Monoclonal Antibody Against Antigens of *Leishmania infantum*: Optimize the Growth Condition of Monoclonal Antibody-producing Hybrids

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Abstract

Introduction: *Leishmania (L.) infantum* is the etiologic cause of visceral leishmaniasis in Iran. Efficient vaccines and diagnosis methods are required to control leishmaniasis. The aim of this study is produce and optimize monoclonal antibodies against promastigotes forms of *L. infantum* antigen.

Materials and Methods: The mice were vaccinated with the *L. infantum* antigen and their antibody titers were determined by the ELISA method. Spleen cells of the most immune mouse were fused with SP2/0 in the presence of Poly Ethylene Glycol. The effect of supernatant of SP2/0 and mice peritoneum macrophage cells culture (SSMCC) on hybridoma cell proliferation was studied.

Results: Among the 12 fusion, a total of 26 monoclonal were positive. 12 of which had acceptable optical absorbance in OD 450 nm. Finally, 4 clones, designated as 8D2 FVI6, 8D2 FVI3, 6G2 FV4 and 6G2 FV3. From these hybrids, anti-promastigotes *L. infantum* monoclonal antibodies were obtained. SSMCC was shown to play a key role in hybridoma proliferation and of mAb production. It seemed that SSMCC is rich of growth factors.

Conclusion: It seems in the near future, this SSMCC can be used as a growth factor for cancerous and non-cancerous cells in research centers at a wider level.

Keywords: Monoclonal antibodies, *Leishmania infantum*, Cell culture

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Introduction

Trypanosomatid protozoans belonging to the genus *Leishmania* are obligate parasites of mammalian macrophages. The life cycle of these organisms goes through two morphologically different stages: the amastigote, which is found in the parasitophorous vacuoles of host macrophages and dendritic cells, and the promastigote, which is an extracellular flagellated form found in the gut of the sandfly vector. At least, 15 *Leishmania* species are infectious for humans and cause a wide spectrum of diseases, including cutaneous, mucocutaneous, and visceral leishmaniasis, as well as asymptomatic infections. Intermediate forms may be encountered, and the same parasite species may cause different forms of disease. Leishmaniasis are prevalent on four continents, and the World Health Organization considers leishmaniasis to be among the major infectious diseases in the world. *Leishmania infantum* is the causative agent of infantile visceral leishmaniasis in the Mediterranean region. *Leishmania* causes several different diseases such as cutaneous leishmaniasis that is self-limiting, and Kala-azar. Due to importance of the disease there is a need for an effective Diagnosis.

*Leishmania infantum* is the etiologic cause of visceral leishmaniasis in Iran. Efficient vaccines and diagnosis methods are required to control leishmaniasis. 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. 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. The aim of this study is produce and optimize monoclonal antibodies against promastigotes forms of *L. infantum* antigen. There are millions of people throughout the world as well as in Iran suffering from Leishmaniasis. Antibodies have been used as therapeutics already in the late 19th century in the form of patient or animal derived sera to treat infectious diseases. With the invention in the 1970’s of the method to produce monoclonal antibodies (mAbs), the idea of a magic bullet, in particular against tumour specific antigens in addition to infectious diseases, started to take a strong hold.

Monoclonal Antibodies are highly beneficial tools for the diagnosis, biochemical and immunopathological identification of *Leishmania* parasites. Leishmaniasis is endemic in 88 countries. Amastigote forms of *Leishmania* are experts at exploiting host cell processes to establish infection. So far, control of these diseases by hygienic methods has not been successful. So treating the disease is the current solution to fight the disease.

The first step in treating the disease is to diagnose the disease early and differentiate it from other diseases. Although there are good practical methods for diagnosis, the sensitivity of these methods is different and some of them do not have high sensitivity and specificity.

Monoclonal antibodies are used in the identification of antigens of various microorganisms as a suitable tool for use in diagnostic, therapeutic and research tests. Monoclonal antibodies are key reagents used in the diagnosis of infectious and non-infectious diseases. 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.

Monoclonal antibodies (mAbs) are important for the identification of species determinants on promastigotes types of *Leishmania* antigens. These parasites have a relatively simple digenetic life cycle, existing in the sandfly vector as promastigotes and in suitable host macrophages as intracellular amastigotes.

Methodology

First, standard strains were cultured and promastigote antigens of *L. infantum* were obtained.

Promastigote culture: A standard Iranian strain of *Leishmania infantum* (MHOM/IR/04/IP-IUN10) (obtained from Pasteure Institute of Iran) 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, Frankfurt, Germany) supplemented with fetal bovine serum (FBS) 10% (Biosera, South America), L-glutamine (2 mM) 1% (Gibco), antibiotics penicillin (100u/ml) and streptomycin (100μg/ml) 1% (Merck, Germany).
Germany). They were incubated in 24°C to reach appropriate concentration. After that, the cultured promastigotes were used to obtain amastigotes antigens.

**Preparation of *L. infantum* antigens:** Harvested amastigotes were counted (4×10⁹ parasite cells) and their antigens were extracted using the freeze (five times at liquid nitrogen) and thaw at 37°C. For preparation of antigens, different dilutions were prepared in several vials. promastigotes antigens were collected and stored at -70°C until use. Since then, BALB/c mice were immunized and antibody titers were determined.

**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 *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 intravenously through the tail.

**Cell fusion**

Sp2/0-Ag14 cells (IBRC C10106) were used for fusion and cultured in special mediums. 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), 2% (Sigma, St. Louis, Missouri, USA) L-glutamine (2 mM), 20% FBS, 5% CO₂, 1% penicillin (100 U/ml) with streptomycin (100μg/ml) and were incubated at 37°C. After 1 week HAT, medium were replaced with HT (Hypoxanthine and thymidine) medium (Sigma). 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.

**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 asoxalate, pyruvate, and insulin (OPI), (Merck) growth factor. Consequently, mAb producing monoclones 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 2×10⁶ 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 h and centrifuged at (4000×g, 20 min). Supernatant was collected and stored at -70°C until use.

**Preparation of cell culture supernatant**

SP2/0 and peritoneum macrophage cells were separately cultured in complete culture medium containing RPMI-1640 (Gibco Co. UK), penicillin (100U/ml) and streptomycin (100μg/ml). This was conducted in the presence of FBS 10% (Gibco Co. UK) and 5% CO₂ in the incubator at 37°C. After proliferation and covering much parts of the flask by cells, the supernatant was discarded and incubated cells were washed twice using centrifuge (8 min, 800 rpm). After 24 h, cells were obtained from the stationary phase and centrifuged for 8 min with 3000 rpm. Subsequently, supernatant was harvested and filtered using 22 μm filters.

**Comparison of the effects of cell supernatant and growth factors on hybridoma**

Before using the supernatants of sp2/0 and peritoneum macrophage cells, the addition amount to complete medium was analyzed and optimized. The content of the sixth fusion hybridoma was divided between 21 plates and 2.5×10⁵ ml cells were positioned in one well of 6-well plates. In the first three plates, complete culture medium containing 10% of sp2/0 cells supernatant was added. In the second set of three plates, complete culture medium containing 10% peritoneum
macrophage cell supernatant was added. In the third three plates, supernatant of SP2/0 and mice peritoneum macrophage cell culture (SSMCC) were added to the complete culture medium equally (10%). In the fourth three plates, only 1% sodium pyruvate was added to the complete culture medium as growth factors. In the fifth three plates, only feeder layer (2000 cells/well) was used and in the sixth three plates, OPI was applied as a growth factor. Finally, three plates that only contained complete culture medium were used as controls. All plates were incubated in an incubator with 5% CO₂, 37˚C temperature and 90% humidity. After 1 week, cells in wells were counted and their average number was calculated.

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.

The mice were vaccinated with the L. infantum antigen and their antibody titers were determined by the ELISA method. Spleen cells of the most immune mouse were fused with SP2/0 in the presence of Poly Ethylene Glycol. The effect of supernatant of SP2/0 and mice peritoneum macrophage cells culture on hybridoma cell proliferation was studied.

Results

Among the 12 fusion, a total of 26 monoclonal were positive.12 of which had acceptable optical absorbance in OD 450 nm. Finally, 4 clones, designated as 8D2 FVI6, 8D2 FVI3, 6G2 FV4 and 6G2 FV3. From these hybrids, anti-promastigotes L. infantum monoclonal antibodies were obtained. SSMCC was shown to play a key role in hybridoma proliferation and of mAb production. It seemed that SSMCC is rich of growth factors.

Conclusion

Since 1975, when Kohler and Milestein discovered monoclonal antibodies, rapid advances in hybridoma technology and the application of monoclonal antibodies have been made. Pan and McMahon-Pratt also developed monoclonal antibodies against Leishmania pipanoi amastigotes in 1988. In 2004, Froes and colleagues developed monoclonal antibodies against Leishmania shagasi amastigotes.

To date, monoclonal antibodies specific to the species of Leishmania mexicana, Brasiliensis and Tropica complex and Donovani have been produced for use in the immune diagnosis and taxonomic classification of Leishmania species. According to various studies on antigens of different species of Leishmania, little information is available about Leishmania infantum.

However, according to the information available in the network, no specific monoclonal antibody against the pro-mastigote form of Leishmania infantum has been prepared in Iran.

Therefore, preparation of a specific monoclonal antibody against the promastigote form of Leishmania infantum of Iranian strain seems to be very necessary. In this study, 12 monoclonal had the desired antibody. Compared to the work of other researchers, our results differ in terms of the number of monoclonal obtained, and this significant difference can be due to the use of our new applied strategies as well as its breadth. Methods the development of monoclonal antibodies against Leishmania infantum promastigotes is important in that it is useful in determining the species of Leishmania, its direct diagnosis in the wound. In cell fusion, one of the most essential factors for hybrid production is the presence of a strong growth factor, in the absence of which hybridoma production is difficult. Hybridoma cells usually grow better in a rich culture medium. Growth factors that are imported from abroad, such as OPI and sodium pyruvate, are firstly very expensive and secondly, they are difficult and time consuming to obtain from abroad. To make monoclonal antibodies cost-effective, biological growth factors can be substituted for chemical growth factors. The use of this material as a growth factor is low cost, easy and practical method and secure.

It seems in the near future, this SCCSM can be used as a growth factor for cancerous and non-cancerous cells in research centers at a wider level.

Acknowledgment

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**Conflict of Interest:** Ezzat Nourizadeh (Responsible Author) hereby undertake not to submit this article to any other publication at the same time and assign the copyright to this publication.
به‌پهنه سازی شیاری رشد هپسیرده‌های مولد آنتی‌بادی مونوکلونال علیه آنتی‌زن لیشمیانا اینفنتوم

عزنوروزاده

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چکیده

همانند محققان، هپسیرده‌های لیشمیانا اینفنتوم عامل اتیپولیزیک لیشمیانا احتیاج دارند. این ایون‌های موتوکلونال بشری برای تشخیص درمان و تبعیض ویژگی‌های انتانی اکلا مورد استفاده قرار گرفته‌اند. هدف از این مطالعه، بهبودی سازی شیاری رشد هپسیرده‌های مولد این بادی موتوکلونال علیه آنتی‌زن لیشمیانا اینفنتوم است.

مواد و روش‌ها: موش‌ها توسط آنتی‌زن لیشمیانا اینفنتوم واپسین شده و تیر نتیجه‌گیری آن توسط روش‌های تولید و هپسیرده‌ی محصوله، ایمنی و فعالیت موش‌های دسته‌بندی و تونیمن‌های دودکننده با سولهای SP2/0 در حضور پی‌این‌گیل کلیک ادام شده. تأیید سپرورفتان سولهای SP2/0 و سولهای ماکروفلاز SP2/0 نشان داد که مایع روبی (سولهای SP2/0 و بیماری ژن‌های اینفانتومی) یک کلیدی در تکنیک هپسیرده و تولید سولهای اینفانتوم موتوکلونال دارد که پیوست ژن‌های هپسیرده رشد است.

نتیجه‌گیری: بنظر می‌رسد در آن‌ها نزدیکی باعث رشد بی‌کنترل در سلول‌های سرطانی و غیرسرطانی در مراکز تحقیقاتی در سطح ویسکر مورد استفاده قرار گیرد.

کلیدواژه‌ها: آنتی‌بادی موتوکلونال، لیشمیانا، اینفنتوم، سلول‌های کلییدی، فعالیت سلول‌های سرطانی

مقدمه

لیشمیانا اینفنتوم توسط نیش پشته خالک جنس Lutzomyia گرفته شده است. این بیماری در 89 کشور جهان که میلیون‌ها نفر در معرض خطر عفونت هستند و تأثیر این بیماری عمدتاً بر جمعیت فقیر در سطح جهان است. 1[ ] این بیماری در چین، اسیای میانه، خاورمیانه، آفریقا، آمریکای جنوبی، بیماری خطرناکی است که از طریق جویانه به انسان منتقل می‌شود.[3]

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پیک بیمار در ایران در این بررسی در محیط‌های کشت در میکروگرم و برای ایمپورتاژیون موش‌ها استفاده شد. این تحقیق از بکارگیری با بکارگیری دانهگاه محقق اردبیلی انجام گرفت.[7]

2.2 کشت سویه‌های استاندارد و تهیه آنتی‌زن: برای کشت سویه‌های لیپوسامیک اینفنتوم نخست از محیط کشت دو فاژی (پی) نمونه و RPMI 1640 L-glutamine (100 μg/ml) انتخاب شد. این نمونه از اکلیل گاز تهیه نشده و انتهای آنها توسط روش انحصار شد.

2.3 روش تهیه آنتی‌زن: سوله با کشت داده شده اکسل پس از اکلیل در نرم‌زیبی، شیوه ای با کاهش شکل در این استفاده در محیط کشت نمونه و در آزمایشات آزمایشگاهی به کار رفته است.

3 کشت بیماری‌ها افزاش در جهان در کشورهای کلیمی، اپوپی، کنگو، کامبیزیا، و سودان در سال‌های 1998 میلادی تا 2016 در جریان افراشته‌ای از طبیعت. لیپوسامیک از مهم‌ترین بیماری‌های واقعی است که در سراسر جهان به‌جایه در کشور ایران در سطح بالایی افراد در این است. لیپوسامیک برای کشیدن چشمه‌های انجام شده هنوز یکی از مشکلات پدیده‌های جانوری و محققان می‌باشد.

نتایج تحقیقات سازمان بهداشت جهانی نشان می‌دهد بیماری لیپوسامیک افراشته در جهان در کشورهای کلیمی، اپوپی، کنگو، کامبیزیا، و سودان در سال‌های 1998 میلادی تا 2016 در جریان افراشته‌ای از طبیعت. لیپوسامیک از مهم‌ترین بیماری‌های واقعی است که در سراسر جهان به‌جایه در کشور ایران در سطح بالایی افراد در این است. لیپوسامیک برای کشیدن چشمه‌های انجام شده هنوز یکی از مشکلات پدیده‌های جانوری و محققان می‌باشد.
**RPMI1640**

### Experimental Protocol

1. **Materials**
   - RPMI1640
   - 

2. **Methods**
   - Preparation of Materials
   - Culture Setup
   - Analysis

3. **Results**
   - Data Interpretation
   - Conclusion

4. **Discussion**
   - Implications
   - Future Directions

5. **Acknowledgments**

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**References**

- [1] Reference 1
- [2] Reference 2
- [3] Reference 3

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**Figure Legends**

- [A] Figure A
- [B] Figure B
- [C] Figure C

---

**Appendix**

- Additional Information
- Supplementary Data

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**Author Contributions**

- [Name 1]
- [Name 2]
- [Name 3]

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**Ethical Approval**

- IRB Approval
- Animal Welfare

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**ORCID iD**

- [ID 1]
- [ID 2]
- [ID 3]
از جاک‌هایی 68 خانه به 26 خانه منطقی شدند و به تدریج کمک‌های یافتن و به قدری که در میان جمعیت در حیات مخرب می‌گوردند تعدادی از سلول‌های انرخ بنا شده در حیات مخرب از سلول‌های و در از ماپراهای استفاده در آزمایش‌های بعدی تکه‌داری شدند.

9.2. روش‌های محاسباتی آماری: برای جزئی‌تر و تحلیل داده‌ها از نرم‌افزار آماری SPSS با نسخه 18 و از آزمون نی تست استفاده شد.

2.1. مقصود آزمایش: تأیید تعدادی از سلول‌های مادری SP2/0 و ماکروفاژ مصنوعی SP2/0 به صورت متناسباتی به سرما به روش راکت‌های سرد و در حیات مخرب از سلول‌های و در از ماپراهای استفاده در امکانات‌های بعدی تکه‌داری شدند.

2.2.2. انجام برای رتبه‌بندی سلول‌های مادری: Limiting dilution

3.1.1. اینستور: سلول‌های تیتراسیون سرم و سلول‌های مادری به صورت راکت‌های سرد و در حیات مخرب از سلول‌های و در از ماپراهای استفاده در امکانات‌های بعدی تکه‌داری شدند.

3.2. تکثیر سلول‌های هیبریدوما: سلول‌های مادری پورین‌دار
2.3 انتخاب هیبریدومهای مولد آنتی‌بادی
شدید: هپتامیکتیکا اینفانتوم: از بین هیبریدهای در حال رشد ۱۶ هیبرید که با آنتی‌ژن پروماستیگوت نیشما در اینفانتوم واکنش مثبت قوی نشان داده پودند و عبار آنتی‌بادی Limiting dilution بالاتری را داشتند انتخب شدند و توسط بررسی شدند سزاری که هورم موردن با آزمون ایا موردر بررسی ۴G1 ۴B4 F III ۱A4 F III ۴C9 FIV ۱D6 FIV ۸E4 F III ۸F4 FV ۶G2 FVI ۶G2 FV ۸E6 FV ۸E4 F III ۸D2 FVI ۸G8 FV ۸G8 FV ۷F6 FIV ۶G2 FV ۸E6 FV ۸E6 FV ۸E4 FIV ۶C4 FIV ۸E6 FV ۶G2 FV ۶C4 FIV ۸D2 FVI ۸G8 FV ۷F6 FIV ۶G2 FV

جدول ۱: هیپرایهای مثبت حاصل از چهار هیپرایه که بالاترین تیتر آنتی‌بادی (Ab) را دارند

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که در هر چاهک یک با نیم کلون قرار گرفت و در پلیت‌های حاوی محفظه کشت با بستر Feeder layer تک کلون‌های تولیدکننده آنتی‌بادی آماده‌سازی شدند. به‌این‌نوع کلون‌های تولیدکننده آنتی‌بادی مذکور مجاز شدند. جدول ۲ نتایج تک‌تک هیبریدوما برای جداسازی تک کلون‌های تولیدکننده آنتی‌بادی توسط روش حد نهایی وقت داشتند. نتایج نشان می‌دهد.

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<tr>
<td>8D2 FV1</td>
<td>6G2 FV6</td>
<td></td>
</tr>
<tr>
<td>8D2 FV2</td>
<td>6G2 FV7</td>
<td></td>
</tr>
<tr>
<td>8D2 FV3</td>
<td>6G2 FV8</td>
<td></td>
</tr>
<tr>
<td>8D2 FV4</td>
<td>6G2 FV9</td>
<td></td>
</tr>
<tr>
<td>8D2 FV5</td>
<td>6G2 FV10</td>
<td></td>
</tr>
</tbody>
</table>

نتایج مقایسه تأثیر مایع روپی کشت سلول‌های (0/2) مادرگروه‌های G3 و sp2/0، و سلول‌های ماکروفاژی از نظر تأثیر با فاکتور رشد OPI قابل مقایسه هستند. و مادر گروه‌های G6 و G9 از هم‌های بیشتر ایست؛ این نشانه‌هایان آن است که مایع روپی (سلول‌های 0/2 و سلول‌های ماکروفاژی) از نظر لایه خاکسار (G5) و OPI (G6) می‌تواند کاست کامل (کنترل بدون فاکتور رشد) (G7) می‌باشد سلول‌های ماکروفاژی (SP2/0) در هر گروه ۳ پلیت خاکسازی کنترل ۲۰۰۰ سلول/کل (حل) در هر چاهک ۱۵۰ میکرومتر تیتر بستر داشتند. در هر چاهک ۵۰ سلول سلول‌های ماکروفاژی (SP2/0) با فاکتورهای مختلف رشد بر روی سلول‌های هیبریدوما با مایع روش ۲ مایع روپی سلول‌های ماکروفاژی (SP2/0) با فاکتورهای مختلف رشد بر روی سلول‌های هیبریدوما ۱ Limiting dilution
References


