

Preparation and Characterization of Monoclonal Antibodies against *Neisseria meningitidis* type A and C

A Thesis

Submitted for the Philosophy Doctorate

(Ph.D.) degree in Microbiology

By

Amanie Mohamed Mahmoud Elbarbary

Senior Researcher - R&D Dept- VACSERA

B.Sc. Microbiology-Chemistry (1992) M.Sc. Microbiology (2002) Faculty of Science Ain Shams University

Supervised by

Prof.Dr. Fawkia Mohamed El Beih Professor of Microbiology Microbiology Department Faculty of Science Ain Shams University Prof. Dr. Mohamed Sayed Salama
Prof of Molecular Biology &
Genetic Engineering
Faculty of Science
Ain Shams University

Prof.Dr. Rafik Tawfik Soliman
Professor of Immunology
Microbiology Department
Faculty of Veterinary Medicine
Cairo University

Dr. Khaled Zakaria El Baghdady Assistant Professor of Microbiology Microbiology Department Faculty of Science Ain Shams University Dr.Mahmoud Abbas Abd El Sadek Senior Researcher & CEO of the Egyptian Organization for Vaccines and Veterinary Drugs

Microbiology Department Faculty of Science Ain Shams University (2015)



Preparation and Characterization of Monoclonal Antibodies against *Neisseria* meningitidis type A and C

Board of Scientific Supervision

Prof.Dr. Fawkia Mohamed El Beih

Professor of Microbiology
Microbiology Department
Faculty of Science
Ain Shams University, Cairo, Egypt.

Prof. Dr. Mohamed Sayed Salama

Professor of Molecular Biology & Genetic Engineering Faculty of Science
Ain Shams University, Cairo, Egypt.

Prof.Dr. Rafik Tawfik Soliman

Professor of Immunology Microbiology Department Faculty of Veterinary Cairo University, Egypt.

Dr. Khaled Zakaria El Baghdady

Assistant Professor of Microbiology Faculty of Science Ain Shams University, Cairo, Egypt.

Dr. Mahmoud Abbas Abd El Sadek

Senior Researcher & CEO of the Egyptian Organization For Vaccines and Veterinary Drugs, Cairo. Egypt.

ACKNOWLEDGEMENT

I dedicate my thanks to **Prof. Dr. Fawkia Mohamed El Beih**, Proffessor of Microbiology in Microbiology Department, Faculty of Science, Ain Shams University for her essential supervision, continual management, sympathetic and very useful aid throughout my thesis.

I would like to express my grateful appreciation and sincere gratitude to **Prof. Dr. Mohamed Sayed Salama**, Prof. of Molecular Biology & Genetic Engineering, Faculty of Science, Ain Shams University for his essential and valuable supervision, sympathetic kind help and great support backed by his vast experience.

I owe special thanks, deepest feeling and sincere gratitude to **Prof. Dr. Rafik Tawfik Soliman**, Prof. of Immunology, Microbiology Department, Faculty of Veterinary, Cairo University for his crucial and close supervision, continuous guidance and great support throughout my thesis.

Deepest gratefulness is indepted to **Ass. Prof. Khaled Zakaria El Boghdady**, Ass. Proffessor of Microbiology, Faculty of Science, Ain Shams University for his continuous guidance, valuable supervision, criticism and support.

I dedicate my thanks to **Dr. Mahmoud Abbas Abd El Sadek** Senior Researcher & CEO of The Egyptian Organization For Vaccines and Veterinary Drugs, Cairo for his sympathetic and continual help throughout the thesis.

Deepest gratefulness is indepted to **Dr. Zeinab Mohamed** and **Dr. Hemaat Moustafa**, the Staff of the Central Laboratory of Monoclonal Antibody Production, VACSERA for their great assistance and their enduring facilities provided throughout the thesis.

The Last but not the least, I decicate my thanks for Prof.Dr. **Moahmed Khaled** chairman of the Microbiology Department, Faculty of Science, Ain Shams University and the entire staff members of Microbiology Dept for their sincere effort done to encourage me during my thesis time.

AMANIE ELBARBARY

CONTENTS

LIST OF ABBREVIATIONS LIST OF TABLES LIST OF FIGURES AIM OF WORK ABSTRACT	Page I II VI VII VIII
1. INTRODUCTION	1
2. LITERATURE REVIEW	5
2.1.Admission of meningococci	5
2.2.Immigration of the N. meningitidis throughout the naso oropharyngeal mucosa	7
2.3. Violence of the <i>Neisseria meningitidis</i> to the naso oropharyngeal mucosa	8
2.4.Endurance of the meningococcus in the bloodstream	9
2.5. Scientific awarding to the danger of meningococcal disease	10
2.6. Functioning of Neisseria meningitidis antigens in patient's body	11
2.6.1.The shocking effect	11
2.6.2.Infectivity with meningococcal meningitis disease	11
2.7.Risk factors caused by meningoccocal infection	12
2.8.Patients on top of danger	13
2.9.Avoidance of the meningococcal disease attack	13
2.9.1.Principal meningococcal avoidance	13
2.10.Identification and revealing of the infecting agent	14
2.11. The typical meningococcal disease diagnostic systems	16
2.12.Therapy	19
2.13. Secondary prophylaxis 2.14 Switches to the production of managinal antihodies against Naissavia maniacitidis antigans	20
2.14. Switches to the production of monoclonal antibodies against <i>Neisseria meningitidis</i> antigens 2.14.1. What are monoclonal antibodies?	21 22
2.14.1.W hat are monocional antibodies: 2.14.2.Knowledge about manufacturing of monoclonal antibodies	23
2.14.2.1.The main constructed MAbs	25 26
2.14.2.1. The main constructed MAbs	26
2.14.2.3.constructed MAbs using the genetics technology	26
2.14.2.4. Constructed human MAbs practices	27
2.14.2.4.1.Using the DNA technologies	27
2.14.2.4.2.Using transgenic mice technology 2.14.2.4.3.Through the usage of phage exhibition capability	28 28
2.14.3. Production of MAbs	28 28
2.14.3.1.Manufacture of MAbs in mice	29
2.14.3.2.Laboratory manufacture of MAbs	30
2.14.3.2.1.Culturing in consignment	30
2.14.3.2.2.Throughout the partially leaking membrane	31
2.14.3.3. Manufacture of MAbs in bulk	32
2.14.3.4.Aspects influencing the fabrication of MAbs	32
2.15.Uses of MAbs in therapeutics and in diagnostics	33
2.15.1.Uses of MAbs in therapeutics	33
2.15.2.Uses of MAbs in diagnostics	35
2.15.3 Uses of MAbs in diagnostics of Noissaria maningitidis antigons	36

3.MATERIALS AND METHODS	38
3.1.Materials	38
3.1.1.Neisseria Meningitidis Antigens Type A and C	38
3.1.2.Animals	38
3.1.3.Cells	38
3.1.4.Chemicals and Biological Reagents	38
3.1.5.Adjuvants	39
3.1.6.ELISA buffers and reagents	39
3.1.6.1.Coating buffer	39
3.1.6.2. Washing buffer	39
3.1.6.3.Blocking buffer	39
3.1.6.4.Conjugates	39
3.1.6.5.Enzyme substrate (ABTS)	40
3.1.6.6.Stopping buffer	40
3.1.7. Plastic wares and Supplies	40
3.1.8. Equipments	42
3.2.Methods	44
PART–I: Immunization section	44
3.2.1. Preparation of Neisseria meningitidis antigens type A and C	44
3.2.1.1.Standardization of the optimum concentration of antigens	44
to elicit the higher immune response in BALB/c mice	
3.2.1.2. ELISA screening according to Sugasawara <i>et al.</i> , 1984	44
3.2.2.BALB/c mice Immunization	45
3.2.2.1.Using the complete and incomplete Freund's oil adjuvant	45
according to Jennie <i>et al.</i> , 1998 and Harold, 2005	45
3.2.2.2.Using the chemically composed Aluminum phosphate (AIPO ₄)	16
according to Gupta and Rost, 2000 and Norman et al., 2002	46
PART-II: Fusion Section	49
3.2.3. Maintenance of Myeloma cell line	47
>Thawing	47
> Freezing of myeloma cells	48
3.2.4.Myeloma cell preparation prior to fusion	48
3.2.4.1.Counting of Myeloma cells and viability test	49
3.2.5.Preparation of feeder cell layer (Mouce peritoneal macrophages)	49
3.2.6. The Fusion procedure according to Basalp and Yucel., 2003	50
3.2.6.1.Splenocytes harvest	50
3.2.6.2.Myeloma cell harvest	51
3.2.6.3. Fusion Method according to Sugasawara et al., 1983	51
PART-III: Cloning Section according to Ravi et al., 2007	53
3.2.7. Expansion of culture prior to cloning (addition of HT media before cloning) 3.2.8 Propagation of parityped magraphages as feeder cells prior to cloning	53
3.2.8.Preparation of peritoneal macrophages as feeder cells prior to cloning	50
by the Limiting dilution method according to Sugasawara <i>et al.</i> , 1985	53 54
3.2.9.Cloning by the Limiting dilution method of positive hybridoma cells 3.2.10. Propagation of positive clones and method of preservation	54 55
3.2.10.1 Propagation of positive clones and method of preservation 3.2.10.1. Propagation of positive clones	55 55
3.2.10.1. Propagation of positive clones 3.2.10.2. Method of preservation of positive clones	55 55
COMO A COMO ACCUMUNA DE PERSON CAMUNE DE PUSITACO CIUNICO	33

PART-IV: Characterization of Monoclonal Antibodies	56
3.2.11.Characterization and Differentiation of some of the resultant antimeningococcal meningitis monoclonal antibodies into type A and type C according to their binding corresponding <i>Neisseria meningitidis</i> antigen type A or C using ELISA according to Poolman <i>et al.</i> , 1995	56
PART- V: Isotyping of Monoclonal Antibodies	
3.2.12. Isotyping of some of the resultant anti- meningococcal meningitis monoclonal antibodies produced by the eight hybridomas producing antibodies according to De Gaspari, 2004	57
Flow chart (1): Standard protocol for monoclonal antibodies production in BALB/C mice	58
4.RESULTS	59
PART- I: Immunization of Mice, Serum Screening,	
Myeloma and Feeder cells Preparation	59
4.1.Immunization of BALB/c mice 4.1.1.Estimation of the standard capsular polysaccharide dose of Neisseria	59
meningitidis antigens type A and C for immunization of BALB/c mice and for coating of ELISA plates	59
4.1.2. Immunization of BALB/c mice with <i>Neisseria meningitidis</i> antigens antigens type A and C	59 59
4.1.2.1. Determination of the best adjuvant type for immunization 4.1.2.2. Result of comparison between the adjuvant effect using the complete and incomplete Freund's oil adjuvant or the aluminum phosphate (AlPO ₄)chemical adjuvant in eliciting higher	59
immune response in immunizing BALB/c mice	60
4.1.3.Preparation of myeloma cells prior to fusion	62
4.1.4.Preparation of feeder layers (Mouse Peritoneal macrophages)	62
PART-II: Fusion and Screening of Hybridomas Supernatants	62
4.2. Result of fusion of myeloma cell line with Neisseria meningitidis antigens immunized spleen cells	62
4.2.1.Microscopical examination of the fusion plates	62
4.2.2. Screening of supernatants from the fusion plates	66
4.2.3.Plucking of hybridomas showing positive ELISA titers	72 72
4.2.3.1.Expansion of highly positive hybridomas in 24 well tissue culture plate 4.2.3.2.Screening for antibody producing hybridomas	12
using ELISA as the second sreening after fusion	79
4.2.3.3. Screening for anti-meningococcal meningitis antibody producing	,,
hybridoma lines using ELISA as the third screening after fusion	82
4.2.3.4. Screening for anti- meningococcal meningitis antigens	
specific antibody producing hybridoma lines using ELISA as	
the fourth screening after fusion	84
PART-III: The Cloning Section	85
4.3. Cloning of positive hybridomas secreting antibodies using the Limiting dilution	
method to anti- meningococcal meningitis specific antibody hybrids	87
4.3.1.Results of screening of the cloned hybridomas using	
ELISA test on selected clones from the six cloning plates	92

PART-IV: Characterization and Specificity of monoclonal antibodies	98
4.4. Characterization of some of the resultant anti- meningococcal meningitis	
monoclonal antibodies into type A and type C according to their specificity	
to Neisseria meningitidis antigen type A or type C using ELISA test	102
PART- V: Isotyping of monoclonal antibodies	107
4.5.Isotyping of the monoclonal antibodies produced	
by the eight hybridomas producing antibodies	107
DISCUSSION	108
CONCLUSION	123
SUMMARY	124
REFERENCES	127
ARABIC SUMMARY	

LIST OF ABBREVIATIONS

ABTS 2,2 azino-bis [3-ethyl benz thiazoline-6-sulphonic

acid].

Ag Antigen

BSA Bovine serum albumin
CFA Freund's Complete Adjuvant

CSF Cerebro spinal fluid

CT scan Computed tomography scan

D.W. Distilled waterDMSO Dimethyl sulphoxideDNA Deoxyribonucleic acid

ELISA Enzyme linked Immunosorbent Assay

F_{ab} Antibody fragment

FACS Fluorescence activated cell sorting

F_c Constant fragment

FDA Food and Drug Administration

GAPDHs Glyceraldehyde 3-phosphate dehydrogenases HAT media Hypothanthine Aminopterine Thymidine media

hrs Hours

HT media Hypothanthine Thymidine media.

I/P Intraperitoneally

IFA Incomplete Freund's adjuvant

Ig Immunoglobulin

in vitro Means that the experiment is carried out in an artificial

environment

In vivo Means that the experiment is carried out in living

organisms

MAbs Monoclonal Antibodies
MLST Multilocus sequence typing
N. meningitides Neisseria meningitidis

nm Nanometer
OD Optical Density

PBST₂₀ Phosphate buffered saline-Tween₂₀

PCR Polymerase chain reaction PEG Polyethylene glycol

R.C.P Royal College of Physicians

rpm Round per minute

Rs media PRMI media supplemented with serum

Rss media PRMI media supplemented with serum including HT

SF serum free

SHS Second hand smoke ST Sequence type Micro liter Micro gram

VACSERA Egyptian Organisation for Biological Products &

Vaccines- DOkki, Cairo.

WHO World Health Organization

LIST OF TABLES

Table No.		Page
(1)	Plan associated with BALB/c mice immunization with Neisseria meningitidis antigens	47
(2)	Effect of using the oil adjuvant (FCA and FIA) and the chemical adjuvant (ALPO ₄) in eliciting the immune response in immunizing BALB/c mice as revealed by the OD value	61
(3)	The distinct selected wells representing ten to fifty percent of growth from three fusion plates using microscopical examination seven days post fusion	64
(4)	Results of screening of supernatants from wells in fusion plate No.1 for antibody producing cells using ELISA test twelve days post fusion as first screening after fusion	67
(5)	Results of screening of wells in fusion plate No.2 for antibody producing cells using ELISA test twelve days post fusion as first screening after fusion	68
(6)	Results of screening of wells in fusion plate No.3 for antibody producing cells using ELISA test twelve days post fusion as first screening after fusion	69
(7)	Evaluation of positive fused cells for antibody production in the three fusion plates using ELISA test 12 days post fusion	70
(8)	The exact values of microscopically examined hybrids and ELISA screening 12 days post fusion	72
(9)	Summary of plucking of hybridomas showing positive ELISA titers from the three fusion plates and their transfer into tissue culture expansion plates	73
(10)	Plate (I_1) plucked hybridomas from fusion plate No.1	74

(11)	Plate I ₂ plucked hybridomas from fusion plate No.1	74
(12)	Plate II III plucked hybridomas from fusion plates number 2 and 3 respectively	75
(13)	Location of positive hybridomas in the first 24 well plate (I_1) and their origine according to their locality in the first fusion plate	76
(14)	Location of positive hybridomas in the second 24 well plate (I_2) and their origine according to their locality in the second fusion plate	77
(15)	Location of positive hybridomas in the third 24 well plate (II III) and their origine according to their locality in the third fusion plate	78
(16)	Positive hybridomas selected from the three 24 well tissue culture expansion plates submitted to the second ELISA testing for anti- meningococcal meningitis antibody producing hybridomas	80
(17)	Evaluation of positive anti- meningococcal meningitis antibody producing hybridoma lines in the supernatants of wells showing hybrid cell growths using ELISA post second screening after fusion	81
(18)	Positive hybridomas selected from the three 24 well tissue culture plates and flasks submitted to the third ELISA testing for anti-meningococcal meningitis antibody production	83
(19)	Positive hybridomas chosen from flasks as well as from the tissue culture expansion plates for antibody production testing as the fourth ELISA screening post fusion	85

(20)	A synopsis of the recuperated specific anti- meningococcal meningitis antibody producing hybridoma lines from the supernatants of flasks showing hybrid cell growths using ELISA as the fourth screening after fusion	86
(21)	Detection of positive clones using microscopic examination and ELISA test of (I_1A_3) cloning plates	88
(22)	Detection of positive clones using microscopic examination and ELISA test of (I_2B_6) cloning plates	89
(23)	Detection of positive clones using microscopic examination and ELISA test of (II III D_2) cloning plates	90
(24)	The data sheet illustrated the exact values of the figure (3) columns	92
(25)	ELISA screening for designated clones from the first five cloning plates showing cells growth	93
(26)	ELISA testing for the designated clones from the sixth cloning plates showing cells growth	94
(27)	Selected clones for expansion from the six cloning plates representing the three cloned hybridomas and showing the highest antibody reproducibility for antimeningococcal meningitis monoclonal antibody	96
(28)	ELISA OD values obtained by the second screening of the cloning supernatants for anti-meningococcal meningitis monoclonal antibody production and their designation abbreviations	97
(29)	The eight selected clones from the 24 well tissue culture expansion plate for continuous propagation and growth and their parent hybridomas origin	99

(30)	The ELISA OD values of the eight selected clones obtained by screening of the cloning supernatants for anti-meningococcal meningitis monoclonal antibody production and their designation abbreviations after splitting into flasks	101
(31)	Characterization and specificity of anti- meningococcal meningitides MAbs developed against <i>Neisseria meningitidis</i> antigen type A using purified <i>Neisseria meningitidis</i> antigen type A as the coating antigen in ELISA test	103
(32)	Characterization and specificity of anti- meningococcal meningitis MAbs developed against <i>Neisseria meningitidis</i> antigen type C using <i>Neisseria meningitidis</i> antigen type AC as the coating antigen in ELISA test	104
(33)	Characterization of the resultant anti- meningococcal meningitis MAbs developed against Neisseria meningitidis antigens	105
(34)	Characterization and differentiation of the resultant anti- meningococcal meningitis monoclonal antibodies propagated clones	106

LIST OF FIGURES

Figure No.		Page
(1)	A typical young hybridoma stabilizing in HAT medium culture in the center of a fusion plate well in the presence of feeder cells twelve days post fusion.	65
(2)	Evaluation between positive microscopically examined hybrids and ELISA screening test in the three fusion plates in relation to time 12 days post fusion	71
(3)	Evaluation of single clones in relation to positive hybrids in the six cloning plates after microscopic examination and ELISA test	91
(4)	A positive secretory single clone at the periphery of a well in a cloning plate after limiting dilution in the presence of feeder cells as a stabilizing and conditioning medium for the single hybridoma for further expansions	95
(5)	The OD values of fifteen single clones representing the second sreening post cloning	98
(6)	The ELISA OD values of the elected eight specific anti-meningococcal meningitis monoclonal antibody producing clones after several splitting in flasks as the third ELISA screening post cloning	100

AIM OF WORK

In spite of the prospective uses of polyclonal antisera in wide medical application dealings, monoclonal antibodies technologies are nowadays favored in terms of their target-specific applications. Monoclonal antibodies technologies are good standardized inventions developed for creating definite serologic reagents that can detect a wide variety of antigens in indefinite quantities.

The aim of the present work was to produce monoclonal antibodies against the meningococcal meningitis antigens serogroup A and C and to characterize them for possible future use as diagnostic kits to detect these two antigens in recently infected Egyptian patients.