Production and Characterization of Murine Monoclonal Antibodies to Leishmania Gp63 Antigen

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ABSTRACT

Background: Production of monoclonal antibodies to *Leishmania* antigens assists the identification and characterization of these organisms. **Objective**: Production of monoclonal antibodies against epitopes on the gp63. **Methods**: Two murine monoclonal antibodies to gp63 were produced and characterized. The reactions of both antibodies with soluble leishmanial antigens, purified gp63 and truncated recombinant gp63 molecules were studied by an ELISA assay. These two antibodies reacted with the crude soluble antigens prepared from 4 reference strains of *Leishmania,* 10 isolates from the patients, purified gp63 and recombinant gp63 molecules. However, no reaction with several non-leishmanial antigens was observed. Reaction of both antibodies with the intact recombinant gp63 and truncated molecules were compared. **Results:** The results indicated that the two antibodies specifically recognize two different epitopes on the gp63 molecule. **Conclusion**: Possible applications of such antibodies in searching for immunogenic epitopes are discussed.

Key words: gp63, Leishmania , Monoclonal Antibody

INTRODUCTION

The surface glycoprotein (gp63) of *Leishmania*, with a molecular weight of 63 KDa is a zinc-dependent protease which is anchored to the membrane and has been purified in soluble form (1). The genes encoding the molecule are clustered on a 650 kb chromosome region (4,9). These genes are differentially expressed during different stages of the life cycle of *L. major* and *L. chagasi* (18,19). The molecule is expressed at a lower level in amastigotes and represents about 0.1% of the total amastigote proteins (15). The role of gp63 in promastigote multiplication and

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Table 1. Reactivity of 5C5 and 4C8 antibodies with soluble antigens of 4 reference strains of Leishmania in ELISA method.

Monoclonal Antibody	Strains of *Leishmania* R Values			
	K-27	5ASKH	IPTI	DD8
5C5	5.8	6.1	6.0	5.9
4C8	6.0	5.8	6.0	6.2

R value is defined as the ratio of OD of the test sample / OD of the negative control in ELISA method

survival in mammalian host and insect vector has been well established (14,20). In this regard, *L. amazonensis* gp63-deficient promastigotes exhibit low macrophage binding and intracellular survival (7,13). The gp63 molecule exerts proteolytic activity on C3b, which may help the organism to resist complement attack (11). A role for gp63 in enhancement of promastigotes interaction with murine and human macrophages has been suggested (2,5,12,14). Mice orally vaccinated with *Salmonella typhimurium* expressing gp63 are reported to be protected against experimental *L. major* infection (21). Experimental leishmaniasis in mice shows that certain gp63 derived peptides are good vaccine candidates that can induce protection both at the T and B cell levels (6). Strains of *Leishmania* vary widely in respect to the gp63 protein expression and gene copy number (16). The complete nucleotide sequence of the gp63 gene has been determined (3). Considering the highly conserved structure of gp63 among *Leishmania* parasites the possibility of generating monoclonal antibodies with the potential to detect shared antigen determinants was investigated. Production of such monoclonal antibodies to gp63 is reported in this study.

MATERIALS AND METHODS

Parasites. Four reference strains of *Leishmania* and 10 isolates from the patients were used in this study. *Leishmania tropica* (MHOM/SU/74/k27), *Leishmania donovani* (MHOM/IN/80/DD8), *Leishmania infantum* (MHOM/SU/80/IPT1), and *Leishmania major* (MHOM/SU/73/5ASKH) strains were kindly provided by Dr. David Evans (London School of Tropical Medicine and Hygiene), and preserved in liquid nitrogen. *Leishmania* species were cultured in RPMI medium supplemented with 20% fetal calf serum or maintained in RPMI 1640 containing 10% fetal calf

Table 2. Reactivity of 5C5 and 4C8 with crude extracts of Leishma in ELISA method.

Monoclonal antibodies	Isolate number R value									
	10	11	12	13	14	15	16	17	20	32
5C5	9.0	8.5	12	10	10.1	10	6.1	8.0	8.2	10
4C8	10	8.5	12	9.0	8.1	13.5	10	8.0	7.8	11

R value is defined as the ratio of the OD of the test sample / OD of the negative control in ELISA method.

Table 3. Reaction of monoclonal antibodies 5C5 and 4C8 with non-leishmanial antigens in ELISA method.

Antigen source	Monoclonal antibody	
	5C5	4C8
Leishmania soluble antigens	+	+
Escherichia coli soluble antigens	–	–
Mycobacterium bovis (BCG) soluble antigens	–	–
Crithidia luciliae soluble Antigens	–	–
Mycobacterium PPD	–	–

serum.

Purified natural gp63 (L. maDor and LD274) were kindly provided by Dr. Arsalan Kharazmi (Staten-Serum Institut Klinisk Microbiologisk afdeling regshospitalet, AFP 7808, Copenhagen, Denmark). The following gp63 samples were employed in this study:

a) Recombinant gp63 expressed in *E coli*; the gene encoding gp63 was cut with the restriction enzymes SmaI, NcoI, and SacI in independent experiments and used for expression of the truncated gp63 products designated as r.SmaI, r.NcoI and r.SacI respectively.

b) Recombinant intact gp63 expressed in *E coli.* All recombinant forms of gp63 were kindly provided by F. Mehboodi (Department of Biotechnology, Pasteur Institute, Iran).

Crude extract of *Leishmania* promastigotes designated as LSA (*Leishmania* Soluble Antigen) was prepared by three cycles of freeze and thawing of promastigotes in PBS buffer, followed by 15 minutes of centrifugation at 5000g. The supernatants containing LSA were stored in liquid nitrogen. Soluble antigens of *E coli, Mycobacterium bovis* (B.C.G.), and *Crithidia luciliae* were also prepared by the same method.

Hybridoma production. Spleen cells were isolated from BALB/c mice immunized by intra peritoneal injection of 10^8 promastigotes in complete freund adjuvant. Immunization was completed by a booster injection of 10^8 promastigotes 3 weeks later, and a final injection of 10^8 promastigotes 5 days before fusion. Hybridomas were produced by fusion of spleen cells with the myeloma cell line p3-x63-ag8.655 by polyethylene glycol according to the method described by Harlow and Lane(10).

Table 4. Reactivity of 5C5 and 4C8 antibodies with gp63 in ELISA method.

Type of gp63	monoclonal antibody R value	
	5C5	4C8
i.r	10.78	10.15
r.NcoI	4.83	0.85
r.Sacl	0.19	0.12
LmaDor	9.57	8.40
LD274	9.30	4.16
LSA	2.94	3.82

R value is defined as the ratio of the OD of the test sample / OD of the negative control in ELISA method.

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

ELISA method. ELISA plates (Nunc, Denmark) were coated with 100 µl of the crude extract of *L. tropica* (k-27), incubated overnight at 25°C, washed three times in washing buffer (0.05% Tween 20 in PBS), and blocked in PBS- casein 2.5% containing 0.05% Tween 20 for 60 minutes. Hybridoma supernatants were added after washing the wells and incubated for a further 60 minutes. Plates were washed as above and HRP-labeled second antibody (Goat anti-mouse, Sigma) was added to each well. Plates were incubated for 45 minutes, washed, and 100 µl of substrate solution (5mg OPD and 5µl H2O2 in 10 ml PBS) was added to each well. After 10 minutes incubation in the dark, the reaction was stopped by adding 25µl of 12.5% H2SO4. The results were read and recorded by ELISA reader Titertech plus-MS2.

RESULTS

Two monoclonal antibodies 5C5 and 4C8 were raised to gp63. The isotypes of 5C5 and 4C8 antibodies were IgM and IgG1, respectively. The reaction patterns of these antibodies with soluble antigens are shown in tables 1 and 2. The results indicated that 5C5 and 4C8 antibodies recognized determinants present on the tested *Leishmania* extracts. The reaction patterns, which are summarized in table 3, indicated that these two antibodies were highly specific for leishmanial antigens, and did not react with any of the non-leishmanial antigen preparations. The reaction patterns of 5C5 and 4C8 monoclonal antibodies with the purified, intact recombinant, and truncated recombinant forms of gp63 in the ELISA assay are demonstrated in table 4. As it is shown, both antibodies recognized recombinant gp63. In addition, ion exchanged purified gp63 strongly reacted with 5C5 and 4C8 monoclonal antibodies. The two antibodies reacted with epitopes on r.SmaI. However, r.NcoI truncated antigen was only recognized by 5C5. The r.SacI lacked both epitopes and was not recognized by either of the monoclonal antibodies.

DISCUSSION

Glycoprotein 63 (gp63) is located over the entire surface of the *Leishmania* parasites and it is suggested that this glycoprotein plays a role in the initiation of infection and virulence of the organism (16). The antigenic determinants are preferentially located on the most divergent regions of the molecule and gp63 may act as a potent B cell immunogen during natural infection (8). Peptides derived from gp63 have been shown to be recognized by T-cells of the CBA mice recovered from *L. major* infection and play a protective role in mouse model of leishmaniasis (17). It has long been accepted that T-cell derived cytokines determine the outcome of *L. major* infection and the role of the antigen itself as a factor for determining the Th1/Th2 responses has been studied in some details. The amino acid sequence of the antigen

may influence the resulting primary response in vivo and the identification of such epitopes may lead to the development of a suitable vaccine inducing the Th1 response. In this study the reactions of two monoclonal antibodies with different forms of gp63 were studied. Considering the DNA nucleotide sequence of gp63 gene (3), the predicted cleavage site for the restriction enzyme NcoI is located to the 3′ end of Asp 357 codon. The proposed cleavage site for the restriction enzyme SmaI is in the 3′ position of the His 429 codon. Restriction enzyme SacI cleaves the gene after the Ala 343 codon.

The results of the reactions of both monoclonal antibodies with the native and truncated forms of gp63 are given in table 4. As it can be deduced, these antibodies react with two perhaps overlapping peptide epitopes located between Ala 343 and His 429. However, the two epitopes seem to be entirely different in structure and location on gp63 molecule.

The reaction patterns of two monoclonal antibodies with the soluble extracts varied between 4 reference strains and 10 isolates from patients. The observed variation in the R values may reflect the variation between strains of *Leishmania* in the copy number of gp63 gene, or in the amount of protein being expressed (6). We suggest that these monoclonal antibodies, raised against two highly immunogenic epitopes, may be useful in characterization of the epitopes and further studies on the host-parasite interactions.

In a preliminary study of the efficiency of 4C8 and 5C5 antibodies, a capture ELISA assay was designed in which ELISA plates were coated with 4C8 or 5C5 antibodies to capture purified form of gp63. The system was employed to evaluate the presence of anti gp63 antibodies in the sera of 13 patients with chronic cutaneous leishmaniasis. All sera were positive for anti- gp63 when gp63 was captured by 4C8 or 5C5 antibodies. Anti gp63 was not detected in the control (normal) sera. The results indicated that such monoclonal antibodies may be employed in the development of sensitive and specific methods or detection of serum antibodies in human leishmaniasis (unpublished data).

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