Dc | 0.518 |
| Ds | 0.377 | Ds | 0.594 | Ds | 0.626 | Ds | 0.518 |
| 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 (OD_{450}) Absorbance of samples at 450 nm.

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.

(OD_{450}) 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 Monoclonies Using Limiting Dilution Method

| Monoclonies | OD | Monoclonies | OD |
|-------------|----|-------------|----|
| 5D2 FVI6 | 1.610 | 3G2 FV6 | 0.989 |
| 5D2 FVI2 | 0.910 | 3G2 FVI | 0.899 |
| 5D2 FVI4 | 1.080 | 3G2 FV7 | 1.670 |
| 5D2 FVI6 | 0.969 | 3G2 FV3 | 2.121 |
| 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.

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

Fusion: Lymphocyte cells from spleen of immunized mice and myeloma cells were fused

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.
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 amazonensis, 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, ?G3 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 FV15, 7D2 FV12) 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 SD2 FV16 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 promastigotes 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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