

STAGE-SPECIFIC AND COMMON ANTIGENS OF *BRUGIA MALAYI* IDENTIFIED WITH MONOCLONAL ANTIBODIES¹

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To identify parasite antigens that trigger protective, pathogenic, and allergic immune responses during filarial infections, we generated a series of monoclonal antibodies to infective larvae, adult worms, and microfilariae of *Brugia malayi*, a human pathogen. Quantitative and qualitative analysis of the reaction patterns of these monoclonal antibodies indicates the existence of stage-specific antigens of *B. malayi*, as well as of antigens shared by different stages of this parasite and by other related and unrelated helminths. These antibodies should provide invaluable tools for the analysis of host-parasite interactions in filariasis at the molecular level.

Immune responses to filarial nematodes play a major role in the pathogenesis of filarial disease syndromes and contribute to the clearance of microfilariae from the circulation (1–3). Filarial helminths also are potent activators of suppressor mechanisms of host immunity, and stimulate hyperproduction of parasite-specific and non-specific IgE (4, 5). The precise nature and identity of parasite antigens that elicit this wide range of immune reactions during filarial infections remains largely unknown.

Efforts to characterize antigens of the multiple stages of *Brugia malayi*, a parasite of man, have been hampered not only by practical difficulties in obtaining sufficient numbers of these worms in various stages of development, but also by the necessity to rely on sera from infected or immunized donors for the identification of filarial antigens. The latter contain antibodies that react with multiple antigenic determinants, some of which are present in related filarial species, as well as in other nematodes (6).

To remedy some of these problems, we developed a large number of monospecific, monoclonal antibodies to antigens of three stages of *B. malayi* present in man:

infective third stage larvae (L3),⁴ adult worms (AW), and microfilariae (MF). We report here the initial characterization of apparently stage-specific as well as of cross-reactive antigens identified by selected monoclonal antibodies.

MATERIALS AND METHODS

Isolation of L3, AW, and MF of *B. malayi*. L3 of *B. malayi* were obtained by mass-dissection of *Aedes aegypti* 12 to 14 days after the mosquitoes had been fed on microfilaremic jirds as described by Ash and Riley (7). AW and MF of *B. malayi* were harvested from peritoneal cavities of infected jirds (8). AW were hand-picked from the peritoneal washings and were segregated by sex. MF were freed from contaminating host cells by a two-step procedure described previously (9).

Preparation of antigenic extracts. AW, MF, and L3 were washed extensively in phosphate-buffered saline (PBS, pH 7.4), lyophilized, and extracted with constant agitation in PBS. Extracts were centrifuged at 20,000 × G for 20 min; the resulting supernatants were stored at –20°C until use. The same procedure was used to prepare antigens from adult *Dirofilaria immitis* (DI) obtained from infected dogs. The protein content of the extracts was measured by the method of Lowry *et al.* (10). Antigenic extracts to be used in transblotting experiments were similarly prepared by dissolving lyophilized worms with 1% sodium dodecyl sulfate (SDS).

Immunization procedures. Six-wk-old BALB/c mice (the Jackson Laboratories, Bar Harbor, ME) were immunized by two i.p. injections 4 wk apart with either 10⁵ MF, 30 L3, or six adult male *B. malayi*. Four days before a scheduled fusion, donor mice were boosted i.v. with 20 µg protein extracted from the same parasite stage as that used in prior immunizations.

Generation of monoclonal antibodies to filarial antigens. The method of Kennett (11) was used to generate hybrids of spleen cells from sensitized mice and from the P3-NSI/AG 4/1 plasmacytoma line. Antibody-producing hybrids were cloned by limiting dilution on a feeder layer of irradiated spleen cells and were grown to large numbers *in vitro* or as ascites tumors *in vivo*.

Radioimmunoassay (RIA) to detect antifilarial antibody. A solid-phase RIA was used to detect antifilarial antibody in ascites fluids or in spent culture supernatants (12). For the initial screening of hybrids, parasite material extracted from the life cycle stage of *B. malayi* to which the spleen cell donors had been sensitized was used as antigen. To determine the specificity of monoclonal antibodies produced, spent supernatants known to contain antibody to one stage of *B. malayi* were assayed for reactivity with extracts of the other two available stages of this parasite. In addition, selected monoclonal antibodies were tested for reactivity with serum from uninfected jirds, and with antigenic extracts of adult DI, muscle stage larvae of *Trichinella spiralis* (a gift of Dr. A. Dessein, Harvard Medical School) or schistosomula of *Schistosoma mansoni* (a gift of Dr. D. Harn, Harvard Medical School).

The results of RIA are expressed as an index of RIA reactivity defined as the ratio of radioactivity retained in antigen-coated wells incubated with monoclonal antibody to that retained in similar wells incubated with irrelevant antibody. A ratio of ≥ 2.5 was considered a positive result.

Qualitative analysis of filarial antigens recognized by monoclonal antibodies. To identify the antigens recognized by selected monoclonal antibodies, we used an immunoperoxidase method to reveal antibody bound to parasite antigen immobilized on nitrocel-

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⁴ Abbreviations used in this paper: MF, microfilariae; AW, adult worms; L3, infective third stage larvae of *B. malayi*; DI, *Dirofilaria immitis*; RIA, radioimmunoassay.

lulose paper. In brief, antigenic extracts of *B. malayi* or DI were first resolved by SDS-polyacrylamide gel electrophoresis as described by Laemmli (13) and were then transferred electrophoretically onto nitrocellulose paper according to the method of Towbin *et al.* (14). Strips of these "Western blots" were incubated overnight at 4°C with ascites fluid containing monoclonal antibody (diluted 1/80 in PBS made to contain 0.5% bovine serum albumin and 0.3% Tween-20). Bound antibody was then visualized by the enzymatic reaction of 3,3'-diaminobenzidine with a mixture of peroxidase-conjugated anti-mouse IgG and IgM (Cappel Laboratories, Cochranville, PA).

RESULTS

Patterns of reactivity of monoclonal antibodies to filarial antigens. The results of six fusions, two each with spleen cells from mice immunized with either L3, MF, or AW of *B. malayi*, are summarized in Table I. Approximately one-third of all antibody-producing cloned hybrids of cells from animals sensitized to L3 or to MF secreted antibody that reacted only with the parasite stage used to immunize the spleen cell donors. Approximately one-half of the clones generated in these fusions reacted with all three stages of *B. malayi*, as well as with antigenic extracts of adult DI, a related filarial species. The remainder of the clones produced antibodies with varying patterns of reactivity.

In fusions with cells from mice immunized with adult male *B. malayi*, five of 27 successfully cloned hybrids produced antibody to the immunizing stage only. Five other clones reacted with antigenic extracts of adult male *B. malayi* and DI; the remainder reacted with the latter two parasite extracts, as well as with MF of *B. malayi*.

Because mice immunized with a single stage of *B. malayi* frequently yielded clones producing antibodies to antigens present also in the heterologous DI extract, representative clones were assayed for reactivity with antigenic extracts of nonfilarial helminths. The results of these experiments are summarized in Table II. None of the monoclonal antibodies that reacted with only one stage of *B. malayi* in initial screening assays cross-reacted with antigens of *Dirofilaria*, *Trichinella*, or *S. mansoni*. In contrast, the majority of monoclonal antibodies that recognized antigenic epitopes of all three

stages of *B. malayi* also reacted with antigenic extracts of a related filariid (DI), a nonfilarial nematode (*T. spiralis*), and a trematode (*S. mansoni*).

Host albumin and other proteins frequently are present in aqueous extracts of filariae such as the ones used in the present study (4, 5). However, none of 27 monoclonal antibodies tested (representing all patterns of reactivity illustrated in Table I) reacted with serum from uninfected jirds.

The majority of the monoclonal antibodies described here appear to react with antigens shared by several stages of *B. malayi*. Additional experiments suggest that these antibodies react with multiple distinct antigenic determinants. Indeed, simultaneous comparison of the degree of reactivity of cross-reactive antibodies from different fusions reveals discordant patterns of reactivity with the four filarial extracts used in these experiments. The results obtained with eight randomly selected clones analyzed in this fashion are illustrated in Table III. Qualitative analysis of parasite antigens recognized by different monoclonal antibodies supports this interpretation (see below).

Qualitative analysis of filarial antigens recognized by monoclonal antibodies. The results reported above suggested that some monoclonal antibodies that we produced reacted with stage-specific antigens of *B. malayi*, and others reacted with antigenic epitopes present in multiple stages of this parasite. To confirm this finding, we used the method of Western blots to identify and characterize the antigens recognized by a number of clones selected on the basis of reactivity patterns obtained by RIA.

We observed a remarkable concordance between the results obtained by RIA and those obtained by Western blotting. Monoclonal antibodies judged to react with stage-specific antigens on the basis of RIA results identified antigens in extracts of the appropriate parasite stage on Western blots, but not in other parasite stages processed in parallel. The epitope recognized by such antibodies often was present on a pair of antigens with different apparent m.w. These results are illustrated in Table IV and in Figures 1 and 2.

In marked contrast, monoclonal antibodies that reacted with multiple stages of *B. malayi* by RIA identified antigenic determinants present in the four filarial extracts tested. In general, the epitope to which such "cross-reactive" antibodies react appeared to be present on a large number of molecules with a wide range of apparent m.w. (Fig. 3).

Qualitative analysis of antigens identified with monoclonal antibodies also confirmed the marked complexity

TABLE I
Patterns of reactivity of monoclonal antibodies to filarial antigens^a

Monoclonal Antibody Reacts with	No. of Antibody-producing Clones Obtained from Spleens of Mice Immu- nized with		
	L3	MF	AW
L3 only	8	0	0
MF only	0	13	0
AW only	0	0	5
L3 + MF	4	1	0
L3 + MF + AW	2	0	0
AW + DI	0	0	5
AW + MF + DI	0	3	14
L3 + MF + AW + DI	11	18	3

^a Only those clones that continued to react with the stage of *B. malayi* used to immunize donor mice were tested.

TABLE II
Specificity of monoclonal antibodies to *B. malayi*

Pattern of Reactivity with <i>B. malayi</i> Antigens ^a	No. clones/No. Tested that React with		
	DI	<i>T. spiralis</i>	<i>S. mansoni</i>
MF only	0/11	0/4	0/2
L3 only	0/3	NT ^b	NT
MF + L3	3/5	0/2	NT
MF + L3 + AW	25/28	3/6	3/4

^a Patterns of reactivity with filarial antigens are shown in Table I.

^b NT, not tested.

TABLE III
Quantitative analysis of reactivity of monoclonal antibodies with different stages of *B. malayi*

Eliciting Antigen ^a	Clone Tested	Index of Reactivity to:			
		MF	AW	L3	DI
L3	1 H11 H9	2.8	2.9	2.8	1.9
L3	4 B12 C1	1.6	3.0	6.6	3.6
L3	4 B12 B2	4.5	2.8	7.1	6.6
AW	2 B8 A5	3.8	4.8	1.0	12.0
AW	5 C8 G10	2.0	4.4	2.4	14.2
MF	2 G10 A11	9.7	8.3	3.7	12.9
MF	3 D10 B9	11.3	2.9	4.8	9.6
MF	3 B9 E1	17.0	6.5	6.4	17.4

^a Eliciting antigen refers to the stage of *B. malayi* used to immunize donor mice.

TABLE IV

Molecular weights of stage-specific antigens of *B. malayi* identified with monoclonal antibodies

Clone No.	Antigen Identified	
	<i>B. malayi</i> stage	M.W. ^a
IC7F10	MF	70,000; 75,000
3A12G9	MF	90,000; 210,000
3D10G6	MF	78,000; 85,000
1H11D1	L3	179,000; 182,000; 220,000
2G3G9	AW	120,000; 135,000

^a Molecular weights are estimated from relative mobility on SDS-polyacrylamide gels.

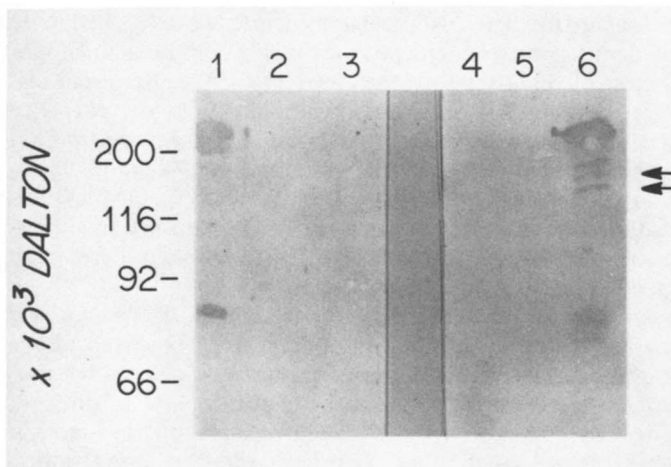


Figure 1. Stage-specific antigens of adult *B. malayi* identified by clone 2G3G9. The figure shows replicate Western blots of antigenic extracts of MF (lanes 3 and 4), infective larvae (lanes 2 and 5), and adult males (lanes 1 and 6) of *B. malayi*. The left strip was incubated with control ascites, the right strip with ascites from mice carrying clone 2G3G9. Arrows indicate two stage-specific antigens.

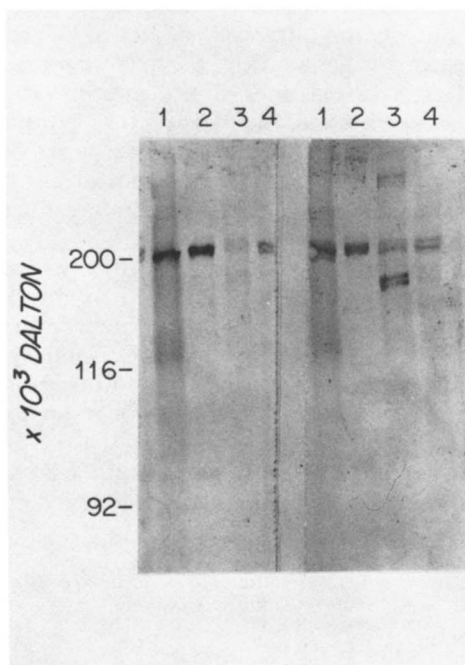


Figure 2. Stage-specific antigens of infective larvae of *B. malayi* identified by clone 1H11D1. Replicate Western blots are shown. The left strip was incubated with control ascites, the right strip with ascites from mice carrying clone 1H11D1. Lane 1: MF; lane 2: AW; lane 3: L3 of *B. malayi*; lane 4: DI

of the antigenic makeup of filarial worms that was suggested by the multiple patterns of reactivity observed in RIA. The results of a typical experiment, shown in Figure

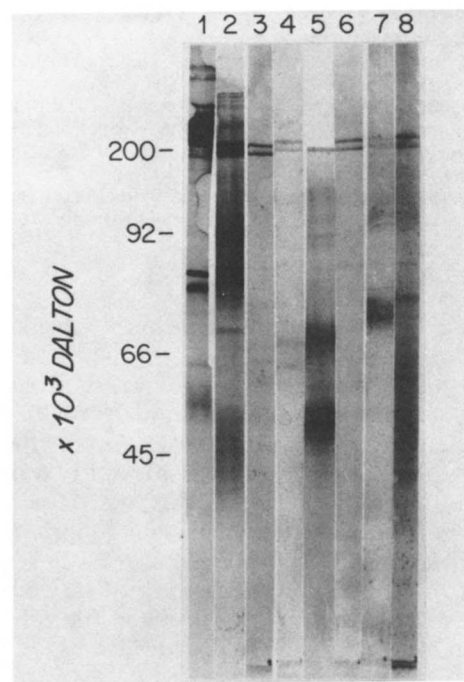


Figure 3. Stage-specific and nonspecific antigens of *B. malayi* MF identified with monoclonal antibodies. Individual strips of the same Western blot of microfilarial extract were incubated with ascites fluid from mice carrying different clones. Lane 1: clone 2B12B9; lane 2: immune serum from a mouse immunized with MF; lane 3: clone 3D10B11; lane 4: clone 1C7F10; lane 5: clone 3D6E11; lane 6: clone 3A12G9; lane 7: clone 3D10G6; lane 8: clone 3B9E12. The antibodies used in lanes 1, 5, and 8 react with all stages of *B. malayi*; those in lanes 3, 4, 6, and 7 react only with microfilarial antigens. The double band (approximate m.w. 210,000) present in all strips results from endogenous parasite peroxidase activity.

3, clearly indicate that the monoclonal antibodies used are directed against epitopes on microfilarial antigens of differing m.w. Similar results were obtained when extracts of infective larvae and adult *B. malayi* were used (results not shown).

DISCUSSION

Filarial nematodes elicit a wide range of protective, allergic, and pathogenic immune reactions that can be both advantageous and deleterious to the infected host (1-4). The nature and origin of parasite antigens that initiate these various types of immune responses remain almost entirely unknown. The limited availability of different stages of filariids that infect man renders a straightforward biochemical approach to this problem impractical, if not impossible. On the other hand, analysis of filarial antigens with sera from infected or immunized donors is hampered by the observation that such sera contain antibodies to multiple filarial antigens, some of which may be shared by other nematodes (1).

As a first step towards the goal of dissecting the complex interactions between *B. malayi* and its host immune system, we developed a series of monospecific antibodies to filarial antigens by applying established methods of somatic hybridization to spleen cells from mice hyperimmunized with a single stage of this parasite. The hybrid clones generated in these experiments produce antibodies with four broad patterns of reactivity. A minority of these antibodies reacts only with the same parasite as that used to immunize the spleen cell donors. Thus, these antibodies appear to recognize stage-specific antigens of *B. malayi*. Whether or not such antigens are also species-

specific has yet to be determined. If this were true, such antibodies could be extremely valuable reagents for use in quantitative diagnostic assays.

A second group of monoclonal antibodies is directed against antigens shared by distinct stages of *B. malayi* but absent from DI, a related filarial species. If future studies confirm that these antigens are absent from *Brugia* spp more closely related to *B. malayi* than to the dog heartworm, antibodies of this type might become useful reagents for the study of zoonotic filariasis.

A third group of antibodies reacts with antigens of adult worms and/or MF common to *B. malayi* and to DI but absent from infective larvae, and a fourth group reacts with all three stages of *B. malayi* tested and with adult DI.

Considering that many humans with filariasis also are infected with other parasites, we assayed *B. malayi*-directed monoclonal antibodies for reactivity with antigenic extracts of other parasites that often are co-endemic with brugian filariasis. It was of great interest that antibodies which react with only one or two stages of *B. malayi* did not cross-react with antigenic extracts of another nematode, *T. spiralis*, and a trematode, *S. mansoni*. In marked contrast, the majority of antibodies that reacted with *B. malayi* and with DI also reacted with these heterologous antigens. Whether these antibodies recognized shared epitopes on somatic or surface antigens is currently under investigation, but it should be noted that sera from patients with *S. japonicum* contain antibodies to some cuticular proteins of *Onchocerca gibsoni* MF (15). Host serum albumin is a major contaminant of the type of parasite extracts used in the present study (6). It was therefore somewhat of a surprise that none of 27 monoclonal antibodies tested reacted with pooled sera from uninfected jirds, the source of MF and adult *B. malayi*. At least one of the antibodies tested in this regard reacts with antigens on the sheath of *B. malayi* MF (16). This finding, along with the results reported by other investigators (6), indicates that surface molecules of filariae are present in the aqueous extracts used in the screening assays. Thus, our results suggest that jird albumin is a poor immunogen for mice, or alternatively, that host proteins are loosely associated with the surface of MF and are removed by extensive washing of the parasite.

Qualitative analysis of filarial antigens recognized by monoclonal antibodies confirmed the results of solid-phase RIA that some antibodies generated during this study identify apparently stage-specific antigens of *B. malayi*. In general, such antibodies reacted with epitopes present on parasite molecules of limited m.w. heterogeneity. In contrast, cross-reacting monoclonal antibodies identified epitopes present on multiple antigens with a wide range of m.w. The significance of this finding has yet to be determined.

It should be noted that all results reported here were obtained with monoclonal antibodies of the IgM or IgG class generated by immunizing donor mice with AW and

MF harvested from the peritoneal cavity of infected jirds. Whether or not immunization with lymph-dwelling adult worms or with MF isolated from the blood of infected animals would yield the same patterns of reactivity was not evaluated.

Of the 87 clones described, 14 have been maintained by continuous *in vitro/in vivo* passage for up to 2 yr. All but one of these long-term lines continue to secrete antibodies with the same apparent reactivity as the original parent clone, whereas one line no longer secretes antibody. Thus, the majority of the clones tested so far appear to be stable. This property enhances the practical usefulness of the monoclonal antibodies they secrete.

It cannot be assumed *a priori* that monoclonal antibodies produced by somatic hybrids of spleen cells of hyperimmunized mice will necessarily detect filarial antigens that play an important role in human filariasis. However, these monospecific reagents should provide invaluable tools to dissect on a molecular level the complex interactions of filarial parasites with their hosts' immune system.

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