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DEVELOPMENT OF MONOCLONAL ANTIBODIES TO
FILARIA SPECIFIC ANTIGENS AND THEIR USE IN
IMMUNODIAGNOSIS OF LYMPHATIC FILARIASIS

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3. Executive Summary

Lymphatic filariasis caused by *Wuchereria bancrofti* and Brugia species affect over 90 million persons worldwide and is a significant cause of morbidity (e.g., elephantiasis of the extremities and hydrocele) in less developed countries. Diagnosis of the disease is generally made by detecting larval forms of the parasite (microfilariae) in the bloodstream of the host. The objective of this study was to develop an antigen detection assay for the diagnosis of this disease.

These studies involved production and characterization of monoclonal antibodies to the filarial parasites and resulted in development of an assay that can be used to detect filarial antigen in sera from infected individuals.

Ms Salwati has become extremely efficient in several immunochemical techniques, including MAB production and Ab purification, biotinylation, and various types of ELISA. In addition, she has learned to run SDS-polyacrylamide gels and perform Western blot analysis and radioimmunoprecipitation. We believe that the skills and experience acquired over the last two years will greatly benefit her laboratory and institution in Jakarta.
4. Research objectives

Lymphatic filariasis caused by *Wuchereria bancrofti* and *Brugia* species affect over 90 million persons worldwide and is a significant cause of morbidity (e.g., elephantiasis of the extremities and hydrocele) in less developed countries. Diagnosis of the disease is generally made by detecting larval forms of the parasite (microfilariae) in the bloodstream of the host. However, a significant proportion of infected individuals either do not have circulating microfilaremia, or have too few to be detected in the 1 ml blood samples generally used for diagnosis. In addition, because these parasites exhibit nocturnal periodicity, e.g., microfilariae can be detected only in late evening when the mosquito vectors are present, blood drawing can then be very tedious. With the development of drug trials, it has become essential to develop more rapid and reproducible methods for diagnosis to aid in both screening for infected individuals and to determine the effect of chemotherapy on parasitemia. In particular, it is important that any screen will also monitor the presence of adult parasites (which in this disease are resident in the lymphatics). Serological analysis provides only limited use as repeated tests are required to differentiate between an active infection and previous exposure to the parasite.

The objective of this study was to develop an antigen detection assay for the diagnosis of lymphatic filariasis. The advantages of a successful assay are:

1) a single blood drawing will suffice to determine the parasitic status of the individual;
2) the assay will detect adult worms in addition to microfilariae;
3) the sensitivity of the assay will be greater than that of directly counting microfilariae;
4) detection will be less labor intensive, with the ability to screen numerous samples by immunological rather than parasitological criteria; and
5) blood drawing will not be restricted to the peak time of microfilaremia and can be conducted at any time.

The approach taken was to develop monoclonal antibodies with specificity for filarial antigens that are excreted and secreted from the adult parasites (excretory/secretory products). These antigens would be expected to be present in the blood of infected individuals and correspond to the degree of infection. Monoclonal antibodies were to be developed by standard methods. Reactivity for parasite antigens was the primary criteria for further investigation, and once isolated, the specificity for the monoclonal antibodies would be determined and the target parasite antigen identified. The final section of the study was to develop an antigen detection assay based on an enzyme linked immunosorbent assay (ELISA) and determine the applicability using first infected animals, and ultimately sera from infected individuals.

5. Methods and Results

1. Production of filarial specific monoclonal antibodies

i) Production of excretory/secretory products

*Brugia malayi* has been adapted to complete the life cycle in Mongolian jirds (*Meriones unguiculatus*). Jirds are infected in the laboratory of Dr John McCall at the University of Georgia at Athens, and are sent to Case Western Reserve University under NIH contract.

To collect excretory/secretory products from *B. malayi*, microfilariae and adults were recovered from the peritoneal cavity of infected gerbils and incubated in serum-free media for 14 days at 37°C. Culture supernatant was collected and parasite material was concentrated by Amicon filtration. Soluble extracts of adult worms (AWE) and microfilariae (MFE)
were prepared as described (1) and used for screening monoclonal antibodies.

**ii) Immunization protocol and production of monoclonal antibodies**

BALB/c mice were immunized subcutaneously with 1.5 μg excretory/secretory products in squalene adjuvant weekly for four weeks. Sera obtained after this time were tested by ELISA using plates coated with 1μg MFE. The mouse showing the strongest reactivity in relation to normal mouse sera was sacrificed. Spleen cells were then fused with B cell hybridoma line P653 by standard techniques. Briefly, hybridomas were selected by growing in HAT media, and positively reacting B cell lines were identified by screening cell supernatants in the MFE-based ELISA. Positive lines were cloned by limiting dilution and reactivity was confirmed.

**iii) Selection and purification of hybridomas**

Ten clones were identified as positive, expanded and stocks were maintained in liquid N2. Of these, two clones were consistently found to react, but with differences in reactivity to MFE as determined by OD492 values: monoclonal antibody designated MAB1 reacted strongly (OD492 = 0.8 - 1.2), whereas MAB2 had intermediate reactivity (OD492 = 0.4-0.6). We reasoned that this consistent difference, which was noted in repeated cell cultures, was more likely to represent differences in the target epitope than differences in antibody concentration. MAB were purified by affinity chromatography using Protein A sepharose CL-4B and isotyped according to Manufacturers directions BioRad, Richmond, CA). Isotype analysis revealed that both hybridomas were IgG2a.
2) **Characterization of target parasite antigens**

i) **Chemical nature of target antigen**

To determine if MAB - specific epitopes were protein or carbohydrate in nature, MFE was treated with either pronase or heat to destroy protein epitopes, or sodium periodate to remove carbohydrate epitopes. To ensure that the enzymatic and chemical treatments were directed at the target antigen and would have no effect on either monoclonal antibody, Pronase and sodium periodate were removed the wells of the ELISA plate before addition of MAB.

Reactivity was reduced >90% after either pronase or heat treatment of MFE prior to addition of either antibody (Figure 1). In contrast, periodate treatment of MFE had no effect on reactivity. Taken together, these results indicate that the target Ag for both MAb is protein and not carbohydrate.

![Bar chart showing reactivity after different treatments](image)

**Figure 1. Chemical nature of target epitopes**
ii) Reactivity with phosphorylcholine

Lal and co-workers have shown that filarial phosphorylcholine is a highly immunogenic molecule that is often the target of antibodies in infected individuals (2). It was therefore important to determine if the MAB reacted with phosphorylcholine epitopes. Plates were coated with 1 μg/well bovine serum albumin (BSA), BSA conjugated to phosphorylcholine (PC-BSA) or B.malayi microfilarial extract (MFE). Plates were then incubated with MAB1, MAB2 or anti-phosphorylcholine MAB CA101 (from Renu Lal). As shown in Figure 2, CA101 reacted with PC-BSA but not BSA, confirming the nature of this Ab. CA101 also reacted with MFE and PC-BSA, whereas MAB1 and MAB2 reacted only with MFE. These results demonstrate that MAB1 and MAB2 do not react with a phosphorylcholine epitope and are consistent with the protein nature of the epitope described in Figure 1.

![Figure 2. Reactivity with phosphorylcholine (PC)](image-url)
iii) The target epitope of MAB2 is filarial chitinase

Continuing studies in this laboratory identified filarial chitinase as a major component of microfilarial ESP. In collaboration with Dr Francine Perler at New England Biolabs, we obtained recombinant *B. malayi* chitinase as a fusion protein with bacterial maltose binding protein (MBP) (3). We tested the reactivity of these monoclonal antibodies against MFE, recombinant chitinase and against MBP as a control. CA101, with a well-defined anti-PC specificity was also included. As shown in Figure 3, CA101 reacted with MFE but not chitinase or MBP. Similarly, MAB1 reacted strongly with MFE and weakly with chitinase and MBP. In contrast, MAB1 reacted strongly with MFE and chitinase but not with MBP.

These results indicate that the target epitope for MAB2 is filarial chitinase. The target epitope of MAB1 is still being investigated, but we have used MAB 2 for development of an antigen detection assay.

![Figure 3. Reactivity with chitinase](image_url)
To confirm the specificity of MAB2 for chitinase, inhibition studies were carried out in which increasing dilutions of chitinase or control MBP were added to the ELISA plate prior to addition of MAB2. As shown in Figure 4, recombinant chitinase inhibited binding of MAB2 in a dose dependent manner whereas MBP even at 100 µg/ml had no effect on binding of MAB2.

![Graph showing inhibition of MAB2 by chitinase and MBP](image)

**Figure 4. Chitinase specificity of MAB2**

2) Development of an antigen detection assay

i) Protocol for chitinase inhibition assay

The basis of the assay was to inhibit binding of biotinylated (b-) chitinase to MAB 2. We used the following conditions for this assay:

1) Coat 'Immunolon 4' plates with 50 µl 10µg/ml MAB 2 overnight, 4°C

2) Block with 10% fetal calf serum 1 hour at 37°C
3) Incubate with 25 µl 0.25µg/ml b-chitinase with 25 µl competing antigen preparation 2h, room temperature (RT)
4) Incubate with streptavadin-peroxidase 1/5000 1h, RT
5) Add OPD substrate, determine percent inhibition

**ii) Native parasite antigen inhibits binding of recombinant chitinase in a dose-dependent manner**

To determine the feasibility and limit of detection of this assay, a soluble extract of adult *B. malayi* worms (AWE) was incubated with MAB2 - coated wells of a microtiter plate prior to addition of b-chitinase. As shown in Figure 5, AWE inhibited binding of b-chitinase in a dose dependent manner. The limit of detection was 0.5 µg/ml.

![Figure 5](image-url)
6) Impact, Relevance and Technology Transfer

These studies have resulted in development of an assay that can be used to detect filarial antigen in sera from infected individuals. This is clearly the next stage of the studies. MAB2 and recombinant chitinase has been sent to Ms Salwati in Jakarta, and she will be able to continue these studies using sera from infected individuals from an endemic area in Indonesia.

She also has the protocol for an alternate assay should there be difficulties in using the chitinase inhibition assay under these conditions. This will involve biotinilating the MAB and performing 2-site ELISA in which the plates are coated with MAB2, antigen is added and b-MAB2 is added prior to detection.

Ms Salwati isolated a number of other monoclonal antibodies which has not yet characterized. These have also been sent to her in Jakarta.

Ervi Salwati has been working under the guidance of Drs. Kazura and Pearlman at Case Western Reserve University in Cleveland. During her tenure here, she has become highly efficient in several immunochemical techniques, including MAB production and Ab purification, biotinylation, and various types of ELISA. In addition, she has learned to run SDS-polyacrylamide gels and perform Western blot analysis and radioimmunoprecipitation. We believe that the skills and experience acquired over the last two years will greatly benefit her laboratory and institution in Jakarta.

7. Project Activities/ Outputs

Ms Salwati attended the annual meeting of the American Society for Tropical Medicine and Hygiene in Atlanta in 1993. Although she did not have a formal presentation, she spoke informally with a number of other scientists and benefited from the experience.

Publication of these results is planned. However, we are awaiting results of field studies before submission.
8. Project Productivity

The project accomplished all the proposed goals with the exception of testing infected individuals. This final goal was not fulfilled as several technical problems arose during the course of her studies. The most time-consuming was that her first hybridoma study did not yield any positive clones and this had to be repeated. Had Ms Salwati's tenure here lasted a few more months, we felt that these could have been completed.

9. Future Work

As described above, future studies will be on infected individuals from an endemic area in Indonesia.

10. Literature Cited