PRODUCTION AND CHARACTERIZATION OF MONOCLONAL ANTIBODIES AGAINST *Onchocerca gibsoni*

PRODUKSI DAN KARAKTERISASI MONOKLONAL ANTIBODI TERHADAP *Onchocerca gibsoni*

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ABSTRACT

Eleven monoclonal antibodies (MAbs) were produced from crossbred Balb/c-Q-bush mice imunized with crude extract *Onchocerca gibsoni* male antigen (1C16, 3D3, 3E12, 5C3, 5E3, 7B5, 9A11, 17D8, A2, B8F6 and D8A8). The target antigens of these MAbs are located on external and internal structures of female and male *O. gibsoni*. Nine MAbs recognized a wide range of proteins with high molecular weight ranging from 66 to 200 kDa and the A2 MAb recognized protein starting from 127 kDa. Two additional MAbs (3D3 and 17D8) displayed low molecular weight target antigens (16 kDa) which originated from spermatocytes. Nine MAbs (1C16, 3E12, 5C3, 5E3, 7B5, 9A11, A2, B8F6 and D8A8) recognized several nematodes. The 3D3 MAb was filariid-specific as it recognized *O. gibsoni* and *Dirofilaria immitis* antigens and 17D8 was *O. gibsoni* specific as it recognized *O. gibsoni* antigen only. Therefore, the high specificity of antibodies 3D3 and 17D8 for recognizing antigen associated with spermatozoa make them a potential Mabs to be used in antigen-capture ELISA diagnostic tool and to access macrofilaricidal effects of new drugs against *O. gibsoni*.

Key words: Monoclonal antibody, *Onchocerca gibsoni*, and ELISA

ABSTRAK

Sebelas monoklonal antibodi (MAb) yaitu 1C16, 3D3, 3E12, 5C3, 5E3, 7B5, 9A11, 17D8, A2, B8F6 dan D8A8 diproduksi dari mencit persilangan Balb/c-Q-bush yang diimunisasi dengan antigen *Onchocerca gibsoni* jantan. Antigen target dari masing-masing monoklonal antibodi terletak di bagian luar dan dalam tubuh cacing jantan dan betina. Sembilan Mab mengenal protein dengan berat molekul yang tinggi mulai dari 66 sampai 200 kDa dan A2 mengenal protein mulai dari 127 kDa. Dua MAb lainnya (3D3 dan 17D8) hanya mengenal antigen dengan berat molekul rendah (16kDa) yang berasal dari spermatosit. Sembilan MAb (1C16, 3E12, 5C3, 5E3, 7B5, 9A11, A2, B8F6 dan D8A8) mengenal beberapa cacing nematoda. Monoklonal antibodi 3D3 spesifik terhadap cacing filarial karena hanya mengenal *O. gibsoni* dan *Dirofilaria immitis*, sedangkan MAb 17D8 hanya mengenal antigen *O. gibsoni*. Oleh karena tingginya spesifisitas dari kedua MAb ini terhadap antigen yang berasal dari spermatozoa *O. gibsoni* membuat Mab tersebut potensial digunakan sebagai bagian dari ELISA antigen untuk alat diagnosa dan untuk menguji keampuhan obat makrofilarisida yang baru.

Kata Kunci: Monoclonal antibody, *Onchocerca gibsoni* dan ELISA

INTRODUCTION

Monoclonal antibodies have contributed greatly to the advancement of diagnosis of infectious diseases including those caused by filarial worms (Weil *et al.*, 1986; More and Copeman 1990). Unfortunately, immuno-diagnostic assay based on monoclonal antibody for human onchocerciasis is not yet available. The assay, if available, could also be used to detect *O. gibsoni* antigens in serum of infected cattle after chemotherapy as a guide to evaluate the adulticidal efficacy of new anti filarial drug.

Difficulties with maintaining *O. volvulus in vitro* or in experimental animals have prompted researchers to use animal models for tertiary drug screens. The bovine parasite *O. gibsoni* closely resembles *O. volvulus* anatomically, biologically and physiologically. These features and the similarity in host-parasite relationship of *O. gibsoni* in cattle and *O. volvulus* in humans have lead a number of researchers to use *O. gibsoni* in cattle as a model for human onchocerciasis (Israel, 1959; Copeman, 1979; Cabrera and Parkhouse, 1986; Franz *et al.*, 1987; Gill *et al.*, 1988 and Harnett *et al.*, 1997).

This article describes the production and characterization of monoclonal antibodies against male *O. gibsoni* as a part of research to develop a diagnostic methods to evaluate microfilaricidal effect of a new drugs.

MATERIAL AND METHODS

Male and fragmented female *O. gibsoni* were recovered by dissection of the nodules obtained from cattle soon after slaughter at a Townsville abattoir, Queenslands, Australia. *Ancylostoma caninum, Toxocara canis, Dyphilidium caninum* and male and female *Dirofilaria immitis* were collected from dogs following euthanasia. The worms were then finely ground in worm solubilization buffer {0.01 M Tris (Tris (Hydroxymethyl) aminomethane) (Sigma Chemicals St. Louis, USA); 0.14 M NaCl (Ajax Chemicals, Sydney, Australia); 1% Sodium deoxycholate (BDH Chemicals Ltd. Boronia, Australia); 1mM PMSF (Phenylmethylsulphonyl fluoride in iso-propanol (Boehringer, Mannheim, F.R.G); 0.5% (w/v) NaN_3 (Sodium azide) (BDH)} and centrifuged at 10,000 x g for 5 minutes. The supernatant fluid was collected and stored at -70°C until used. Protein concentration was determined using a BCA protein assay kit and used as a guide to the amount of antigen present.

The entire method of monoclonal antibody production starting from immunization, fusion and selection of was following the method developed by Harlow and Lane (1989). Ten cross-breed Q-bush and BALB/C mice were intraperitoneally immunized three times with 100 µg *O. gibsoni* male antigen. The first immunization was with double emulsion of Freund's complete adjuvant (Sigma) and PBS/2% Tween 80 (Sigma) followed by 21 days later with second immunization with antigen in incomplete Freund's Adjuvant (Sigma) and the third was antigen only 30 days after the second. The mouse with the highest titre of antibody was killed for fusion.

Spleen cells were recovered from immunized mouse and fused with mouse myeloma cells (SP2/0) in the presence of 50% PEG 4000. The fused cells were plated out into ten of 96-well plates containing CSL-MDM media with 20% bovine donor serum (BDS), 1x OPI and 1x HAT (hypoxanthine, aminopterine and thymidine, Sigma) and incubated in 37°C incubator supplied with 5% CO_2. After seven days, wells containing hybridomas were screened for antibodies against *O. gibsoni* antigens or phosphorylcholine by indirect ELISA. Phosphorylcholine conjugated to bovine serum albumin (PC-BSA) was kindly supplied by Dr W. Harnett, University of Strathclide, Strathclide, U.K. Hybridomas producing antibody against the former and not the latter were transferred to a 25 cm^3 flask containing CLS-MDM and 20% BDS only. The cells were frozen before cloning. The hybrid cells were singled cloned by limiting dilution at least three times to ensure their monoclonality. Cloned cells were aliquoted into 1 mL Nunc Cryotubes (Nunc, Roskilde, Denmark) and frozen in liquid nitrogen in CSL-MDM supplemented with 20% BDS and 10% DMSO (dimethyl sulfide, Sigma).

Immunostaining procedure used was according to Sainte-Marrie (1961). Briefly, nodules were sectioned 5 m thick and dried on glass slides. Ascitic fluid diluted one in one hundred was applied to the slide and incubated at room temperature for 1 hour. Goat anti-mouse conjugated to horseradish peroxidase (Bio Rad) was applied for 1 hour. After washing as before, DAB substrate solution (1.6 mM 3,3,5,5-tetramethyl-benzidine dihydrochloride (Sigma), 0.03% (v/v) H_2O_2 (Sigma) in PBS) was applied for 5 minutes and counter stained with haematoxylin. The histological sections were photographed using a Zeiss photomicroscope (Zeis, Heidelberg, F.R.G.) with Ilford Pan F black and white film (Ilford, Mobberley, U.K.). A # 45 Wrattan filter was used to increase contrast of the image.

Protein from male *O. gibsoni* were electrophoretically separated using Mini Protean gel II (Bio-Rad) under reducing condition by addition of mercapthoethanol. After separation, parasite proteins were electrophoretically transferred onto a nitrocellulose membrane using a Transblot apparatus (Bio-Rad). The nitrocellulose membrane was cut with strips corresponding into each well of SDS-PAGE. The strips were then incubated with optimal dilution of monoclonal antibody from ascitic fluid. The membrane

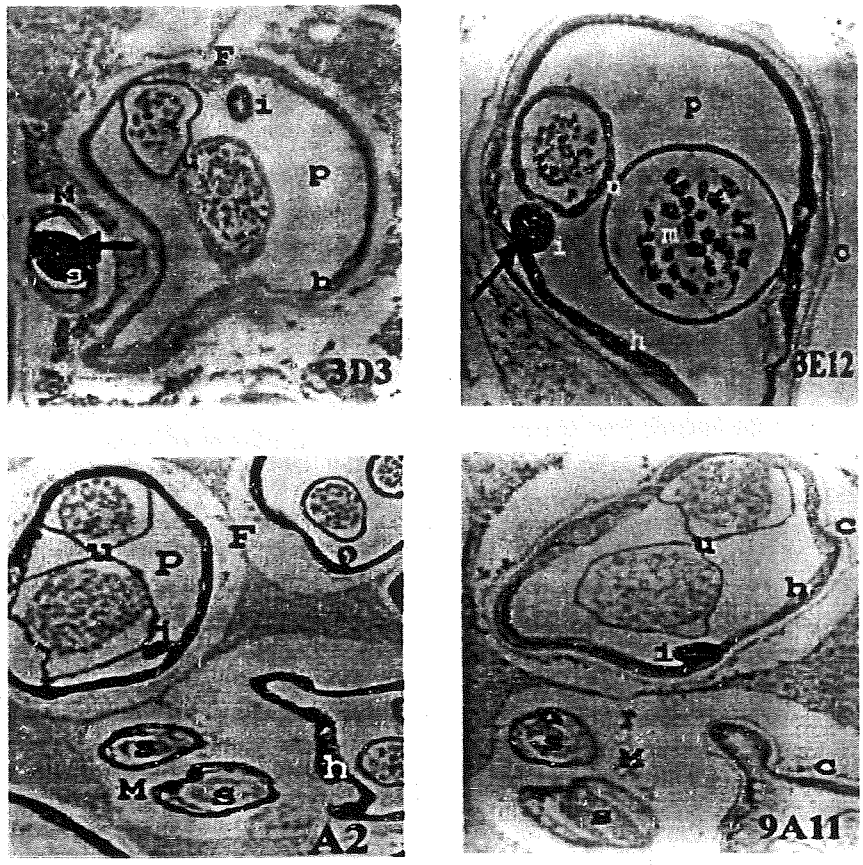

Figure 1. Examples of immunolocalization with four monoclonal antibodies to show target antigens located on external and internal structures of female and male *Onchocerca gibsoni*. Target antigen of 3D3 is restricted in the spermatocytes (arrow) (100x). Target antigen of 3E12 is broadly located in hypodermis, gut, coiled microfilariae and embryos. Note Intense staining of intestine (arrow) (300x). Target antigen of A2 is located in the hypodermis at the interface of cuticle and hypodermis, the wall of proximal sections of uterus and intestine. Note intense staining on the hypodermis and intestine (55x). Target antigens of 9A11 are restricted to the intestine and' to lesser degree the hypodermis (260x)'

Legend: c= cuticle; F= female; h= hypodermis; I= intestine; M= male; m= coiled microfilariae; n= nodular connective tissue; p= pseudocoelemic; s= spermatocytes; u=uterus

was incubated with horseradish peroxidaseconjugated to goat anti-mouse (Bio-Rad) for 1 h, soaked in DAB substrate solution for 5 minutes and washed in water.

RESULTS AND DISCUSSION

Eleven vigorous hybridomas producing monoclonal antibodies (1C16, 3D3, 3E12, 5C3, 5E3, 7B5, 9A11, 17D8, A2, B8F6 and D8A8) against *O. gibsoni* male antigens were selected from several fusions. They all survived freezing and thawing and none of the antibodies recognized phosphorylcholine in indirect ELISA.

Reactivity of the 11 monoclonal antibodies with antigens of *O. gibsoni* and other helminths vary greatly. Nine monoclonal antibodies had broad specificity over the variety of parasite antigens tested, whereas 17D8 was confined to *O. gibsoni* male antigens and 3D3 was specific for *O. gibsoni* male and *D. immitis* male antigens.

Most of the monoclonal antibodies produced in this study recognized organs of both male and female *O. gibsoni* while two (3D3 and 17D8) recognized only testis or spermatozoa (target antigens of four monoclonal antibodies are shown as an examples in Figure 1). Only 3E12 recognized coiled uterine microfilariae. Monoclonal antibody 1C16 stained hypodermis and gut from both sexes as well as the content of uterus and testis. The staining pattern of 3E12 on worm sections was broad. Target antigens were located on hypodermis, gut, uterus, early stages of embryos and coiled microfilariae. The gut was intensely stained. External and internal structures of both male and female worms were recognized by 5C3. In the female, the 5C3 recognized hypodermis and the cuticle-

hypodermis interface. There was intense staining on the gut and sperm-associated protein situated in the proximal part of uterus. The sperm-associated protein persisted on embryos up to the 8-cell stage. In males, in addition to hypodermis target epitopes of 5C3 were mainly found in testis. The target antigens of 5E3 were broadly dispersed. It strongly recognized the gut and hypodermis of both sexes. Staining was also found on testis to a lesser degree.

The target protein of 7B5 was broadly dispersed except more mature embryos and microfilariae. Monoclonal antibody 7B5 strongly recognized hypodermis, gut, sperm-associated protein in testis and uterus as well as the wall of the uterus. The sperm associated protein persisted on embryo up to 8-cell stage. Monoclonal antibody 9A11 target antigen was located mainly in gut and to a lesser degree in the hypodermis. No other organs were recognized by the 9A11. Monoclonal antibody 17D8 strongly identified sperm-associated proteins. It did not bind to other tissues. It appeared to bind to mature sperm but not to immature sperm. Early dividing stages of embryos were stained but not later stages. Monoclonal antibody A2 strongly recognized a discrete line corresponding to the interface between cuticle and hypodermis as well as the hypodermis itself. It gave a strong staining pattern on the outer wall of the gut and to less extent on the inner wall of gut and wall of the uterus. In the male, the cuticle-hypodermal interface and gut were recognized by A2, but not testis and its contents. Target epitopes of B8F6 and D8A8 were mainly located on gut and to a lesser degree the hypodermis. They did not recognize other tissues.

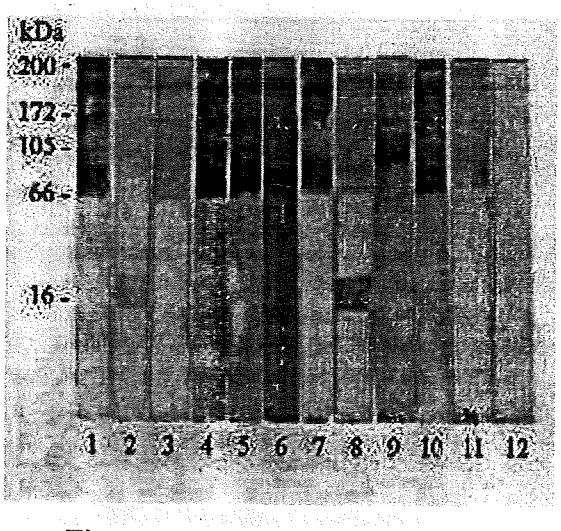

Figure 2. Reaction of each monoclonal antibody to a crude deoxycholate *O. gibsoni* male protein in immunobloting study. Lanes 1 to 12 represent monoclonal antibodies 1C16, 3D3, 3E12, 5C3, 5E3, 7B5, 9A11, 17D8, A2, B8F6, D8A8 and unrelated monoclonal antibody to Dengue virus. Numbers on the left of the figure refer to molecular weight markers in kilodalton (kDa).

The eleven monoclonal antibodies examined by Western blot recognized proteins ranging in size from 18 kDa to 200 kDa (Figure 2). Two monoclonal antibodies designated 3D3 and 17D8 recognized the same size of protein (18 kDa). Protein with low molecular weight was also detected by 17D8. Others recognized a broad range of high molecular weight protein ranging from 66.2 kDa to 200 kDa (1C16, 3E12, 5C3 9A11, A2, B8F6 and D8A8). The monoclonal antibody 5E3 recognized 2 major antigens, 16kDa and 66.2 kDa, as well as minor bands in between these two and higher than 66.2 kDa.

Males rather than females *O. gibsoni* were used as the source of antigen to vaccinate mice for production of hybridomas. Use of males were intended to avoid production of monoclonal antibodies specific for microfilariae that might result if females were used, and also to maximize the chances of producing monoclonal antibodies directed against somatic antigens present only in adults. It was hoped in this way to avoid confusion that might arise through the use of a monoclonal antibody that would recognize a change in level of circulating antigen resulting from destruction of microfilariae only without a concurrent macrofilaricidal effect.

Of the eleven monoclonal antibodies which were selected for categorization, nine exhibited broad specificity for antigens in *O. gibsoni* and other helminths against which they were screened. Such broad specificity by a majority of the monoclonal antibodies produced is to be expected as helminths are known to share common antigens (Cabrera and Parkhouse, 1987). However, this phenomenon hampers attempts to produce specific antibody to the genus *Onchocerca* (Forsyth *et al.*, 1984). Nevertheless, one antibody, 17D8 was specific for antigens from *O. gibsoni* males and another 3D3 only recognized antigen from *O. gibsoni* and *D. immitis* males.

Immuno localization of target antigens of each monoclonal antibody in histological section of *O. gibsoni* shows that most of the monoclonal antibodies were widely distributed in the hypodermis, gut and genital organs. As a consequence, it can be concluded that they all are potential candidates for use as a capture antibody in antigen detection ELISAs for diagnosis of filariasis and evaluation of macrofilaricidal efficacy. Antibodies 3D3 and 17D8, in contrast, had target antigens restricted to testis and embryos up to the 8-cell stage, suggesting that the antigen may be localized in spermatozoa.

Western blot analysis revealed that both 3D3 and 17D8 recognized antigen of 16 kDa thus supporting conclusion by Maizels *et al.* (1983); Kaushal *et al.*

(1984) and Cabrera and Parkhouse (1986 and 1987) that low molecular weight antigens are more species-specific than those of higher molecular weight. A study by More and Copeman (1990) also identified a monoclonal antibody raised against male *O. gibsoni* that has a low molecular weight target antigen specific for *O. gibsoni* located inside testis and preserved on embryos up to the 16-cell stage.

The high specificity of antibodies 3D3 and 17D8 for recognizing antigen associated with spermatozoa may be a useful attribute when they are used in antigen-ELISA for inferring macrofilaricidal effects on *O. gibsoni* as such antigens may be released to the circulation from degenerating males but are unlikely to be significantly released from intact males.

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