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Original Article

Production and Partial Characterization of Monoclonal Antibodies to Leishmania

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Abstract

Background: Monoclonal antibody technology allows identification of amastigote-specific antigen in human tissue biopsies and search for a suitable vaccine candidate, which may induce a long lasting immunity.

Objectives: Production and partial characterization of seven monoclonal antibodies against *Leishmania tropica* promstigotes.

Methods: Seven murine monoclonal antibodies of the IgG isotype were produced against the stationary stage promastigotes of *Leishmania tropica*.

Results: Extensive cross reactivity was found within 4 reference strains and isolates from the patients. Non-Leishmanial antigens were not recognized by any of the antibodies. One antibody recognized a diffuse band 64 to 86 kDa, and the other 6 recognized 1, 2, or more bands in immunoblotting of soluble antigens of promastigotes. The reaction of these 7 antibodies with the soluble extracts of 4 reference strains of Leishmania and 10 isolates from patients was studied.

Conclusion: Although these antibodies were not suitable for identification purposes, the characterization of shared epitopes may be considered in studying the antigenic structure of these organisms. Further experiments may confirm the idea that some of these epitopes are suitable vaccine candidates.
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Keywords • Leishmania • antibodies, monoclonal • antigen

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Introduction

Monoclonal antibodies have been used extensively in experiments designed for detection and characterization of Leishmania antigens.[1-4] These antigens may be involved in parasite invasion of host macrophages and establishment of immunity [5-7]

Identification of Leishmania isolates is considered as an important approach to parasite control and this has been made possible by employing monoclonal antibodies.[8,9,2] Stage-specific monoclonal antibodies have been used in experiments on parasite differentiation.[10]

Monoclonal antibody technology allows identification of amastigote-specific antigen in human tissue biopsies,[11] and search for a suitable vaccine candidate, which may induce a long lasting immunity.[12,13] This report describes the production of seven monoclonal antibodies against *Leishmania tropica* promstigotes. We studied the reactions of these antibodies with soluble extracts prepared from different strains of Leishmania. Western bolt analysis of antigens indicated the presence of epitopes shared by multiple components.

Materials and Methods

Parasites

Four reference strains of Leishmania; *L. tropica* k-27, *L. infantum* IPTI, *L. donovani* DD8, and *L. major* 5ASKH strains were kindly provided by Dr. Evans (London School of Tropical Medicine and Hygiene), and preserved in liquid nitrogen. Ten *Leishmania spp.* were isolated from skin lesions of patients with cutaneous leishmaniasis in the Department of Parasitology of Shiraz University of Medical Sciences, Shiraz, Iran.

All the organisms were propagated in RPMI-1640 supplemented with 15-20% fetal calf serum.

L. tropica K-27 was used as the source of antigen for immunization of BALB/c mice.

Preparation of the antigens

Washed stationary phase promastigotes were processed in three cycles of freezing and thawing. The suspension was centrifuged at 5000 g for 20 minutes. The supernatant was stored in liquid nitrogen as soluble antigens of Leishmania.

Hybridoma production

Spleen cells were isolated from BALB/C mice immunized by intraperitoneal injection of 10^8 promastigotes in complete Freund's adjuvant. Immunization was completed by a booster injection of 10^8 promastigotes three weeks later, and a final injection of 10^8 promastigotes five days before fusion. Hybridomas were produced by fusion of spleen cells with the myeloma cell line P3-C63-Ag8.655 by polyethylene glycol according to the method described by Harlow.[14]

Hybridomas were selected in HAT-RPMI medium (Gibco), 10% FCS. Antibody secreting cells were cloned using limiting dilution method. Culture supernatants of the cloned cells were collected as the source of monoclonal antibody.

Seven cloned hybridomas were selected for further studies. The results of immunoblotting suggested that they reacted to distinct epitopes and were not identical. Isotypes were determined by sandwich ELISA method using the commercial Iso-2 kit.

ELISA method

ELISA plates (Nunc, Denmark) were coated with 100 µl of soluble antigens of Leishmania at the concentration of 2.5×10^6 promastigotes buffer (0.05% Tween 20 in PBS). Plates were blocked in 2.5% casein, 0.05% Tween 20 in PBS for 60 minutes.

Supernatant of the hybridoma culture fluids were collected and added to each well without dilution.

Plates were incubated for two hours at 37 °C, washed three times and horse raddish peroxidase (HRP) conjugated second antibody (Goat-anti-mouse, Sigma) added to each well, and incubated for 45 minutes. Plates were washed and 100 µl of substrate (5 mg. of orthophenylene diamine (OPD), 5 µl of H_2O_2 and 10 ml of PBS) was added to each well. After ten minutes of incubation in the dark, the reaction was stopped by 25 µl. of 12.5% H_2SO_4.

The results were read and recorded by ELISA reader Tittertech plus-MS_2. The ratio of the optical density of the negative control was determined as R-value. R-values ≥3 were considered as positive.

Western blot analysis

Antigens were separated by SDS-PAGE according to the method described by Harlow,[14] and transferred to nitrocellulose sheets by electrophoresis according to the procedure described by Maizles.[15]

The sheets were cut into strips and quenched with 0.05% Tween 20, 2.5% BSA for 60 minutes. The strips were incubated in culture supernatants containing monoclonal antibodies for two hours at 25 °C, washed three times in PBS-Tween followed by a single wash in PBS.

Substrate (10 ml of 50 mM Tris; pH=7.6, 5 mg DAB 5 µl H_2O_2) was added and incubated for 10 minutes in the dark. The reaction was stopped by rinsing the strips in distilled water.

Reaction of antibodies with non-leishmanial antigens

Soluble antigens from crithidia, mycobacterium (BCG), and *Escherichia coli* were tested for any possible reaction with monoclonal antibodies using ELISA.

The principles of the method are described above. These antigens were not recognized by any of the monoclonal antibodies.

Results

Hybridomas were selected by the reaction of their supernatant with the soluble antigens prepared from promastigotes of *L. tropica* K-27 by ELISA method. Further experiments were performed to study the

Table 1: Reactivity of monoclonal antibodies with soluble promastigote antigens from different strains of Leishmania in ELISA

	Leishmania strain			
Monoclonal antibody	**K-27**	**5ASKH**	**DD8**	**IPTI**
9A9	6.0	6.5	5.5	6.7
9H7	6.8	7.0	7.1	6.9
8C11	8.0	8.2	8.6	8.5
8B11	7.8	8.0	8.0	7.8
9A2	7.8	8.0	7.9	7.5
9F1	10.0	9.0	9.9	10.0
8A5	10.0	9.8	10.1	9.5

R-value is the ratio of OD of the test sample/OD of negative control.

Table 2: Reactivity of monoclonal antibodyi es with soluble promastigote antigens from 10 isolates of Leishmania in ELISA

	R value	
Monoclonal antibody	**Range**	**Average**
9A9	8.2-14.0	10.7
9H7	2.0-7.0	3.7
8C11	11.0-16.2	13.9
8B11	6.8-12.2	13.9
9A2	10.5-13.7	11.7
9F1	9.0-15.3	12.8
8A5	12.8-17.5	14.7

R value is the ratio of OD of the test sample/OD of negative control.

interaction of these antibodies with Leishmania antigens.

The reactions of seven monoclonal antibodies with soluble extracts of four strains of Leishmania are shown in Table 1. As the results indicated all monoclonal antibodies reacted with antigens prepared from promastigotes of four reference strains of Leishmania in the ELISA method. Table 2 represents the reactions of seven monoclonal antibodies with soluble antigens of promastigotes of ten isolates of Leishmania. Antibody 9H7 did not react strongly with antigens prepared from some of the Leishmania promastigotes tested (number 11, 13, 20 and 32).

Antigen location was determined using each antibody on promastigotes from different phases of growth. The results of immunoperoxidase staining indicate that the pattern of labeling does not vary with different phases of growth. Three patterns of enzyme labeling are shown in Figures 1, 2, and 3.

The results of immunoblotting indicated that one antibody recognized a diffuse broad band. Antibody 9H7 recognized a single band. The other five antibodies recognized more than one band (Fig. 4). This may suggest the presence of shared epitopes on different antigens of Leishmania organisms. Degradation of the antigen into fragments in the process of antigen preparation is also possible.

Discussion

The results of this study indicate that extensive cross-reactivity exists between the antigens of Leishmania isolates. Six out of seven antibodies reacted with all Leishmanias studied. One antibody, 9H7, reacted only weakly with Leishmania isolates number 11, 13, 20 and 32. This antibody reacted with a 50 kDa antigen presenting as a weakly labeled band. Antibody 9A2 recognized epitopes on antigens with molecular weights between 64 and 89 kDa. The results of other investigations indicate that glycolipids show a characteristic diffuse migration in SDS-PAGE,[16-18] that is consistent with the migration

Figure 1. Immunoperoxidase labeling of *Leishmania* promastigotes from different *in vitro* growth phases with monoclonal antibodies 9A9, 8B11, and 8A5.

Figure 2. Immunoperoxidase labeling of *Lesihmania* promastigotes from different *in vitro* growth phases with monoclonal antibody 9A2.

Figure 3. Immunoperoxidase labeling of *Leishmania* promastigotes from different *in vitro* growth phases with monoclonal antibodies 8C11 and 9F1.

pattern of the antigens detected by monoclonal antibody 9A2. Antibody 9F1 recognized an epitope present on proteins with molecular weights of 46, 63, 69 and 83 kDa. Antibody 8B11 recognizes the epitope on multiple components corresponding to the molecular weights of 38, 41, 46 and 83 kDa. The epitope that is recognized by antibody 9A9 is detected on components with molecular weights of 41, 42, 44 and 51 kDa. Antibody 8C11 reacts with an epitope shared between antigens of 40, 46, and 48.9 kDa. Shared epitopes of different antigens of Leishmania have been reported.[19]

The significance of common epitopes on different antigens is not clear in this stage. It is reasonable to conclude that different antigens may have shared epitopes and that two or more monoclonal antibodies recognize a single antigen. Monoclonal antibodies 9H7 and 8C11 both recognize epitopes on a 48.9 kDa antigen, but the epitope recognized by 8C11 also exists on antigens with molecular weight of 40 and 46 kDa. This indicates that the epitopes recognized by these two antibodies are not identical. Although existence of these shared epitopes and cross-reactivity may not be useful for typing Leishmania isolates, these antibodies may be useful in experiments on identification of shared epitopes as potential candidate antigens for vaccine development or suitable targets for chemotherapeutic agents.

These antibodies may also provide help for the affinity purification of antigens.

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Figure 4. Western bolt analysis of soluble antigens of *Leishmania* promastigotes: soluble antigens from promastigotes sperated on SDS-PAGE (Top), transferred to nitrocellulose paper and probed with monoclonal antibodies (Bottom). Monoclonal antibodies: Lane 1: Anit-gp63; lane 2: 9F1; lane 3: 9A2; lane 4: 8B11; lane 5: 9A9; lane 6: 9H7; lane 7: 8C11; lane 8: negative control.

References

1 de Ibarra DE, Howard JD, Snary D: Monoclonal antibodies to *Leishmania tropica major,* Specificities and antigen location. *Parasitology 1982;***85:** 523-31.

2 Jaffe CL, Bennett F, Grialdi JRG, et al: Production and characterization of species-specific monoclonal antibodies against *Leishmania donovani* for immunodiagnosis. *J Immunol 1984;* **131:** 440-7.

3 Jaffe CL, Surfstein R: Species-specific antibodies to *Leishmania tropica* (minor) recognized somatic antigens and exometabolites. *J Immunol 1987;***139(4):**1310-19.

4 Schnur LF, Sarfstein R, Jaffe CL: Monoclonal antibodies against Leishmania membranes react

with specific excreted factor (EF). *Ann Trop Med Parasitol 1990;***84:**447-56.

5 Ali N, Afrin F: Protection of mice against visceral Leishmania by immunization with promastigote antigen incorporated into liposomes. *J Parasitol 1997;***85:**70-5.

6 Infante RB, Perez M, Di Prisco-Fuenmayor MC, et al: Determination of phagocytosis of *Leishmania spp*. by human polymorphonuclear leukocytes using anti-leishmania monoclonal antibodies. *J Invest Allergol Clin Immunol 1991;***1:**209-12.

7 Rachamin N, Jaffe CL: Pure protein from *Leishmania donovani* protects mice against both cutaneous and visceral leishmaniasis. *J Immunol 1993*;**150:**2322-31.

8 Ardehali S, et al: Characterizarion of Leishmania isolated in Iran. 1. Serotyping with species specific monoclonal antibodies. *Acta Trop 2000;***75:** 301-7.

9 Catolara SB et al: Use of monoclonal antibodies for the identification of *Leishmania spp.* isolated from humans and wild rodents in state of Capeche, Mexico. *Mem Inst Oswaldo Cruz 1999;* **94:**305-9.

10 Jaffe CL, Rechamin N: Amastigote stage-specific monoclonal antibodies against *Leishmania major*. *Infect Immun 1989;* **57:**3770-7.

11 Kenner JR, et al: Immunohistochemistry to identify Leishmania parasites in fixed tissues. *J Cutan Pathol 1999;***26:**130-6.

12 Kemp M et al: Production of interferon- gamma and interleukin-4 by human T cells recognizing Leishmania lipophosphoglycan-associated protein. *Immunol Lett 1993;***38:**137-44.

13 Yang DM, Rogers MV, Liew FY: Identification and characterization of host-protective T-cell epitopes of a major surface glycoprotein (Gp63) from *Leishmania major*. *Immunology 1991;***72:**3-9.

14 Harlow ED, Lane D: Antibodies: A laboratory manual. First ed. Cold Spring Harbour. **1988:** 471-510.

15 Marizels RM: *Parasite Antigens Parasite Genes*. Cambridge University Press. Cambridge. **1991:** 54-7.

16 Glazer TA, et al: An antigenically distinct lipophosphogolycan on amastigotes of *Leishmania major*. *Mol Biochem Parasitiol 1991;***45:**337-44.

17 Handman E, Greenblatt CL, Goding JW: An amphipathic sulphated glycoconjugate of Leishmania: characterization with monoclonal antibodies. *EMBO J 1984;***3:**2310-3.

18 Turco SJ, Wilkerson MA, Clawson DR: Expression of an unusual acidic glycoconjugate in *Leishmania donovani*. *J Biol Chem 1984;* **259:** 3883-6.

19 Maizels RM, et al: Shared carbohydrate epitopes on distinct surface and secreted antigens of the parasitic nematode *Toxocara canis*. *J Immunol 1987;***139:**207-14.