PRODUCTION OF A HUMAN RECOMBINANT ANTIBODY AGAINST SEROTYPE A CANDIDA ALBICANS

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Abstract- After using 3 different generations of antibody including human and non-human hyperimmune sera, monoclonal antibodies and Chimeric antibodies, more recently a newer approach has been developed in which the antibody genes are cloned directly from a patient peripheral B-lymphocytes and expressed in a host like E. coli. In this study the Candida albicans serotype A (NCTC 3135) mannan was purified using a modified Fehling method and used for selection of human recombinant antibody (hrAb) from a C. albicans phage antibody library. After four rounds of affinity selecting (panning), 2 predominant clones were chosen by DNA fingerprinting and ELISA. A 248 amino acid DNA fragment coding for anti-C. albicans mannan scFv was sequenced and cloned in a PBAD TOPO cloning vector to produce a soluble and phage free antibody. The analysis of antibody sequences by V Base Index (DNAPLOT) confirmed the human antibody origin with the VH4 family in V segment of heavy variable chain and VL3 (Lambda 3) in J segment of the light variable chain. This antibody fragment was purified using Immobilised Metal Affinity Chromatography (IMAC) and inmmunoblotted as a 31kDa recombinant protein.

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Key words: Candida albicans, Human recombinant antibody, scFv, and protein purification

INTRODUCTION

Disseminated candidiasis and candidemia were once rare infections, seen only in special care units, but currently they are the 4th most common nosocomial infections with high mortality rates at the end of 20th and the beginning of the 21st century. Candidemia is now the fourth most leading cause of blood stream infections in many intensive care units (1). Despite adequate treatment, the mortality rate is still high, ranging from 50 to 80% (2, 3). The other major problem in management of patients with systemic candidiasis is the development of resistance to many antifungal agents. There is also a shift towards the infections caused by non- albicans species that show different sensitivity to antifungal agents. For example, *C. glabrata* (*T. glabrata*) and *C. krusei* are less sensitive to fluconazole and *C. lusitaniae* and *C. tropicalis* are generally resistant to amphotericin B (4).

The recent advances in antibody engineering have made feasible the production of recombinant antibody for therapeutic and diagnostic purposes. In this study, mannan, the most important candidal cell wall antigen was purified and used for screening of a *C. albicans* antibody library to select a novel human monoclonal anti-*C. albicans* mannan single chain FV (scFV) antibody which can be used for diagnostic or therapeutic purposes in systemic candidiasis cases. After sequencing, a 248 amino acid coding for antibody DNA was re-cloned into the pBAD vector to prepare the purified soluble and phage free scFv. Finally the protective effect of this recombinant antibody was assessed *in vitro* (by broth microdilution test) and *in vivo* by a murine model of systemic candidiasis, which their results will be published in next paper.

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MATERIALS AND METHODS

Mannan purifications

The modified Fehling method was used for extracting of mannan from *C. albicans* serotypes A and B (NCTC 3153 and NCTC 3156) (8). The purified mannans were analysed by using Immunoblot kit (Bio-Rad) for immunodetection of carbohydrates as followed: a concentration of 10 µg/ml mannan in distilled water was prepared and electrophoresed at 110-120 mA. The carbohydrate bands were trans-blotted on the nitrocellulose transfer membrane (Millipore, Watford, UK). The carbohydrate on membrane was firstly biotinylated and then conjugated with streptavidin alkaline phosphatase and finally developed by Sigma Fast 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium solution (Sigma).

Selection of ScFv against Mannan

A *C. albicans* phage antibody library (kindly supplied by Department of Medical and Molecular Microbiology, University of Manchester, UK) was used for screening of an anti-*C. albicans* mannan ScFv antibody. Briefly, the serotype A *C. albicans* mannan was bound to Nunc Immunotube (life Technologies, Paisbury, UK) at a concentration of 10 µg/ml in PBS and the free carbohydrate-binding sites were blocked with 3% skimmed milk in PBS (PBSM).

The phage-containing culture supernatant (about 10^8 pfu/ml) was mixed with an equal volume of blocking solution and incubated in the tubes for 2 hours at 30°C.

The unbound phage antibodies were removed by 4 times washing with PBST (PBS with 0.05% Tween 20), followed 4 times with PBS. The bound phage antibodies were eluted with adding log-phase *E. coli* TG1.

After 4 rounds enrichment, the Bst N1 digest enzyme was used for detecting of the successful enriched clones and finally screened for high affinity binder with ELISA. The transformed clones' DNA was purified by using the Promega Wizard Plus SV Miniprep DNA purification kit (Promega) and sent for sequencing.

Sub-cloning of the antibody gene in pBAD TOPO Cloning vector

Firstly a PCR with phagemid primers (R1: 5-CCATGATTACGCCAAGCTTTGGAGCC-3'; R2: 5'-CGCATCTAAAGTTTTGTCGTCTTTC-3') was performed on clones that enriched with mannan. The PCR product was then used as a template for second PCR using the human antibody specific primers, (Hu Jλ3 forward and Hu VH4a backward primers).

The second PCR products (744 bps DNA fragment coding for anti-*C. albicans* mannan antibody) were cloned into a pBAD TOPO cloning vector (Invitrogene, Sandiego, CA). The vector was then transformed to TOPO10f′ cells (see the kit's catalogue) and grew overnight onto a LB (2×TY Brtoth) agar containing 50µg/ml ampicillin. The transformed *E. coli* were analyzed by PCR and sequencing.

Production and purification of recombinant antibody

The transformed *E. coli* cells were grown in 1 litre LB broth containing 100 µg/ml ampicillin with vigorous shaking at 37°C to an optical density (OD) 600 about 0.5. The cells were then induced with 0.02% sterile L-arabinose for at least 4 hours.

The cell pellet was frozen at -20°C overnight and crashed with a Press (Xpress, AB Biox, Sweden). The recombinant protein (soluble ScFv antibody) was purified by Immobilized Metal Affinity Chromatography (IMAC) using QIA purification system using Nickel resin (see the Kit's catalogue, Quiagene, UK) and different concentrations of imidazole as elution solution.

The chromatography fractions were electro-phoresed on a pre-cast Bis-Trise poly acryl amid gel (Invitrogene) and stained with Coomassie brilliant blue staining for analysing of the protein profiles. Also immunodetected by Anti His-hydrogen peroxidase conjugate on the radiography film by Enhance chemiluminescence method.

This purified scFv then was used for *in vitro* and *in vivo* assessment of its therapeutic potential effects, which was not included in the present paper.

RESULTS

The Polysaccharide (mannan) analysis

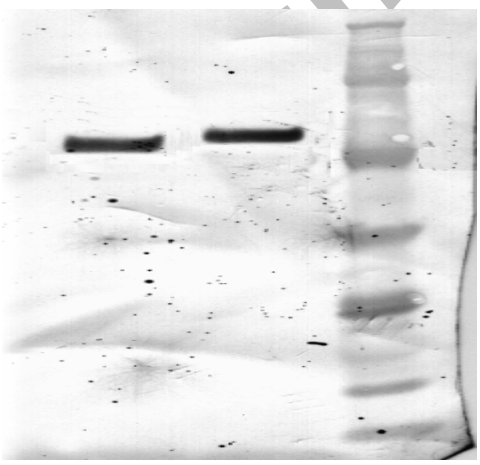
After electrophoresis of purified *C. albicans* serotype A mannan on the SDS-PAGE (pre-cast Trise-base Novex gel), the carbohydrate bands were immobilised on a piece of nitrocellulose membrane (Millipore).

The carbohydrate bands were labelled using the biotinylation and then immunodetected by Streptavidin-Alkaline phosphatase conjugates.

Figure 1 shows serotype A mannan and serotype B mannan as about 66 and 63 kDa bands respectively.

Screening of anti-C. albicans mannan serotype A scFv.

After 4 rounds of panning C. *albicans* phage antibody library against mannan, the high affinity scFv binders were selected using ELISA. A 744 bp DNA coding for anti-*C. albicans* mannan scFv was re-cloned in pBAD TOPO Cloning vector for preparing of soluble and phage free recombinant antibody that the transformed clones was analyzed by PCR, and sequencing (Fig. 2). Figure 3 shows the nucleic acid and predicted amino acid sequences of anti-*C. albicans* mannan A scFv. As can be seen from figure 3, this antibody fragment consisting of human variable heavy and light chains, which connected together with a linker.



Man B Man A Marker

Fig. 1. Western blotting profiles of mannan serotype A and B

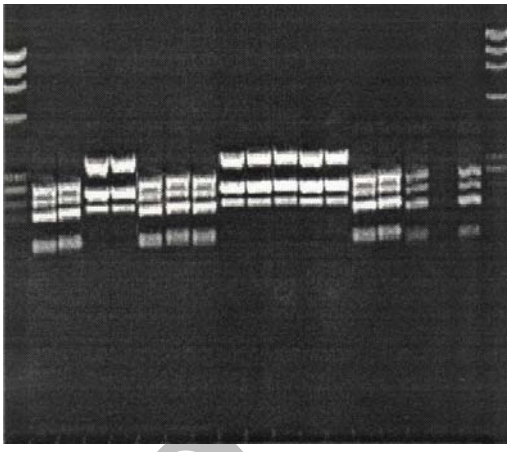


Fig. 2. DNA fingerprinting of clones producing recombinant antibody

Each part of these variable regions contained 4 frame works and 3 CDR regions. Analysis of VH and VL nucleotide sequences in V Base Index (DNAPLOT) (*WWW.mrc-cpe.cam.ac.uk*) showed the VH4a family in V segment of variable heavy and Vλ3 family of human antibody in variable light chain of recombinant antibody.

Purification of anti-C. albicans mannan antibody

By growing of transformed *E. coli* in large volume broth cultures, the expression of recombinant

Antibody was induced with 0.02% L arabinose for four hours. The cells collected and crashed in a Xpress and the Recombinant antibody was purified by Immobilised Metal Affinity Chromatography on a nickel column.

In figure 4 lane M shows the Multimark, Multicolored molecular weight standard marker (185, 98, 52, 31, 19, 17,11, 6kDa from up to down), lane CL shows protein profiles in cleared lysate, lane FT protein profiles in flow through solution, Lanes W1 to W4 different washing solutions, and lanes E1-E4 different elution solutions. As can be seen from the figure 4 lane E1 shows the most purified recombinant protein as a 31kDa protein, which was used for diagnosis of systemic candidiasis in 82 coded patient sera and also for in vitro and in vivo assessment of its therapeutic effects that their results were not included in current study.

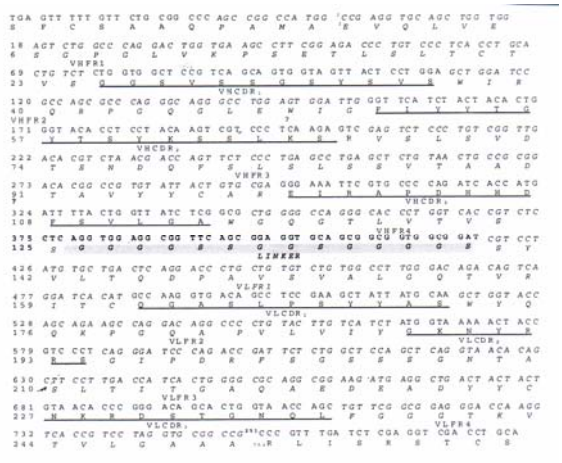


Fig. 3. The complete nucleotide sequences and predictable amino acid sequences of anti-mannan C *albicans.*

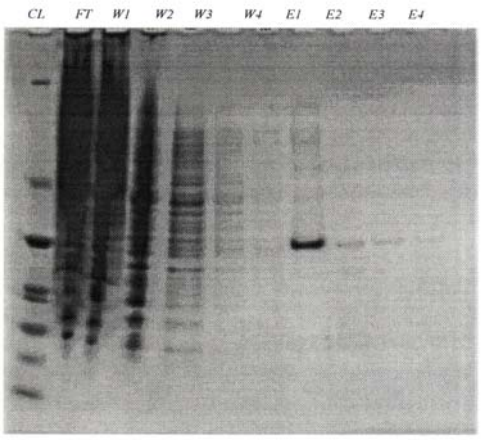


Fig. 4. SDS PAGE profiles of purification fractions

DISCUSSION

Despite current advances made in therapeutic antifungal drugs such as fluconazole, itraconazole, and systemic liposomal-based amphotericin B, the incidence of systemic candidiasis has increased. There are many reports that showed a shift to increasing in the incidence of systemic candidiasis caused by the no-*albicans Candida* strains (1). Also many studies showed the resistance of *Candida* strain to different antifungal agents. For example *C. tropicalis* and *C. lusitaniae* are less sensitive to amphotericin B (4). *C. krusei*, *T. glabrata*, and about 45% of *C. albicans* isolated from patients who treated with fluconazole are resistant to fluconazole and itraconazole and itraconazole (4).

Recombinant antibodies and their fragments represented more than 30% of all biological proteins undergo in clinical trials for diagnosis and therapy at the present, and the engineered therapeutic antibodies were approved for the first time as the ideal cancer-targeting reagents (5). For purification of mannan, the hot water extraction method was used in current study because this method was simple and preserved the complete whole carbohydrate components of mannan (6).

For selective precipitation of mannan from other carbohydrates, the modified Fehling method was used to avoid the cleavage of acid labile bonds of mannan by alkaline pH of Fehling solution (7).

Immunoblotting of mannan extracted from serotype A and B *C. albicans* showed an approximate 65-66kDa and a lower size of 63kDa for serotype A and B respectively (Fig. 1). The higher molecular weight of mannan serotype A is as a result of additional β-linked residues in acid stable part of mannan of this serotype that there is not in serotype B (8).

The molecular weight of mannan extracted from serotype A *C. albicans* in current study (66 kDa) is the same as its molecular weight reported in other studies (9, 10, 11 and 12).

Anti- *C. albicans* mannan scFv prepared in current study is the first antibody fragment against *C. albicans* mannan which prepared by panning of a *C. albicans* antibody library on the immobilised antigen (mannan). The specific binding to mannan was determined by ELISA and its sequencing showed human single fragment variable with the highest homology to VH4 family and Vλ3 family of human antibody germ-line in VH and VL respectively. After re-cloning of 744 bp nucleic acid sequences encoding for this antibody DNA into pBAD TOPO Cloning vector, the soluble and phage free antibody was purified by using Immobilised Metal Affinity Chromatography (IMAC).

There are several methods that can be used for screening of a specific phage antibody from an antibody library, such as panning an immobilised antigen, panning by using specific elutes, using biotinylated antigens, and panning by affinity purification on a column. Any method, which isolates

the bound clones from the unbound and unspecific clones, can be used for selection of a specific phage antibody. Several rounds of binding the specific phage to the target antigen and removing the non-specific phage by washing resulted in the selection of the specific high affinity page antibodies (13).

In the current study the panning on the immobilised antigen (mannan A and mannan B) method on a surface was used for affinity selection of specifically phage antibody (the phage that represented anti-*C. albicans* mannan scFv and bound specific with mannan). Several extensive washings removed the non-specific (non-binding) phages and the bound phages were eluted from the surface by re-infecting of log phase *E. coli*. The re-growing of bacteria on the selective culture (LB agar containing 100μg/ml ampicillin that only the infected clones by phage are resistant) was used for three further rounds of enrichment that produced anti- *C. albicans* mannan antibodies.

The nucleic acid sequence of the VH and the VL part of recombinant antibody were analysed in the V Base Index (MRC Centre for Protein Engineering, Cambridge, UK) using the sequence alignment software DNAPLOT *(http.wwww.mrc-cpe.cam.ac.uk)*.

The highest homology of the recombinant VH nucleic acid sequences in the current study was seen with VH4 family of the human antibody germ-line. Analysing the VL nucleic acid sequences in V Base index showed the Lambda (λ) light chain with the greatest homology with Vλ3 family in antibody V segment of scFv light chain.

In order to determine the variability of the recombinant antibody sequences with the human germ-line sequences, the amino acid sequences of recombinant antibody CDRs were compared with germ-line amino acid sequences that shown in table 1. The recombinant antibody amino acid sequences from different CDRs especially VHCDR3 and VLCDR3 are different from the germ-line CDR sequences that could be as a result of mutation of this genes after rearranging for specific antigen (here mannan of *C. albicans*). Winthrop *et al.* developed a phage display antibody library for targeting the breast cancer, used the same solid surface, and immobilized antigen for selection of Anti-MUC scFv antibody (15).

Table 1. Comparison of amino acid sequences of recombinant antibody CDRs and human germ-line CDRs*

CDRs	Recombinant AC antibody sequences	Most homologue germ-line AC sequences
VH CDR1	GGSVSSGSYSVS	G**YS**W**S**
VH CDR2	FIYYTGYTSYKSS LKS	S**IY**--- HSGST**Y**YNP**SLKS**
VH CDR3	EIRAPDRAPDHH DFS	----YF**D**YWGQGT
VL CDR1	QGASLPSYYAS	**QG**-D**S**-L**R**S**Y**-**YAS**
VL CDR2	GKNYRRS	**GK**----**NNR**P**S**
VL CDR3	NKRDSTGNQL	**N**RS**DSSGN**H

*The equal amino acid sequences are shown with bold letters.

Williamson and Matthews (14) used the panning method, using the immobilised antigen on a surface for affinity selection of specific phage antibodies (the same as method that was used in this study). The isolation of antibody fragments from a large pool of antibody libraries is one of the most successful applications of phage display. It is also an ideal method for displaying of proteins and epitopes in protein and drug research (13).

The successful selection of high affinity antibody from a large pool of antibody fragments can be achieved by the expression of antibody fragments (Fab or Fv) on the surface of a bacteriophage. Typically, each round of panning caused a1000 fold increase in the specifically binding phage antibody (16).

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