[Jpn. J. Parasitol., Vol. 43, No. 3, 229–236, June, 1994]

Characterization of Monoclonal Antibodies Against Microfilariae of *Wuchereria bancrofti*

MASASHI KOBAYASHI[1)], ELIZABETH MALAGUEÑO DE SANTANA[2)], LUCIANO T. MONTENEGRO[2)] AND PAULO J. MIRANDA[2)]

(Accepted for publication; July 20, 1994)

Abstract

Hybridoma cell lines, which secreted antibodies directed against either the soluble fraction of microfilaria or the surface of the sheath of microfilaria, were produced by fusion of spleen cells of *Wuchereria bancrofti* microfilariae-immunized mice with NS-1 myeloma cells. Fourteen cloned antibody-secreting cell lines were successfully established. Two of these clones were characterized. One clone (1G10) secreted an IgM antibody that had a high specificity for recognizing the microfilaria but did not react against adult worm. Another clone (2G01) directed against the sheath of microfilaria was found to produce IgG_1, but did not show binding to adult filaria.

Key word: *Wuchereria bancrofti*, monoclonal antibody, microfilaria, filariasis

Introduction

Lymphatic filariasis caused by *Wuchereria bancrofti* is estimated to affect 100 million people in the tropics (Sasa, 1976). Definite diagnosis of the disease depends on parasitological examination or positive serological tests. However, both methods are not always efficient since blood examination is insensitive and requires investigations in the middle of the night due to the nocturnal periodicity of microfilariae, and serological examinations are confused by cross-reactivities among nematode parasite infections. Furthermore, antigens used in immunological diagnosis are prepared from heterologous filariae such as *Brugia malayi* or *Dirofilaria immitis* (Hege and Ridley, 1977; Grove and Davis, 1978; Ottesen *et al.*, 1982). Despite the development of a technique that introduced the homologous materials in serum examination, the fact that a continuous supply of *W. bancrofti* is difficult limits its practical use. Hence, monoclonal antibody specific for *W. bancrofti* is essential in order to develop not only sensitive but also species-specific serological diagnosis. Several laboratories have reported serological diagnoses utilizing monoclonal antibodies (Karen *et al.*, 1985; Weil *et al.*, 1987). However, most of monoclonal antibodies were produced against different species of filaria. Recently, monoclonal antibodies raised against a circulating antigen form the patient of bancroftian filariasis (Lal *et al.*, 1987) and microfilariae ES antigen of *W. bancrofti* (Reddy *et al.*, 1989) were reported. Each monoclonal antibody-based ELISA in bancroftian filariasis showed less satisfactory result in specificity, nevertheless these monoclonal antibodies were produced against homologous antigen.

In this study, we produced mouse monoclonal antibodies against *W. bancrofti* microfilaria and analyzed their specificity.

Materials and Methods

Human subjects. An epidemiological survey for bancroftian filariasis was carried out in a community of Jaboatão, Perunambuco, Brazil. This area has a high prevalence of filariasis, with a microfilaremia rate of 10% and a clinical disease rate of 2% (authors' unpublished observations). Microfilaremia was detected by examining 20 μl of blood obtained by finger-prick under microscopy at night between 22:00 and 24:00. A 10-ml blood sample

[1)]*Department of Parasitology, School of Medicine, Chiba University, Chiba, Japan.*
[2)]*Laboratório de Imunopatologia Keizo Asami, Universidade Federal de Pernambuco, Recife, PE., Brazil.*
小林　仁（千葉大学医学部寄生虫学教室）
Malagueño, E. S., Montenegro, L. T., Miranda, P. J. (LIKA, UFPE, Recife, PE, Brazil)

was taken from each of the microfilariae carriers. One ml of the sample was used for quantifying the microfilaremia by the Nuclepore filtration technique (Denis and Kean, 1971), and 4 ml was used for serum. The remaining 5 ml of blood was used as microfilaria sources.

Microfilaria. W. bancrofti microfilariae were obtained by filtration of blood through the Nuclepore membrane with 3μm porosity. *B. malayi* microfilariae were collected by peritoneal washing of *Meriones unguiculatos* previously infected with *B. malayi*, which was kindly supplied by Dr. R. Maeda, Teikyo Univ. School of Med., Tokyo, Japan. *D. immitis* microfilariae were obtained from blood of naturally infected dog by the filtration technique. Agarose gel concentration method (Nogami *et al.*, 1982) was employed when further purification was required.

Antigens. Ten mg of lyophilized microfilaria was sonicated with 100 mg of glass powder in 1 ml of veronal buffer containing 0.1% Triton X-100 and 2 mM PMSF (phenylmethylsulphonylfluoride). Following incubation for 48 hrs at 4C, the supernatant of 17,000g was used as microfilaria antigen. Other antigens used in this study were prepared as described previously (Kobayashi *et al.*, 1987).

Production of monoclonal antibody. BALB/c mice were injected i.v. with 10^5 *W. bancrofti* microfilaria, and boosted with 10^4 of the parasites 3 weeks later. Five days after the last injection, spleen cells were removed and were fused with NS-1 myeloma cells according to conventional methods of hybridoma production (Kennet, 1979). Supernatants of resultant hybridomas were screened by ELISA and IFA on microfilaria of *W. bancrofti*. Hybridomas secreting antibody against microfilaria of *W. bancrofti* were cloned twice and then injected into pristane-treated BALB/c mice for ascites production (McKearn, 1980).

Immunofluorescence assay. Intact microfilariae were incubated with PBS containing 0.1 u/ml papain (Sigma) for 1 hr at 37C. and washed twice with PBS. Fifty μl of microfilaria suspension containing 100–200 microfilariae was overlaid on a chamber of HTC glass (Cel-Line Associates Inc., NJ., US) and dried at room temperature. Microfilariae fixed on the glass were incubated with 30 μl of hybrid cell supernatant. After 1 hr of contact at 37C., excess antibodies were removed by 3 washes with PBS, followed by incubation with 30 μl of 1:100 diluted fluorescence-conjugated anti-mouse immunoglobulins (Cappel Lab., PA, US). Reactions were washed, counterstained with Evans blue (1:10,000 dilution), and examined under a fluorescence microscope.

Enzyme linked immunosorbent assay (ELISA). ELISA was carried out as described previously (Niimura *et al.*, 1988) with minor modifications. Peroxidase-conjugated sheep anti-mouse immunoglobulins (Serotec, OX, England) in a dilution of 1:2,000 and ABTS as a substrate (Matsuda *et al.*, 1984) were used in this study. The titer was expressed as the reciprocal of the highest sample dilution in which the absorbance was two times greater than that of normal mouse serum at 1:20 dilution.

Peroxidase-antiperoxidase technique. Fixed paraffin sections of human lymph node containing adult *W. bancrofti* were kindly supplied by Dr. Figueredo-Silva, LIKA, UFPE, PE, Brazil. A Vectastain™ ABC kit (Vector Lab., CA, US) was used in this study. This method has been described in detail by others (Hsu *et al.*, 1981).

Surface treatment of microfilariae with various detergents. Frozen microfilariae of *W. bancrofti* and living microfilariae of *B. malayi* were exposed to various detergent treatments after preincubation as described above: 2% (w/v) Triton X-100, 2% (w/v) Triton X-114, or 1% (w/v) sodium deoxycholate. All solutions were prepared in PBS. After overnight incubation at 4C in one of the solutions, worms were washed extensively with PBS prior to immunofluorescence analysis. Microfilariae were also treated with 20 mM sodium metaperiodate on ice in the dark, washed, and assayed by IFA as described above.

Gel electrophoresis and western blotting. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed on gel of 12.5% polyacrylamide (Laemmli, 1970). As indicated in the leg-ends to the figures, the samples were heated for 3 min at 95C and reduced with 1% 2-mercaptoethanol. Protein content was about 7 μg per track. The proteins separated by electrophoresis were transferred to 0.45 μm nitrocellulose papers as described by Towbin *et al.* (1979), and the mem-

branes were blocked in TBS-Tween containing 5% dried milk for 2 hr at room temperature. The nitrocellulose membranes were incubated with monoclonal antibodies diluted to 1:100 for 2 hr at room temperature. The membranes were then washed and further incubated with 1:500 dilution of biothinylated sheep anti-mouse Ig (Amersham) for 20 min at room temperature. After incubation, nitrocellulose strips were again washed and immersed in a 1:3,000 dilution of streptavidin alkaline phosphatase complex (Amersham) for an additional 20 min at room temperature. Alkaline phosphatase activity was determined with a mixed solution of 0.03% NBT and 0.015% BCIP in 100 mM diethanolamine buffer (100 mM, pH9.5) containing 5mM magnesium chloride.

Determination of isotypes. Isotypes of monoclonal antibodies were identified by agar double-diffusion technique (Ouchterlony, 1949) using specific rabbit antisera to mouse Ig classes (Miles Lab., IL., US).

Results

Antibodies reacting with the extract of microfilariae or with surface antigens were produced in *W. bancrofti*-inoculated BALB/c mice and were readily detected 3 weeks after inoculation. Microfilariae were still observed in peripheral blood of mouse 1 week after inoculation. Spleen cells of *W. bancrofti* microfilariae inoculated-mice on day 26 of inoculation were fused with NS-1 myeloma cells. Hybridomas producing antibodies to microfilaria antigens were detected by ELISA by using extract of microfilariae or by IFA by using papain-treated microfilariae of *W. bancrofti*. Fourteen stable, independently arising clones with high binding activity were obtained.

Characterization of monoclonal antibodies to W. bancrofti microfilaria. Culture supernatants were screened for monoclonal antibodies against *W. bancrofti* microfilariae by ELISA and IFA. Supernatants of thirteen hybrids revealed the presence of antibodies against antigenic determinants in *W. bancrofti* microfilariae by IFA. Two of these clones apparently reacted against the surface of exsheathed microfilariae and the others against the sheath remaining around the microfilariae. However, these antibodies did not react with sonicated homogenates of whole microfilariae of *W. bancrofti* as examined by ELISA. Another supernatant was shown to be positive by ELISA with the extract of *W. bancrofti* microfilariae but had a negative fluorescence reaction with *W. bancrofti* microfilariae. We determined the immunoglobulin class of the positive supernatants. These results are shown in Table 1. One clone with the most intense fluorescence reaction on the surface of microfilaria (2G01.3A01.1B08) and the ELISA positive clone (1G10.1A05.1E04) were expanded as ascites tumors.

Identification of target antigens. In order to determine the molecular property of antigens that reacted with 1G10 and 2G01, immunoblottings were carried out using the extract of *W. bancrofti* microfilariae. 1G10 recognized 2 molecules of 60 Kd and 50 Kd, whereas 2G01 reacted with a number of molecules of 75Kd, 58Kd, 48Kd as well as some smaller ones (Fig. 1).

Reactivity of the monoclonal antibodies against W. bancrofti *adult worm.* Sections of *W. bancrofti* adult worms in human lymphatic vessel were treated with 1:400 dilution of 2G01 and 1G10 ascites fluids, respectively, and then the avidin-biothin-peroxidase complex (ABC kit) procedure was carried out. Uni-

Table 1 Screening of monoclonal antibodies against microfilaria bancrofti

Antibody source	ELISA (OD)	IFA	IgG class
1A01, 1A12, 2B05	0.325	+	IgG1
1A08, 1C05	0.315	+	IgG1
1B03, 1B09, 1A01	0.208	+	IgG1
1B06, 1A06, 1H02	0.480	+	IgM
1G10, 1A05, 1E04	>2.0	–	IgM
1H05, 1B02	0.211	+	IgG2b
1H08, 1C01, 1E05	0.290	+	IgM
2B04, 1F06, 1A02	0.333	+	IgG1
2D07, 3G07, 1A04	0.289	++	IgG2a
2G01, 3A01, 1B08	0.278	+++	IgG1
2G03, 1C04	0.255	+	IgG2b
2G12, 2D05, 1H08	0.209	+	IgG1
3D05, 1C01, 1D11	0.296	+	IgM
3E05, 1A09, 1C12	0.252	++	IgM
Fusion serum 1:40	1.366	+++	
Normal serum	0.204	–	
Culture medium	0.095	–	

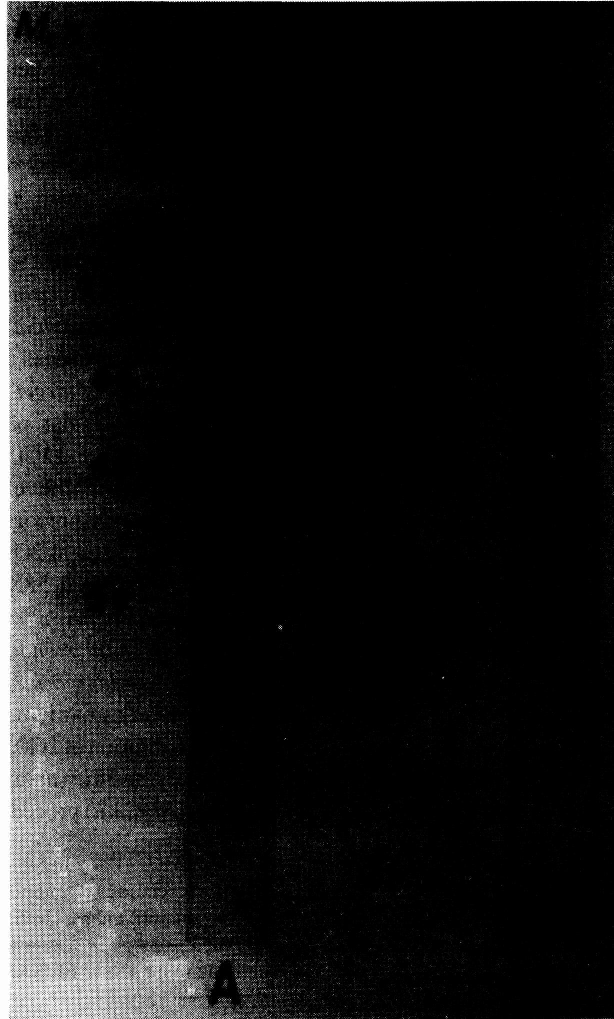

Fig. 1 Immuno-blotting of microfilarial extract and ascites fluid containing monoclonal antibodies, 1G10 (lane A) and 2G01 (lane B). Calibration of molecular weight was made with β-galactosidase (116K), bovine serum albumin (66K), egg albumin (45K) and carbonic anhydrase (29K).

form staining was seen not only on those microfilariae migrated into the tissue but also on those remaining in the uterus of adult female when 1G10 was used. However, only faint reaction was observed on the microfilariae when 2G01 was used (Fig. 2). It was revealed that *W. bancrofti* adult worms did not have any antigenic determinants recognized by the two monoclonal antibodies.

Cross-reactivities of monoclonal antibodies against other Filaria spp. The 1G10 ascites fluid reacted slightly by ELISA with the crude extract from both *B. malayi* and *D. immitis* microfilariae with a titer of 1:100, whereas a titer of 1:1,600 was determined when the crude extract of *W. bancrofti* microfilariae was used. The 2G01 ascites fluid was negative when antigens from *B. malayi* and *D. immitis* microfilariae were used. When the ascites fluid of 2G01 at a 1:100 dilution was employed as

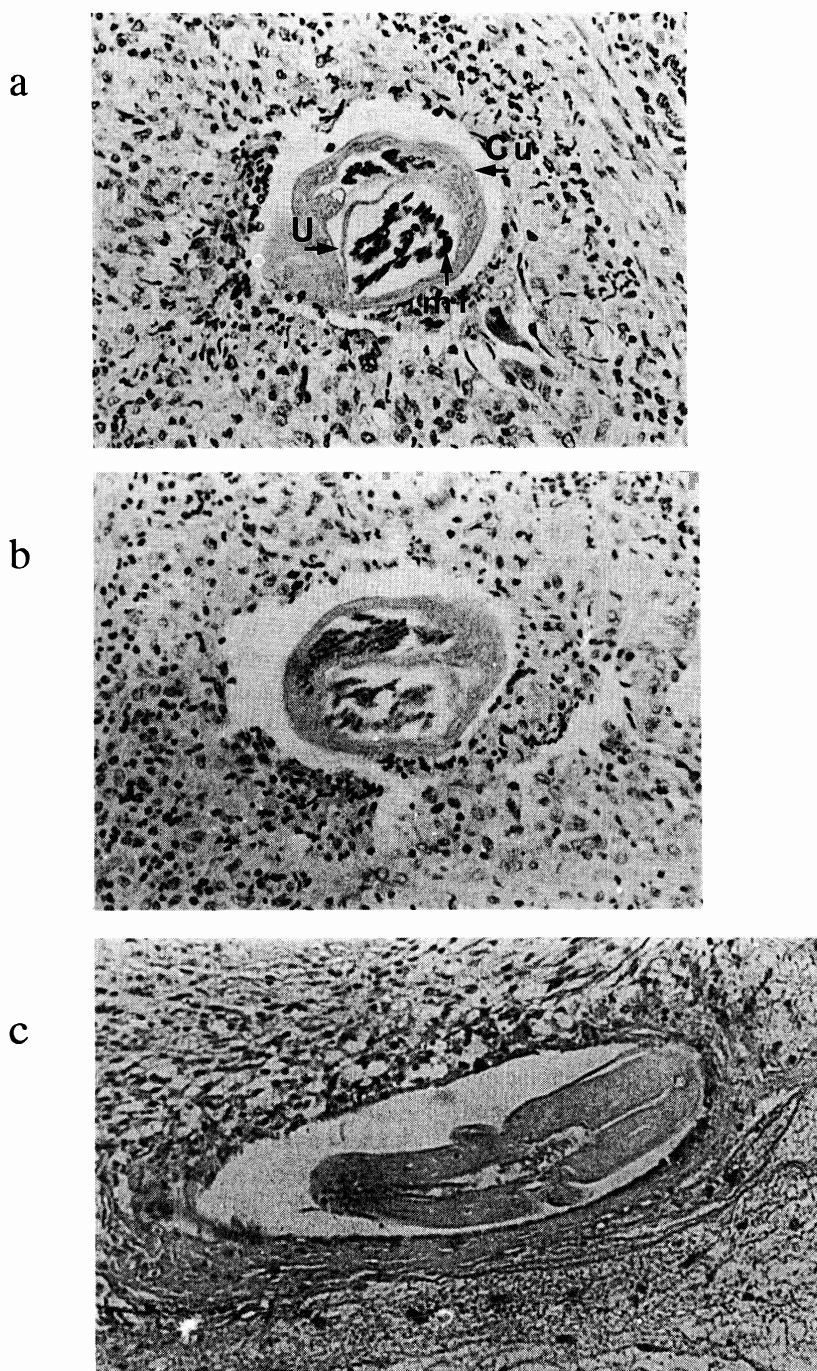

Fig. 2 Immuno-peroxidase staining of adult female *W. bancrofti* in a lymphatic vessel. a) Treated with monoclonal antibody 1G10. Microfilariae within the uterus are stained strongly but none of organs of adult is stained, uterus (U), cuticle (Cu), mf (microfilaria); b) Treated with monoclonal antibody 2G01. Slight reactions are observed on microfilariae in the uterus; c) Treated with normal mouse serum (control).

Table 2 Effect of various reagents against antigen distributed on the sheath of microfilaria

Reagent	Result of IFA with 2G01	
	W. bancrofti	*B. malayi*
Sodium deoxicholate	+++	– (+)
Triton X-100	– (–)	– (–)
Triton X-114	– (–)	– (–)
Sodium metaperiodate	– (+)	– (–)

IFA was carried out at a dilution of 1:400 (100).

primary antibody with IFA, weak binding of fluorescence was observed on both *B. malayi* and *D. immitis* microfilariae whereas strong reaction was observed at a 1:400 dilution on *W. bancrofti* microfilariae. On the other hand, no species of microfilariae was able to bind to ascites fluid of 1G10 at a 1:100 dilution when examined by IFA.

The cross-reactivity of the monoclonal antibodies against other helminth antigens was examined by ELISA. The antigens used were *Ascaris lumbricoides, Strongyloides stercoralis, Toxocara canis, Trichinella spiralis, Fasciola hepatica, Schistosoma mansoni*, and *Taenia saginata.* None of the wells coated with these antigens at different concentrations (2 μg/ml, 5 μg/ml and 10 μg/ml) bound with either of two ascites fluids at a dilution of 1:100.

Effect of various reagents on the antigen recognized by 2G01 antibody. The effect of various treatments with detergents was monitored by IFA. As indicated in Table 2, the epitope was no longer detectable after incubation in non-ionic detergents Triton X-100 or Triton X-114. However, the treatment with sodium deoxycholate failed to significantly reduce the fluorescent signal.

Treatment of bancroftian microfilariae and brugian microfilariae with low concentrations of sodium periodate decreased the binding of 2G01 in IFA. That is namely at a dilution of 1:100, reactions were observed only on bancroftian microfilariae. This suggests that the determinants recognized by 2G01 are carbohydrate, or at least, that carbohydrate plays a role in the structure of the antigen molecule.

Discussion

In the present study, we successfully produced hybridoma-derived monoclonal antibodies for microfilariae of *W. bancrofti.* These monoclonal antibodies were specific for the soluble fraction of microfilariae as shown by ELISA with extracts from microfilariae, or for materials distributed on the sheath and/or cuticle of microfilariae as shown by IFA. Two monoclonal antibodies that reacted against the soluble fraction of microfilariae (1G10) and against the sheath of microfilariae (2G01) were characterized.

The 1G10 monoclonal antibody which was found to be of the IgM class, showed no cross-reactivity with the extract of other species of microfilariae by either ELISA or immunoblotting. Furthermore, it did not react with adult *W. bancrofti*, and thus had greater specificity for microfilariae of *W. bancrofti* than the previous monoclonal antibodies by Sutant *et al.* (1985), Parab *et al.* (1988) and Weil *et al.* (1987). Antigenic analysis using immunoblotting revealed that the 1G10 antibody recognized two molecules of 60 Kd and 50 Kd. The specific determinant molecule recognized by 1G10 was soluble and located in the internal body of microfilariae. The antigen was detectable not only in microfilariae but in embryo in uterus of the female *W. bancrofti* (Fig. 2). However, it was not observed in any part of the adult worm. Therefore, it is reasonable that we failed to detect the antigen in other helminths by either ELISA or immunoblotting. In addition, the stage-specific monoclonal antibodies have been described for *W. bancrofti* microfilaria (Lal *et al.* 1987; Reddy *et al.*, 1989). Monoclonal antibodies designated CA_{86} and CA_{101} by Lal *et al.*, in spite of the products against circulating antigen from bancroftian filariasis patient are dangerous to assert species-specific since these monoclonal antibodies were screened by *B. malayi* antigen. Wb E34 by Reddy *et al.* (1989) was raised against Es antigen of *W. bancrofti* microfilariae. However, indirect ELISA using this monoclonal antibody showed a lot of false positive reactions in other filariasis (eg, malayan filariasis and onchocercosis). Furthermore, distribution of target antigen in the microfilaria recognized by Wb E34 was obscure.

The monoclonal antibody 2G01 which was found to be IgG_1, bound to the surface of the sheath membrane of microfilariae. Concrete evidence was obtained by indirect fluorescence on acetone fixed

microfilariae. The staining was bright and was unequivocally located on the sheath. In fact, with this assay it was anticipated that this antibody would be directed to the membrane antigen. In contrast, the 2G01 antibody only cross-reacted weakly with the sheath of *B. malayi*. Further work will be necessary to determine whether these antigens reside on identical proteins.

The fact that the 2G01 monoclonal antibody did not produced a positive ELISA reaction against veronal buffer extract of microfilariae indicates that the antigen molecule was insoluble or that structural destruction of the antigen took place when the molecule was released from membrane. However, the binding activity of 2G01 monoclonal antibody to the sheath of microfilariae was subsequently lost when treated with Triton-X100 or Triton-X114, although it was not influenced by the treatment with sodium deoxyacholate. This suggests that the antigen molecule has a hydrophobic residue and is solubilized by non-ionic detergents. 2G01 recognized multiple bands of 75, 58 and 48 Kd. As demonstrated previously by IFA and immunostaining on the adult section of *W. bancrofti*, 2G01 was microfilaria stage-specific and did not react with adult filaria. Sodium metaperiodate treatment of microfilariae caused reduced binding of 2G01, suggesting that the target antigens are carbohydrate or that carbohydrate plays a role in the structure of the antigen molecule and in the expression of the target epitope.

The production of these antibodies may be useful not only for application in a clinical setting but also for understanding the biology of microfilariae.

Acknowledgements

The authors would like to thank Dr. J. Figueredo-Silva for providing cross section of adult of *W. bancrofti*, and Dr. R. Maeda for supplying the Jirds infected with *B. malayi*. This work was supported in part by a Grant-in-Aid from the Ministry of Education, Science and Culture of Japan and supported by the Japan International Cooperation Agency.

References

1) Denis, D. T. and Kean, B. H. (1971): Isolation of microfilariae: Report of a new method. J. Parasitol., 57, 1146–1147.
2) Grove, D. I. and Davis, R. S. (1978): Serological diagnosis of bancroftian and malayan filariasis. Am. J. Trop. Med. Hyg., 27, 508–513.
3) Hedge, E. C. and Ridley, D. S. (1977): Immunofluorescent reactions with microfilariae: 1. Diagnostic evaluation. Trans. Roy. Soc. Trop. Med. Hyg., 71, 304–307.
4) Hsu, S. M., Raine, L. and Fanger, H. (1981): Use of avidin-biothin-peroxidase complex (ABC) in immunoperoxidase techniques; a comparison between ABC and unlabeled antibody (PA) procedures. J. Histochem. Cytochem., 29, 577–580.
5) Kennet, R. (1979): Cell fusion, in *Methods in Enzymology*, Academic Press, New York, 345–349.
6) Kobayashi, M., Niimura, M., Yokogawa, M. and Yamaguchi, T. (1987): Studies of immunological tests for acute human trichinosis. Jpn. J. Parasitol., 36, 248–253.
7) Laemmli, U. K. (1970): Cleavage of structural proteins during the assembly of the head of the bacteriophage T_4. Nature, 227, 680–685.
8) Lal, R. B., Paranjape, R. S., Briles, D. E., Nutman, T. B. and Ottesen E. A. (1987): Circulating parasite antigen(s) in lymphatic filariasis: Use of monoclonal antibodies to phosphocholine for immunodiagnosis. J. Immunol., 138, 3454–3460.
9) Matsuda, H., Tanaka, H., Blas, B. L., Noseñas, J. S., Tokawa, T. and Ohsawa, S. (1984): Evaluation of ELISA with ABTS, 2,2-azino-di-(3-ethylbenzthiazoline sulfonic acid), as the substrate of peroxidase and its application to the diagnosis of schistosomiasis. Jpn. J. Exp. Med., 54, 131–138.
10) McKearn, T. J. (1980): Method for growing hybridomas in rats or mice. in *Monoclonal Antibodies*, Plenum, New York, 403–404.
11) Niimura, M., Kobayashi, M. and Kojima, S. (1988): A mouse monoclonal antibody that binds to on α-stichocyte of *Trichinella spiralis*. Parasitol. Res., 74, 271–276.
12) Nogami, S., Tanaka, H. and Tomisato, M. (1982): Isolation of microfilariae using agarose gel. Jpn. J. Parasitol., 31, 47–54.
13) Ottesen, E. A., Weller, P. F., Lunde, M. N. and Hussain, R. (1982): Endemic filariasis on a pacific island. II. Immunologic aspects: Immunoglobulin, complement, and specific antifilarial IgG, IgM and IgE antibodies. Am. J. Trop. Med. Hyg., 31, 953–961.
14) Ouchterlony, O. (1949): Antigen antibody reactions in gels. Acta Pathol. Microbial. Scand., 26, 507–511.
15) Parab, P. B., Rajasekariah, G. R., Chandrashekar, R., Alkan, S. S., Braun, D. G. and Subrahmanyam, D. (1988): Characterization of a monoclonal antibody against infective larvae of *Brugia malayi*. Immunol., 64, 169–174.
16) Reddy, M. V. R., Parkhe, K. A., Piessens, W. F. and Harinath, B. C. (1989): *Wb* E34 monoclonal antibody: Further characterization and diagnostic use in bancroftian filariasis. J. Clin. Lab. Anal., 3, 227–281.
17) Sasa, M. (1976): A global survey of epidemiology control. in *Human Filariasis*, University Park Press,

Baltimoa.

18) Sutanto, I., Maizels, R. M. and Denham, D. A. (1985): Surface antigens of filarial nematode: Analysis of adult *Brugia pahangi* surface components and their use in monoclonal antibody production. Molec. Biochem. Parasitol., 15, 203–204.

19) Towbin, H., Taehelin, T. and Gordon, J. (1979): Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA, 76, 4350–4354.

20) Weil, G. J., Jain, D. C., Stanthanam, S., Malhotra, A., Kumar, H., Sethumadhavan, K. V. P., Liftis, F. and Ghosh, T. K. (1987): A monoclonal antibody-based enzyme immunoassay for detecting parasite antigenemia in bancroftian filariasis. J. Infect. Dise., 156, 350–355.