The role of aspergillus antigens and specific anti-aspergillus antibodies in the diagnosis of invasive aspergillosis

Detection and identification of anti-Aspergillus IgG antibodies and Aspergillus antigens associated with invasive infection.

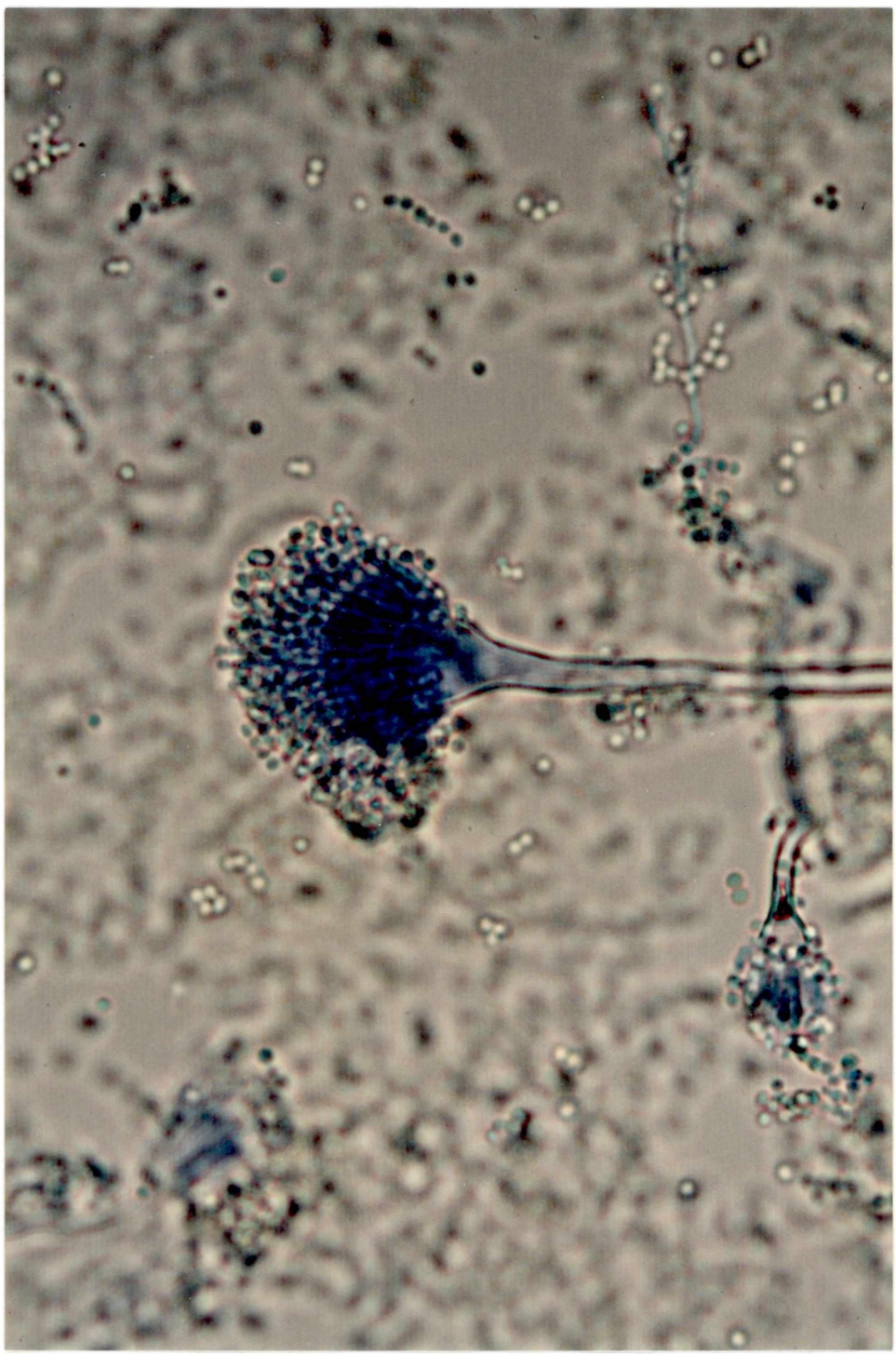
Kenneth Adrian Haynes BSc.

Charing Cross and Westminster Medical School

17 Horseferry Road, London SW1P 2AR.

Thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy (PhD). University of London. December 1990.

FRONTPIECE.



Declaration.

I certify that the work presented in this thesis is my own unless otherwise stated.

Kenneth Adrian Haynes.

Acknowledgements.

I would like to thank Dr. Tom Rogers, Senior Lecturer in Medical Microbiology, for developing my interest in mycology and agreeing to supervise this project. His door has always been open for discussion and he has bent over backwards to obtain funds to support the work and to allow me to attend scientific meetings.

Without his help and guidance this thesis would not have been possible. I look forward to a long period of collaboration and I promise that I will try to get Ireland international tickets for him.

I would also like to thank Professor Donald Mackenzie and Dr. Veronica Hearn from the Mycological Reference Laboratory for help in intiating this project.

My thanks also go to Dr. Rosemary Barnes, Dr. Yvonne Young and Dr. Debra Hyatt who have all been research registrars attached to the Westminster pediatric bone marrow transplant team during the course of this work. They were all willing to discuss my work at length and with good humour.

Thanks also to all members of the Department of Medical Microbiology at Westminster, both past and present for putting up with me stealing their equipment, reagents and disposables.

I am grateful, although my liver may not be, to Dr. Jean-Paul Latge of the Unite de Mycologie, Institut Pasteur, Paris, France for allowing me to work in his laboratory, sleep on his floor, many hours of discussion and introducing me to the wonderful city in which he lives. I would also like to mention the other members of the Unite de Mycologie who have always made me feel welcome in Paris, Anne Beauvais, Jacqueline Sarfati, Jean-Paul Debeaupuis, Michel Diaquin and Mohammad Mouhktakil.

My deepest thanks though are reserved for my one time fiancee and now wife Dana Arnold. She has always been there and you cannot ask for more! Good luck.

Financial support for this work came from the Leukeamia Research Fund and the Westminster Joint Research Committee.

CONTENTS.

Title page.	i
Frontpiece.	ii
Declaration.	iii
Acknowledgements.	iv
Contents.	v
Abstract.	vi
Table of Contents.	ix
Abbreviations.	xviii
List of Tables.	xx
List of Figures.	xxiii
Chapter One.	1
Chapter Two.	27
Chapter Three.	39
Chapter Four.	55
Chapter Five.	92
Chapter Six.	128
Chapter Seven.	195
Chapter Eight.	244
Bibliography.	252
Publications arising from this work.	273

ABSTRACT.

Invasive aspergillosis (IPA) is a life threatening infection which is occurring with increased frequency in the immunocompromised, especially patients with haematological malignancy, those undergoing bone marrow or solid organ transplantation and in individuals receiving long-term corticosteroid therapy.

The diagnosis of this infection is currently difficult. It is dependent on the demonstration of hyphae in, or culture of the fungus from, deep tissue sites. The procedures required to obtain the neccessary biopsy specimens are often contraindicated in the 'at risk' patient population. A simple, reliable and non-invasive diagnostic technique is required in order to provide support for the initiation of empirical antifungal therapy at an early stage of infection. It is hoped that the prompt initiation of treatment will improve the poor prognosis that has been associated with IPA.

A serological study was undertaken of 121 patients undergoing either bone marrow transplantation or remission induction therapy for leukaemia. Nineteen of these patients developed microbiologically and clinically documented IPA, 12 had clinical courses stronly suggestive of invasive fungal infection, 8 had other invasive mycoses while the remaining 82 had no evidence of fungal involvement.

The detection of specific anti-*Aspergillus* immunoglobulin-G antibodies by both enzyme-linked immunosorbent assay (ELISA) and immunoblotting did not aid the diagnosis of IPA.

Three antigen detection methods were evaluated: two inhibition ELISAs and a latex agglutination test (LAT). One of the ELISAs and the LAT were based on monoclonal antibodies (Mab) to *Aspergillus fumigatus* galactomannan (GM), while the second ELISA employed a high titre human anti-*Aspergillus* antiserum as detector antibody.

All three methods proved to be of value in diagnosing IPA. Furthermore the Mab based ELISA detected antigen at an earlier stage of the infection and was positive in more specimens than the other assays. Antigen titres in all patients fluctuated widely. No relationship was seen between antigen concentration and the administration of antifungal therapy. A terminal rise in antigen titre was seen in only 7/18 fatal cases of IPA. Antigen was detected more frequently in urine than in serum.

Antigen was also detected in all 12 patients with clinically suspected fungal infection.

Only a single specimen from the patient groups with no evidence of IPA was positive in one of the ELISA assays. However, 7% of specimens from these patients were positive by the LAT.

The finding that 12.6% of sera and 7.6% of urines from

the patients with IPA were positive for antigen by the ELISA based on the human antiserum but negative in the Mab ELISA prompted an investigation to determine if non-GM antigens were present in body fluids during this infection.

These studies revealed that seven non-GM antigens with apparent molecular masses of 44 kilodaltons (kDa), 38 kDa, 29 kDa, 18 kDa, 14 kDa, 13 kDa and 11 kDa were present in the urine of IPA patients. The 18 kDa and 11 kDa antigens appeared in 90% and 100% of urines analysed respectively.

Homology was seen between the 29 kDa, 18 kDa and 11 kDa antigens in patients' urine specimens and components of a water soluble extract of A.fumigatus NCPF 2109 which were of a similar molecular mass.

These antigens are likely, along with GM, to represent immunodominant antigens of Aspergillus involved in IPA.

Table of Contents.

Chapter One. General Introduction. Pages 1 - 26.

1.1 Brief history and description of the genus *Aspergillus*. 1

1.2 Aspergilli and man. 6

1.3 Aspergilli and human disease. 6

1.4 *Aspergillus* virulence factors. 9

1.5 Host defences against aspergilli. 13

1.5.1 Alveolar macrophages. 14

1.5.2 Polymorphonuclear leucocytes. 14

1.5.3 Acquired immunity. 16

1.6 Risk factors for the development of IPA. 17

1.6.1 Extrinsic risk factors. 17

1.6.2 Intrinsic risk factors. 18

1.7 Diagnosis of IPA. 21

1.7.1 Antibody detection. 22

1.7.2 Antigen detection. 23

1.8 Aims of this work. 24

Chapter Two. Patients. Pages 27 - 38.

2.1 Overall description. 27

2.2 Proven invasive aspergillosis (IPA). 28

2.3 Clinical fungal infection (CFI). 28

2.4 Other fungal infections (OFI). 33

2.5 No evidence of fungal infection (NEFI). 33

Chapter Three. General methods. Pages 39 - 54.

3.1 Fungal strains. 39

3.2 Culture of fungi for preparation of antigen fractions. 39

3.3 Preparation of Aspergillus antigen fractions. 41

3.3.1 Culture filtrate antigen (CF). 42

3.3.2 Water soluble antigen (WS). 42

3.3.3 Ethanol precipitate antigen (EP). 44

3.3.4 ConA bound antigen (BF). 45

3.3.5 Galactomannan. 46

3.4 Determination of protein content. 46

3.5 Determination of carbohydrate content. 47

3.6 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). 47

3.7 Electroblotting of SDS-PAGE gels. 48

3.8 Staining of blots. 49

3.8.1 Ponceau-S stain. 49

3.8.2 India ink stain. 50

3.9 Estimation of molecular mass. 50

3.10 Buffer and reagent composition. 51

3.10.1 Starting buffer. 51

3.10.2 Eluting buffer A. 51

3.10.3 15% polyacrylamide gel composition. 51

3.10.4 4% polyacrylamide gel composition. 51

3.10.5 Sample buffer. 52

3.10.6 Running buffer. 52

3.10.7 Transfer buffer. 52

3.10.8 Phosphate buffered saline. 52

3.10.9 Carbonate buffer (0.06M, pH 9.6). 52

3.10.10 Phosphate, citric acid buffer (0.07M, pH 5.0). 53

3.10.11 Phosphate buffer (50mM, pH 7.4). 53

3.10.12 Coupling buffer. 53

3.10.13 Acetate buffer (0.1M, pH 4.0). 53

3.10.14 Tris buffer (0.1M, pH 8.0). 53

3.10.15 20% polyacrylamide gel composition. 53

3.10.16 Eluting buffer B. 54

Chapter Four. Detection of anti-Aspergillus antibodies by enzyme-linked immunosorbent assay. Pages 55 - 91.

4.1 Introduction. 55

4.2 Aims. 62

4.3 Patients, materials and methods. 64

4.3.1 Patients. 64

4.3.2 Control sera. 64

4.3.3 ELISA for anti-Aspergillus IgG antibody. 65

4.3.4 Validation of assays and determination of positive results. 66

4.3.4.1 Validation. 66

4.3.4.2 Determination of positive results. 67

4.3.5 Use of peripheral wells in microtitre plates. 68

4.3.6 Inter- and intra- assay variation. 68

4.4 Results. 70

4.4.1 Comparison of antigen fractions WS2109, WS2008, CF2109, CF2008, EP2109 and EP1028 in their ability to discriminate between anti-Aspergillus IgG antibody positive and negative control sera. 70

4.4.2 Dilution curves of anti-Aspergillus IgG antibody positive and negative control sera. 74

4.4.3 Detection of anti-Aspergillus IgG antibody in patients. 74

4.4.3.1 IPA patients. 74

4.4.3.2 CFI patients. 79

4.4.3.3 OFI patients. 81

4.4.3.4 NEFI patients. 81

4.4.4 Comparison of the different patient groups. 81

4.5 Discussion. 85

Chapter Five. Detection of anti-Aspergillus IgG antibodies by immunoblotting. Pages 92 - 127.

5.1 Introduction. 92

5.1.1 Detection of specific anti-Aspergillus IgG antibodies to individual Aspergillus antigens or fractions of known composition. 93

5.1.2 Detection of specific anti-Aspergillus IgG antibodies by immunoblotting. 96

5.2 Aims. 99

5.3 Patients, materials and methods. 101

5.3.1 Patient and control sera. 101

5.3.2 Antigen. 101

5.3.3 SDS-PAGE. 101

5.3.4 Electroblotting of SDS-PAGE gels. 102

5.3.5 Staining of blots. 102

5.3.5.1 Ponceau-S and India ink staining. 102

5.3.5.2 Immunostaining. 102

5.3.6 Estimation of M_r. 103

5.4 Results. 104

5.4.1 India ink staining of WS2109 blots. 104

5.4.2 Reactivity of ABPA and blood donor sera with WS2109 blots. 104

5.4.3 Reactivity of patient sera with WS2109 blots. 108

5.4.4 Comparison of immunoblot pattern and anti-*Aspergillus* IgG ELISA result. 115

5.4.5 Immunoblot patterns of pre- and post-seroconversion sera. 115

5.5 Discussion. 120

Chapter Six. Detection of *Aspergillus* antigen by a latex agglutination test and two ELISAs. Pages 128 - 194.

6.1 Introduction. 128

6.2 Aims. 141

6.3 Patients, materials and methods. 143

6.3.1 Patients. 143

6.3.2 Control sera and urines. 143

6.3.3 Antigens. 143

6.3.4 Preparation of sera and urines for antigen testing by ELISA. 144

6.3.5 Polyclonal inhibition ELISA (PAb). 144

6.3.5.1 Antigen coating of microtitre plates. 144

6.3.5.2 Detector antiserum. 146

6.3.5.3 Antigen control. 148

6.3.5.4 Inhibition ELISA. 148

6.3.6 Monoclonal antibody ELISA (MAb). 150

6.3.7 EBA2, monoclonal antibody to A.fumigatus GM. 150

6.3.8 Latex agglutination test (LAT). 151

6.3.8.1 Sensitivity of the LAT. 151

6.3.8.2 Control antigen. 151

6.3.8.3 Performance of the LAT. 152

6.3.8.4 Repeat testing. 152

6.3.9 Statiatical analysis. 152

6.4 Results. 153

6.4.1 PAb ELISA. 153

6.4.1.1 Control levels. 153

6.4.1.2 Inter- and intra- assay variation. 153

6.4.1.3 BF2109 antigen titration. 153

6.4.1.4 IPA patient results. 154

6.4.1.5 CFI patient results. 159

6.4.1.6 OFI and NEFI patients results. 162

6.4.1.7 Value of the PAb ELISA in diagnosing IPA. 162

6.4.2 MAb ELISA. 164

6.4.2.1 Control levels. 164

6.4.2.2 Inter- and intra- assay variation. 164

6.4.2.3 GM titration. 164

6.4.2.4 IPA patient results. 164

6.4.2.5 CFI patient results. 169

6.4.2.6 OFI and NEFI patient results. 170

6.4.2.7 Value of the MAb ELISA in diagnosing IPA. 170

6.4.3 Comparison of the PAb and MAb ELISAs. 170

6.4.4 Latex agglutination test. 171

6.4.4.1 Control specimens. 171

6.4.4.2 IPA patient results. 173

6.4.4.3 CFI patient results. 173

6.4.4.4 OFI and NEFI patient results. 175

6.4.4.5 Repeat testing. 175

6.4.4.6 Value of the LAT in diagnosing IPA. 175

6.4.5 Comparison of all three antigen detection methods. 181

6.4.6 Fluctuation of antigen levels. 184

6.5 Discussion. 185

Chapter Seven. *Aspergillus* antigen detection by immunoblotting. Page 195 - 243.

7.1 Introduction. 195

7.2 Aims. 204

7.3 Patients, materials and methods. 206

7.3.1 Patient specimens. 206

7.3.1.1 Serum. 206

7.3.1.2 Urine. 206

7.3.2 Control specimens. 207

7.3.3 Urine preparation. 207

7.3.4 Antigens. 208

7.3.5 Antisera and monoclonal antibody. 208

7.3.6 Preparation of IgG fraction from high titre human anti-*Aspergillus* IgG sera. 210

7.3.7 Affinity chromatography. 211

7.3.7.1 Preparation of affinity columns. 211

7.3.7.2 Use of columns. 212

7.3.8 Dot-blotting. 213

7.3.9 SDS-PAGE. 214

7.3.10 Electroblotting of SDS-PAGE gels. 214

7.3.11 Immunostaining of blots. 215

7.3.12 Estimation of M_r. 215

7.3.13 Preparation of monospecific antibodies from polyclonal antisera. 215

7.4 Results. 217

7.4.1 Dot-blotting experiments. 217

7.4.1.1 Serum. 217

7.4.1.2 Urine. 217

7.4.2 Immunostaining of WS2109 extract. 223

7.4.3 Immunostaining of NEFI control urine. 223

7.4.4 Immunostaining of IPA patient urines. 225

7.4.4.1 PAb-,MAb- urine pool. 225

7.4.4.2 PAb+,MAb- urine pool. 225

7.4.4.3 PAb-,MAb+ urine pool. 226

7.4.4.4 PAb+,MAb+ urine pool. 226

7.4.4.5 Individual IPA patient urines. 228

7.4.5 Homology between *Aspergillus* antigens detected in urine and components of the WS2109 and CF1028 extracts. 231

7.4.5.1 Absorbtion experiments. 231

7.4.5.2 Monospecific antibody experiments. 234

7.5 Discussion. 236

Chapter Eight. Concluding remarks. Pages 244 - 251. 244

Bibliography. Pages 252 -272.

Publications arsing from this work. 273

Abbreviations.

A_{number}	absorbance at that wavelength
ABPA	allergic bronchopulmonary aspergillosis
anti-CW	rabbit antiserum to cell wall fraction of Aspergillus fumigatus NCPF 2109
BALF	bronchoalveolar lavage fluid
BF	concanavalin A binding fraction
CF	culture filtrate antigen
CFI	clinically suspected fungal infection
CIE	counterimmunoelectrophoresis
CV	coefficient of variation
EBA1	IgM monoclonal antibody to GM
EBA2	IgM monoclonal antibody to GM
ELISA	enzyme-linked immunosorbent assay
EP	ethanol precipitate antigen
GM	galactomannan
IPA	invasive aspergillosis
Ig	immunoglobulin
Da	daltons
LAT	latex agglutination test
MAb	monoclonal antibody based ELISA
MPO	myeloperoxidase
M_r	molecular mass
MRL	Mycological Reference Laboratory
NCPF	National Collection of Pathogenic Fungi

NEFI	no evidence of fungal infection
NFM	non fat milk in PBST
NPV	negative predictive value
OFI	other fungal infections
PAb	polyclonal antibody based ELISA
PBS	phosphate buffered saline
PBST	PBS containing 0.05% (v/v) Tween 20
PMN	polymorphonuclear leucocytes
PPV	positive predictive value
RIA	radioimmunoassay
SD	standard deviation
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
TCA	trichloroacetic acid
W1	high titre human anti-Aspergillus serum
WS	water soluble antigen
%I	percentage inhibition

List of Tables.

Table Number	Table Title.	Page Number
I.i	The spectrum of disease caused by aspergilli.	10
II.i	Details of patients with proven invasive aspergillosis (IPA).	29
II.ii	Details of patients with clinically suspected fungal infection (CFI).	34
II.iii	Details of patients with other fungal infections (OFI).	37
III.i	Protein to carbohydrate ratios for antigen fractions used in this study.	43
IV.i	Detection of specific anti-*Aspergillus* IgG antibodies in non-invasive pulmonary aspergillosis.	57
IV.ii	Detection of specific anti-*Aspergillus* IgG antibodies in invasive aspergillosis.	59
IV.iii	Detection of specific anti-*Aspergillus* IgG antibodies in all 121 patients by WS2109 ELISA.	75
IV.iv.i	Detection of specific anti-*Aspergillus* IgG antibodies by WS1209 ELISA: Seroconversion and number of positive sera from individual IPA, CFI and OFI antibody positive patients.	78
IV.iv.ii	Detection of specific anti-*Aspergillus* IgG antibodies by WS1209 ELISA: Seroconversion and number of positive sera from individual NEFI antibody positive patients.	82
IV.v	Chi-squared analysis of anti-*Aspergillus* IgG antibody detection by WS2109 ELISA as a diagnostic method for IPA.	84
V.i	Characterized antigens of *Aspergillus* spp which have been used to detect specific anti-*Aspergillus* antibodies.	94

V.ii	Reactivity of ABPA and blood donor sera with antigens in the WS2109 extract.	105
V.iii	Reactivity of IPA and CFI patient sera with antigens in the WS2109 extract.	109
V.iv	Reactivity of OFI and NEFI patient sera with antigens in the WS2109 extract.	112
V.v	Reactivity of anti-Aspergillus IgG ELISA antibody positive and negative sera from all patient groups with antigens in the WS2109 extract.	116
V.vi	Appearance of antibodies to antigens in the WS2109 extract in the 17 patients who seroconverted to anti-Aspergillus IgG positivity by ELISA.	118
VI.i	Detection of Aspergillus antigens in IPA.	129
VI.ii	Detection of Aspergillus antigen in serum and urine of all study patients using the PAb ELISA.	156
VI.iii	Number of days by which antigen detection preceded or postdated a conventional diagnosis of IPA.	160
VI.iv	Number of days by which antigen detection preceded or postdated a clinical suspicion of fungal infection in CFI patients.	161
VI.v	Sensitivity, specificity, positive and negative predictive values for the PAb ELISA, MAb ELISA and LAT in the diagnosis of IPA.	163
VI.vi	Detection of Aspergillus GM in serum and urine of all study patients using the MAb ELISA.	166
VI.vii	Comparison of PAb and MAb ELISA results for individual serum and urine specimens, from IPA and CFI patients, tested by both assays.	172
VI.viii	Detection of Aspergillus GM in sera of all patients by the latex agglutination test.	174

VII.i	Composition of a culture filtrate antigen prepared from a 48 hour fermenter culture of A.fumigatus 1028.	196
VII.ii	Proposed structures of Aspergillus GM.	197
VII.iii	Antigen fractions and antisera which have been employed in the detection of Aspergillus antigen in experimental and human IPA.	201
VII.iv	Rabbit antisera used in this study.	209
VII.v.i	Reactivity of anti-Aspergillus IgG affinity chromatography fractions and unfractionated antigen positive and negative sera with antisera to different Aspergillus extracts and EBA1.	219
VII.v.ii	Reactivity of anti-Aspergillus IgG affinity chromatography fractions and unfractionated antigen positive and negative concentrated urine with antisera to different Aspergillus extracts and EBA1.	222
VII.vi	Antigen content of the PAb-,MAb-; PAb+,MAb-; PAb-MAb+; PAb+,MAb+ urine pools, NEFI control urine pool and the 10 individual IPA patient urines revealed with anti-CW.	230

List of figures.

Figure Number	Figure Title.	Page Number
Frontpiece	Conidial head of *A.fumigatus* stained with lactophenol cotton blue.	ii
1.1	Typical heads of aspergilli.	2
1.2	Colonies of *A.fumigatus*, *A.flavus*, *A.niger* and *A.terreus*.	4
1.3	Typical life cycle of *Aspergillus* spp.	5
2.1.1	Clinical details of IPA patients numbers 1-7.	30
2.1.2	Clinical details of IPA patients numbers 8-13.	31
2.1.3	Clinical details of IPA patients numbers 14-19.	32
2.2.1	Clinical details of CFI patients numbers 20-25.	35
2.2.2	Clinical details of CFI patients numbers 26-31.	36
4.1.1	Discrimination between anti-*Aspergillus* IgG antibody positive and negative sera received from the MRL.	71
4.1.2	Discrimination between all anti-*Aspergillus* IgG antibody positive and negative control sera.	72
4.2	Dilution curves of anti-*Aspergillus* IgG antibody positive and negative control sera in WS2109 ELISA.	73
4.3	Anti-*Aspergillus* IgG titres in antibody positive IPA patients.	77
4.4	Anti-*Aspergillus* IgG titres in antibody positive CFI patients.	80
5.1	Immunoblots of ABPA sera against WS2109 antigen.	106

5.2	Immunoblots of blood donor sera against WS2109 antigen.	107
5.3	Immunoblots of IPA patients' sera against WS2109 antigen.	110
5.4	Immunoblots of CFI patients' sera against WS2109 antigen.	111
5.5	Immunoblots of OFI patients' sera against WS2109 antigen.	113
5.6	Immunoblots of NEFI patients' sera against WS2109 antigen.	114
5.7	Immunoblots of anti-*Aspergillus* IgG antibody negative and positive sera from all patient groups against WS2109 antigen.	117
5.8	Immunoblots of individual patient sera pre- and post- seroconversion by ELISA to anti-*Aspergillus* IgG antibody positive against WS2109 antigen.	119
6.1	Principle of inhibition ELISAs.	145
6.2	Immunoblots of WS2109 and WS2008 antigens.	147
6.3	Titration of BF2109 antigen in serum and urine by PAb ELISA.	155
6.4	Maximum %I detected in all patients by PAb ELISA.	157
6.5	Mean antigen concentration in the serum and urine of IPA and CFI patients detected by the PAb ELISA.	158
6.6	Titration of GM in serum and urine by MAb ELISA.	165
6.7	Maximum %I detected in all patients by MAb ELISA.	167
6.8	Mean GM concentration in the serum and urine of IPA and CFI patients detected by the MAb ELISA.	168
6.9.1	*Aspergillus* antigen detection and clinical course of IPA patient number 14.	176

6.9.2 Aspergillus antigen detection and clinical course of IPA patient number 17. 177

6.9.3 Aspergillus antigen detection and clinical course of IPA patient number 19. 178

6.9.4 Aspergillus antigen detection and clinical course of CFI patient number 21. 179

6.9.5 Aspergillus antigen detection and clinical course of CFI patient number 22. 180

6.10 Comparison of Aspergillus antigen detection and conventional methods in the early diagnosis of IPA. 182

6.11 Comparison of Aspergillus antigen detection and conventional methods as early indicators of CFI. 183

7.1 Concentration of beta (1->5) galactofuranose polymers of various lengths required to give 50% inhibition of binding of EBA2 monoclonal antibody to EP1028 in ELISA. 199

7.2 Reactivity of various anti-Aspergillus antisera and EBA1 monoclonal antibody to GM with anti-Aspergillus IgG affinity column serum bound and non-bound fractions plus unfractionated Aspergillus antigen positive and negative sera. 218

7.3 Reactivity of various anti-Aspergillus antisera and EBA1 monoclonal antibody to GM with anti-Aspergillus IgG affinity column urine bound and non-bound fractions plus unfractionated Aspergillus antigen positive and negative urines. 221

7.4 Immunoblots of NEFI urine pool stained with pre-immunization serum, anti-CW and EBA1 monoclonal antibody to GM. 224

7.5 Immunoblots of IPA patient urine pools stained with pre-immunization serum, anti-CW and EBA1 monoclonal antibody to GM. 227

7.6 Immunoblots of individual IPA patient urines stained with pre-immunization serum and anti-CW. 229

7.7 Immunoblots of WS2109 antigen, individual IPA patient urines and NEFI urine pool stained with EBA1 monoclonal antibody to GM. 232

7.8 Immunoblots of IPA patient urine (U10) and WS2109 antigen stained with anti-CW, non-bound anti-CW (WS) and non-bound anti-CW (CF). 233

7.9 Immunoblots of IPA patient urine (U10) stained with monospecific antibodies to the 11 kDa, 18 kDa and 29 kDa antigens of WS2109. 235

CHAPTER ONE: GENERAL INTRODUCTION

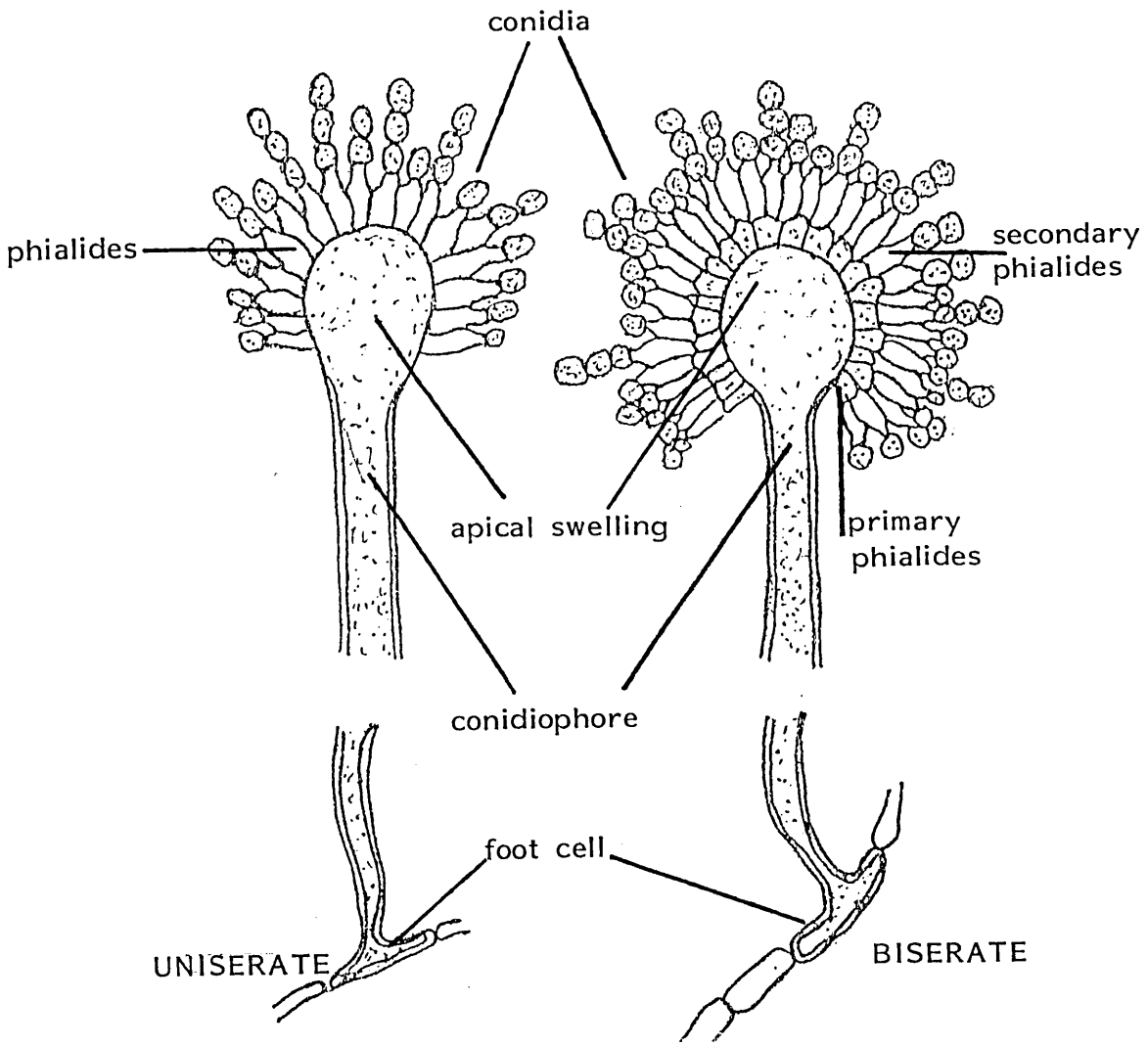
1.1 Brief history and description of the genus Aspergillus.

The genus Aspergillus was first described in 1729 by Pier Antonio Micheli, the Director of the Public Gardens in Florence, when he published his monograph Nova Plantarum Genera (Micheli, 1729). In it he described for the first time, among other molds, the genus Aspergillus. He may have named the genus thus because of their remarkable resemblance to the aspergillum, a device used to shower the catholic faithful with holy water. It is certainly true that the choice of name was apt as aspergilli have "sprinkled and bespattered" (from the latin aspergo) the entire Earth. In addition to their normal habitat of decaying vegetation Aspergillus conidia have been recovered from temperate and desert soils, the upper atmosphere and polar regions.

Aspergilli are mainly imperfect filamentous fungi with septate, hyaline, branched and multinucleate hyphae. Occasional thick-walled foot cells occur in the hyphae from which arise characteristically long and erect conidiophores. These conidiophores produce a terminal swelling from which conidiogenous cells (phialides) arise. Species vary on the size and shape of the conidial head and by having one or two series of phialides (Fig 1.1). Large numbers of conidia develop basipetally on the phialides and form dry chains

Figure 1.1 Typical heads of aspergilli.

After Rapper and Fennell (1965).



which are easily dislodged. Species also vary in the size, shape and colour of the conidia. Indeed, colony colour (due to conidial colour) is an important aid to the identification of species. Aspergillus fumigatus produces dark green colonies, while those of A.flavus are light green, A.niger black and A.terreus brown (Fig 1.2). The variation seen among the aspergilli has led to the establishment of about 200 currently scientifically recognised taxa (Kwon-Chung, 1988), most of which are saprophytic soil fungi. However, to date, 17 species have been implicated as aetiologic agents of human disease.

Members of the genus Aspergillus can be delineated from closely related fungi eg Penicillium by the simultaneous production of phialides across the apical vesicle of the conidiophore and the presence of a foot cell (Rapper and Fennell, 1965).

Some species have no known perfect (sexual) state eg, A.niger, whereas others do reproduce sexually and are members of genera within the ascomycete family Eurotiaceae, eg teleomorphs of the A.fumigatus group can be found in the genus Neosartorya. These sexual states are characterized by the production of small (100-200μm), spherical cleistothecia which contain a large number of eight-spored asci. A typical life cycle is shown in Fig 1.3.

Figure 1.2 Colonies of A.fumigatus, A.flavus, A.niger and A.terreus.

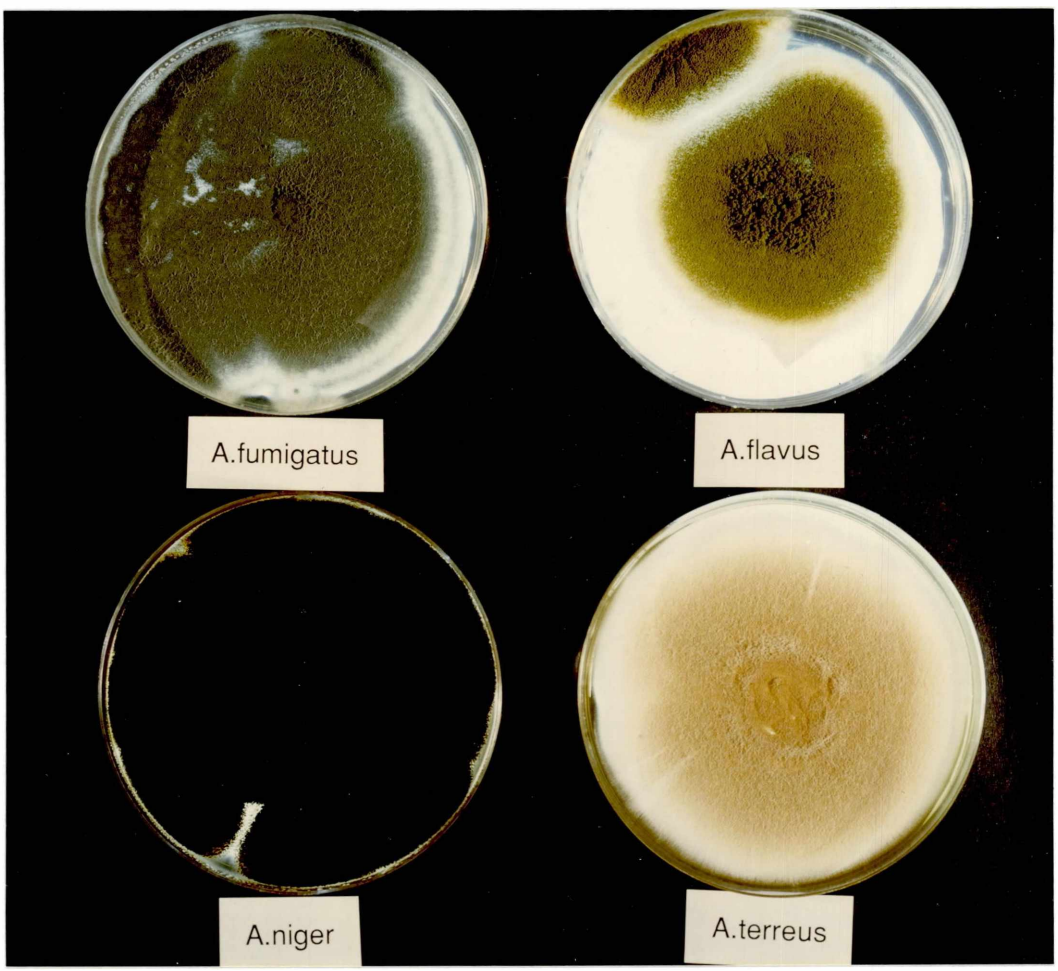
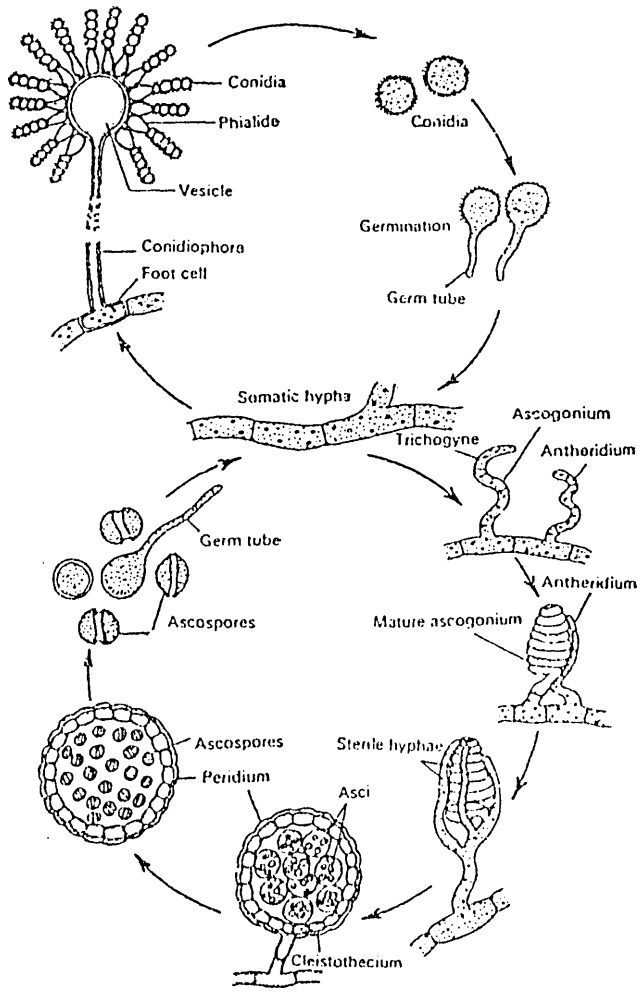


Figure 1.3 Typical life cycle of Aspergillus spp. From Dude (1983).



1.2 Aspergilli and man.

Aspergilli have long been used by man to his advantage, in the production of fermented foodstuffs, beverages (most notably Japanese rice wine 'sake') and organic acids, especially citric acid. More recently these fungi have been used to produce industrial enzymes and as research tools in the field of genetics and the molecular biology of gene expression.

Other associations, not sought by man, are usually disadvantageous. Aspergilli, in common with many other molds, cause economic loss through bio-deterioration of food and other organic substrates. They also cause disease in farmed animals eg avian aspergillosis.

1.3 Aspergilli and human disease.

Aspergilli also affect man directly by causing human disease. The first cases of human infection attributed to these fungi were reported in the mid-nineteenth Century (Bennett, 1844; Slutyer, 1847), whilst a decade later the classic paper of Virchow describing four cases of bronchial and pulmonary aspergillosis established *Aspergillus spp* as pathogens of man (Virchow, 1856). The most common pathogen is *A.fumigatus* followed by *A.flavus* and *A.niger*. Other species which have been implicated in human disease include *A.nidulans*, *A.terreus*, *A.candidus*, *A.oryzae* and members of the *A.glaucus* group.

The spectrum of Aspergillus associated diseases in man is large, ranging from saprophytic colonization to invasive infection (Table I.i). The work of this thesis is concerned primarily with the invasive form of aspergillosis. However, I feel it is pertinent to give a brief description of the other forms of aspergillosis:

1. Colonization occurs when aspergilli grow in the upper respiratory tract with no concomitant disease seen. It is debateable whether colonization represents a disease state. However it is generally accepted that conidia must gain access to the upper respiratory tract before Aspergillus related diseases can develop. Colonization therefore definitely forms part of the complex interactions that occur between these fungi and humans.

2. Aspergillomas are mycetomas caused by aspergilli (most commonly A.fumigatus, A.flavus and A.niger) which usually occur in pre-existing lung cavities (Glimp and Bayer, 1983).

3. Aspergilli possess many potent allergens (Kauffmann et al, 1985; van der Heide et al, 1985; Longbottom, 1986a) and can cause both extrinsic (due to conidial inhalation) and intrinsic (due to growth of hyphae in the airways) hypersensitivity. Asthma, extrinsic allergic alveolitis and eosinophilic pneumonitis can all be caused by aspergilli. However, the most common allergic manifestation due to these fungi is allergic

bronchopulmonary aspergillosis (ABPA) (Greenberger, 1986). It is characterized by pulmonary eosinophilia, haemoptysis and moderate or severe asthma.

4. Invasive infection due to aspergilli is characterized by hyphal invasion of the tissues. Two forms have been recognised:

a) Invasive aspergillosis (IPA) usually occurs in severely neutropenic patients, individuals receiving long term corticosteroid therapy or patients with an inherited impairment of immune function (see below). Occassionaly IPA can occur in apparently healthy individuals with no known predisposing factors (Karam and Griffin, 1986). The primary focus of infection is usually the lung although other sites, most notably the sinuses, have been documented. Inhalation of the conidia is followed by germination and invasion of the lung parenchyma by hyphae. Haematogenous dissemination to other organs can follow (Aisner et al, 1977).

b) Chronic necrotising pulmonary aspergillosis is seen in patients with functioning immune systems who have a pre-existing pulmonary disease. Lung invasion occurs at much slower rates than in IPA and the disease very rarely develops into IPA.

5. Chronic and acute mycotoxicosis can be caused by aspergilli, either by direct ingestion of contaminated foodstuffs or by inhalation of toxin-containing conidia.

Aflatoxins, produced by A.flavus are extremely hepatotoxic and are also among the most potent carcinogens known to man.

It should be borne in mind that this classification of diseases caused by the aspergilli, especially those of pulmonary origin, is not hard and fast and that passage of individual patients from one form to another can, and does, take place.

1.4 Aspergillus virulence factors.

As has already been mentioned aspergilli are normally saprophytic soil fungi. In this enviroment a large number of organisms obtain food by phagocytic ingestion. It is therefore no suprise that aspergilli produce metabolites which are capable of impeding phagocytosis.

Mullbacher (1985) identified 3 components in a 5-day old liquid culture of A.fumigatus which inhibited the phagocytosis of carbon particles by thioglycollate induced peritoneal macrophages. One of these compounds was identified as the low molecular mass (M_r) (approximately 500 dalton (Da)) mycotoxin, gliotoxin. This was capable of completely inhibiting phagocytosis within 15min. However, none of the three components were detected in the liquid medium until 72h of culture. It would therefore seem unlikely that these metabolites could prevent the phagocytosis of resting conidia or germlings as hyphal

Table1 I.i The spectrum of disease caused by aspergilli.

1. Saprophytic colonization
2. Aspergilloma (fungus ball)
3. Allergic lung disease
Asthma
Extrinsic allergic alveolitis
Eosinophilic pneumonitis
Allergic bronchopulmonary aspergillosis
4. Invasive aspergillosis
Invasive aspergillosis
pulmonary aspergillosis
disseminated aspergillosis
Chronic necrotising pulmonary aspergillosis
5. Mycotoxicosis
Acute mycotoxicosis
Chronic mycotoxicosis

production usually begins soon after conidial swelling (approximately 2h). However, they may help to protect the hyphal organism from phagocytic cell damage.

In contrast, Robertson and co-workers in Edinburgh have demonstrated an active component(s) of A.fumigatus conidia which was released within 2min of placing the conidia into liquid media. This soluble factor was capable of: a) inhibiting the release of superoxide anion and H_2O_2 by mouse peritoneal exudate cells [32% polymorphonuclear leucocytes (PMN) and 63% macrophages] (Robertson et al, 1987a); b) preventing the internalization of both red blood cells and A.fumigatus conidia after attachment to mouse peritoneal cell surfaces (Robertson et al, 1987b), and c) reduced the migration of these cells and human PMNs towards the chemoattractant zymosan activated serum (Robertson et al, 1987c). The active component of the conidial diffusate was removed by dialysis indicating that its M_r was <14 kDa.

Washburn et al (1986) have also described an active component(s) of A.fumigatus which was quickly released from the conidia. This component, which had a M_r of >10 kDa, inhibited the C3b dependent phagocytosis and killing of Aspergillus conidia by human peripheral blood monocytes. The factor did not appear to affect the CR1 or CR3 complement receptors on the monocyte's cell surface and the authors suggested that it may have acted by either inhibiting activation of the alternative complement pathway

or by preventing deposition of C3b onto the fungal cell surface. The soluble component had only a small effect on the activation of the classical complement pathway. A.flavus also released an active component(s) which behaved in the same manner whereas A.niger did not (Washburn et al, 1986). This finding may partially explain the more frequent observation of A.fumigatus and A.flavus as pathogens in IPA. In vivo production of this active component might increase pathogenicity by preventing the generation of opsonins and chemoattractants by the hosts immune system.

In addition to the soluble components released/produced by Aspergillus conidia, germinated hyphae secrete a large number of proteases eg glucoronidase, chymotrypsin, catalase and elastase (Reiss, 1986; Kurup et al, 1986a). These enzymes were probably required by the fungus for the degredation of protein substrates in the soil however, they may also act to potentiate pathogenicity in man (Jonsson and Martin, 1964; Miyaji and Nishimura, 1979). Indeed, Kothary et al (1984) have demonstrated that elastase producing strains of A.fumigatus caused increased mortality and were much more invasive in the lungs of cortisone treated mice than non-elastase producing strains. The authors concluded that elastase was a "significant virulence factor in infections caused by A.fumigatus".

A.fumigatus and A.flavus are also known to produce endotoxins (Tilden et al, 1961), mycotoxins such as

aflatoxin and fumagatin (Bardana, 1981), tremorgenic toxins (Yamazaki et al, 1971), oxalic acid (Benoit et al, 1985) and C substance (Longbottom and Pepys, 1964) all of which may play a part in the development of Aspergillus disease. However the specific role of these, and many other metabolites produced by aspergilli (Frisvad, 1987) in the pathogenesis of IPA remain unknown.

1.5 Host defences against aspergilli.

The production of so many virulence factors by the aspergilli begs the question: why does everybody not develop IPA? The reason is that humans have evolved a sophisticated response to this ubiquitous component of their environment.

On inhalation, the host is initially faced with metabolically inactive resting conidia. If this stage is not killed the host is then confronted with metabolically active swollen conidia (germlings) and finally an invasive hyphal organism that is too large to be ingested by phagocytic cells. At each stage the fungus displays distinct surface properties eg antigens which can elicit different host responses.

The role of phagocytic cells and acquired immunity in relation to host defence against aspergilli will now be discussed.

1.5.1 Alveolar macrophages. At 2-3.5μm Aspergillus conidia are extremely small; they are also very hydrophobic in nature (Cole et al, 1982). These two properties may help to explain the ease with which they can reach the terminal bronchi following inhalation where the first phagocytic cell encountered by them is the resident alveolar macrophage. These cells are capable of killing Aspergillus conidia (Schaffner et al, 1982; 1983; Waldorf et al, 1984; Kurup, 1984). Unopsonised conidia are bound to the alveolar macrophage via its cell surface mannose receptor (Kan and Bennett, 1988). Phagocytosis proceeds rapidly, within 15min, and is followed by phagolysosomal fusion. Killing of the conidia is achieved within 2h (Merkow et al, 1968; 1971).

Killing of Aspergillus conidia by alveolar macrophages proceeds both in the presence and absence of oxygen and must be myeloperoxidase (MPO) independent as these cells lose the ability to syntyhesize this enzyme in the process of maturation from blood monocytes. Levitz and Diamond (1984) have suggested that a ferrous ion-H_2O_2-iodide system is responsible for the killing of resting conidia by alveolar macrophages. They have shown that this cytotoxic system is comparable, in conidiacidal activity, to the MPO system of neutrophils when tested in cell-free conditions.

1.5.2 Polymorphonuclear leucocytes. PMNs are capable of phagocytosing resting Aspergillus conidia (Lehrer and Jan,

1970), but their ability to effect killing of these propagules has not been resolved. Two groups of workers have demonstrated that PMNs possess conidiacidal activity (Pagani et al, 1981; Fietta et al, 1984), whereas others have been unable to do so (Lehrer and Jan, 1970; Levitz and Diamond, 1985). However this point may be of academic interest only as resting conidia are incapable of inducing PMN chemotaxis. In contrast, swollen metabolically active germlings, which are highly susceptible to PMN killing, can activate the alternative complement pathway thereby inducing PMN migration (Waldorf and Diamond, 1985). Therefore it would appear likely that the majority of conidia would have swollen and become susceptible to PMN conidiacidal activity prior to large scale PMN migration into the lung.

If germlings are not removed then hyphae are produced and the host is faced with an organism that is too large to be phagocytosed. Aspergillus hyphae are not capable of activating complement but they do produce factors which are chemotactic for PMNs (Waldorf and Diamond, 1985). Once PMNs are recruited to the site of growing hyphae they can, in the absence of opsonins, spread over the fungal surface and cause cell damage and probably death (Diamond et al, 1978; Diamond and Clark, 1982; Schaffner et al, 1982). Peripheral blood monocytes are also capable of killing Aspergillus hyphae (Diamond et al, 1983).

The killing of Aspergillus hyphae and germlings by PMNs

is critically dependent on oxidative mechanisms. Anaerobiosis, catalase, histidine and tryptophan all inhibit hyphal killing thereby suggesting a primary role for MPO mediated mechanisms (Diamond and Clark, 1982; Schaffner et al, 1986). Cationic proteins prepared from human PMNs were not capable of killing Aspergillus hyphae (Diamond and Clark, 1982).

1.5.3 Acquired immunity. Only limited animal studies have been reported on the role of acquired immunity in host defence against IPA.

Two groups of workers have demonstrated a possible role for acquired immunity in host resistance to IPA. Lehmann and White (1976) demonstrated that mice which had been previously infected with A.fumigatus conidia were less susceptible to re-infection than mice which had not been previously infected. The exact nature of this acquired immunity was not stated but it was not humoral in origin. In a subsequent study Corbel and Eades (1977) proposed a role for T-lymphocyte dependent cell-mediated immunity in host resistance to IPA. This was based on their observation that adult mice were less likely than young mice from the same strain to develop IPA when treated with cortisone but were just as susceptible when treated with an anti-mouse lymphocyte serum which severely impaired lymphocyte function. However, Williams et al (1981) have shown that

congenitally athymic nude (nu/nu) mice, which lack normal T-lymphocyte function do not have an increased susceptibility to experimentally induced IPA compared to heterozygous (nu/+) mice which have functioning T-lymphocytes.

No work has been published on the role of acquired immunity in human resistance to IPA. Cell mediated immune responses have not been demonstrated in patients with ABPA (Slavin et al, 1987). However, perhaps the most compelling evidence that acquired immunity is not critical to host defences against IPA is the observation that patients infected with human immunodeficiency virus, which causes dysfunction of cells with the $CD4^{+}$ cell surface marker (mainly T-helper cells), do not develop IPA as an opportunistic infection (Schaffner, 1984).

1.6 Risk factors for the development of IPA.

Risk factors for the development of IPA can be divided into two categories: extrinsic ie environmental and intrinsic ie characteristics of the individual patient which result from treatment regimens or naturally occurring disease states.

1.6.1 Extrinsic risk factors. It is generally accepted that the human aquisition of *Aspergillus* conidia is via inhalation into the upper respiratory tract; therefore the presence of any conidia in the atmosphere of clinical areas

housing susceptible patients (see below) represents an extrinsic risk factor for the development of IPA.

The number of conidia in outdoor air shows a seasonal variation which peaks in the winter (Stix, 1977), possibly due to the increase in available substrates eg leaf litter. However, the ratio of conidia in outdoor, compared to indoor, air remains approximately 3.8:1 (Richards, 1954). Obviously any decrease in this ratio, brought about by an increase in the internal conidial load, for example due to building work (Barnes, 1988), faulty air-conditioning (Burton et al, 1972) or the presence of potted plants (Staib et al, 1985) represents an increase in this extrinsic risk factor. Aspergillus conidia have a low settling velocity (0.002m/sec). This may exacerbate the problem as once settled conidia have been disturbed they can remain airborne for long periods (Noble and Clayton, 1963) and are therefore more likely to be inhaled.

1.6.2 Intrinsic risk factors. IPA strikes across different age, sex and racial groups with equanimity.

The pivotal role of the alveolar macrophage and PMN in the hosts defences against Aspergillus infection, as described above, means that before invasive infection can become established the fungus must break through the double defence offered by these two types of phagocytic cell. Clearly, therefore any reduction in the concentration or

functional capacity of alveolar macrophages or PMNs will result in an increased risk for IPA.

Patients suffering from diseases in which phagocytic cell functions are impaired eg, chronic granulomatous disease are at increased risk for IPA (Cohen et al, 1981). The phagocytic cells of these patients are unable to generate reactive oxygen intermediates which results in a reduced capacity for microbial killing (Johnston and Baehner, 1971). However, their peripheral blood monocytes have been shown to kill Aspergillus hyphae and this may go some way to explaining the usually indolent nature of IPA seen in these patients (Cohen et al, 1981).

IPA is also an increasing problem in the immunocompromised host (Fraser et al, 1979) and is the major infective cause of death in some transplant centres (Barnes and Rogers, 1988). Patients with haematological malignancy (Meyer et al, 1973) and those receiving solid organ (Burton et al, 1972) or marrow transplants (Watson, 1983) are particularly at risk of developing IPA. These patients often receive immunosuppressive drugs eg busulphan, daunorubicin, cytosine arabinoside, 6-thioguanine and cyclophosphomide and/or high dose radiotherapy as part of their treatment regimens. A side effect of this therapy is the induction of profound neutropenia which is probably the single most important risk factor for the development of IPA (Barnes, 1988). Over 70% of patients contracting the infection have

PMN counts of 0.5 x 10^9/l or less (Young et al, 1970).

The duration of neutropenia is also an important risk factor (Myers and Atkinson, 1983), with the incidence of IPA rising from 1% per day after the initial drop in PMN concentration to 4.3% per day by day 24 of neutropenia (Gerson et al, 1984). In patients who suffer prolonged neutropenia, ie greater than 28 days duration, the incidence of IPA observed was above 50% (Gerson et al, 1985).

Furthermore, it has been demonstrated that the alveolar macrophages of marrow transplant recipients are replaced by those of the donor and initially do not function normally; in addition even when their PMN counts have risen these cells still function abnormally for some time (Winston et al, 1982). This continuing dysfunction of the phagocytic cells may account for the fact that in a review of bone marrow transplantation at 17 centres documented invasive fungal infection due to Apergillus spp was seen in 43/606 (7.1%) marrow recipients (Watson, 1983).

The long-term use of high dose adrenal corticosteroids to prevent and treat graft rejection or graft versus host disease is also an important risk factor for the development of IPA (Sandhu et al, 1970; Barnes, 1988). In a review of 98 cases of IPA, 87% had recieved corticosteroid therapy (Young et al, 1970). Use of these drugs does not prevent phagocytosis of the conidia (Schaffner et al, 1982) but, phagolysosomal fusion is prevented and killing completely

ablated (Merkow et al, 1968), thereby resulting in an increased risk for the individual patient.

1.7 Diagnosis of IPA.

If the treatment of IPA is to be successful then diagnosis, and initiation of antifungal therapy, has to be made early in the course of infection (Aisner et al, 1977; Burch et al, 1987). However, even with appropriate treatment, survival is not guaranteed (Young et al, 1970; DeGregorio, 1982). Failure to diagnose and treat almost always results in a fatal outcome.

The diagnosis of IPA however, has long been recognised as a major problem. Clinical symptoms are often non-specific or may even be absent during the early stages of infection (Herbert and Bayer, 1981). Microbiological cultures of blood (Young et al, 1974) and urine (Meyer et al, 1973) are usually negative, even with extrapulmonary dissemination. Culture positive respiratory specimens may represent colonization rather than invasion (Young et al, 1970; Meyer et al, 1973). The value of respiratory surveillance cultures for *Aspergillus* as an indicator of IPA remains unclear; in one study only 9% of patients with positive *Aspergillus* sputum cultures developed IPA (Fisher et al, 1981). However, two recent reports have suggested that the culture of aspergilli from respiratory specimens can be highly predictive of invasive disease (Yu et al, 1986; Meunier,

1987).

The generally poor diagnostic capability of microbiological and clinical methods has led to the development of numerous serological techniques for the diagnosis of IPA. These can be divided into two areas: a) the detection of specific anti-*Aspergillus* antibodies and b) the detection of *Aspergillus* antigens in body fluids.

1.7.1 *Antibody detection*. The usefulness of specific anti-*Aspergillus* antibody detection in the diagnosis of IPA is the subject of controversy. Young and Bennett (1971) could not detect anti-*A.fumigatus* antibodies in 15 haematological patients with proven infection. However, other workers have been more successful in detecting specific anti-*Aspergillus* antibodies in patients with IPA. The reported sensitivities of antibody detection as a method of diagnosing IPA, in various patient groups eg haematological malignancy and transplant recipients, has ranged from 33% (Gold et al, 1980) to 100% (Holmberg et al, 1980). Seroconversion, during infection, has also been documented in patients with IPA. Trull et al (1985) in a study of 19 renal transplant recipients with IPA observed seroconversion in 5/10 from whom serial specimens were available and, Schaefer et al (1976) showed seroconversion to anti-*A.fumigatus* antibody positivity in 7/10 patients. However, in the latter study seroconversion was also seen in six patients with no

evidence of fungal infection. The usefulness of antibody detection in the diagnosis of IPA has still to be proven. However, one would expect a reduced humoral response to the invading fungus in the majority of patients at high risk of developing IPA due to the nature of their underlying illness or treatment regimens.

1.7.2 Antigen detection. Demonstration of microbial antigen in body fluids has been used successfully as an alternative strategy to antibody detection in the serological diagnosis of other fungal infections notably cryptococcosis (Neill et al, 1951; Kauffman and Blumer, 1983), histoplasmosis (Wheat et al, 1986) and coccidioidomycosis (Weiner, 1983).

Detection of Aspergillus antigen in both human and experimental infection has been reported by many workers (Table VI.i).

The early work of one group (Lehmann and Reiss, 1978; Reiss and Lehmann, 1979) suggested that the circulating antigen was a galactomannan (GM) polysaccharide of approximate M_r 25-75 kDa which may have been associated with protein in vivo. The majority of work since has concentrated on carbohydrate and glycoprotein antigen fractions, usually prepared from A.fumigatus. Antigen is known to circulate in the ng/ml range and therefore sensitive serological techniques are necessary for detection. Enzyme-linked immunosorbent assay (ELISA) has

been the preferred method of many groups (Richardson et al, 1979; Sabetta et al, 1985; Dupont et al, 1987; Wilson et al, 1987; Fujita et al, 1988). The tests developed to date have a high specificity with only a few false positive results reported. However, sensitivities have varied from 18% (Dupont et al, 1987) to 100% (Reiss and Lehmann, 1979; Shaffer et al, 1979b).

IPA due to species of Aspergillus other than A.fumigatus have been diagnosed by some of these tests even though the detector antigens and antibodies were prepared from, or raised to, A.fumigatus (Weiner et al, 1983; Sabetta et al, 1985; Talbot et al, 1987; Wilson et al, 1987). This is understandable, as many workers have shown cross-reactivity between antigens of A.fumigatus and other aspergilli, in particular A.flavus (Kim and Chaparas, 1979; Shaffer et al, 1979a; Fujita et al, 1988).

1.8 Aims of this work.

Currently, a definitive diagnosis of IPA rests on the histological or microbiological demonstration of aspergilli in tissue biopsy specimens (Kahn and Jones, 1986a). Obtaining these specimens in the critically ill is often hazardous and can be associated with significant morbidity and occasional mortality. The performance of biopsy procedures may be contraindicated, therefore many cases of IPA are not diagnosed, or confirmed, until autopsy.

Improved methods of diagnosis are required as a matter of urgency. The work of this thesis has been directed at achieving this goal and mainly addresses the serological diagnosis of IPA.

I have carried out a study of 121 'high risk' patients undergoing marrow transplantation or remission induction therapy for leukaemia. 19 of these patients developed documented IPA and 12 others had clinical courses which were strongly indicative of invasive fungal infection.

I have assayed serial samples of serum from all these patients, by ELISA, for anti-A.fumigatus immunoglobulin (Ig) G antibodies to assess the contribution this may play in the serological diagnosis of IPA and analysed the data in relation to the conflicting reports that have appeared in the literature. The nature of each individual's anti-Aspergillus IgG profile was then determined, by immunoblotting, to see if this could aid diagnosis.

As previous reports have suggested that antigen detection may be a promising approach to the diagnosis of IPA, I have evaluated three different methods of antigen detection. Two ELISA inhibition assays (one based on a monoclonal antibody to A.fumigatus GM and the other on a high titre human anti-Aspergillus serum) and a latex agglutination test (incorporating another monoclonal antibody to A.fumigatus GM) were evaluated. The human anti-Aspergillus assay was included in order to determine if non-

GM antigens were present in specimens from patients with IPA.

The last part of the thesis deals with the characterization of the non-GM antigens present in the urine of patients with IPA which were revealed by the preceding ELISA work.

It is hoped that this work may go some way towards throwing light onto the grave and increasingly important question of IPA diagnosis.

CHAPTER TWO: PATIENTS.

2.1 Overall description.

121 patients seen either at the Westminster Hospital or the Westminster Childrens Hospital were entered into this study. The patients were under the clinical care of either Dr. K. Hugh-Jones, Dr. D. McCarthy, Dr. D. Samson or Professor A.J. Barrett to whom I am especially grateful for allowing me to study these patients.

All 121 patients became severely neutropenic (neutrophil count $<0.5 \times 10^9/l$) at some stage during hospitalization and underwent either bone marrow transplantation (73) or remission induction therapy (48). Their underlying diseases were haematological malignancies (79), inborn errors of metabolism (39), soft tissue malignancies (2) and congenital immunodeficiency (1).

The mean age of the patients was 17.8 years (range 0.4-63) and there were 59 males and 62 females.

Bacterial and viral infections occurred with a similar frequency to that reported from other units (Watson, 1983) although there was a reduced incidence of cytomegalovirus infections in these patients due to their lower age.

Patients could be placed into one of four groups based on clinical, microbiological and histological findings and these are described below.

2.2 Proven invasive aspergillosis (IPA).

Nineteen patients (nine male and ten female) with an average age of 15.2 years (range 0.5-63) developed IPA. Clinical details of these individuals are outlined in Table II.i and given in more detail in Figs 2.1.1 to 2.1.3.

In each of 14 patients the diagnosis of IPA was confirmed by demonstration of hyphae in and culture of an Aspergillus sp from deep tissue (eg lung) specimens obtained at post-mortem. In the remaining five patients a post-mortem was not performed and the diagnosis was based on the following criteria: i) fever >38°C for >5 days unresponsive to systemic antibacterial antibiotics and, ii) repeated isolation of the same Aspergillus sp from upper respiratory tract specimens (four patients) or arterial blood cultures (one patient) in the face of pulmonary infiltrates.

The causative organism of IPA was A.fumigatus (14 patients), mixed A.fumigatus/A.flavus (2), A.flavus (1), A.glaucus group (1) and the remaining isolate was not speciated.

2.3 Clinical fungal infection (CFI).

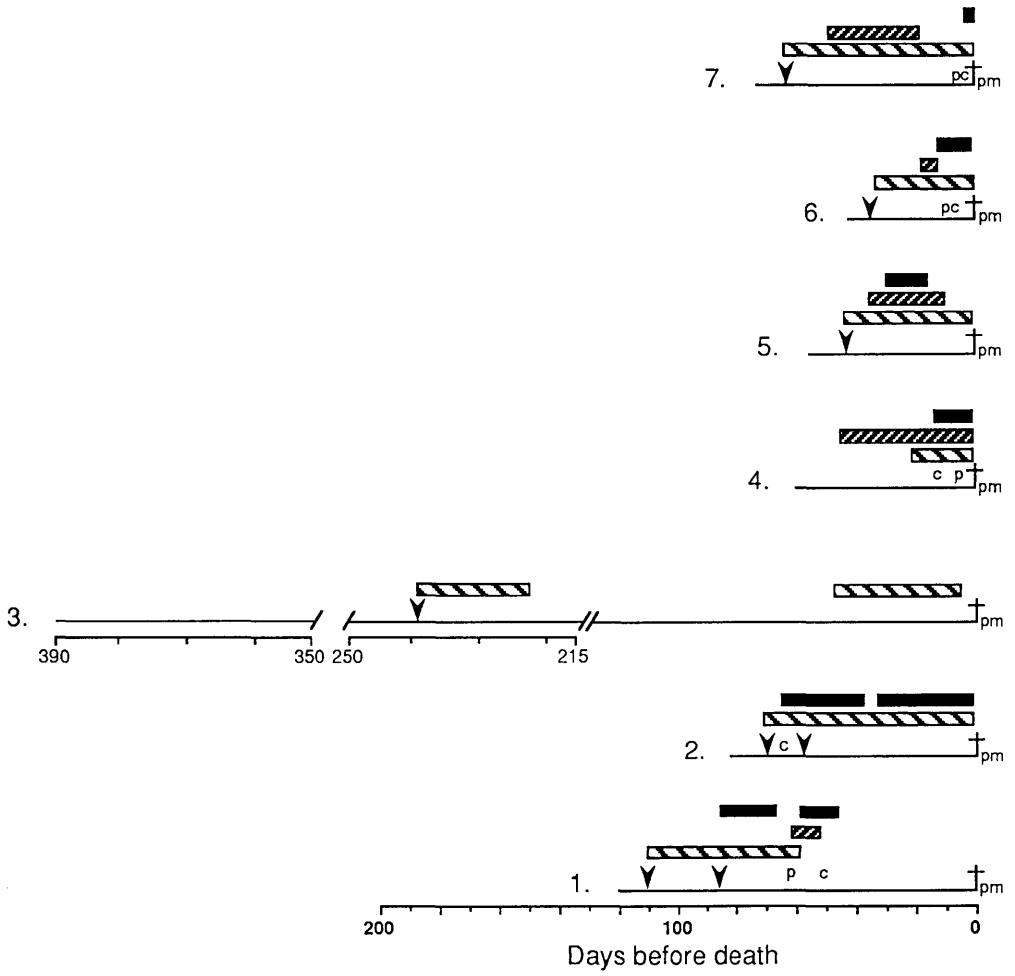
Twelve patients (8 male and 4 female) were included in the CFI group based on the following criteria: i) fever >38°C for >5 days unresponsive to systemic antibacterial antibiotics and, ii) failure to demonstrate any bacterial, viral or protozoal pathogen (Barnes and Rogers, 1988). In nine of these patients pulmonary

Table II.i Details of patients with proven invasive aspergillosis (IPA).

Patient	Age yrs	Sex	Underlying Disease	Performance, administration or detection of: BMT	Steroids	Pul Inf	Anti-Fungals	Site/Species Aspergillus cultured	
1	0.5	F	Osteopetrosis	+	+	+	+	pericardial fluid PM	glaucus group
2	39	F	AML	+	-	-	+	nasal biopsy PM	flavus
3	21	M	ALL	+	-	-	-	PM	fumigatus
4	5	F	ALL	-	+	+	+	sputum PM	fumigatus
5	3	F	Hurlers	+	+	-	+	PM	fumigatus
6	34	F	ALL	+	+	+	+	sputum PM	fumigatus
7	9	F	Gauchers	+	+	+	+	sputum PM	fumigatus
8	13	F	ALL	+	+	-	+	PM	unspec
9	2	F	Beta thal	+	+	+	-	pharyngeal wash	fumigatus
10	0.5	F	Hurlers	+	+	+	+	BAL PM	fumigatus
11	63	M	ALL	-	-	+	+	BAL	fumigatus
12	2	M	AML	-	-	+	+	PM	fumigatus
13	4	M	Fanconis	+	+	-	+	BAL PM	fumigatus
14	2	M	Hurlers	+	-	+	+	BAL	fumigatus flavus
15	3	M	Myelodysplasia	+	-	+	+	arterial blood cult	fumigatus
16	11	M	ALL	+	+	+	+	bron asp PM	fumigatus
17	32	M	CML	+	+	-	+	bron asp PM	fumigatus
18	9	F	ALL	+	+	+	-	sputum PM	fumigatus flavus
19	37	M	ALL	+	-	+	+	bron asp	fumigatus

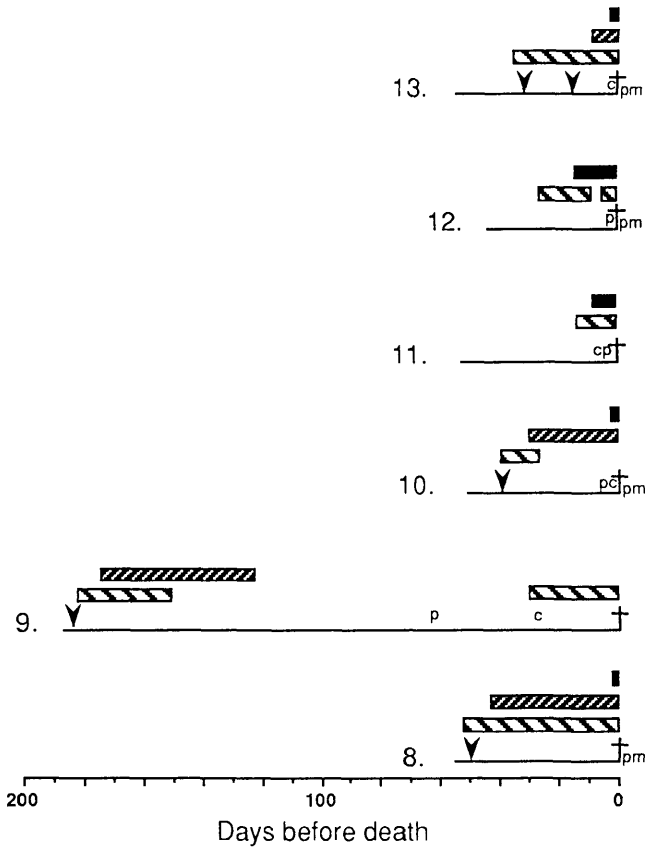
key. AML, acute myeloid leukaemia; ALL, acute lymphoblastic leukaemia; Beta Thal, beta thalassemia major; CML, chronic myeloid leukaemia; Pul Infs, pulmonary infiltrates; PM, post mortem tissue specimen; BAL, bronchoalveolar lavage fluid; bron asp, bronchial aspirates; unspec, unspeciated; +, performed, administered or detected; -, not performed, not administered or not detected.

Figure 2.1.1 Clinical details of IPA patients numbers 1-7.



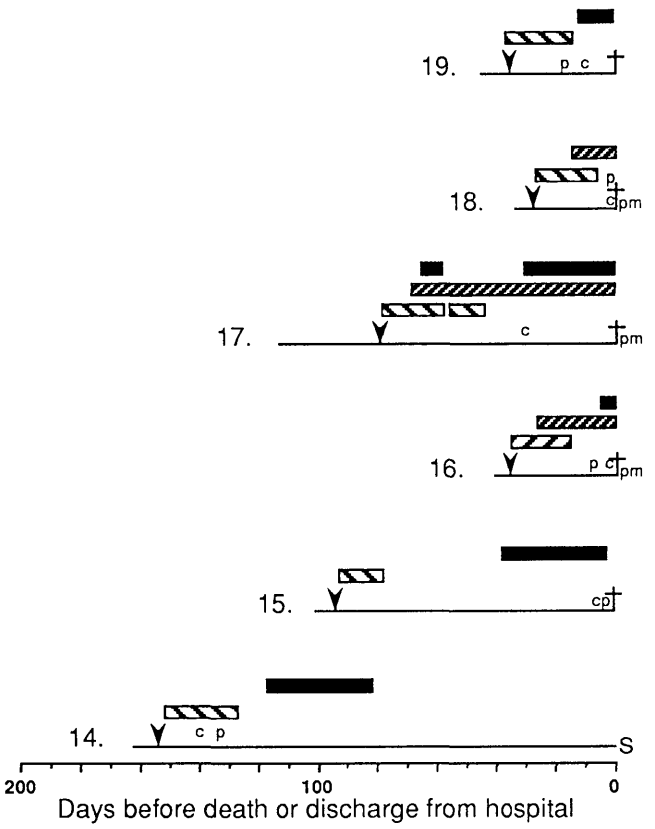
Y, bone marrow transplant; p, first appearance of pulmonary infiltrates; c, first culture of Aspergillus; pm, post-mortem culture of Aspergillus; † death; periods of neutropenia; steroid administration; empirical antifungal therapy. figures indicate patient number.

Figure 2.1.2 Clinical details of IPA patients numbers 8-13.



Y, bone marrow transplant; p, first appearance of pulmonary infiltrates; c, first culture of Aspergillus; pm, post-mortem culture of Aspergillus; † death; periods of neutropenia; steroid administration; empirical antifungal therapy. figures indicate patient number.

Figure 2.1.3 Clinical details of IPA patients numbers 14-19.



Y, bone marrow transplant; p, first appearance of pulmonary infiltrates; c, first culture of Aspergillus; pm, post-mortem culture of Aspergillus; s, survival and release home; †, death; periods of neutropenia; steroid administration; empirical antifungal therapy.
figures indicate patient number.

infiltrates were documented at the same time.

The average age of these patients was 20.2 years (range 2-52) and their clinical details are given in Table II.ii and Figs 2.2.1 and 2.2.2. Fungus was not cultured from any of these patients.

2.4 Other fungal infections (OFI).

Eight patients (three male and five female) with an average age of 19.3 years (range 1-51) were included in this group. Seven of the patients had disseminated invasive candidosis (all Candida albicans) and the other had invasive mucormycosis caused by Rhizpous oryzae (this fungus has been entered into the National Collection of Pathogenic Fungi as NCPF 2712). Each case was documented by isolation of the fungus from deep tissue specimens obtained at post-mortem. In no patient was an Aspergillus spp isolated at any time.

Clinical details of these patients are given in Table II.iii.

2.5 No evidence of fungal infection (NEFI).

This was the largest group and contained 82 patients (39 male and 43 female) with an average age of 16.8 years (range 0.4-61).

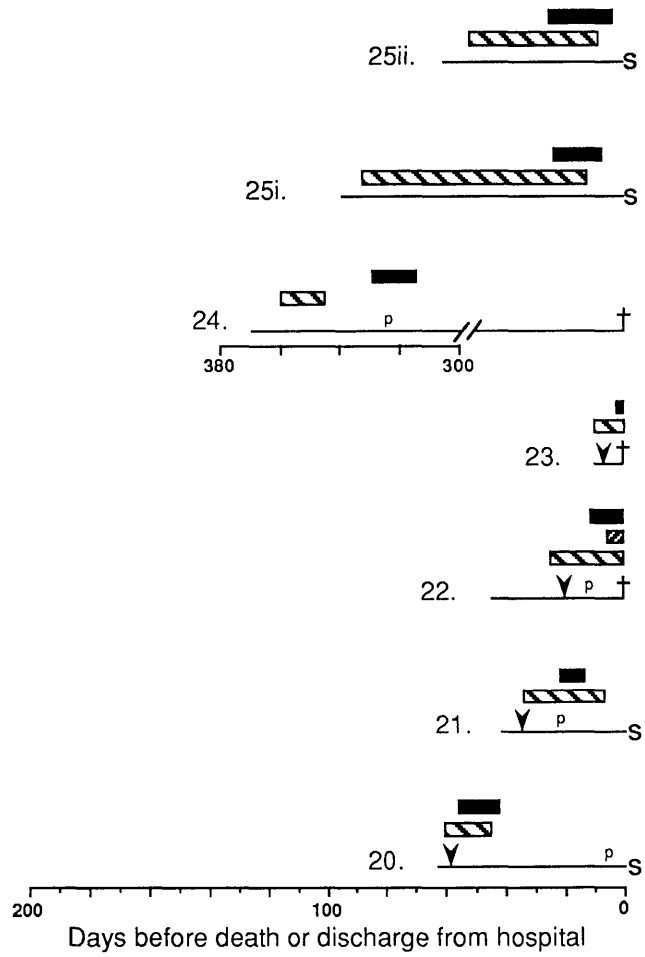
Of these 82 patients, 53 had haematological disorders; acute lymphoblastic leukaemia (30), acute myeloid leukaemia (14), chronic myeloid leukaemia (3), Hodgkins disease (2), aplastic

Table II.ii Details of patients with clinically suspected fungal infection (CFI).

Patient	Age yrs	Sex	Underlying Disease	Performance, administration or detection of			
				BMT	Steroids	Pul Infs	Anti-fungals
20	18	F	ALL	+	-	+	+
21	5	M	Beta thal	+	-	+	+
22	4	F	Hurlers	+	+	+	+
23	37	M	ALL	+	-	-	+
24	38	F	Myeolo-dysplasia	-	-	+	+
25*	52	M	AML	-	-	-	+
26	4	M	Beta thal	+	+	+	+
27	20	M	AML	-	-	+	+
28	20	F	AML	-	-	-	+
29	2	M	ALL	+	-	+	+
30	36	M	ALL	+	-	+	+
31	7	M	ALL	-	-	+	+

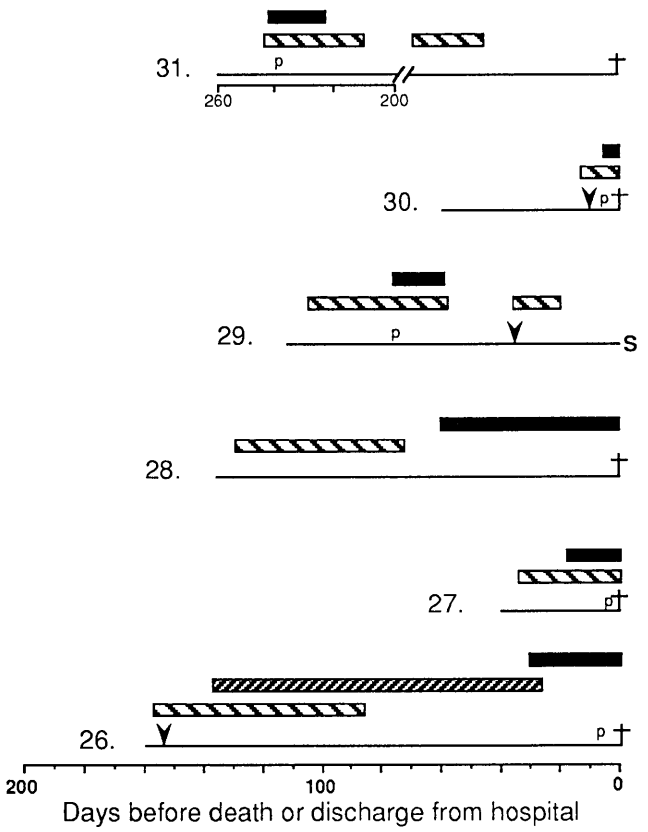
key. see Table II.i
* this patient had two separate clinically suspected fungal infections.

Figure 2.2.1 Clinical details of CFI patients numbers 20-25.



Y, bone marrow transplant; p, first appearance of pulmonary infiltrates; c, first culture of Aspergillus; pm, post-mortem culture of Aspergillus; s, survival and release home; †, death; periods of neutropenia; steroid administration; empirical antifungal therapy.
figures indicate patient number.
patient number 25 had two episodes of clinically suspected fungal infection.

Figure 2.2.2 Clinical details of CFI patients numbers 26-31.



Y, bone marrow transplant; p, first appearance of pulmonary infiltrates; c, first culture of Aspergillus; pm, post-mortem culture of Aspergillus; s, survival and release home; †, death; periods of neutropenia; steroid administration; empirical antifungal therapy.
figures indicate patient number.

Table II.iii Details of patients with other fungal infections (OFI).

Patient	Age	Sex	Underlying Disease	Performance, administration or detection of: BMT	Steroids	Pul Infs	Anti-fungals	Infection	Organism
32	9	F	ALL	+	+	-	+	Cand	C.alb
33	9	M	AML	+	+	+	+	Cand	C.alb
34	12	F	ALL	+	-	-	+	Cand	C.alb
35	3	F	Hurlers Synrome	+	+	-	+	Cand	C.alb
36	0.6	F	Hurlers Syndrome	+	+	-	+	Cand	C.alb
37	29	F	AML	-	-	-	+	Cand	C.alb
38	51	M	ALL	+	+	-	+	Cand	C.alb
39	40	F	CGL	-	+	+	+	Mucor	R.oryzae

key. Cand, invasive candidosis; Mucor, invasive mucormycosis; C.alb, Candida albicans; R.oryzae, Rhizopus oryzae; CGL, chronic granulocytic leukaemia. see table II.i for rest of key.

anaemia (1), chronic granulocytic leukaemia (1), non Hodgkins lymphoma (1), myeloma (1); 26 had inborn errors of metabolism; Beta thalassemia major (8), Hurlers syndrome (8), Gauchers disease (5), Lesch Nyham syndrome (1), Maroteaux Lamy syndrome (1), metachromatic leukodystrophy (1), osteopetrosis (1), Wiskott Aldrich syndrome (1); 2 had soft tissue malignancies; ewings syndrome and neuroblastoma stage III and the last patient had severe combined immunodeficiency.

Forty four underwent bone marrow transplantation and the other 38 received remission induction therapy.

CHAPTER THREE: GENERAL METHODS.

This chapter contains methods and the composition of reagents which are used throughout this thesis. All other methods, which are unique to certain portions of the work, are contained within individual chapters.

All single component buffers and reagents are described in the text while others are referred to by a code in parentheses eg "{3.10.1}". These buffers and reagents are listed in section 3.10 below.

3.1 Fungal strains.

The following fungal strains were used for the production of various antigenic fractions during the course of this work: *Aspergillus fumigatus* NCPF 2109

Aspergillus fumigatus 1028

Aspergillus flavus NCPF 2008.

The NCPF strains were received from the National Collection of Pathogenic Fungi, Mycological Reference Laboratory, Public Health Laboratory Service, Colindale, London (MRL).

Strain 1028 was obtained from Dr. J-P. Latge, Unite de Mycologie, Institut Pasteur, Paris, France.

3.2 Culture of fungi for preparation of antigen fractions.

For the production of the water soluble and culture

filtrate antigens A.fumigatus NCPF 2109 and A.flavus NCPF 2008 were cultured in a 2% (w/v) glucose, 1% (w/v) special peptone liquid medium.

Aspergillus conidia, which had been stored frozen at -70°C in 10% (v/v) glycerol, were thawed, inoculated onto Sabourauds dextrose agar slopes and incubated at 37°C for 48h. Conidia were harvested into a small amount of sterile distilled water containing 0.01% (v/v) Tween 20, filtered through sterile gauze, to remove mycelial debris, counted in a haemocytometer and suspended at 10^6/ml in sterile distilled water.

One ml of conidial suspension was added to 300ml of culture medium and incubated at 37°C for 72h with constant aeration (this was achieved by use of a magnetic flea stirring at 100 revolutions per minute [rpm]). This starter culture was transferred to 3l of the same medium and incubated for a further four days in the same manner.

The mycelial mat was separated from the culture medium by filtering through Whatman No. 1 paper (Whatman UK, Maidstone, Kent) on a buchner funnel. The mycelial mat was washed with at least 3l of sterile distilled water and frozen at -70°C until use. The culture filtrate was also retained.

A.fumigatus NCPF 2109 and A.fumigatus 1028 were also grown in a fermenter system to provide culture filtrate for preparation of ethanol precipitate antigens.

Freshly prepared conidia (10^6) were inoculated into 50ml of Sabouraud liquid medium and incubated at 25°C for 72h, with stirring at 100 rpm. This was added to 1.4l of the same medium in a 2l fermenter (Bio-Lafitte, Poissy, France) and cultured for a further 48h at 25°C with an air supply of 0.5l/min and stirring at 600 rpm. This starter culture was used to seed 20l of Sabouraud liquid medium in a fermenter (Chemap, Zurich, Switzerland). This culture was incubated as above for 42h to 45h. The culture medium was separated from the mycelia by filtration through Whatman No.1 paper, dialysed extensively against distilled water, filtered (0.5µm, Flow Laboratories, Rickmansworth, Herts) and lyophilised. The fermentation work was done in the laboratory of Dr. J-P. Latge.

3.3 Preparation of Aspergillus antigen fractions.

Antigen fractions from all Aspergillus spp were prepared in the same way.

Water soluble and culture filtrate antigens were prepared from A.fumigatus NCPF 2109 (designated WS2109 and CF2109 respectively) and A.flavus NCPF 2008 (WS2008 and CF2008).

Ethanol precipitate antigens were prepared from A.fumigatus NCPF 2109 (EP2109) and A.fumigatus 1028 (EP1028). Concanavalin A (conA) bound antigens were prepared from WS2109 (BF2109). GM was purified from EP1028.

The protein to carbohydrate ratios for all antigen fractions, except GM which contained <1.0% N (J-P. Latge, personal communication), are shown in Table III.i.

3.3.1 Culture filtrate antigen (CF). Immediately after culture the filtrate from seven day flask cultures of A.fumigatus NCPF 2109 and A.flavus NCPF 2008 were centrifuged (12,000 x g for 30min), dialysed extensively against distilled water, centrifuged again (12,000 x g for 30min) and filtered (0.5µm, Flow Laboratories). The CF was freeze dried and stored lyophilised until use. CF from the fermenter cultures of A.fumigatus NCPF 2109 and A.fumigatus 1028 were prepared in the same way.

3.3.2 Water soluble antigen (WS). The frozen mycelial mat from flask cultures of A.fumigatus NCPF 2109 and A.flavus NCPF 2008 were allowed to thaw thoroughly at room temperature before antigen preparation began. 50-100g wet weight of mycelium was suspended in 200ml of pre-cooled (4°C) volatile buffer [0.4% (w/v) NH_4HCO_3, p.H. 7.3] and homogenised in a kitchen blender at maximum speed for 30sec. This was repeated three times. The resulting mycelial slurry was increased in volume to 500ml by the addition of further pre-cooled volatile buffer.

This mixture was added at a ratio of 2:1 to pre-cooled

Table III.i Protein to carbohydrate ratios for antigen fractions used in this study.

Antigen Fraction	Protein:carbohydrate ratio
WS2109*	1.64
WS2008	2.31
EP2109	2.01
EP1028	2.80
CF2109*	0.41
CF2008	0.62
BF2109*	0.33

All ratio results were obtained from a single culture except those marked * which were the mean ratios from three different antigen batches.

0.1mm diameter glass ballontini beads in a 250ml teflon container, placed into a Vi-4 cell disintegrator (Edmund Buhler, Tubingen, West Germany) and disrupted for 4min. The cell disintegrator was cooled to -10°C throughout.

Mycelial damage was checked by light microscopy. If >80% of mycelial walls were disrupted the fungal extract was separated from the beads on a sintered glass funnel (each 250ml bead-slurry mixture was washed with 100ml of cold sterile distilled water), if not the mixture was shaken for a further 2min and then separated.

The fungal extract was centrifuged for 60min at 12,000 x g, the resulting supernatant was carefully removed and dialysed extensively against sterile distilled water at 4°C and filtered (0.5µm, Flow Laboratories). The resulting extract was lyophilised and designated WS.

3.3.3 Ethanol precipitate antigen (EP). CFs from the fermenter cultures of A.fumigatus NCPF 2109 and A.fumigatus 1028 were prepared as described in section 3.3.1 except that the extracts were not freeze dried. These antigens were concentrated approximately x10 under vacuum on a Rotavapor (Laboratoriums-Technik AG, Flawil, Switzerland), filtered through Whatman No.1 paper, centrifuged at 12,000 x g for 30min and mixed with four volumes of ice cold 95% ethanol. This mixture was allowed to stand at -20°C overnight and then centrifuged at 12,000 x g for 20min. The resulting

precipitate was washed three times with ice cold 95% ethanol, air dried and then lyophilised.

3.3.4 ConA bound antigen (BF). 100mg of WS2109 antigen was reconstituted into 5ml of starting buffer {3.10.1}. ConA Sepharose 4B (Sigma Chemical Co., Poole, Dorset) was washed on a sintered glass funnel with ten volumes of the same buffer and then mixed with the reconstituted WS2109 fraction. The mixture was rotated end over end for 18h at 4°C and placed into a disposable polypropylene column with a 35µm porous polyethylene bed support (Bio-Rad, Watford, Herts).

Unbound molecules were eluted in starting buffer at a flow rate of 0.5ml/min and 3ml fractions were collected in an LKB 7000 Ultrarac fraction collector (LKB Instruments Ltd., Croydon, Surrey). The absorption at 280nm (A_{280})of each of the fractions was measured in a Lambda 5 UV/VIS spectrophotometer (Perkin Elmer Ltd., Beaconsfield, Bucks.). When the A_{280} was equal to background levels the column was washed with ten volumes of starting buffer.

Bound molecules were eluted with eluting buffer A {3.10.2} at a flow rate of 0.25ml/min. Fractions were collected and monitored as above.

Fractions that bound to conA and had an A_{280} greater than background levels were pooled and freeze dried.

3.3.5 Galactomannan. 50mg of EP1028 was placed into a large glass test tube and covered with approximately 10ml of anhydrous hydrazine. This was heated at 100°C for 24h, cooled and the hydrazine poured off. The precipitate was washed with 95% ethanol and centrifuged at 12,000 x g for 15min and dissolved into 20ml of distilled water. 550mg of $NaNO_2$ and 3ml of glacial acetic acid were added and the mixture was left at room temperature overnight. The resulting precipitate was dialysed against running tap water for 48h and against three changes of distilled water over a 24h period.

The hydrazine and $NaNO_2$ extraction procedure was repeated and after extensive dialysis against distilled water the dialysate was filtered (0.2μm, Flow Laboratories) and freeze dried. This was designated GM.

3.4 Determination of protein content.

Protein concentrations were determined by a modification of the Coomassie blue dye binding method proposed by Spector (1978).

The reagent was prepared by adding 100mg of Coomassie Blue G250 (Sigma Chemical Co.) to 50ml of 95% ethanol. This was mixed with 100ml of 85% phosphoric acid, made up to 1l with distilled water, filtered through Whatman No.1 paper and stored in a dark bottle at room temperature. The reagent was discarded after two weeks.

10µl of sample was mixed with 125µl of the reagent in a microtitre plate and mixed on a rotor for 20min. The absorbance at 595nm (A_{595}) was read in a Titertek multiscan spectrophotometer (Organon Technica, St. Neots, Cambs).

Standards from 0-200mg/ml of non fat milk (Sainsbury's, London) were run with each assay and a standard curve of the A_{595} versus concentration was used to determine the protein content of test samples.

3.5 Determination of carbohydrate content.

Carbohydrate concentration was determined by the department of Chemical Pathology, Westminster Hospital using the hexokinase method ie:

Glucose + ATP -----> Glucose 6 phosphate + ADP

Glucose 6 Phosphate + NADP -----> Gluconate 6 phosphate + NADPH.

The reduction of NADP to NADPH was measured at 340nm in a Hitachi 717 multiple analyser (Hitachi Instruments, Japan).

3.6 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE).

SDS-PAGE was performed according to the discontinuous buffer system of Laemmli (1970).

1.5mm thick, 15% acrylamide resolving gels {3.10.3} were cast in a Protean II minigel electrophoresis system

(Bio-Rad). The resolving gel was overlayed with a 4% acrylamide stacking gel {3.10.4}. All reagents used in the preparation of SDS-PAGE gels were of electrophoresis grade and purchased from Bio-Rad.

100µl of substrate to be electrophoresed was added to 25µl of x5 sample buffer {3.10.5} and 10µl of 0.1% (w/v) bromophenol blue. The samples were boiled for 5min, cooled and centrifuged at 10,000 x g for 2min. 30µl of the sample supernatant was added to each well.

Running buffer {3.10.6} was stored, at 4°C, as a x5 stock solution and diluted with distilled water just prior to use. Buffer was discarded after each run. The same buffer was used in both upper and lower reservoirs.

Electrophoresis was carried out at 175V until the dye front reached the bottom of the gel (approximately 45min).

3.7 Electroblotting of SDS-PAGE gels.

Electroblotting of the separated antigen from SDS-PAGE gels was done onto polyvinylidene difluoride, PVDF, (Immobilon, Millipore, Bedford, MA, USA) membranes essentially as described by Towbin et al (1979).

Gels were equilibrated in transfer buffer {3.10.7} for 15min. A scotchbrite pad was wetted in transfer buffer and laid onto the black side of a blotting cassette (Bio-Rad). Wet filter paper was placed on top of the pad and the acrylamide gel was laid on top of this. Great care was taken

to ensure the gel was not distorted. The PVDF membrane was rinsed in 100% methanol, washed in distilled water and transfer buffer and then placed onto the gel. All air bubbles were removed by gentle pressure in order to ensure good contact between the gel and the nylon. The blotting sandwich was completed with another layer of filter paper and a second scotchbrite pad and the cassette was then closed.

The cassette was placed into a Transblot cell (Bio-Rad) containing approximately 3l of transfer buffer. The gel was orientated towards the negative electrode and transfer effected at 30V overnight followed by 60V for 1h.

5l of transfer buffer was prepared at a time, and the 3l used on each run was returned to the remaining 2l. The buffer was stored at 4°C and used for up to five runs.

3.8 Staining of blots.

3.8.1 Ponceau-S stain. Transfer efficiency was checked by staining blots with 0.3% (w/v) Ponceau-S (Sigma Chemical Co.) in 0.3% (w/v) trichloroacetic acid (TCA). Blots were stained for 5min on a gel rocker. Partial destaining was effected with 0.3% (w/v) TCA. At this stage the M_r standards (see 3.9) run on each gel were cut from the blot and dried between filter paper sheets. The rest of the blot was totally destained with three changes of phosphate buffered

saline (PBS) {3.10.8}.

3.8.2 India ink stain. Blots were placed into 0.1% (v/v) India ink (Pelikan AG, Hannover, Germany) in PBS containing 0.05% (v/v) Tween 20 (PBST) and incubated at room temperature (approximately 23°C), on a gel rocker, for 1h. Blots were destained in two changes of PBST over 4h and dried between filter paper sheets.

3.9 Estimation of molecular mass.

M_r standards were included on every gel. These were phosphorylase b, 97.4 kDa; bovine serum albumin, 66.2 kDa; egg albumin, 45 kDa; carbonic anhydrase, 31 kDa;. soybean trypsin inhibitor, 21.5 kDa and lysozyme, 14.4 kDa.

The distance migrated by each of the standards from the start of the resolving gel was measured on the Ponceau-S stained blot and used to construct a graph of M_r versus distance migrated using the software programme 'Cricket Graph' (Cricket Software, Malvern, Pennsylvania, USA). The M_r of unknown bands was determined by measuring the distance they migrated and referring to the graph. An exponential curve was fitted to the data to allow determination of M_r of unknown molecules just outside the range of the standards. M_r determination of each band was taken as the average of at least three runs.

3.10 Buffer and reagent composition.

3.10.1 Starting buffer.

1mM Tris-HCl, pH 7.2
1mM $CaCl_2$
1mM $MnCl_2$
0.25M NaCl

3.10.2 Eluting buffer A.

1mM Tris-HCl, pH 7.2
1mM $CaCl_2$
1mM $MnCl_2$
0.25M NaCl
0.2M methyl alpha-D-mannopyranoside

3.10.3 15% polyacrylamide gel composition.

5.0ml	3.0M Tris-HCl pH 8.0
10.0ml	30% (w/v) acrylamide
2.0ml	1% (w/v) bis-acrylamide
2.6ml	distilled water
200.0µl	10% (w/v) sodium dodecyl sulphate

These reagents were added and the mixture was thoroughly degassed in a side arm flask using a venturi pump as a vacuum source. The mixture was polymerised with:

200.0µl	10% (w/v) ammonium persulphate
30.0µl	N,N,N',N'-Tetramethylethylenediamine (TEMED).

3.10.4 4% polyacrylamide gel composition.

2.5ml	1.0M Tris-HCl pH 6.8
1.0ml	30% (w/v) acrylamide
1.0ml	1% (w/v) bis-acrylamide
5.4ml	distilled water
100.0µl	10% (w/v) sodium dodecyl sulphate

These reagents were added and the mixture was thoroughly degassed in a side arm flask using a venturi pump as a vacuum source. The mixture was polymerised with:

100.0µl	10% (w/v) ammonium persulphate
20.0µl	TEMED.

3.10.5 Sample buffer.

345 mg sodium dodecyl sulphate
5.1 ml glycerol
2.0 ml 2-mercaptoethanol
3.0 ml 1.0M Tris-HCl pH 6.8

This was made as a x5 stock solution and added to substrate to be electrophoresed at a ratio of 1:4.

3.10.6 Running buffer.

5.0 g sodium dodecyl sulphate
72.0 g glycine
15.0 g Tris base

This was made as a x5 stock solution and diluted 1:5 just prior to use.

3.10.7 Transfer buffer.

25mM Tris base
192mM glycine
20% (v/v) methanol

This was made 5l at a time and used for upto 5 runs.

3.10.8 Phosphate buffered saline (PBS).

80.0 g NaCl
11.5 g Na_2HPO_4
2.0 g KCl
2.0 g KH_2PO4

This was made as a x10 stock solution and diluted 1:10 just prior to use.

3.10.9 Carbonate buffer (0.06M, pH 9.6).

4.53 ml 1.0M $NaHCO_3$
1.82 ml 1.0M Na_2CO_3

This was made up to 100ml with distilled water.

3.10.10 Phosphate, citric acid buffer (0.07M, pH 5.0).

24.7 ml 0.1M citric Acid
25.3 ml 0.2M Na_2HPO_4

This was made up to 100ml with distilled water.

3.10.11 Phosphate buffer (50mM, pH 7.4).

81.0 ml 0.2M Na_2HPO_4
19.0 ml 0.2M NaH_2PO_4

This was made up to 400ml with distilled water.

3.10.12 Coupling buffer.

0.1M $NaHCO_3$ pH 8.3
0.5M NaCl

3.10.13 Acetate buffer (0.1M, pH 4.0).

0.1M $CH_3COO.Na$ pH 4.0
0.5M NaCl

3.10.14 Tris buffer (0.1M, pH 8.0).

0.1M Tris-HCl, pH 8.0
0.5M NaCl

3.10.15 20% polyacrylamide gel composition.

5.0ml	3.0M Tris-HCl pH 8.0
12.6ml	30% (w/v) acrylamide
2.0ml	1% (w/v) bis-acrylamide
200.0µl	10% (w/v) sodium dodecyl sulphate

These reagents were added and the mixture was thoroughly degassed in a side arm flask using a venturi pump as a vacuum source. The mixture was polymerised with:

200.0µl	10% (w/v) ammonium persulphate
30.0µl	TEMED.

3.10.16 Eluting buffer B.

5mM	glycine-HCl pH 2.3
0.5M	NaCl
0.05% (v/v)	Tween 20

CHAPTER FOUR: DETECTION OF ANTI-ASPERGILLUS ANTIBODIES BY ENZYME-LINKED IMMUNOSORBENT ASSAY.

4.1 Introduction.

Detection of specific antibodies has proved useful in the diagnosis of many fungal diseases e.g. histoplasmosis (Sathapatayavongs et al, 1983) and coccidioidomycosis (Drutz and Catanzaro, 1978).

This approach has also been used in an attempt to diagnose the various forms of aspergillosis. However it is complicated by the fact that aspergilli are ubiquitous fungi and the majority of humans have a low, but detectable, level of specific anti-*Aspergillus* antibodies (Baradana et al, 1972b; Schonheyder and Andersen, 1982; 1984b; Richardson et al, 1984). Methods therefore need to be sensitive enough to differentiate between this background level and any possible increase in specific anti-*Aspergillus* antibodies seen in *Aspergillus* related infections. To this end a large number of antigen preparations including culture filtrates (metabolic) (Longbottom and Pepys, 1964; Kauffman et al, 1983; van der Heide et al, 1984), mycelial extracts (somatic) (Malo et al, 1977; Mohan et al, 1980; Wilson and Hearn, 1983a), acid precipitated fractions (Bardana et al, 1972a) and intact germlings or young hyphae (Warnock, 1974; Gordon et al, 1977; Schonheyder et al, 1982) have been extensively studied in a large number of test systems for the serodiagnosis of non-invasive pulmonary aspergillosis by

the detection of specific anti-Aspergillus antibodies (Table IV.i). These methods have included double diffusion (Longbottom and Pepys, 1964; Calvanico et al, 1981; Kauffman et al, 1983; Piechura et al, 1983), counter-immunoelectrophoresis (CIE) (Malo et al, 1977; Kauffmann and deVries, 1980b) indirect immunofluoresence (Gordon et al, 1977; DeMagaldi and Mackenzie, 1984), radioimmunoassay (RIA) (Patterson et al, 1978; Dewair and Baur, 1984) and ELISA (Richardson et al, 1982; 1983; Wilson and Hearn, 1983b; Khan et al, 1984a; 1984b; Kauffmann et al, 1986).

The heterogeneity of methodologies and reagents employed by different laboratories makes comparisons of results difficult. However, approximately 75-100% of patients with aspergilloma and 66-100% with ABPA have been reported to have elevated levels of specific anti-Aspergillus IgG antibodies (Hearn, 1988) (Table IV.i). Patients with ABPA also have a raised level of specific anti-Aspergillus IgE (Brummond et al, 1987).

The presence of specific antibodies can therefore give valuable information to clinicians when clinical and/or radiological evidence suggests a diagnosis of non-invasive aspergillosis.

The invasive form of aspergillosis however, differs from the mycetomatous and allergic conditions in that the majority of patients who develop the infection are severely immunocompromised either as a result of their underlying

Table IV.i Detection of specific anti-Aspergillus IgG antibodies in non-invasive pulmonary aspergillosis.

Reference	Detection method	Antigen preparation	Patients pos/tested	Controls pos/tested	Sens	Spec
Longbottom 64	DD	CF	56/57	14/185	98	92
''	IE	CF	56/57	11/185	98	94
Bardana 72b	ABA	TCA	21/26	3/54	81	94
Warnock 74	IIF	germlings	15/15	0/41	100	100
Malo 77	DD	CF x 3	42/50	1/24	84	96
Malo 77	CIE	CF x 3	36/46	1/24	78	96
Patterson 78	RIA	AS	30/38	0/19	79	100
Kim et al 79	DID	various	18/19	0/20	95	100
Calvanico 81	DD	glycoprotein	24/32	0/11	75	100
Mishra 83	ELISA	AS	35/35	5/123	100	96
Khan 84b	ELISA	CF	15/15	0/50	100	100
DeMagaldi 84	ELISA	ME	26/31	1/30	84	97
Schonheyder 84a	ELISA	470 kDa	24/26	0/113	92	100
''	ELISA	250 kDa	25/26	0/113	94	100
''	ELISA	<50 kDa	25/26	0/113	94	100
Kauffmann 86	ELISA	CF	20/20	0/8	100	100
Kurup 86	ELISA	CF	28/28	0/20	100	100
Harvey 86b	ELISA	Ag 7	29/30	1/15	97	93
Harvey 87	ELISA	Ag 13	25/25	5/30	100	83
Brummond 87	ELISA	CF	25/25	0/18	100	100
Leung 88	dot blot	ME	22/22	0/10	100	100

key. pos, positive; Sens, sensitivity; Spec, specificity; DD, double diffusion; IE, immunoelectrophoresis; ABA, antigen binding assay; IIF, indirect immunofluoresence; CIE, counter immunoelectrophoresis; RIA, radioimmunoassay; DID, double immunodiffusion; ELISA, enzyme linked immunosorbent assay; CF, culture filtrate; TCA, trichloroacetic acid precipitate; AS, ammonium sulphate precipitate; ME, mycelial extract.

diseases or because of immunosuppresive therapeutic regimens (Rinaldi, 1983). These patients usually have a poor humoral response to infectious agents during immunosuppression, but patients suffering from acute leukaemias (though not lymphomas, chronic leukaemias or myelomas) generally recover their ability to mount an antibody response when immunosuppression is stopped (Libansky, 1967). Methods have therefore been developed to detect specific anti-Aspergillus antibodies with a view to diagnosing IPA (Table IV.ii).

Young and Bennett (1971) used four different antibody detection methods and two metabolic extracts of A.fumigatus in a study of patients with haematological malignancy. Specific anti-Aspergillus antibody could not be detected using three of the methods and it was impossible to distinguish between patients with IPA and non-infected cancer patients, who were used as controls, by the fourth technique.

Similarly, two other groups of workers have been unable to differentiate individuals with IPA from non-infected patients. Both groups employed similar metabolic and somatic antigens of A.fumigatus but used different detection methods. Bardana et al (1975) used an immunodiffusion test whereas Gold et al (1980) developed a passive haemagglutination assay. The patients, underlying diseases were not stated in the first study. In the second all

Table IV.ii Detection of specific anti-Aspergillus IgG antibodies in invasive aspergillosis.

Reference	Detection method	Antigen	Patient group	Patients pos/tested	Controls pos/tested	Sens	Spec
Young 71	DD,IE	CF	Haem Mal	0/10	0/10	0	100
''	IF	CF	Haem Mal	10/10	10/10	100	0
Bardana 75	ID	CF/ME	Unknown	4/7	0/34	57	100
Schaefer 76	ID	CF	Haem Mal	7/10	9/70	70	85
Marier 79	RIA	CF/ME	Various	15/19	7/58	79	88
Gold 80	PH	CF/ME	Cancer	18/55	19/122	33	84
Holmberg 80	ELISA	AS	Haem Mal	8/10	0/12	80	100
Mishra 83	ELISA	AS	Unknown	6/8	5/123	75	96
Matthews 85	BLOT	40kDa	Haem Mal*	11/140	0/10	86	100
Trull 85	ELISA	COMM.	Renal/ Hepatic	16/19	18/205	84	90

key. pos, positive; Sens, sensitivity; Spec, specificity; DD, double diffusion; IE, immunoelectrophoresis; IF, indirect immunofluoresence; ID, immunodiffusion; RIA, radioimmunoassay; PH, passive haemagglutination; ELISA, enzyme linked immunosorbent assay; BLOT, immunoblotting; CF, culture filtrate; ME, mycelial extract; AS, 75% ammonium sulphate precipitate; 40kDa, 40kDa antigen band of mycelial extract; COMM, antigens from commercial sources; Haem Mal, haematological malignancies; Renal/Hepatic, liver/kidney transplant recipients; IEM, inborn errors of metabolism.
*included 2 patients without haematological malaginancies.

patients had neoplastic disease.

In contrast to these findings a number of studies have demonstrated that the presence of anti-Aspergillus antibodies was diagnostic for IPA. The methods and antigens described in these reports varied considerably but they all gave similar sensitivities (75% to 84%) and specificities (84% to 100%) for the diagnosis of IPA (Table IV.ii).

In an extended study of 80 leukaemic patients, in which an immunodiffusion test based on a metabolic extract of A.fumigatus was used, seroconversion to anti-Aspergillus precipitin positive, or a rise in antibody titre, was seen in 7/10 patients with proven IPA whereas only 6/70 patients with no evidence of fungal involvement seroconverted (Schaefer et al, 1976).

It has been reported that germling (unbranched) hyphae of A.fumigatus can bind IgG from healthy individuals (Schonheyder and Andersen, 1982). However, Gordon et al (1977) used them successfully as an antigen source for an indirect immunofluoresence test. An antibody titre >1:64 was regarded as indicative of Aspergillus infection and this level was demonstrated in 4/5 patients with IPA compared to only 1/25 control subjects. The underlying disease of the IPA patients and controls was not stated.

The more sensitive technique of RIA has also been applied to the diagnosis of IPA by the detection of anti-Aspergillus IgG (Marier et al, 1979). Both somatic and

mycelial antigens from A.fumigatus and A.flavus were employed in the solid phase RIA described by these workers. Interestingly the A.flavus assay was slightly more sensitive than the A.fumigatus method: 15/19 (78%) patients with IPA had detectable anti-Aspergillus IgG by the former method compared to 14/19 (74%) by the latter. Serial sera were available on ten of these patients and five seroconverted to antibody positive. Only 7/58 (12%) control patients with a variety of other fungal and bacterial infections had a positive antibody test.

Three laboratories have reported the successful use of ELISA for the detection of anti-Aspergillus antibody in the diagnosis of IPA. Trull et al (1985) used four commercially available Aspergillus antigens (Mercia Brocades and Bencard) in a study of solid organ tranplant recipients. Specific anti-Aspergillus IgG antibodies were detected in up to 16/19 (84%) cases of proven IPA depending on the antigen used whereas a maximum of 10/71 (14%) non-infected patients were positive. The other two groups each employed a similar ammonium sulphate precipitated fraction of A.fumigatus. Mishra et al (1983) demonstrated specific anti-Aspergillus IgG in 6/8 (75%) IPA patients compared to 5/123 (4%) control patients. The nature of the IPA patients was not stated but individuals in the control group either had a variety of pulmonary diseases or were healthy individuals. Holmberg et al (1980) detected antibody in 8/10 haematological patients

with proven IPA compared with only 1/12 (8%) non-infected neutropenic individuals.

It has been suggested that the difference in the nature of patient populations studied may have contributed to the discrepancy in results which have been reported in the literature. However, studies of similar patient populations have produced results suggesting that the detection of anti-Aspergillus antibody may (Holmberg et al, 1980) or may not (Young and Bennett, 1971) be useful in the diagnosis of IPA. Furthermore, studies on different patient populations have produced consistent reports indicating the usefulness of anti-Aspergillus antibody detection in the diagnosis of IPA (Schaefer et al, 1976; Matthews et al, 1985).

4.2 Aims.

The aim of this section of the thesis was therefore to determine whether anti-Aspergillus IgG antibody detection, by ELISA, was capable of diagnosing IPA in the patient population described in Chapter 2.

An ELISA method was chosen for a number of reasons: a) increased sensitivity compared to other methods (Kauffman et al, 1983; Trull et al, 1985); b) ease of processing a large number of specimens and, c) the relative nontoxicity of reagents used compared to those required for RIA.

It was decided to assay only for anti-Aspergillus IgG antibodies as these are the predominant Ig class produced in

response to Aspergillus infection (Kauffman et al, 1986; Brummond et al, 1987).

Three fractions from A.fumigatus NCPF 2109, one from A.fumigatus 1028 and two from A.flavus NCPF 2008 were used as it is known that both species of aspergilli (Froudist et al, 1989) and antigen fractions from the same species (De Magaldi and Mackenzie, 1984) differ in their ability to bind anti-Aspergillus antibodies.

4.3 Patients, materials and methods.

4.3.1 Patients.

Serum samples were collected from the 121 patients described in chapter 2.

A total of 1711 serum specimens were tested for anti-*Aspergillus* IgG antibodies. The number of sera assayed from each of the four patient groups were as follows: IPA 406 (number of sera available on each patient 5 to 57, median 15); CFI 257 (2 to 44, 16); OFI 145 (4 to 43, 12) and 903 from the NEFI individuals (1 to 39, 9).

4.3.2 Control sera.

Three strongly positive anti-*Aspergillus* IgG sera and three anti-*Aspergillus* IgG negative sera were a kind gift from Dr. V.M. Hearn, MRL. The anti-*Aspergillus* IgG antibody content of these controls had been established by the routine double immunodiffusion and ELISA methods used at the MRL. These sera were tested by all six ELISAs.

Sera were also obtained from 24 ABPA patients (these were a kind gift from Dr. Penny Fitzharris, Brompton Hospital, London) and 25 women who were seen at a ante-natal clinic for rubella screening. All 49 of these specimens were assayed by the WS2109 ELISA and ten anti-*Aspergillus* IgG positive sera from the ABPA group and ten anti-*Aspergillus* IgG negative sera from the pregnant women were chosen. These

20 sera, in addition to the six from the MRL, served as anti-Aspergillus IgG positive and negative controls for this section of the thesis.

4.3.3 ELISA for anti-Aspergillus IgG antibody.

Chequerboard titrations were performed to determine optimal concentrations for all reagents.

Dilution curves of the three positive and three negative anti-Aspergillus IgG sera obtained from the MRL were performed in order to determine the dilution giving optimal discrimination between the two sets of controls.

100µl of antigen (WS2109; CF2109; EP2109; WS2008; CF2008 or EP1028), at 5µg/ml protein, was coated onto flat bottomed microtitre plates (M129A, Dynatech, Billingshurst, West Sussex) in 0.06M carbonate buffer, pH9.6 {3.10.9} overnight at 4°C. The plates were washed x3 in PBST and free binding sites were blocked with 5% (w/v) non-fat milk (Sainsbury's) in PBST (NFM) for 60min at 37°C.

The plates were again washed x3 with PBST and 100µl of test or control sera, diluted 1:250 in 0.1% (w/v) NFM, was added to duplicate wells. The plates were incubated for 60min at 37°C and then washed x3 in PBST. 100µl of horseradish peroxidase conjugated anti-human IgG (Dako, High Wycombe, Bucks), diluted 1:2000 in 0.1% (w/v) NFM, was added to each well and incubated at 37°C for 60min.

The plates were washed again x3 with PBST and then

100µl of freshly prepared substrate/chromogen solution (0.2% (w/v) o-phenylene diamine, dihydrocholride in 0.07M phosphate, citric acid buffer, pH5.0 {3.10.10} containing 0.03% (v/v) H_2O_2) was added to each well. The plates were incubated in the dark for 15min. The reaction was stopped with 100µl of 1N H_2SO_4 and the absorbance at 492nm (A_{492}) was read in a Titertek multiscan spectrophotometer (Organon Technica).

A control, in which 0.1% (w/v) NFM replaced primary antibody was included on all plates in order to detect non-specific binding of the peroxidase conjugated anti-human IgG to the Aspergillus antigen extract.

4.3.4 Validation of assays and determination of positive results.

5/13 anti-Aspergillus IgG antibody positive, and 5/13 anti-Aspergillus IgG negative control sera were chosen at random and assayed on each ELISA run (Richardson et al, 1984). These controls were used to validate the assay run and also as a reference level to determine the amount of anti-Aspergillus IgG antibody in test sera, as described by Hearn et al (1985).

4.3.4.1 Validation. Results from assays were included only if the following three criteria were met: i) the ratio of the mean A_{492} values for the anti-Aspergillus IgG positive

to negative controls was >5.0; ii) the difference in the individual test/control duplicate A_{492} values did not differ by >10% of the lower reading and iii) the $\log_{10}$ standard deviation (SD) of the five negative control sera A_{492} was <0.171.

This $\log_{10}$ limit was determined by assaying five anti-Aspergillus IgG negative control sera (each group of five being picked at random from the 13 available) on five separate ELISA runs. The absolute A_{492} of each serum was converted to its $\log_{10}$ value and the SD of each group of five was calculated. The $\log_{10}$ mean of these five SDs was 0.106 and the acceptable level of SD for subsequent ELISAs was determined by multiplying the square root of the 95% value of the appropriate F distribution (1.61) by this value; i.e. 1.61 x 0.106 = 0.171.

4.3.4.2 Determination of positive results. Test sera were considered positive for anti-Aspergillus IgG antibody if their mean $\log_{10}$ A_{492} value exceeded m + k x 0.106: where m was the mean $\log_{10}$ of the five negative controls assayed and k was a suitable numerical factor (2.449, see below).

Therefore, a test serum was positive for anti-Aspergillus IgG antibody if its mean $\log_{10}$ A_{492} value was > m + 2.449 x 0.106 = m + 0.260.

The value of k was determined, for a false positive rate of 1%, by multiplying the 98% quantile of the normal

distribution by $(1+1/n)^{1/2}$ where n was the number of negative controls (Hearn et al, 1985). Thus k = 2.326 x $(6/5)^{1/2}$ = 2.449.

4.3.5 Use of peripheral wells in microtitre plates.

It has been suggested that the peripheral wells of microtitre plates perform in a different manner to the central wells (Kricka et al, 1980). To test if the M129A microtitre plates performed uniformly, six anti-Aspergillus IgG positive control sera were assayed, in quintuplicate, in external and internal wells of the same microtitre plate. This was repeated with six anti-Aspergillus IgG negative control sera.

The mean and SD of the five individual A_{492} values for each serum was calculated and the results from internal and external wells compared using the students t-test. No statistical difference ($p>0.1$) was seen between sera tested in internal as compared to external wells, thus all wells were used.

4.3.6 Inter- and intra- assay variation.

Inter-assay variation was determined by assaying four anti-Aspergillus IgG positive and four anti-Aspergillus IgG negative sera in quintuplicate, on three different ELISA runs. Intra-assay variation was determined by testing the same sera in 10 wells of the same microtitre plate.

The mean and SD of each set of results was calculated and then the co-efficients of variation (CV) were determined. All CVs were <10% and considered acceptable.

4.4 Results.

4.4.1 Comparison of antigen fractions WS2109, WS2008, CF2109, CF2008, EP2109 and EP1028 in their ability to discriminate between anti-Aspergillus IgG antibody positive and negative control sera.

When the three positive and three negative anti-*Aspergillus* IgG antibody controls (received from the MRL) were assayed using all the antigen fractions, only WS2109 ($p = 0.0011$, paired t-test) and WS2008 ($p = 0.034$) ELISAs were capable of differentiating the two sets of controls (Fig 4.1.1). Subsequent assay of the other antibody controls showed that the WS2109 ($p = 0.0001$), WS2008 ($p = 0.005$), CF2008 ($p = 0.0065$), EP2109 ($p = 0.0115$) and EP1028 ($p = 0.0002$) ELISAs were capable of differentiating between the mean values of the two sets of controls. However, with the exception of the WS2109 ELISA, all antigen fractions showed a large amount of overlap between individual sera in the two control groups (Fig 4.1.2). In addition the lower 95% confidence limit of the anti-*Aspergillus* IgG positive controls was not greatly different from the upper 95% confidence limit of the negative controls except for the WS2109 ELISA. It was decided therefore only to assay patients' sera using the WS2109 ELISA.

Both the EP antigens from *A.fumigatus* 1028 and *A.fumigatus* NCPF 2109 as well as the *A.flavus* NCPF 2008 WS

Figure 4.1.1 Discrimination between anti-Aspergillus IgG antibody positive and negative sera received from the MRL.

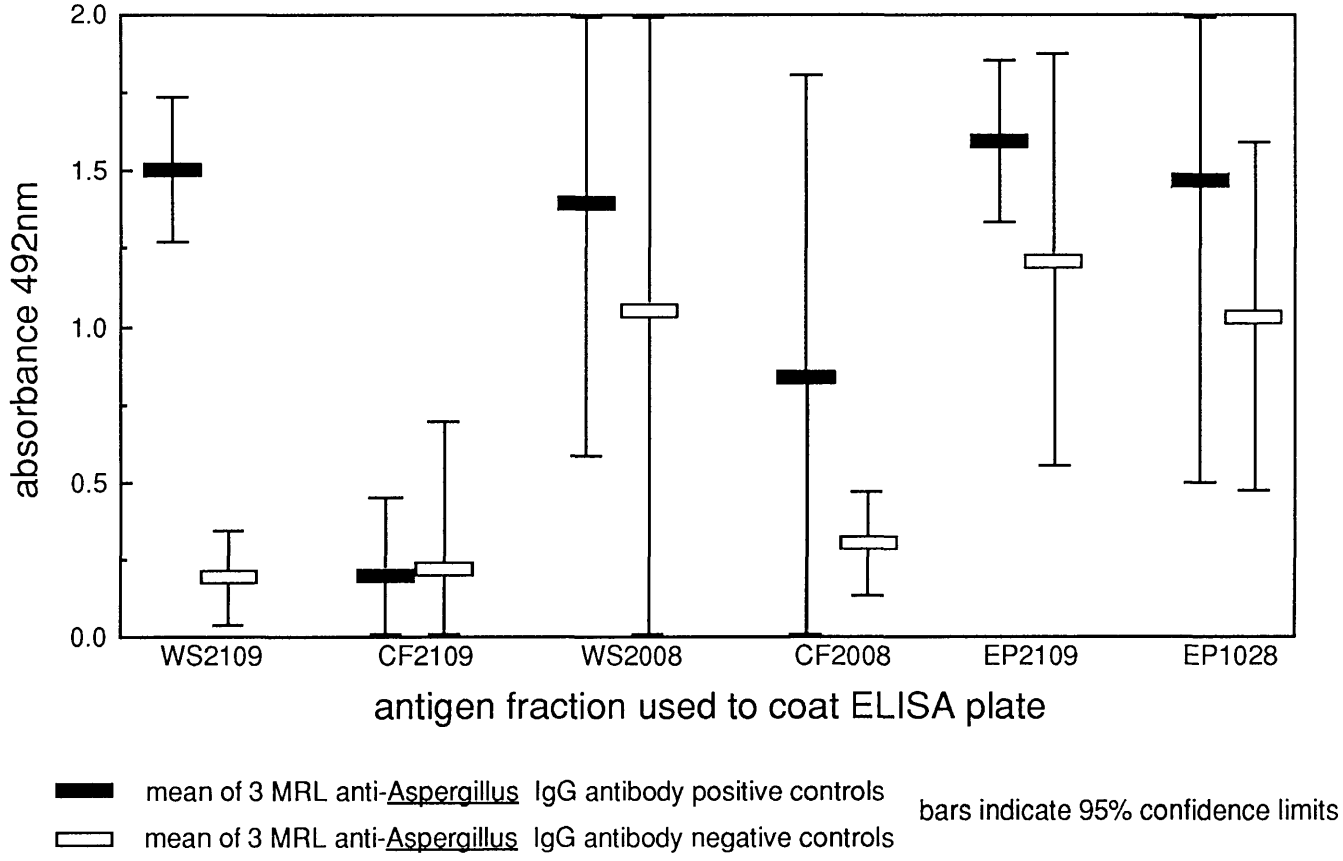


Figure 4.1.2 Discrimination between all anti-Aspergillus IgG antibody positive and negative control sera.

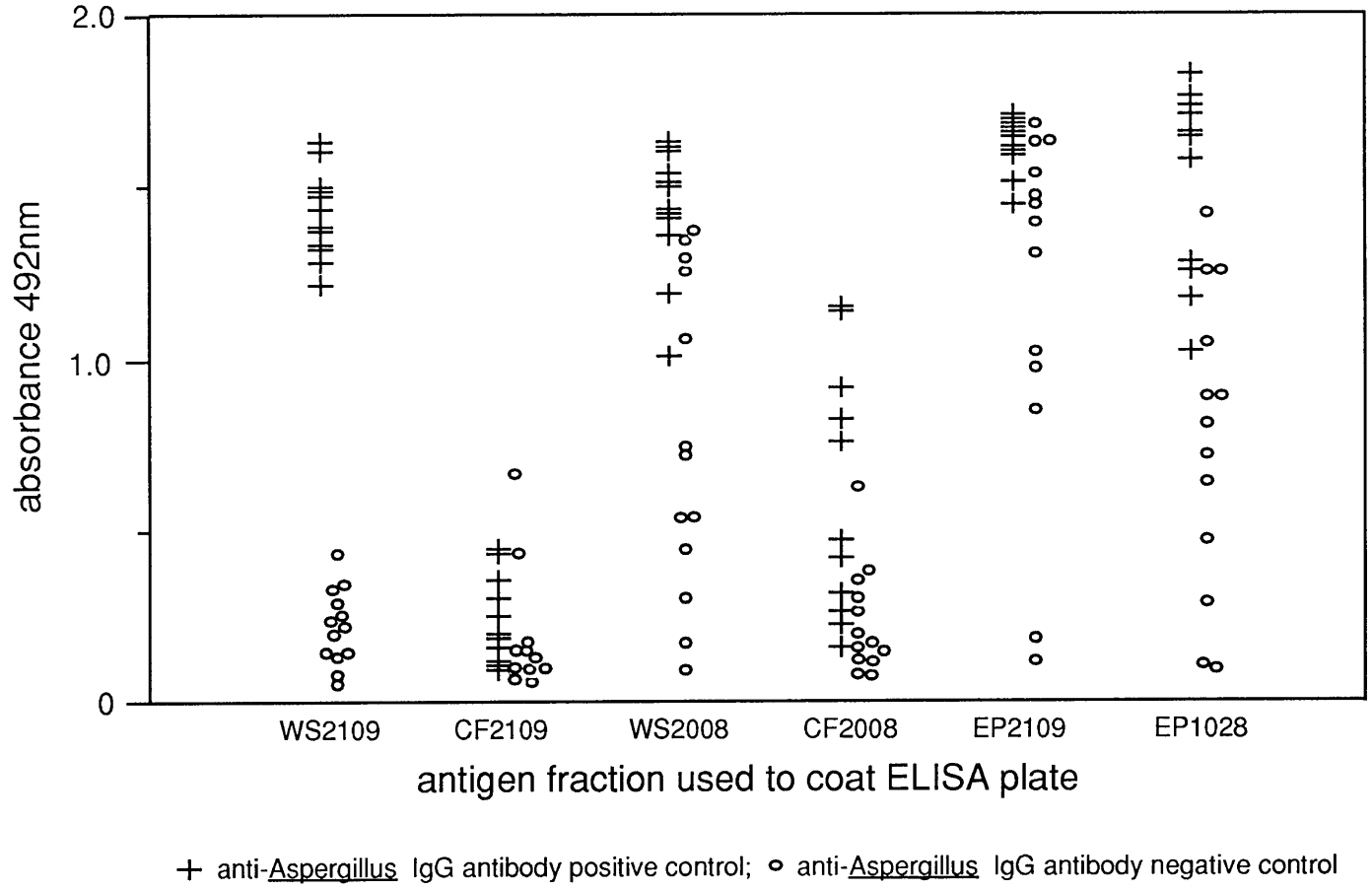
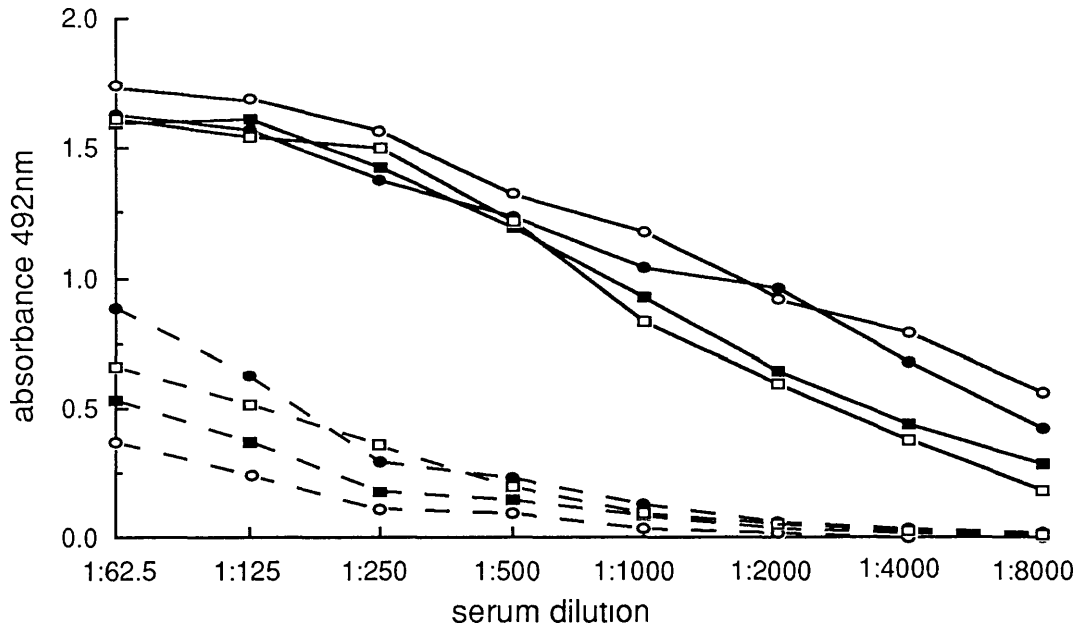


Figure 4.2 Dilution curves of anti-Aspergillus IgG antibody positive and negative control sera in WS2109 ELISA.



unbroken lines, anti-Aspergillus IgG positive sera; dashed lines, anti-Aspergillus IgG negative sera

● □ ○, MRL anti-Aspergillus controls; ■ , mean of all control sera

extract gave a high number of "false" positive results, whereas both CF fractions gave almost uniformly negative results.

4.4.2 Dilution curves of anti-Aspergillus IgG antibody positive and negative control sera.

The 13 anti-Aspergillus IgG negative and 13 anti-Aspergillus IgG positive contol sera used throughout this work gave similar absorbance curves when assayed in serial dilutions (Fig 4.2). It was therefore considered acceptable to test all sera at a single dilution (Shale and Faux, 1985). Good discrimination between the anti-Aspergillus IgG positive and negative control sera was seen over the range 1:100 to 1:1000. The dilution 1:250 was chosen to assay sera.

4.4.3 Detection of anti-Aspergillus IgG antibody in patients.

The overall results for detection of anti-Aspergillus IgG in all patients and sera assayed are shown in Tables IV.iii, IV.iv.i and IV.iv.ii. The results are presented in more detail by patient group below.

4.4.3.1 IPA patients. Five of the 19 patients with IPA had anti-Aspergillus IgG antibodies detectable by the WS2109 ELISA (Table IV.iii). One patient was positive when admitted

Table IV.iii Detection of specific anti-Aspergillus IgG antibodies in all 121 patients by WS2109 ELISA.

Patient group	Number of patients positive / tested	(%)	Number of sera positive / tested	(%)
IPA	5 / 19	(23)	50 / 406	(12)
CFI	4 / 12	(33)	45 / 257	(18)
OFI	2 / 8	(25)	10 / 145	(8)
NEFI	18 / 82	(22)	67 / 903	(8)

to hospital (number 2) and the remaining four (numbers 6, 10, 13, 15) seroconverted to anti-*Aspergillus* IgG antibody positive (Table IV.iv.i) while in hospital.

Patient 2 was anti-*Aspergillus* IgG positive throughout the period proceeding BMT. Post-transplantation this woman developed a long term *A.flavus* infection of the paranasal sinuses; she also became severly neutropenic. Her antibody titres fell slowly post BMT and did not recover before death (Fig 4.3.i). *A.fumigatus* was never isolated from this individual.

Patient 13 was also anti-*Aspergillus* IgG positive prior to BMT. However this patient was initially anti-*Aspergillus* IgG negative (Fig 4.3.iv).

Patients 6 and 10 seroconverted to antibody positive post-transplantation (Fig 4.3.ii and 4.3.iii).

No serum was available on patient 15 until 30 days post BMT. At this time the patient was anti-*Aspergillus* IgG negative, but was not neutropenic (white cell count of 2.6 x 10^9/l). This individual seroconverted 74 days post BMT (Fig 4.3.v).

After becoming antibody positive all four patients who seroconverted (numbers 6, 10, 13 and 15) had falling anti-*Aspergillus* IgG levels. They all died from proven *A.fumigatus* infections.

None of the other IPA patients had detectable anti-*Aspergillus* IgG antibodies: all but one (number 14) of these

Figure 4.3 Anti-Aspergillus IgG titres in antibody positive IPA patients.

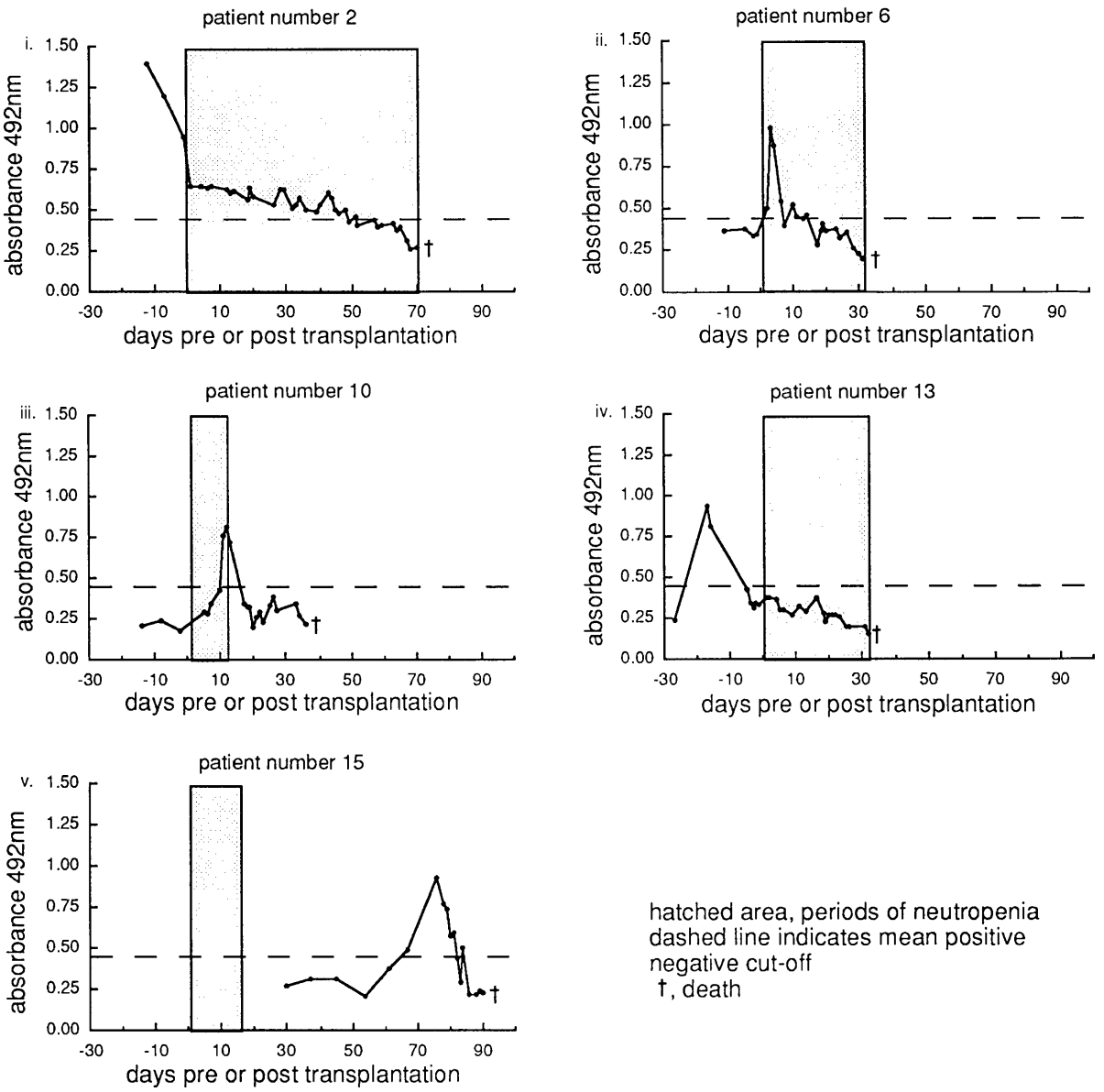


Table IV.iv.i Detection of specific anti-Aspergillus IgG antibodies by WS2109 ELISA: Seroconversion and number of positive sera from individual IPA, CFI and OFI antibody positive patients.

Patient number	Patient group	Number of sera positive / tested	(%)	Seroconversion
2	IPA	26	36 (72)	-
6	IPA	9	22 (41)	+
10	IPA	4	21 (19)	+
13	IPA	3	26 (12)	+
15	IPA	8	13 (62)	+
20	CFI	12	26 (46)	+
21	CFI	12	16 (75)	-
25	CFI	13	32 (41)	+
31	CFI	8	10 (80)	+
36	OFI	7	43 (16)	-
39	OFI	3	12 (25)	-

key. -, no seroconversion; +, seroconversion to anti-Aspergillus IgG positive.

14 patients died.

4.4.3.2 CFI patients. Four of the 12 CFI patients had detectable anti-Aspergillus IgG antibody by the WS2109 ELISA (Table IV.iii). Three of these seroconverted to antibody positive (numbers 20, 25 and 31), while the other patient (number 21) was positive on initial testing (Table IV.iv.i).

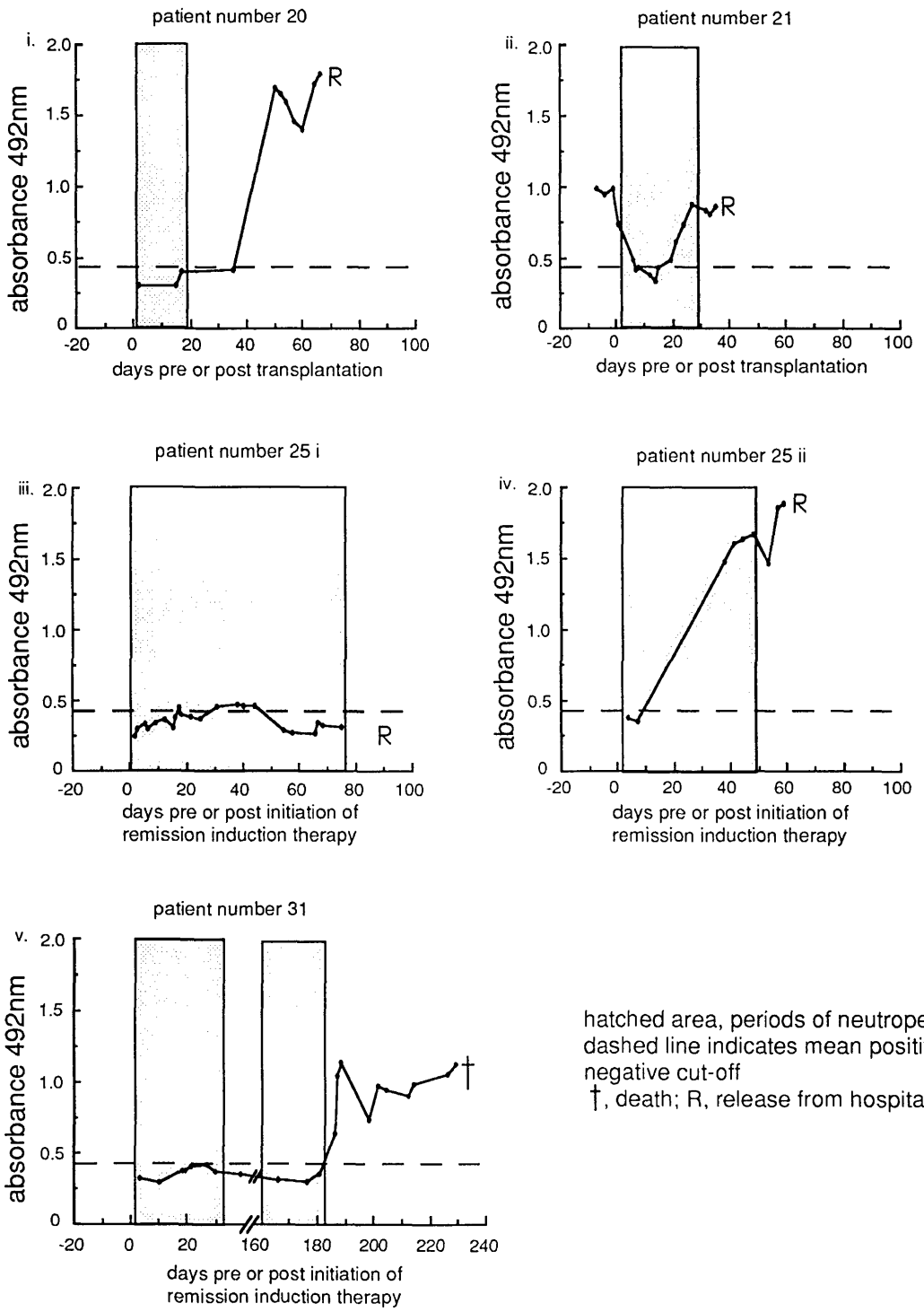
Patient 21 had anti-Aspergillus IgG antibodies prior to BMT: these antibodies returned post-transplant (Fig 4.4.ii) and the patient was discharged from hospital alive and well.

Patient 20 seroconverted to anti-Aspergillus IgG antibody positive post-transplantation and had increasing titres of antibody (Fig 4.4.i) until released home.

Patients 25 and 31 did not undergo marrow transplatation but received remission induction therapy. Patient 25 had two seperate episodes of CFI. During the first episode this patient seroconverted but their antibody titres remained at a low level and eventually reverted to anti-Aspergillus IgG negative (Fig 4.4.iii). In the second episode, which followed approximately four months later, antibody titres were initially negative but became positive and remained high until release from hospital (Fig 4.4.iv).

Patient 31 was antibody negative at time of admission but became anti-Aspergillus IgG positive after a period of neutropenia. His antibody level remained high until death (Fig 4.4.v).

Figure 4.4 Anti-Aspergillus IgG titres in antibody positive CFI patients.



hatched area, periods of neutropenia
dashed line indicates mean positive negative cut-off
†, death; R, release from hospital.

The other 8 CFI patients had no anti-Aspergillus IgG antibody detectable by the WS2109 ELISA; one of these patients (number 29) survived while all the others died.

4.4.3.3 OFI patients. Anti-Aspergillus IgG was detected in only two of these patients (Table IV.iii). Both patients were antibody positive on initial testing (Table IV.iv.i) prior to BMT or the initiation of remission induction therapy. Antibody returned on engraftment or the completion of therapy.

4.4.3.4 NEFI patients. Sixty four of the 82 NEFI patients had no detectable anti-Aspergillus IgG antibodies (Table IV.iii). However, 18 patients were antibody positive; eight in the first serum sample available and the other ten seroconverted during the course of their hospitalization (Table IV.iv.ii).

4.4.4 Comparison of the different patient groups.

Chi-squared analysis of the ELISA data showed that there was no difference in the prevalence of anti-Aspergillus IgG antibody between the IPA or CFI patients when compared with the OFI and NEFI groups (Table IV.v). The sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) for the diagnosis of IPA by detection of anti-Aspergillus IgG antibody in the IPA

Table IV.iv.ii Detection of specific anti-Aspergillus IgG antibodies by WS2109 ELISA: Seroconversion and number of positive sera from individual NEFI antibody positive patients.

Patient number	Number of sera positive / tested	(%)	Seroconversion
64	2 / 5	(40)	+
70	1 / 19	(5)	+
71	11 / 19	(58)	-
76	1 / 7	(14)	+
77	5 / 32	(16)	+
78	3 / 5	(60)	-
79	3 / 3	(100)	-
83	2 / 4	(50)	-
86	8 / 12	(67)	+
89	6 / 18	(33)	-
90	7 / 7	(100)	-
91	4 / 14	(29)	-
94	1 / 10	(10)	+
96	1 / 1	(100)	-
109	1 / 7	(14)	+
115	2 / 6	(33)	+
116	1 / 3	(33)	+
121	1 / 2	(50)	+

key. see table 4.4.1

group compared to the OFI and NEFI groups was 26%, 78%, 20% and 83% respectively.

Table IV.v Chi-squared analysis of anti-*Aspergillus* IgG antibody detection by WS2109 ELISA as a diagnostic method for IPA.

Comparison groups	Chi-squared statistic	Probability
IPA vs OFI/NEFI	0.148	p>0.1
CFI vs OFI/NEFI	0.733	p>0.1
IPA/CFI vs OFI/NEFI	0.587	p>0.1

4.5 Discussion.

Many techniques have been described for the detection of specific anti-*Aspergillus* antibodies (see introduction). These tests, especially precipitin techniques such as double diffusion, are both sensitive and specific for the diagnosis of ABPA and aspergilloma where a strong humoral response to the fungus is seen (Hearn, 1988).

However, in patients with IPA, both the underlying disease and treatment regimen cause immunosuppression hence a strong humoral response is usually not seen. This has been reflected in the generally disappointing results obtained using relatively insensitive techniques such as passive haemagglutination (Gold et al, 1980), immunodiffusion (Bardana et al, 1975) and immunoelectrophoresis (Young and Bennett, 1971) to detect anti-*Aspergillus* antibodies as a method of diagnosing IPA. However, one group of workers did publish fairly encouraging results (sensitivity 70%, specificity 85%) for an immunodiffusion test (Schaefer et al, 1976).

More sensitive methods have therefore been applied to the detection of specific anti-*Aspergillus* antibodies. RIA has been used with some success in the diagnosis of IPA (Marier et al, 1979). ELISA systems have also been developed to detect anti-*Aspergillus* IgG (Greenberger and Patterson, 1982; Richardson et al, 1982). These assays are similar in sensitivity and specificity to RIAs. They have the

additional advantages of lower cost, increased stability of reagents and greater safety as the use of radiochemicals is not required.

Two reasons for the increased sensitivity of ELISAs over techniques such as CIE for the detection of anti-Aspergillus antibodies can be put forward: i) the capacity of antigens that do not precipitate in gels to bind to the surface of microtitre plates and ii) the predominance of non-precipitating antibodies produced in response to the fungus (Hearn et al, 1985). However, it must be stated that in a small number of precipitin positive sera anti-Aspergillus IgG antibodies could not be detected by ELISA (Kauffman et al, 1983). This may be due to the inability of every precipitating antigen to bind to microtitre plate surfaces due to competition from other molecules in the antigen extract (Kenny and Dunsmoor, 1983).

There are several problems associated with the use of ELISAs to detect anti-Aspergillus IgG. The major one being the ubiquitous nature of aspergiili. This means that the majority of individuals would have been exposed to these fungi and therefore possess some level of specific IgG antibodies (Bardana et al, 1972b; Warnock, 1974); thus any point used to discriminate between anti-Aspergillus IgG positive and negative will be more or less arbitrary. As the distribution of anti-Aspergillus IgG in the normal population is positively skewed (Richardson et al, 1984) it

was not acceptable to take the mean plus 1.96 SD as the cut-off point between the two groups. An alternative based on the relationship between a panel of negative control sera and the samples being tested has been proposed (Hearn et al, 1985). This was the approach adopted in this thesis and therefore the positive/negative cut-off point was critically dependent upon the negative control sera used. Richardson et al (1984) have shown that the most appropriate negative control panel was one where individual sera were chosen at random from a panel of precipitin negative specimens. This was done in these experiments.

Another problem is that absorbance values in ELISA do not neccessarily reflect antibody concentration, but rather antibody affinity and therefore these assays should be treated as only semi-quantitative (Lew, 1984). The dilution curves of all anti-Aspergillus IgG positive controls used in this thesis were similar, as were the curves for the negative control sera (Fig 4.2). This similarity suggests that there was a quantitative relationship between anti-Aspergillus IgG concentration (dilution) and absorbance value in the WS2109 assay system and that comparison of sera at a single dilution was valid (Shale and Faux, 1985).

Previous reports have produced conflicting evidence on the influence that the choice of antigen extract, used in ELISAs, has on the ability to detect anti-Aspergillus IgG. Richardson and Warnock (1984) demonstrated that four

commercial extracts of A.fumigatus were all equally capable of detecting anti-Aspergillus IgG antibodies whereas DeMagaldi and Mackenzie (1984) showed that antibody detection was directly influenced by the antigen extract used to coat microtitre plates. The results presented in this chapter supports the view that the choice of antigen fraction critically effected the ability to detect anti-Aspergillus IgG antibodies. The WS2109 extract was the only antigen fraction assayed that was capable of discriminating between anti-Aspergillus IgG positive and negative control sera (Fig 4.1.1 and 4.1.2). This may be due to the observed differences in individual antigen content of A.fumigatus extracts (Kim et al, 1978; Kim and Chaparas, 1978; Reed, 1978; Hearn and Mackenzie, 1979; Hearn et al, 1980a). Similarly, the observed heterogeneity of antigenic composition seen when identical extracts are prepared from different species of Aspergillus (Gerber and Jones, 1973; Piechura et al, 1985); different strains of the same species (Hearn and Mackenzie, 1980; Hearn et al, 1980b; Kurup et al, 1983; Mishra, 1984; Wallenbeck et al, 1984; Calvo et al, 1985) or even different batches from the same strain (Kurup et al, 1977) presumably influences the detection of anti-Aspergillus antibodies.

The WS2008, EP2109 and EP1028 extracts exhibited a high degree of non-specific binding of IgG from the negative control sera. This may have been due to the presence of

cross-reacting antibodies in these sera to antigens in the fungal extracts. Cross-reactivity has been demonstrated between Aspergillus antigens and extracts from other fungi (Suzuki et al, 1967; Tee et al, 1987) especially Candida spp (DeMagaldi and Mackenzie, 1984).

The WS2109 ELISA, although capable of distinguishing between sera containing anti-Aspergillus IgG and those without, was of no use in the diagnosis of IPA having a sensitivity of only 26% and a specificity of 78%.

These findings contrast with the results obtained by other workers who have used ELISA successfully to diagnose IPA (Holmberg et al, 1980; Mishra et al, 1983; Trull et al, 1985). As was stated earlier the nature of a patient's underlying disease directly influences their ability to mount a humoral response once immunosuppression has been stopped. In one previous study (Holmberg et al, 1980) the nature of the patients was not described. The IPA patients in another study (Trull et al, 1985) underwent solid organ transplants and may therefore have been more likely to mount a humoral response once immunosuppression was stopped than the majority of the neutropenic patient population studied here. This may help to explain the difference in results observed. In a third report the patient population was essentially similar to the one studied here (Mishra et al, 1983); however, the number of control patients was relatively small (n=12) which may have resulted in an

underestimation of the true level of antibody positivity, in the population as a whole, by these workers. This would have affected the specificity of their test.

It has been suggested that the presence of pre-existing anti-Aspergillus IgG antibodies predisposes individuals to IPA once they become immunosuppressed (Trull et al, 1985). Again the results presented here do not support this view; only two patients with proven IPA (numbers 2 and 13) had pre-existing anti-Aspergillus IgG antibodies whereas ten patients with no evidence of aspergillosis (OFI and NEFI groups) had pre-existing anti-Aspergillus IgG.

It has also been claimed that the maintenance of, or an increase in, specific anti-Aspergillus IgG antibody levels heralded recovery whereas a falling titre was a poor prognostic indicator (Matthews et al, 1985). Although not reaching statistical significance the data presented here support this claim; all 25 patients with proven or suspected IPA who died had no anti-Aspergillus IgG or falling titres. Conversely 3/6 who survived had rising titres.

In conclusion the results suggest that the detection of anti-Aspergillus IgG antibodies can not be used to make a positive diagnosis of IPA (PPV = 26%) but, in line with other reported studies (Table IV.ii) our NPV (83%) was moderately high. It is possible therefore that a negative ELISA result may be helpful in eliminating IPA as a potential diagnosis. However, it has to be stressed that

these negative results must be treated with the utmost caution and interpreted in the light of other laboratory and clinical findings eg the presence or absence of Aspergillus antigen; pulmonary infiltrates; fungus in surveillance cultures and pyrexia unresponsive to antibiotics with no evident aetiological agent.

CHAPTER FIVE: DETECTION OF ANTI-ASPERGILLUS IgG ANTIBODIES BY IMMUNOBLOTTING.

5.1 Introduction.

Detection of anti-*Aspergillus* IgG antibodies by ELISA was not capable of discriminating between patients with IPA and non-infected patients (chapter 4). However, antigen extracts similar to the WS2109 antigen used in this ELISA have been shown to be complex mixtures of antigens (Wilson and Hearn, 1983a; Piechura et al, 1983). There is the possibility therefore, that the detection of the overall anti-*Aspergillus* IgG level by ELISA could mask differences in antibody response to individual antigens. If this were the case the detection of these specific antibodies could be used as a diagnostic method for IPA.

There is some evidence in the literature to support the view that a differential antibody response to aspergilli is seen in ABPA and aspergilloma, but very little work has been done on IPA. Two approaches can be taken to determine if antibody response to specific antigens of aspergilli are diagnostic; i) the purification of single antigens or fractions of known composition from crude extracts for use in antibody detection methods or ii) the use of immunological techniques which allow the separation of individual antigens in a crude extract and subsequent detection of specific antibody binding by eg immunoblotting.

5.1.1 Detection of specific anti-Aspergillus IgG antibodies to individual Aspergillus antigens or fractions of known composition. Increased binding of specific antibodies has been noted to certain antigen fractions of A.fumigatus eg ammonium sulphate precipitates (Kim et al, 1978) and conA binding antigens (Wilson and Hearn, 1983a) when compared with crude extracts from which they were derived. Using both hydrophobic interaction and size exclusion chromatography Schonheyder and co-workers identified a number of antigen fractions which reacted specifically with antibodies in the sera of patients with ABPA or aspergilloma (Schonheyder and Andersen, 1983; 1984a; 1984b; Schonheyder et al, 1985). These fractions had apparent M_rs of approximately 470 kDa, 250 kDa, 160 kDa, 160-50 kDa and 50-25 kDa. All fractions appeared to contain more than a single antigen. The 250 kDa fraction exhibited catalase activity and the 50-25 kDa fraction neutral protease activity.

Furthermore, the purification of specific antigens, for use in antibody detection tests, has resulted in increased diagnostic sensitivity in ABPA and aspergilloma (Table V.i). The majority of this work has been done by two groups; Longbottom and co-workers in London and Kurups team in Wisconsin.

Longbottom and co-workers using the techniques of crossed immunoelectrophoresis and self-crossed immunoelectrophoresis have identified a large number of

Table V.i Characterized antigens of *Aspergillus* spp which have been used to detect specific anti-*Aspergillus* antibodies.

Reference	Antigen	Source	Comment
Tran Van Ky 67; 68	C Ag	CF	catalase
Calvanico 81	CS2 (180 K)	ME	45 K sub units
Piechura 83	CS2 (130-190 K)	ME	see text for description
Schonheyder and Andersen 83; 84a; 84b	250 K	ME	catalase
	160-50 K	ME	neutral protease
Matthews 85	40 K	ME	Ab response in IPA patients
Kurup 86b	20, 40, 80 K	CF	acidic glycoproteins
Harvey and Longbottom 86a	Ag 7 (150-200 K)	CF	heat stable 36 K sub units
Harvey and Longbottom 87	Ag 13 (70 K)	CF	catalase
Taylor and Longbottom 88	Ag 5 (35 K)	CF	heat labile
Brouwer 88	18, 24-26, 36-38 K	Com	Ab response in precipitin pos patients
Leung 88	70 K	ME	Ab response in ABPA patients
Kurup 89	<14 K	CF	Ab response in ABPA patients

key. K, M_r in kilodaltons; ME, mycelial extract; CF, culture filtrate; Com, commercial antigen from Bencard; Ab, antibody; IPA, invasive aspergillosis.

Aspergillus antigens which react with antibodies from ABPA patients. Two of these antigens designated Ag7 (Harvey and Longbottom, 1986a; 1986b) and Ag13 (Harvey and Longbottom, 1987) were major antigens reacting with antibodies in 29/30 and 25/25 ABPA patients respectively. However, Ag13 also reacted with 5/12 patients with farmers lung. Ag7 was identified as a high M_r (150-200 kDa) heat stable (100°C for 5min), acidic glycoprotein capable of binding to the lectin conA and composed of 36 kDa subunits (Harvey and Longbottom, 1986a). Ag13 was identified as a catalase of apparent M_r 70 kDa which was heat labile and bound to conA (Harvey and Longbottom, 1987). This antigen was the same as the 'C' antigen isolated in the early work of Tran Van Ky and co-workers (1966; 1968). DeMagaldi and Mackenzie (1984) demonstrated specific anti-C antigen IgG antibodies in 12/14 ABPA patients and 15/17 aspergilloma patients.

In addition to the major antigens Ag7 and Ag13 Longbottom's group have isolated two major allergens of A.fumigatus designated Ag3 (Longbottom, 1986b) and Ag5 (Taylor and Longbottom, 1988) one of which (Ag5) also bound IgG antibody. Ag5 was heat labile, did not bind to conA and had an apparent M_r of 35 kDa.

The isoelectric focusing work of Kurup's group revealed both CF and WS extracts of A.fumigatus to be extremely complex mixtures of proteins (Piechura et al, 1983; 1985). From a WS extract of A.fumigatus these workers isolated a

component (designated CS2), by gel filtration, which reacted with 24/30 sera from aspergillosis patients but with none of 11 control patients who were either healthy individuals or had other fungal infections. The structure of this antigen fraction remains unclear. Initial reports suggested that it was a tetrameric acidic glycoprotein with an isoelectric point of 5.2-5.6 and subunits of approximately 45 kDa (Calvanico et al, 1981). Subsequently, two structures for the antigen were proposed: i) a dimer of two unequal subunits with apparent M_rs of 80-85 kDa and 50-55 kDa or ii) a trimer of three unequal subunits with apparent M_rs of 41 kDa, 50 kDa and 80 kDa respectively (Piechura et al, 1983).

In a later report (Kurup et al, 1986b) these workers identified three acidic glycoproteins (isoelectric point 6.0-6.5) which all bound to conA and had apparent M_rs of 20 kDa, 40 kDa and 80 kDa. Using a biotin-streptavidin ELISA system they demonstrated increased levels of specific IgG antibodies to these antigens in 27 ABPA and aspergilloma patients compared to 24 control subjects.

5.1.2 Detection of specific anti-Aspergillus IgG antibodies by immunoblotting. The use of SDS-PAGE to separate complex antigenic mixtures on the basis of their electrophoretic mobilities is a common practice in biological research. If this is followed by transfer onto a solid substrate eg

nitrocellulose (Towbin et al, 1979) the antibody response of an individual can be 'dissected' by probing the blots with that person's serum. This approach has been adopted in an attempt to diagnose various forms of aspergillosis, especially ABPA and aspergilloma.

Brouwer (1988) attempted to relate anti-Aspergillus ELISA and precipitin results to the band patterns found in immunoblots with sera from patients with chronic obstructive lung disease and healthy blood donors. A commercial antigen of A.fumigatus (Bencard, Beecham Pharma, Amstelveen, Netherlands) was used throughout the work. Of 100 ELISA and precipitin negative sera, 85 had two to four weak bands at M_rs between 90 kDa and 120 kDa while the remaining 15 exhibited stronger reactions with antigens at 36 kDa, 50 kDa and between 100 kDa and 120 kDa. In contrast, a much more pronounced response to a large number of bands between 17 kDa and 150 kDa was seen with 20 strongly precipitin positive sera. The major reaction was to antigens with apparent M_rs of 18 kDa, 24-26 kDa and 36-38 kDa. However, the individual band patterns were very varied. A single blot of a patient with IPA was demonstrated which contained a number of bands. It was not possible to approximate the M_r of individual bands as no scale was placed on the figure (Fig 2, lane 7; Brouwer, 1988). However, all staining was to antigens with M_rs above 38 kDa.

In an immunoblot study, designed primarily to determine

allergens of A.fumigatus, Leung and co-workers (1988) demonstrated that the major protein bands of a mycelial extract had apparent M_rs of 70 kDa, 66 kDa and 45 kDa. In addition, a large number of other bands were seen between 30 kDa and 110 kDa. When sera were used to probe this extract 20/22 ABPA patients had specific IgG antibodies to a 70 kDa antigen. 0/10 controls had detectable IgG antibody. The relationship of this antigen to Ag13 (Harvey and Longbottom, 1986a) or C antigen (Tran Van Ky et al, 1966; 1968) is unknown.

In a similar study Kurup et al (1989) identified a large number of antigens in a CF extract of A.fumigatus with M_rs from <10 kDa to >92 kDa. Individual sera from patients with ABPA or aspergilloma reacted with between 3 and 19 of these bands. Only one serum from six controls reacted with a single antigen. The M_r of individual bands was not stated by the authors, but they did say that the majority of staining, with ABPA sera, was to antigens with M_rs <14 kDa.

There has been one study reported of the use of immunoblotting to detect anti-Aspergillus IgG antibodies as a diagnostic method for IPA (Matthews et al, 1985). A mycelial extract of A.fumigatus was used as the antigen source and results of immunoblotting were quantified by using densitometry. Sera from 10 healthy blood donors were used as controls; these sera only contained IgG antibodies

to two antigens with apparent M_rs of 83 kDa (3 sera) and 63 kDa (2 sera). In contrast sera from 14 patients with IPA contained specific antibodies to a maximum of nine individual antigens with M_rs between 88 kDa and 33 kDa. IgG antibodies to a single antigen with an apparent M_r of 40 kDa were demonstrated in 11 of these 14 patients. The authors suggested that possession of antibodies to this antigen was a good prognostic indicator since the two IPA patients who survived had low but persistent levels of specific IgG throughout whereas all 12 who died had absent or falling titres of antibody to this antigen.

5.2 Aims.

Because of these encouraging results I decided to evaluate the role that detection of specific IgG antibodies to individual antigens of A.fumigatus could play in the diagnosis of IPA.

SDS-PAGE followed by immunoblotting was chosen as the approach to be adopted as the separation of antigens, inherent in this method, would allow quicker screening of a large number of sera than purification of individual antigens from a crude extract and the subsequent detection of specific antibodies. This method has the added advantage that if successful it could be used as a guide to the purification of relevant antigens.

The WS extract from A.fumigatus NCPF 2109 was chosen as

the antigen source because it was the only antigen capable of discriminating anti-Aspergillus IgG positive and negative sera by ELISA.

5.3 Patients, materials and methods.

5.3.1 Patient and control sera.

The following sera were used as primary antibody to stain the WS2109 blots (see below): i) two from each of the 14 IPA and 8 CFI patients who had no anti-*Aspergillus* IgG detectable by the WS2109 antibody ELISA; ii) one serum from each of the 6 OFI and 62 NEFI patients who were anti-*Aspergillus* IgG negative by ELISA; iii) two sera from each of the 5 IPA, 4 CFI, 2 OFI and 20 NEFI patients who were anti-*Aspergillus* IgG positive by ELISA including pre- and post-seroconversion sera from the 17 individuals who seroconverted during their hospitalization; iv) each of the 24 ABPA sera and, v) sera from 20 apparently healthy blood donors.

5.3.2 Antigen.

WS antigen was prepared from *A.fumigatus* NCPF 2109 as described in section 3.3.2.

5.3.3 SDS-PAGE.

WS2109 was reconstituted into sterile distilled water at a concentration of 2mg/ml protein. This was then mixed with sample buffer and electrophoresed as described in section 3.6.

CHARING CROSS & WESTMINSTER
CHARING CROSS
MEDICAL SCHOOL LIBRARY

5.3.4 Electroblotting of SDS-PAGE gels.

The separated WS2109 antigens were transferred onto nylon membranes as described in section 3.7.

5.3.5 Staining of blots.

5.3.5.1 Ponceau-S and India ink staining. Blots were stained with ponceau-S and India ink as described in sections 3.8.1 and 3.8.2 respectively.

5.3.5.2 Immunostaining. Blots were cut into strips equating to the tracks of the SDS-PAGE gel.

Free binding sites were blocked by incubation with 5% (w/v) NFM for 60min at 25°C on a rotary shaker at 50 rpm.

The blots were rinsed with 0.1% (w/v) NFM (this was used as the diluent for all sera and antibodies) and incubated with primary antibody, diluted 1:100, for 4h as above.

The blots were washed x3 with PBST and then incubated at 25°C for 60min with a horseradish peroxidase conjugated anti-human IgG (Dako) diluted 1:1000.

After two 2min washings each in PBST, PBS and 50mM phosphate buffer, pH 7.4 {3.10.11} blots were equilibrated in chromogen (0.4% (w/v) 3,3'-diaminobenzidine tetrahydrochloride in 50mM phosphate buffer, pH 7.4) without H_2O_2 for 5min. Bands were revealed by placing the blots in

fresh chromogen containing 0.01% (v/v) H_2O_2. The reaction was stopped with distilled water and blots were dried between filter paper sheets.

5.3.6 Estimation of M_r.

The M_r of antigens in the WS2109 extract was determined as described in section 3.9.

5.4 Results

5.4.1 India ink staining of WS2109 blots.

Staining of the WS2109 blots for total protein revealed a large number of bands between 100 kDa and 17 kDa. Distinct bands were visible with apparent M_rs of 82 kDa, 77 kDa, 63 kDa, 61 kDa, 53 kDa, 47 kDa, 44 kDa, 38 kDa, 34 kDa, 29 kDa, 27 kDa, 24 kDa, 21 kDa, 19 kDa and 17 kDa (Fig 5.1, lane 1; Fig 5.2, lane 1).

5.4.2 Reactivity of ABPA and blood donor sera with WS2109 blots.

Most ABPA sera were strongly reactive with a large number of antigens in the WS2109 extract over the entire M_r range (Fig 5.1; Table V.ii). Antibodies to the antigen bands at 82 kDa, 77 kDa and 44 kDa were present in 100%, 100% and 88% of the ABPA sera tested respectively. The least reactive bands were at 63 kDa (25% of sera tested) 47 kDa (33%), 27 kDa (33), 24 kDa (29%) and 21 kDa (21%). One ABPA serum was almost totally non-reactive in immunoblotting against the WS2109 extract (Fig 5.1 lane 6), although this specimen's ELISA value was not significantly different from those of the other sera.

Blood donors' sera were generally less reactive than those from ABPA patients (Fig 5.2; Table V.ii). However, as with the ABPA sera, antibodies to the entire M_r range of

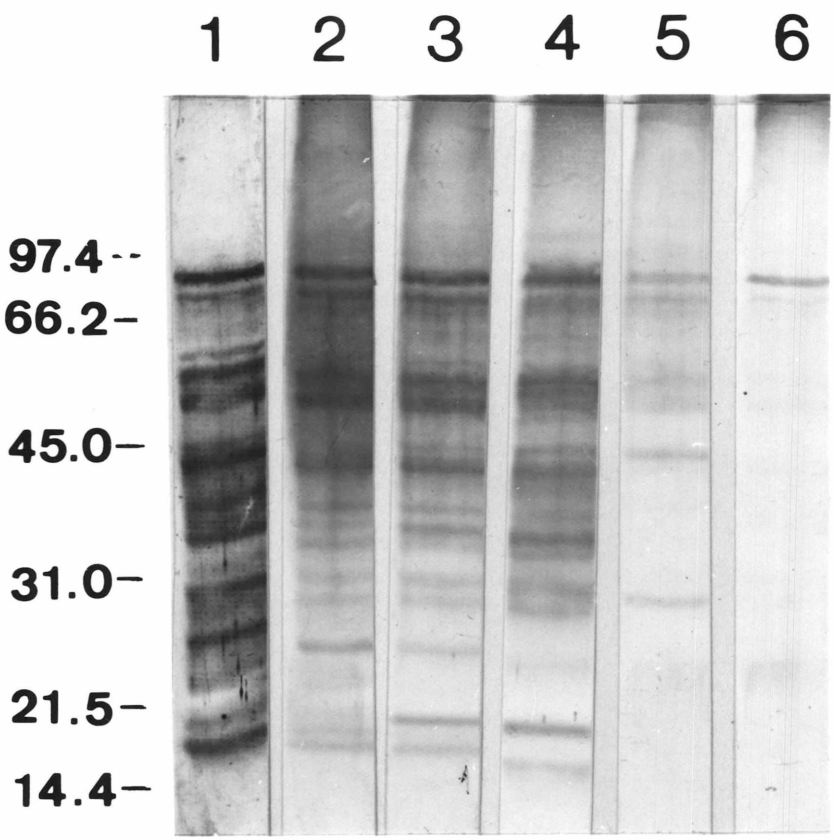
Table V.ii Reactivity of ABPA and blood donor sera with antigens in the WS2109 extract.

antigen M_r (kDa)	Examples of ABPA sera shown in Fig 5.1 lane 2	3	4	5	6	total	Examples of blood donor sera shown in Fig 5.2 lane 2	3	4	5	6	total
82	+	+	+	+	+	24	+	+	+	+	+	20
77	+	+	+	+	+	24	+	+	+	+	-	19
63	+	+	-	-	-	6	+	+	+	-	-	8
61	+	+	+	+	-	19	+	+	+	+	-	12
53	+	+	-	+	W	16	+	+	+	+	-	13
47	-	+	+	+	-	8	+	-	-	-	-	3
44	+	+	+	-	W	21	+	+	-	-	+	16
38	+	+	-	-	-	11	+	-	-	-	-	7
34	+	+	+	-	-	13	+	-	-	-	-	2
29	+	+	+	-	-	12	+	W	W	-	-	5
27	+	+	+	-	-	8	+	W	W	-	-	4
24	+	+	-	-	-	7	-	+	-	-	-	2
21	-	-	-	-	-	5	-	-	-	-	-	1
19	-	+	+	-	-	13	W	-	-	-	-	3
17	+	+	+	-	-	15	+	-	-	-	-	7
13	-	-	-	-	-	4	-	-	-	-	-	2
11	-	-	-	-	-	2	-	-	-	-	-	1

key. -, no reaction with antigen; W, weak reaction; +, positive reaction; total, number of sera which reacted with an antigen of this M_r out of the 24 ABPA and 20 blood donor sera tested.

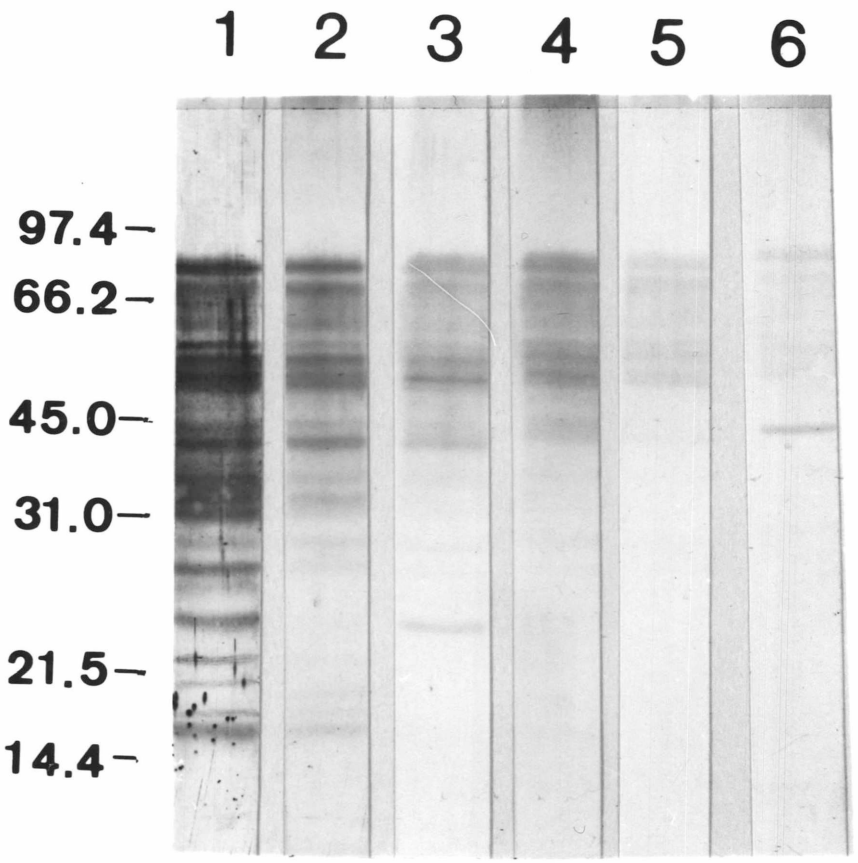
Anti-*Aspergillus* IgG WS2109 ELISA results: ABPA sera from lane number 2, 1.190; 3, 1.505; 4, 0.940; 5, 0.896; 6, 1.289; blood donor sera from lane number 2, 0.781; 3, 0.328; 4, 0.670; 5, 0.243; 6, 0.181.

Figure 5.1 Immunoblots of ABPA sera against WS2109 antigen.



key. lane 1, India ink stain of WS2109; lanes 2-6, various ABPA patient sera. See Table V.ii for WS2109 ELISA results. M_r standards are in the left margin.

Figure 5.2 Immunoblots of blood donor sera against WS2109 antigen.



key. lane 1, India ink stain of WS2109; lanes 2-6, various blood donor sera. See Table V.ii for WS2109 ELISA results. M_r standards are in the left margin.

antigens were present in at least one serum, although the majority of antigens below 44 kDa were relatively unreactive with blood donor sera. The exception was a single band with an apparent M_r of 17 kDa. The most reactive antigens were those with apparent M_rs of 82 kDa (reacting with 100% of sera tested), 77 kDa (95%) and 44 kDa (80%).

No correlation was seen between ELISA antibody levels and reactivity in immunoblots with either ABPA or blood donor sera (Fig 5.1; Fig 5.2).

5.4.3 Reactivity of patient sera with WS2109 blots.

Sera from all the patient groups reacted with a large number of antigens in the WS2109 extract over the entire M_r range (Fig 5.3 to 5.6; Tables V.iii and V.iv). However the intensity of band staining was generally much lower than was seen with the ABPA sera.

The most reactive antigens in the WS2109 extract were those at 82 kDa and 44 kDa binding antibody from 113 (93%) and 103 (85%) of the 121 patients studied respectively. The least reactive antigens were at 17 kDa (16 patients, 13%), 21 kDa (15, 12%) and 24 kDa (11, 9%).

It was not possible to discriminate between the different patient groups on the basis of anti-*Aspergillus* IgG band patterns produced against the WS2109 extract (Fig 5.3 to Fig 5.6; Tables V.iii and V.iv).

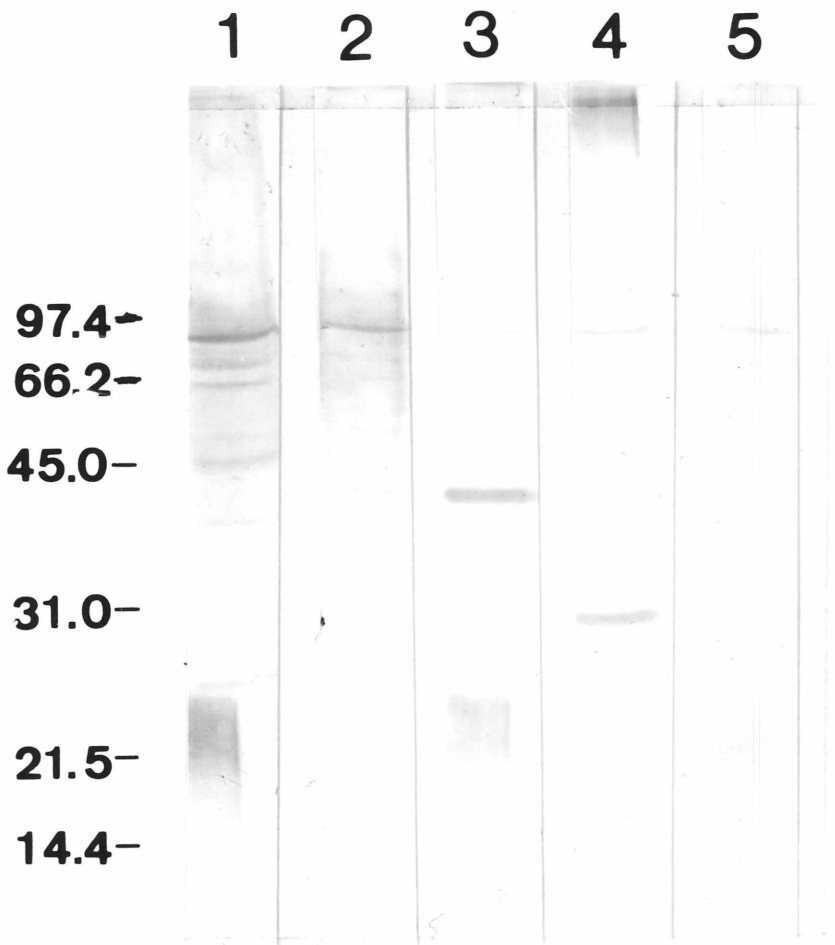
Table V.iii Reactivity of IPA and CFI patient sera with antigens in the WS2109 extract.

antigen M_r (kDa)	Examples of IPA sera shown in Fig 5.3 lane 1	2	3	4	5	total	Examples of CFI sera shown in Fig 5.4 lane 1	2	3	4	5	total
82	+	+	+	+	+	19	+	+	-	-	W	10
77	-	-	-	-	W	6	-	-	-	-	-	1
63	+	W	W	-	W	14	+	+	-	W	W	8
61	-	-	-	-	-	5	-	-	-	-	-	4
53	W	W	-	-	-	11	W	-	-	-	-	3
47	+	W	-	-	-	8	-	-	-	-	-	5
44	-	+	+	+	-	17	+	+	W	+	+	12
38	+	-	W	-	-	8	-	-	-	W	-	4
34	-	W	-	-	-	8	W	-	-	W	-	7
29	-	W	W	+	W	10	W	+	W	W	+	9
27	-	-	-	-	-	3	W	W	-	W	-	4
24	+	+	+	-	-	3	-	W	-	W	-	2
21	-	-	-	W	-	2	+	W	-	-	W	3
19	W	-	-	-	-	2	-	+	-	+	+	5
17	W	-	-	-	W	4	-	-	-	-	-	0
13	-	-	-	-	-	7	+	+	-	+	+	10
11	W	W	+	+	W	8	+	+	-	+	+	7

key. -, no reaction with antigen; W, weak reaction; +, positive reaction; total, number of patients from whom at least one serum reacted with an antigen of this M_r out of the 19 IPA and 12 CFI patients.

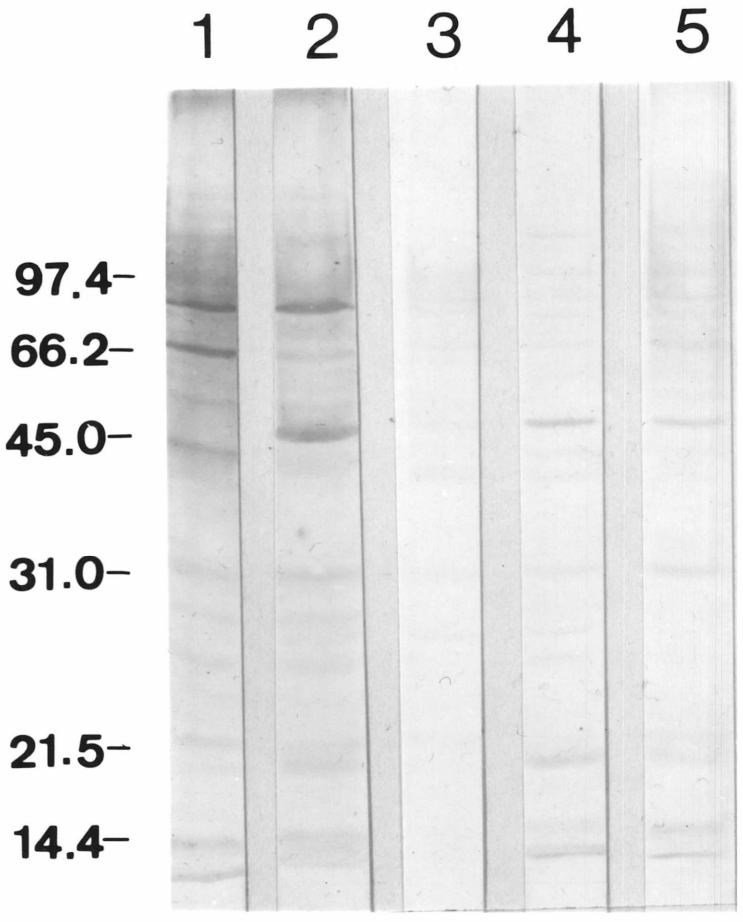
Anti-Aspergillus IgG WS2109 ELISA results for IPA patients (patient number in parentheses) in lane 1, 0.870 (6); 2, 0.952 (2); 3, 0.387 (3); 4, 0.264 (8); 5, 0.194 (16) and CFI patients in lane 1, 0.693 (20); 2, 0.312 (22); 3, 0.278 (28); 4, 0.991 (21); 5, 0.244 (29).

Figure 5.3 Immunoblots of IPA patients' sera against WS2109 antigen.



key. lane 1, IPA patient number 6; lane 2, IPA patient number 2; lane 3, IPA patient number 3; lane 4, IPA patient number 8; lane 5, IPA patient number 16. See Table V.iii for WS2109 ELISA results.
M_r standards are in the left margin.

Figure 5.4 Immunoblots of CFI patients' sera against WS2109 antigen.



key. lane 1, CFI patient number 20; lane 2, CFI patient number 22; lane 3, CFI patient number 28; lane 4, CFI patient number 21; lane 5, CFI patient number29. See Table V.iii for WS2109 ELISA results.
M_r standards are in the left margin.

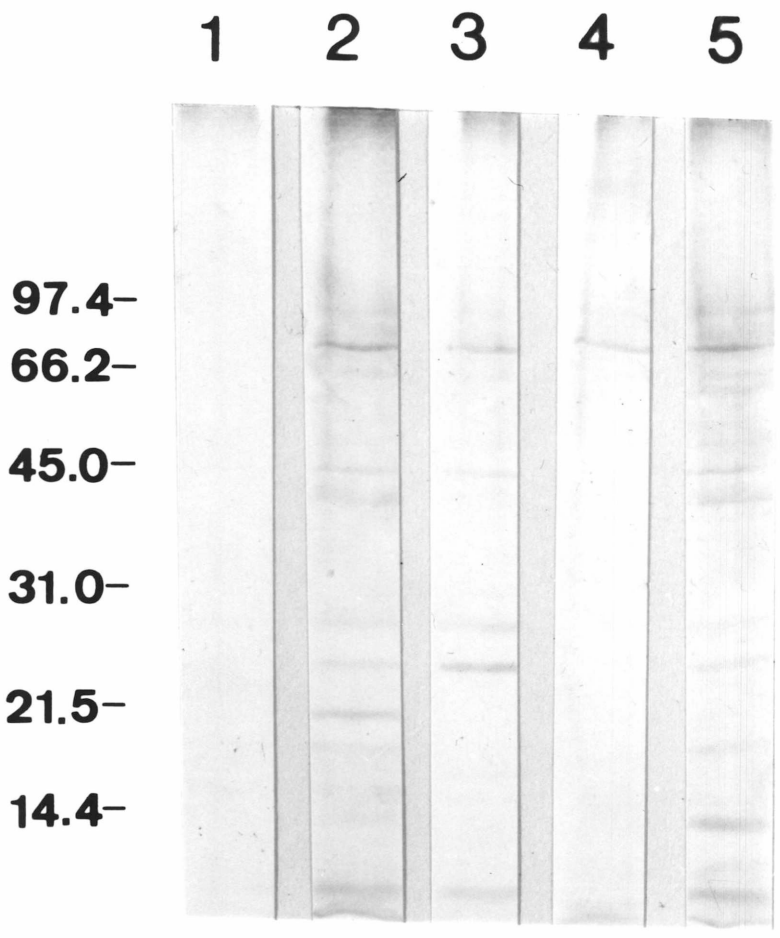
Table V.iv Reactivity of OFI and NEFI patient sera with antigens in the WS2109 extract.

antigen M_r (kDa)	Examples of OFI sera shown in Fig 5.5 lane 1	2	3	4	5	total	Examples of NEFI sera shown in Fig 5.6 lane 1	2	3	4	5	total
82	-	+	+	+	+	7	+	W	W	+	W	77
77	-	-	-	-	-	0	+	+	-	-	+	39
63	-	W	-	-	W	3	+	-	-	+	-	26
61	-	-	-	-	W	3	+	-	W	-	-	31
53	-	-	-	-	-	1	W	-	-	-	-	51
47	-	W	W	-	W	5	-	-	+	-	-	12
44	-	W	-	-	W	5	W	W	-	+	W	69
38	-	-	-	-	-	2	-	-	-	-	-	23
34	-	-	-	-	-	2	W	-	-	W	-	26
29	W	W	W	-	W	4	+	W	-	W	-	14
27	-	W	+	-	W	4	+	-	-	W	W	11
24	-	-	-	-	-	0	-	-	-	W	-	6
21	-	+	-	-	W	2	-	-	-	-	-	8
19	-	W	-	-	W	2	+	-	W	-	-	13
17	-	-	W	-	-	1	-	-	-	-	W	11
13	-	-	-	-	+	3	+	+	W	-	W	53
11	-	+	+	-	+	6	-	-	-	-	-	4

key. -, no reaction with antigen; W, weak reaction; +, positive reaction; total, number of patients from whom at least one serum reacted with an antigen of this M_r out of the 8 OFI and 82 NEFI patients.

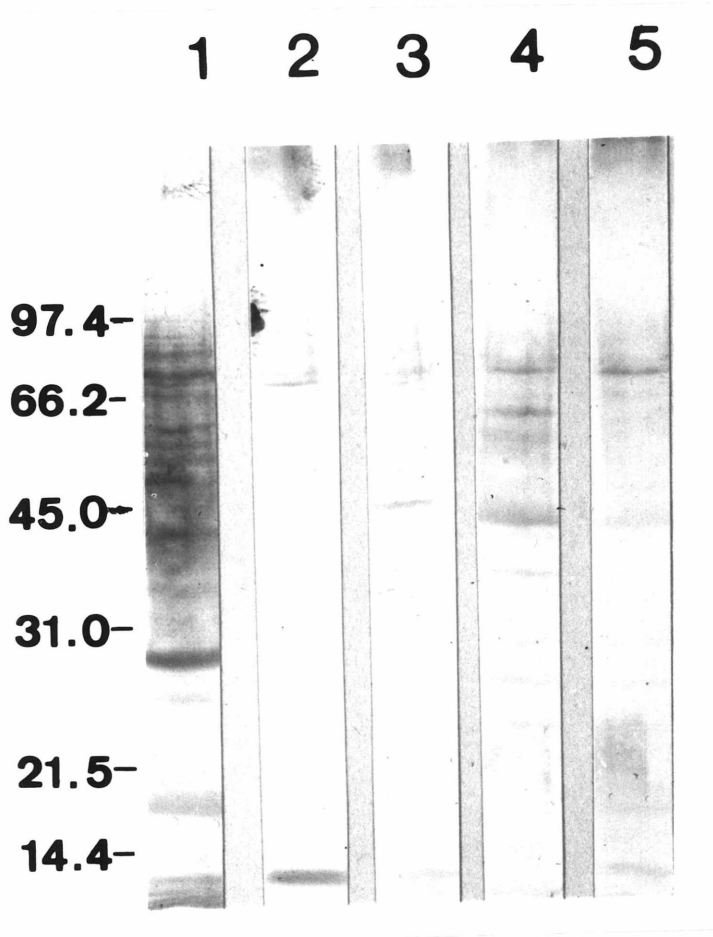
Anti-Aspergillus IgG WS2109 ELISA results from OFI patients (patient number in parentheses) in lane 1, 0.181 (32); 2, 0.304 (37); 3, 0.386 (38); 4, 0.195 (33); 5, 0.683 (36) and NEFI patients in lane 1, 0.756 (76); 2, 0.129 (44); 3, 0.667 (96); 4, 0.302 (48); 0.236 (65).

Figure 5.5 Immunoblots of OFI patients' sera against WS2109 antigen.



key. lane 1, OFI patient number 32; lane 2, OFI patient number 37; lane 3, OFI patient number 38; lane 4, OFI patient number 33; lane 5, OFI patient number 36. See Table V.iv for WS2109 ELISA results.
M_r standards are in the left margin.

Figure 5.6 Immunoblots of NEFI patients' sera against WS2109 antigen.



key. lane 1, NEFI patient number 76; lane 2, NEFI patient number 44; lane 3, NEFI patient number 96; lane 4, NEFI patient number 48; lane 5, NEFI patient number 65. See Table V.iv for WS2109 ELISA results.
M_r standards are in the left margin.

5.4.4 Comparison of immunoblot pattern and anti-Aspergillus IgG ELISA result.

Immunoblot reactivity did not correlate well with results of the anti-*Aspergillus* IgG ELISA; similar band patterns were often seen with ELISA antibody positive and antibody negative sera (Fig 5.7, Table V.v). However, the few sera (7) which were almost totally unreactive on immunoblots all had ELISA absorbance values <0.250.

5.4.5 Immunoblot patterns of pre- and post- seroconversion sera.

Probing of the WS2109 blots with sera from 17 individual patients taken both pre- and post- seroconversion to anti-*Aspergillus* IgG positivity, by WS2109 ELISA, did not reveal the consistent appearance of new antibodies to any specific antigen (Fig 5.8, Table V.vi). Nearly all sera revealed the same antigen pattern pre- and post-seroconversion.

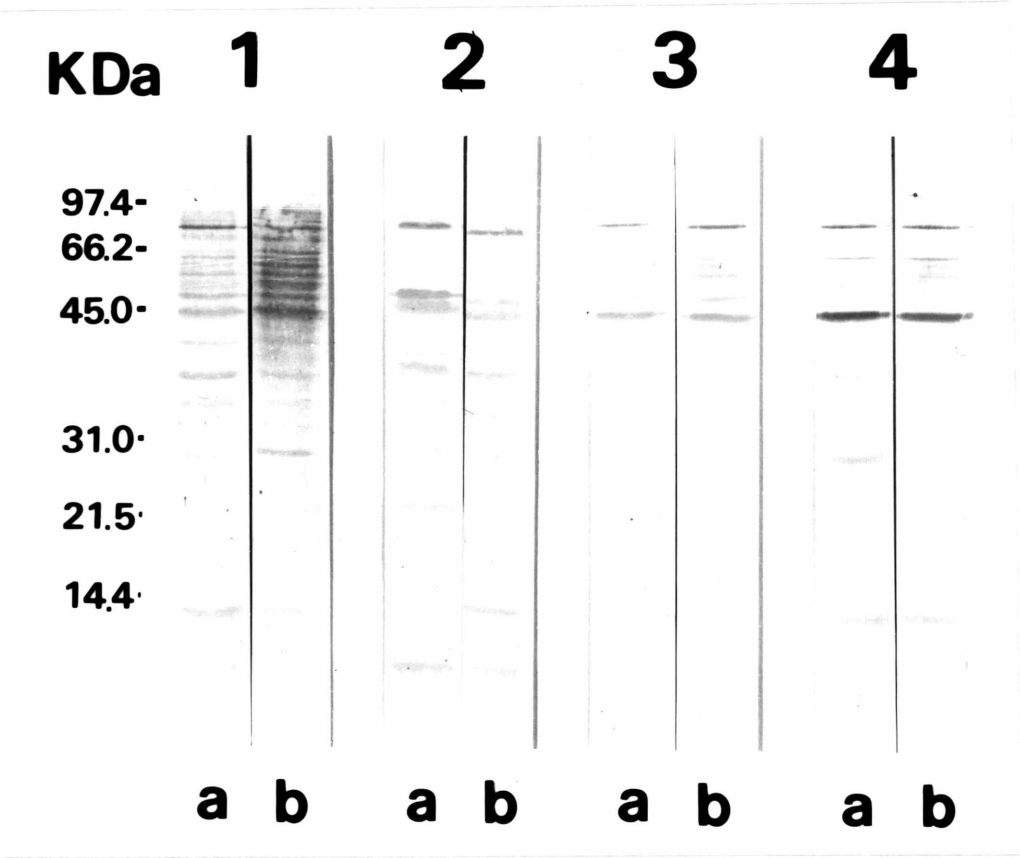
Table V.v Reactivity of anti-Aspergillus IgG ELISA antibody positive and negative sera from all patient groups with antigens in the WS2109 extract.

antigen M_r (kDa)	Examples of antibody positive sera shown in Fig 5.7 lane 1b	2b	3b	4b	total	Examples of antibody negative sera shown in Fig 5.7 lane 1a	2a	3a	4a	total
82	+	+	+	+	27	+	+	+	+	84
77	+	-	W	-	8	W	-	-	-	34
63	+	-	W	+	15	W	W	W	+	36
61	+	W	W	W	11	+	-	W	-	32
53	+	-	-	-	17	+	+	-	-	49
47	+	+	+	-	10	+	+	+	-	20
44	+	+	+	+	26	+	+	+	+	79
38	W	+	+	+	12	+	+	W	+	25
34	+	+	+	+	11	+	+	W	+	32
29	+	W	-	+	12	+	W	-	+	25
27	-	-	-	-	6	-	-	-	-	16
24	-	-	-	-	3	-	-	-	-	8
21	-	-	-	W	4	-	-	-	W	11
19	-	-	-	-	10	-	-	-	-	12
17	-	-	-	-	6	-	-	-	-	10
13	+	+	W	+	20	+	W	W	+	53
11	+	+	W	-	11	+	+	W	-	28

key. -, no reaction with antigen; W, weak reaction; +, positive reaction; total, total number of anti-Aspergillus IgG antibody positive or negative patients from whom at least one sera reacted with an antigen of that M_r.

Anti-Aspergillus IgG WS2109 ELISA results for antibody positive patients (patient number in parentheses) in lane 1b, 0.813 (10); 2b, 1.110 (31); 3b, 0.586 (39); 4b, 0.861 (71) and antibody negative patients in lane 1a, 0.222 (14); 2a, 0.194 (27); 3a, 0.358 (35); 4a, 0.147 (53).

Figure 5.7 Immunoblots of anti-Aspergillus IgG antibody negative and positive sera from all patient groups against WS2109 antigen.



key. lanes 1a-4a, anti-Aspergillus IgG ELISA negative patients; lanes 1b-4b, anti-Aspergillus IgG ELISA positive patients. lane 1a, IPA patient number 14; lane 2a, CFI patient number 27; lane 3a, OFI patient number 35; lane 4a, NEFI patient number 53. lane 1b, IPA patient number 10; lane 2b, CFI patient number 31; lane 3b, OFI patient number 39; lane 4b, NEFI patient number 71; See Table V.v for WS2109 ELISA results.
M_r standards are in the left margin.

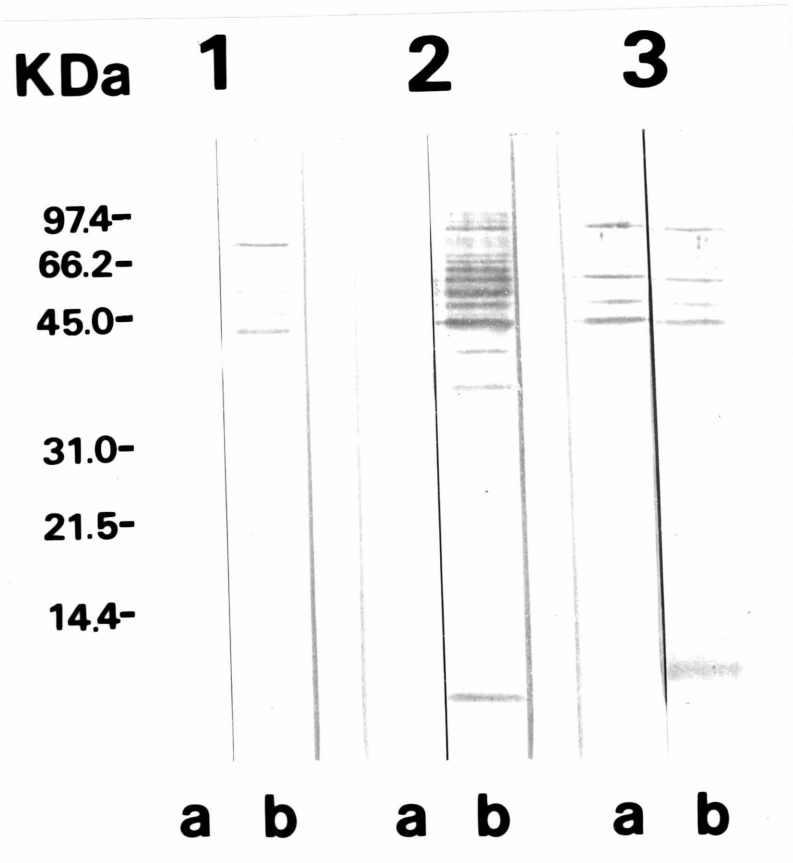
Table V.vi Appearance of antibodies to antigens in the WS2109 extract in the 17 patients who seroconverted to anti-*Aspergillus* IgG positivity by ELISA.

antigen M_r (kDa)	Examples of patients who seroconverted to antibody positive shown in Fig 5.8 lanes 1a pre	1b post	2a pre	2b post	3a pre	3b post	total pre	total post
82	W	+	+	+	+	+	16	16
77	-	-	-	-	-	-	3	4
63	-	+	-	+	-	-	8	10
61	-	W	+	+	-	-	6	7
53	-	+	-	+	+	+	9	12
47	W	+	+	+	+	+	6	6
44	W	+	+	+	+	+	14	15
38	-	W	-	+	W	W	7	9
34	W	+	+	+	W	W	9	9
29	-	W	-	-	-	-	2	6
27	-	-	-	-	-	-	2	3
24	-	-	-	-	-	-	1	1
21	-	W	-	-	-	-	0	2
19	-	-	-	-	-	-	2	6
17	-	-	-	-	-	-	1	4
13	-	W	W	W	-	-	9	13
11	W	W	W	+	-	-	3	7

key. -, no reaction with antigen; W, weak reaction; +, positive reaction; pre, prior to seroconversion; post, after seroconversion; total, total number of patients from whom at least one serum reacted with antigen of that M_r pre or post seroconversion.

Anti-*Aspergillus* IgG ELISA results for patients (patient number) in lane 1a, 0.348 (13); 1b, 0.951 (13); 2a, 0.401 (25); 2b, 1.408 (25); 3a, 0.321 (64); 3b, 1.071 (64).

Figure 5.8 Immunoblots of individual patient sera pre- and post- seroconversion by ELISA to anti-Aspergillus IgG antibody positive against WS2109 antigen.



key. lanes 1a-3a, pre- seroconversion; lanes 1b-3b, post seroconversion. lanes 1a and 1b, IPA patient number 13; lanes 2a and 2b, CFI patient number 25; lanes 3a and 3b, NEFI patient number 64. See Table V.vi for WS2109 ELISA results.
M_r standards are in the left margin.

5.5. Discussion.

Electrophoresis of the WS2109 antigen extract revealed a complex mixture of molecules (Fig 5.1, lane 1; Fig 5.2, lane 1). At least 15 distinct bands between 82 kDa and 17 kDa were seen when the antigen was stained with India ink. In addition less discrete staining was seen at >100 kDa. No bands were seen at less than 17 kDa with the India ink total protein stain suggesting that all proteinaceous components of the extract had higher M_rs than this. The complexity of the antigen extract demonstrated by this work is in agreement with previous reports of similar A.fumigatus fractions where a large number of antigens have been found (Hearn et al, 1990).

The majority of ABPA sera reacted strongly with a large number of the antigens in the WS2109 extract. This was to be expected as reported experience in these patients has shown an elevated level of specific anti-Aspergillus IgG antibodies (see Table IV.i). The most reactive antigens in the WS2109 extract demonstrated by this work were those at 82 kDa, 77 kDa and 44 kDa.

The relationship, if any, of these antigens to molecules reported by other workers is unknown although antigens of similar M_r have been reported. Piechura et al (1983) described an antigen isolated from a WS extract of A.fumigatus which was thought to have sub-units of 80-85 kDa and 50-55 kDa. Antibodies to antigens of 82 kDa and 53 kDa

were seen in 100% and 67% of the ABPA patients studied here respectively. In later work the same authors described an antigen with an apparent M_r of 80 kDa which also specifically bound antibodies in ABPA sera (Kurup et al, 1986b).

To my knowledge no workers have described an antigen with apparent M_r of 77 kDa as being of importance in ABPA. However, it is possible that this antigen was a component of the 160-50 kDa fraction described by Schonheyder and Andersen (1983). It is also possible that the 44 kDa antigen described here was a component of the 25-50 kDa fraction described by these workers (Schonheyder and Andersen, 1983). The somatic extracts from which these two antigens were fractionated were prepared from the same strain of A.fumigatus (NCPF 2109) and in a similar way to WS2109. The 44 kDa antigen also has a similar M_r to the sub-units (45 kDa) of the tetrameric glycoprotein initiallly described by Calvanico et al (1981).

In addition to the three major antigens described in this work some of the other antigens to which anti-Aspergillus IgG antibodies were found have similar M_rs to those of previously described molecules. In a large study of patients with specific anti-Aspergillus antibodies Brouwer (1988) stated that the major IgG response was to three antigens with apparent M_rs of 18 kDa, 24-26 kDa and 36-38 kDa. Specific anti-Aspergillus IgG antibodies were seen to

antigens of similar M_rs ie 17 kDa, 24 kDa and 38 kDa in 63%, 29% and 46% of the ABPA patients respectively.

Some workers however have reported antibodies in ABPA patients to antigens which were not demonstrable in this work. The most obvious antigen to which antibodies were not detected in the present study was the catalase or 'C' antigen which has a M_r of 70 kDa. Specific IgG antibodies to this antigen have been documented in patients with ABPA by a large number of workers (Tran Van Ky et al, 1968; DeMagaldi and Mackenzie, 1984; Harvey and Longbottom, 1987). This finding was puzzling as it has been demonstrated that WS extracts prepared from A.fumigatus NCPF 2109, in the same manner as was done in this thesis, did contain the 70 kDa catalase antigen (Hearn et al, 1990). It may be possible that the particular batch used in these experiments did not contain this antigen as it is well known that extracts of Aspergillus can vary both quantitatively and qualitatively even when prepared under identical conditions (Kurup et al, 1977).

Another discrepancy between these results and a previous immunoblot study (Kurup et al, 1989) was that only a minority of these ABPA patients had specific anti-Aspergillus IgG antibodies which were reactive with antigens whose apparent M_r was <14 kDa. The reason for this is unkown as there were two antigens in the WS2109 extract with M_r <14 kDa ie 13 kDa and 11 kDa. These antigens were not revealed

with the India ink stain but were reactive with 4 and 2 ABPA sera respectively.

Notwithstanding the discrepancies in the antibdoy response to specific antigens seen with the ABPA sera, the major difference between the results presented in this thesis and those of previous studies was in the response seen in apparently healthy controls.

Most workers have demonstrated little or no binding of antibody from control sera to western blots of Aspergillus antigens. Brouwer (1988) showed that the majority of anti-Aspergillus ELISA and precipitin negative sera reacted weakly with a maximum of four antigens between 90 kDa and 120 kDa. A minority also reacted with two antigens with apparent M_rs of 36 kDa and 50 kDa. Other reports have demonstrated even less reactivity with either no specific binding (Leung et al, 1988) or binding to only a single antigen (Kurup et al, 1989). However, in the experiments presented here, when blood donor sera were used to stain the WS2109 blots a large number were reactive with the separated antigens. As with ABPA sera the most reactive antigens were those at 82 kDa, 77 kDa and 44 kDa reacting with 100%, 95% and 80% of blood donor sera respectively. It was not possible to distinguish ABPA patients and blood donors by the band pattern they produced against the WS2109 antigen but in general there was much less binding to the lower M_r antigens (<38 kDa) by the blood donor sera.

It was unlikely that the dilution of serum used to stain the blots caused non-specific staining as other workers have used similar dilutions eg 1:100 (Kurup et al, 1989) and 1:50 (Brouwer, 1988) in their experiments and shown only limited binding.

As humans are universally exposed to aspergilli their immune systems have to deal with these fungi. In the general introduction to this thesis evidence was presented that suggested both alveolar macrophages and neutrophils were capable of killing these fungi. It is possible that these cells can also present antigens from the fungus to antibody producing cells and that the antigens at 82 kDa, 77 kDa and 44 kDa represent immunodominant antigens in the normal healthy population. In contrast, the lower M_r antigens (<38 kDa) appear to stimulate antibody production only after prolonged exposure to the fungus, as is the case in ABPA or aspergilloma.

The majority of neutropenic patients, irrespective of their clinical condition, produced remarkably similar immunoblot patterns. The staining was generally weaker than the ABPA and blood donor sera but antibodies to the 82 kDa and 44 kDa antigens were seen in a large number of these patients ie 93% and 85% respectively. However, a smaller proportion of these patients had antibody to the 77 kDa antigen.

It was not possible to differentiate patients with IPA

or CFI from those with no evidence of aspergillosis on the basis of immunoblot band pattern.

In the only previous report of immunoblotting to detect antibodies as a diagnostic method for IPA all reactive bands had apparent M_rs >33 kDa (Matthews et al, 1985). These workers demonstrated that antibodies to a 40 kDa antigen were present in 11/14 patients with IPA but in none of 10 controls. I was unable to detect specific antibodies to this antigen in the patients studied here despite using a similar antigen preparation, electrophoretic, blotting and staining techniques. However, given the known variability of antigen extracts prepared from Aspergillus spp it was possible that the WS2109 extract did not contain this antigen. This possibility was supported by the fact that no antigen at this M_r was seen on the India ink stain of the WS2109 extract.

Notwithstanding the fact that they were able to differentiate between IPA patients and controls by antibody detection Matthews et al (1985) concluded that the level of specific antibody was very low and that the test may not be clinically useful. The results presented in this section of the thesis supports this view.

It has been shown that ELISA results correlate well with other immunological methods such as RIA (Dupont et al, 1987) and double immunodiffusion (Sepulveda et al, 1979). However, differences were seen when ELISA and immunoblotting

were compared (Brouwer, 1988). Sera positive for anti-*Aspergillus* IgG by ELISA were negative by blotting in 50% of cases and conversely 9% of ELISA negative sera were positive for anti-*Aspergillus* IgG by immunoblotting. The work done here supports this data. No correlation was seen between WS2109 ELISA results and the band pattern produced by immunoblotting. Both ELISA positive and negative sera gave essentially similar blot patterns.

This finding was not surprising as the discrimination between positive and negative results in ELISA is usually a compromise (section 4.5). Cut-off points are usually determined by reference to values in apparently healthy controls. When applied to the detection of anti-*Aspergillus* antibodies, individuals within the normal ELISA range will still have some specific antibody as a result of ubiquitous exposure to this fungus (Bardana et al, 1972b; Schonheyder and Andersen, 1982; 1984b). The presence of this innate anti-*Aspergillus* antibody may have been revealed on immunoblots.

It was though initially surprising to find that patients who seroconverted to antibody positive by ELISA did not produce specific antibodies 'de novo' to a particular antigen. However, in the majority of patients studied here, especially bone marrow recipients, antibody response to previously unseen antigens is not very good until approximately six months after engraftment. Therefore it is

likely that the major response will be to previously recognised antigens. This would be reflected by increased overall binding in an ELISA but would not be revealed on immunoblots.

In conclusion it is possible to state that: i) immunoblotting for the detection of anti-Aspergillus IgG was not capable of diagnosing IPA; ii) the predominant antibody response in healthy individuals appears to be to higher M_r components of aspergilli particularly the antigens at 82 kDa, 77 kDa and 44 kDa and, iii) lower M_r antigens are immunogenic only after prolonged exposure to the fungus.

CHAPTER SIX: DETECTION OF ASPERGILLUS ANTIGEN BY A LATEX AGGLUTINATION TEST AND TWO ELISAs.

6.1 Introduction.

The inability to diagnose IPA by the detection of specific anti-*Aspergillus* IgG means that another approach must be adopted. Microbial antigen has been demonstrated in the body fluids of individuals suffering from a large number of infectious diseases since the first report of circulating substances in experimental *Streptococcus pneumoniae* infection in the early part of this Century (Dochez and Avery, 1917).

In fungal infections antigen detection has been especially useful in the diagnosis of cryptococcosis where the major immunoreactive circulating antigen is a high M_r glucoronoxylomannan (Kaufman and Blumer, 1983). Circulating antigen has also been shown to be present in invasive candidosis (Reiss et al, 1986a; Matthews et al, 1987; Matthews and Burnie, 1988), coccidioidomycosis (Galgiani et al, 1984) and histoplasmosis (Wheat et al, 1986).

This approach has also been attempted for the diagnosis of IPA (Table VI.i). The first report of the detection of *Aspergillus* antigen in body fluids was in 1977 when antigenaemia was demonstrated in experimental murine infection. Two precipitin lines were formed by CIE of infected mouse serum against a rabbit hyperimmune antiserum

Table VI.i Detection of Aspergillus antigens in IPA.

Reference	Detection method	Patients pos/tested	Controls pos/tested	Sens	Spec	PPV	NPV
Reiss & Lehmann 79	CIE	6/6	N	100	NA	NA	NA
Shaffer et al 79b	RIA	3/3	0/21	100	100	100	100
Weiner 80	RIA	4/7	0/69	57	100	100	96
Sabetta et al 85	ELISA	11/19	0/42	58	100	100	84
Dupont et al 87	ELISA	2/12[1]	N	17	NA	NA	NA
	RIA	7/13[2]	N	54	NA	NA	NA
LePape & Deunff 87	ELISA	9/10	0/10	90	100	100	91
Talbot et al 87	RIA	16/22	3/31	73	90	84	82
Fujita et al 88	ELISA	16/18	1/155	89	99	94	99
Johnson et al 89	ELISA	4/5	0/17	80	100	100	94
Dupont et al 90	LAT	14/15	0/54	93	100	100	98

key. pos, positive; Sens, sensitivity; Spec, specificity; PPV, positive predictive value; NPV negative predictive value; N, ,no controls tested; NA not applicable; CIE, counter immunoelectrophoresis; RIA, radioimmunoassay; ELISA, enzyme-linked immunosorbent assay; LAT, latex agglutination test.
[1], when sera assayed; [2], when urines assayed.

raised to both mycelial and commercial (Bencard antigens 1 and 2) extracts of A.fumigatus (White et al, 1977). Mice were infected either intravenously or by inhalation with conidia of A.fumigatus NCPF 2085 after immunosuppression with cortisone. Antigen was detected after 72h in serum pooled from groups of mice and after 96h from individual mice. Antigenaemia was not detected in all mice, and some which were heavily infected were antigen negative by CIE. The only information given on the nature of the circulating antigen was that CIE showed two precipitin lines.

This work was extended two years later when a comparison of CIE and ELISA for the detection of antigenamia in experimentally infected mice was undertaken (Richardson et al, 1979). 72h post-infection antigen was detected by ELISA in 19 mice (100%) compared with 11 by CIE (58%); after a further 24h the numbers positive by ELISA had fallen to 8 (66%) and risen to 12 (86%) by CIE. The authors suggested that this was due to a change in the nature of the circulating antigen during infection.

In contrast another group of workers were unable to detect antigenaemia, using CIE, in experimentally infected rabbits when they used a number of hyperimmune antisera to various A.fumigatus extracts. However, when serum from an infected animal was used as immunogen the resulting antiserum, which recognised a single component of a crude mycelial extract of A.fumigatus, was capable of detecting

antigenaemia and antigenuria 60-120h post-infection (Lehmann and Reiss, 1978). The circulating antigen recognised by this antiserum was destroyed by sodium metaperiodate hydrolysis but not by an agarose bound pronase, suggesting that it was partially or totally carbohydrate.

Subsequently a GM polysaccharide (galactose:mannose 1.17:1) was purified from A.fumigatus mycelium (Reiss and Lehmann, 1979). This antigen had an apparent M_r of 25-75kDa and was capable of reacting with the antiserum described above. However, the circulating antigen present in infected rabbits was unable to pass through a membrane with a M_r cut-off of 125kDa. This discrepancy in size was said to be due to the complexing of GM with protein *in vivo*.

Sera from 3/3 patients with proven IPA and 3/3 with suspected IPA were antigen positive when tested by this CIE method.

In addition to the early work of the Atlanta group described above two other laboratories have used purified GM in their detection assays. One group prepared GM as described by the Atlanta workers and the antigen extracted had similar properties: it was resistant to boiling and treatment with pronase, it had a M_r between 50-100 kDa and bound to conA. When used in an inhibition ELISA with a hyperimmune antiserum to an A.fumigatus cell wall fraction the level of sensitivity of the assay was 10ng/ml of GM. The

inhibition ELISA was evaluated in an experimental model of IPA. Sera from 4/4 infected rabbits contained GM at concentrations from 95ng/ml to 350ng/ml; two control rabbits were GM negative. The assay was not inhibited by C.albicans mannan at concentrations up to 1µg/ml (deRepentigny et al, 1987). No reports on the use of this inhibition ELISA in human IPA have appeared in the literature.

Dupont et al (1987) prepared GM according to the method of Bennett (1985). This antigen contained 0.7% nitrogen, 0.17% phosphate and only galactose and mannose on monosaccharide analysis. The GM was conjugated with BSA and used to coat microtitre plates for an inhibition ELISA. The detector antibody was a rabbit hyperimmune antiserum raised to A.fumigatus by intravenous injection of conidia.

This ELISA was used to detect GM in both serum and urine from human and experimental IPA. When serum from rabbits infected with A.fumigatus was tested, 4/12 were GM positive after 2 days. The level of GM ranged from 108ng/ml to 356ng/ml. However, GM could not be detected in a further eight rabbits infected with A.flavus (cf Weiner and Coats-Stephens, 1979).

In contrast urinary antigen was found whenever the first sample was taken in 16 rabbits with lethal IPA (14 A.fumigatus, 2 A.flavus) and in 4/15 animals with non-lethal IPA. The GM concentration in the urine ranged from 24ng/ml to 1900ng/ml. The GM present in rabbit urine was shown to

have a wide M_r range, as demonstrated by gel filtration, of between 70 kDa and 10kDa, which centred on 18kDa.

When the ELISA was evaluated in human infection, GM was seen in the serum of only 2/12 patients with proven IPA compared with 7/13 cases when urine was assayed. The concentration of GM found in the two positive sera was 150ng/ml and 500ng/ml; the concentration in urine was lower ranging from 1ng/ml to 83ng/ml. An RIA was also evaluated using the same GM and antiserum and the results of the two assays were essentially interchangable.

In addition to the work with purified GM described above other groups have employed crude carbohydrate enriched fractions from A.fumigatus in an attempt to diagnose IPA by antigen detection.

A series of papers from the same laboratory have reported on the use of a competitive RIA for the detection of circulating Aspergillus antigen. These workers initially established experimental IPA in rabbits and found that the principal humoral response in animals, who survived, was to four Aspergillus antigens (Weiner and Coats-Stephens, 1979). Two of these contained carbohydrate as they were sensitive to periodate hydrolysis. One of the periodate sensitive antigens, a cell wall carbohydrate, was purified from a crude mixture of disrupted A.fumigatus mycelium by chromatography on Sephacryl S200. The immunoreactive components (M_r 150-180 kDa) were radiolabelled and then

fractionated by gel filtration and affinity chromatography. The resulting antigen bound to conA and was probably polymeric as gel filtration chromatography sized the antigen between 45 kDa and 300kDa. A detailed analysis of the chemical composition of this antigen(s) was not undertaken.

This radiolabelled antigen was used in a competitive RIA with a hyperimmune rabbit antiserum to a cell wall extract of A.fumigatus. The RIA was capable of detecting 3.1ng/ml of free antigen or 500ng/ml of antigen in absorbed rabbit serum. When applied in a rabbit model of IPA 78% of 51 infected rabbits were antigen positive whereas none of 25 animals with invasive candidosis or 76 normal controls were positive. The mean (+/- standard error of the mean (SEM)) concentration of antigen in rabbit serum was 11.7µg/ml (+/- 3.92µg/ml) 48h post-infection compared with 0.59µg/ml (+/- 0.04µg/ml) at day eight. Heating of the sera, to be assayed, with 0.04M citrate buffer increased the sensitivity of the RIA from 20% to 53% suggesting that the reactive epitopes were occluded by protein in vivo (Weiner and Coats-Stephens, 1979).

In an evaluation of the RIA in human infection 16/21 sera from 4/7 patients with IPA were antigen positive (Weiner, 1980). The mean (+/- SEM) serum antigen concentration found in patients was 335ng/ml (+/- 37ng/ml); much lower than that reported for rabbits, but the level in a positive pleural fluid was high (1551ng/ml). None of 69

control sera, including 11 from patients with non-invasive forms of aspergillosis, were antigen positive. Patients with IPA caused by A.flavus as well as A.fumigatus had detectable antigenaemia (Weiner et al, 1983).

This RIA has also been applied to the detection of Aspergillus antigen in bronchoalveolar lavage fluid (BALF), as IPA is often restricted to a pulmonary focus (Andrews and Weiner, 1981). 91% of BALFs from rabbits, defined as having major pulmonary involvement, were antigen positive. However, in a group of rabbits with minor pulmonary involvement only 2/20 unconcentrated BALF specimens contained antigen. In a small number of animals the BALF was positive while the serum was negative (14%); in a larger group (33%) the opposite was seen ie positive serum, negative BALF. This was reflected in the mean concentration of antigen seen in BALF (22.9ng/ml) compared to serum (5.47µg/ml). Overall when both BALF and serum were examined the sensitivity of the assay was 90% and the specificity was 100% (Andrews and Weiner, 1981).

The latest report of this RIA was in a study of 79 haematological patients (Talbot et al, 1987). The sensitivity was 74%; specificity 90%; PPV 82%; NPV 85% and it would have been the first indicator of IPA in 17/22 (77%) episodes.

Two reports of similar competitive RIAs, based on two different antigens, have been published by another group

(Schaffer et al, 1979a; 1979b). Initially an alkali extract of defatted A.fumigatus mycelium was used. This extract contained approximately 84% carbohydrate and 4% protein. Three precipitin lines were produced in double immunodiffusion against a hyperimmune rabbit antiserum raised to a crude mycelial extract of the same A.fumigatus strain. This 'polysaccharide rich alkali extract' was radiolabelled and used in a competitive RIA. Sera to be tested were deproteinized by centrifugation prior to assay. 4/6 rabbits experimentally infected with A.fumigatus were antigen positive by this RIA which had a limit of sensitivity of 500ng/ml (Shaffer et al, 1979a). Similar carbohydrate antigens prepared in the same way from A.flavus, A.niger and A.carneus could inhibit binding of the A.fumigatus antigen in this RIA, whereas polysaccharide antigens from Cryptococcus neoformans and C.albicans could not.

In the second report a GM enriched alcohol precipitate was prepared by the method of Sakaguchi et al (1969). This extract was further purified by chromatography and carbohydrate containing fractions were pooled. The resulting antigen, designated M39, contained 10% protein and 74% carbohydrate. Only one band was seen in agar gel diffusion against antisera raised to mycelial extracts of A.fumigatus, A.carneus, A.flavus and A.niger. The RIA was used to test six serum samples from two patients with proven IPA; five

were antigen positive. All five sera from a patient with suspected IPA were also antigen positive. No sera from any of 21 control patients contained detectable antigen. The RIA was not inhibited by C.albicans mannan or polysaccharides from Blastomyces dermatitidis and Coccidioides immitis. Sera to be tested were deproteinized with perchloric acid, dialysed and reconstituted into PBS at 1/10th the original volume - in effect a 10 fold concentration. Antigen concentrations detected in serum ranged from 50ng/ml to >1µg/ml. However, the lower levels were also observed in sera from healthy controls and patients not infected with Aspergillus sp. The limit of sensitivity of the assay was 10ng/ml to 100ng/ml (Shaffer et al, 1979b). No further report of these RIAs have appeared in the literature.

Carbohydrate enriched fractions from A.fumigatus have also been used in the equally sensitive ELISA technique to detect Aspergillus antigen. A somatic antigen from the mycelium of A.fumigatus containing 47% carbohydrate and 47% protein was preciptated with TCA. After centrifugation the supernatant was collected and designated 'Aspergillus carbohydrate antigen' (Sabetta et al, 1985). A hyperimmune antiserum was prepared by immunizing rabbits with A.fumigatus conidia. Both reagents were used in an inhibition ELISA to detect Aspergillus antigenaemia. Sera to be tested in the ELISA were precipitated with TCA to remove protein, therefore the authors concluded that the antigen

detected was '*Aspergillus* carbohydrate antigen'. In an experimental model of IPA, antigenaemia was detected in 5/6 infected rabbits but not in any of eight control animals. When applied retrospectively to a study of 19 patients with proven IPA antigenaemia was detected in 11 individuals. The concentration of '*Aspergillus* carbohydrate antigen' ranged from 5ng/nl to 50ng/ml. Antigenaemia was not detected in any of 42 control patients.

A sandwich ELISA method has also been described for the detection of antigenaemia in IPA. Carbohydrate containing fractions of an *A.fumigatus* mycelial extract were prepared by chromatography and used as a reference antigen (Fujita et al, 1988). The detector antibody was an IgG fraction obtained from a hyperimmune rabbit antiserum raised to *A.fumigatus* conidia. Specimens to be tested were precipitated with EDTA to remove protein components, the authors claimed that they were therefore detecting only carbohydrate antigens. 16/18 patients with IPA were antigen positive compared with only 1/155 non-infected controls. The concentration of carbohydrate antigen in patients ranged from 1.6ng/ml to 17ng/ml.

As was stated above the majority of work has concentrated on GM and carbohydrate fractions of *A.fumigatus*. However there are several reports in the literature where crude preparations from *A.fumigatus*, which contained both carbohydrate and protein and/or glycoprotein

antigens, have been used in inhibition ELISAs (LePape and Deunff, 1987; Wilson et al, 1987; Johnson et al, 1989). A positive result in these assay systems could have been due to the presence of any antigen that was capable of inhibiting binding of the detector antiserum to antigen coated microtitre plates.

In a recent study (Johnson et al, 1989) an unfractionated CF antigen prepared from A.fumigatus, which was substantially glycoprotein in nature, and a high titre human anti-Aspergillus serum from a patient with ABPA, were used in an inhibition ELISA. This was evaluated in 22 bone marrow transplant recipients, five of whom developed proven or suspected IPA. Four of these patients were antigenaemic whereas all 17 individuals with no evidence of fungal involvement were antigen negative. The concentration of antigen seen in the patients' serum ranged from approximately 80ng/ml to 800ng/ml. The limit of sensitivity of the assay was 68ng/ml.

A crude unfractionated mycelial extract from A.fumigatus has also been used in an inhibition ELISA as coating antigen and either a rabbit hyperimmune antiserum to A.fumigatus cell walls or a human anti-Aspergillus serum was employed as the detector antibody (Wilson et al, 1987). When evaluated in a retrospective study of nine patients with IPA antigenaemia was detected in eight. However, the rabbit and human ELISAs did not always concur. When applied

prospectively to a study of 315 sera recieved from 200 'at risk' patients 38 contained detectable antigen (24 patients). When a single serum specimen from a patient was tested the level of positivity was 3.5%, but positivity rose to 51.3% when two or more sera were assayed.

An antigen level of 100ng/ml was the lowest concentration that these authors considered 'strongly suggestive of fungal invasion'. Levels up to 4µg/ml were seen.

In one patient almost daily sera were available. The concentration of antigen fluctuated widely. In addition a double antibody sandwich ELISA utilising an IgG fraction from a rabbit anti-Aspergillus antiserum was used to assay the same samples. The results of the two assays did not always coincide suggesting that they were detecting different antigens.

A conA binding fraction of a crude mycelial extract has been used to coat microtitre plates for an inhibition ELISA. The detector antibody was prepared by ligating the conA bound fraction onto an affinity column. A rabbit anti-Aspergillus antiserum was incubated with the column and non-bound antibodies were washed off. Those antibodies specifically bound to the glycoprotein fraction were then eluted from the column and used as the detector antibody (LePape and Deunff 1986).

When evaluated in a mouse model of IPA this inhibition

ELISA detected antigenaemia in 10/10 infected mice whereas 0/10 controls were antigen positive. The concentration of circulating glycoprotein antigen in mice ranged from 28ng/ml to >500ng/ml. In human infection 9/10 patients had detectable glycoprotein antigen ranging in concentration from approximately 25ng/ml to >500ng/ml. Antigen was not detected in any of 10 control patients.

6.2 Aims.

The aims of this section of the thesis were to determine: i) if *Aspergillus* antigen detection could predict the development of IPA; ii) if diagnosis by the detection of antigen preceded diagnosis by conventional methods; iii) if antigen could be detected more often in urine than serum; iv) if GM was the only antigen of *Aspergillus* present during IPA and v) if antigen detection correlated with the clinical course of the infection.

To this end a number of important general points can be made, based on the published literature, on *Aspergillus* antigen detection: i) GM is the only defined antigen known to circulate during IPA; ii) a difference in the antigen detection method or the reagents employed will affect the results obtained; iii) antigen levels fluctuate widely; iv) the concentration of antigen is low and therefore sensitive methods of detection are required; v) the testing of multiple samples increases the chances of a positive test

and vi) urine appears to be a more promosing specimen to test than sera or BALF.

The design of this section of the thesis has tried to take account of all these variables by the use of different Aspergillus antigen fractions and antibodies in three antigen detection tests (two inhibition ELISAs and a latex agglutination test [LAT]) and by the regular testing of both serum and urine specimens.

Both inhibition ELISAs were performed mostly on specimens which were prospectively collected however, a small number of sera had been stored at -70°C for a short period before this study began. The LAT was not available until the latter stages of this work and testing was therefore done retrospectively on specimens that had been stored at -70°C.

6.3 Patients, materials and methods.

6.3.1 Patients.

Serum samples were available on all 121 patients described in Chapter 2.

Urine specimens were available on eight patients with IPA (numbers 1, 2, 3, 11, 12, 14, 17 and 19); nine with CFI (numbers 20-25, 27, 29 and 31); two with OFI (numbers 37 and 38) and 75 with NEFI.

6.3.2 Control sera and urines.

Sera were collected from 100 voluntary blood donors and urines from 81 medical students; these specimens were used as controls for this section of the thesis. In addition 19 sera containing high levels of rheumatoid factor and 52 sera received for routine rubella antibody screening were used as controls for the LAT.

6.3.3 Antigens.

WS and BF antigen fractions were prepared from A.fumigatus NCPF 2109, EP from A.fumigatus 1028 and GM from EP1028 as described in sections 3.3.2, 3.3.4, 3.3.3 and 3.3.5 respectively.

6.3.4 Preparation of sera and urines for antigen testing by ELISA.

Sera to be tested were diluted 1:5 in PBST and heated to 80°C for 30min. After cooling sera were centrifuged at 10,000 x g for 5min.

Urines were dialysed overnight against distilled water and tested neat without heating.

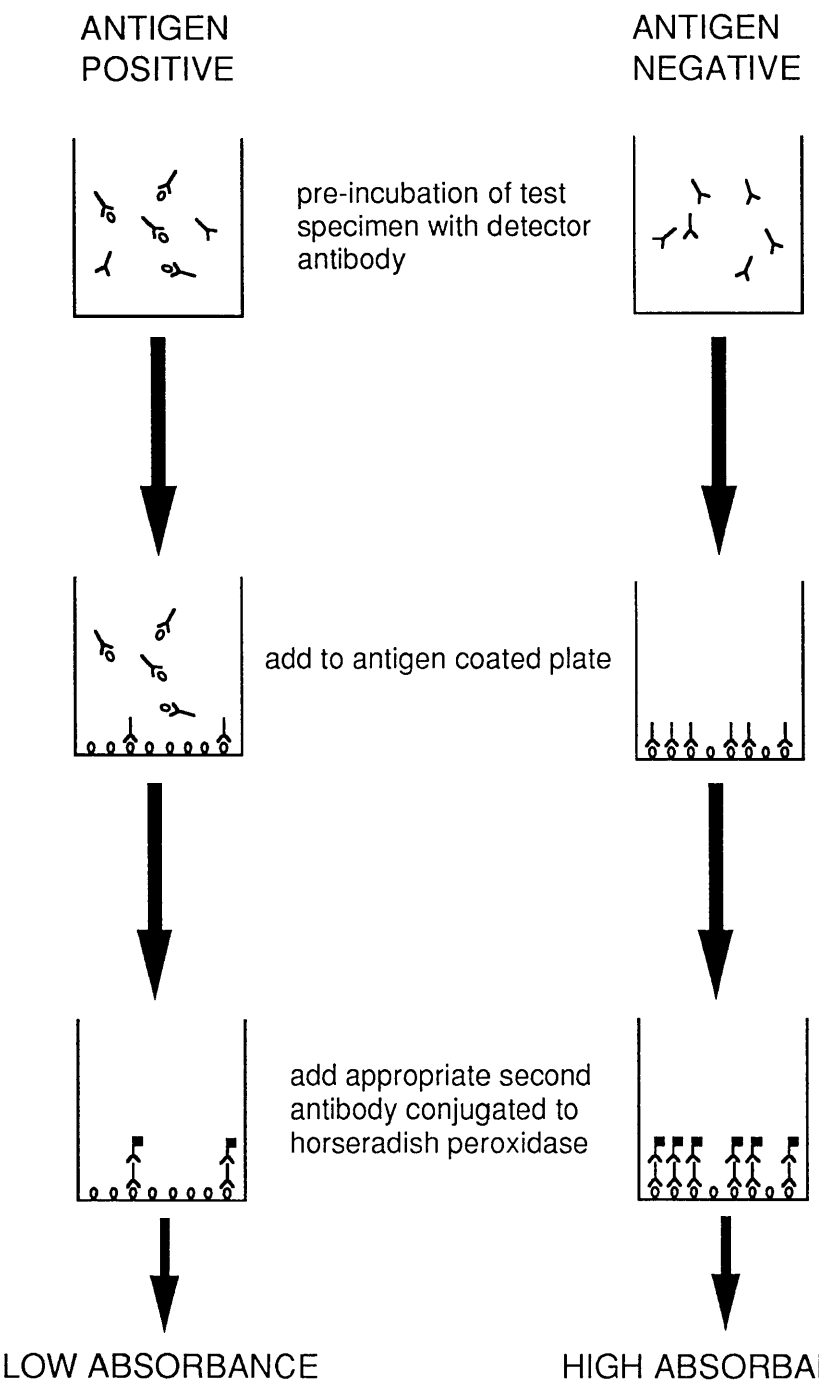
6.3.5 Polyclonal inhibition ELISA (PAb).

This assay was based on the principle (as was the MAb ELISA described in 6.3.6) that pre-incubation of a test specimen which contained antigen with an appropriate detector antibody would result in removal of a proportion of that antibody. This would be reflected in a reduction of binding by the detector antibody to a similar antigen coated onto microtitre plates and a reduction in absorbance after incubation with an appropriate horseradish peroxidase conjugated secondary antibody. If no antigen was present in the test specimen antibody binding would not be reduced (Fig 6.1).

Optimal concentration of all reagents was determined empirically by chequerboard titrations.

6.3.5.1 Antigen coating of microtitre plates. WS2109 was added to carbonate buffer {3.10.9} at 1-5µg/ml protein. 100µl of antigen solution was placed into each well of a

Figure 6.1 Principle of inhibition ELISAs.



key, ° antigen; detector antibody; second antibody (conjugated to horseradish peroxidase.

M129A microtitre plate (Dynatech) and incubated at 37°C for 2h. Plates were washed x3 with PBST, blocked with 5% (w/v) NFM for 60min at 37°C, rinsed in PBST and used immediately.

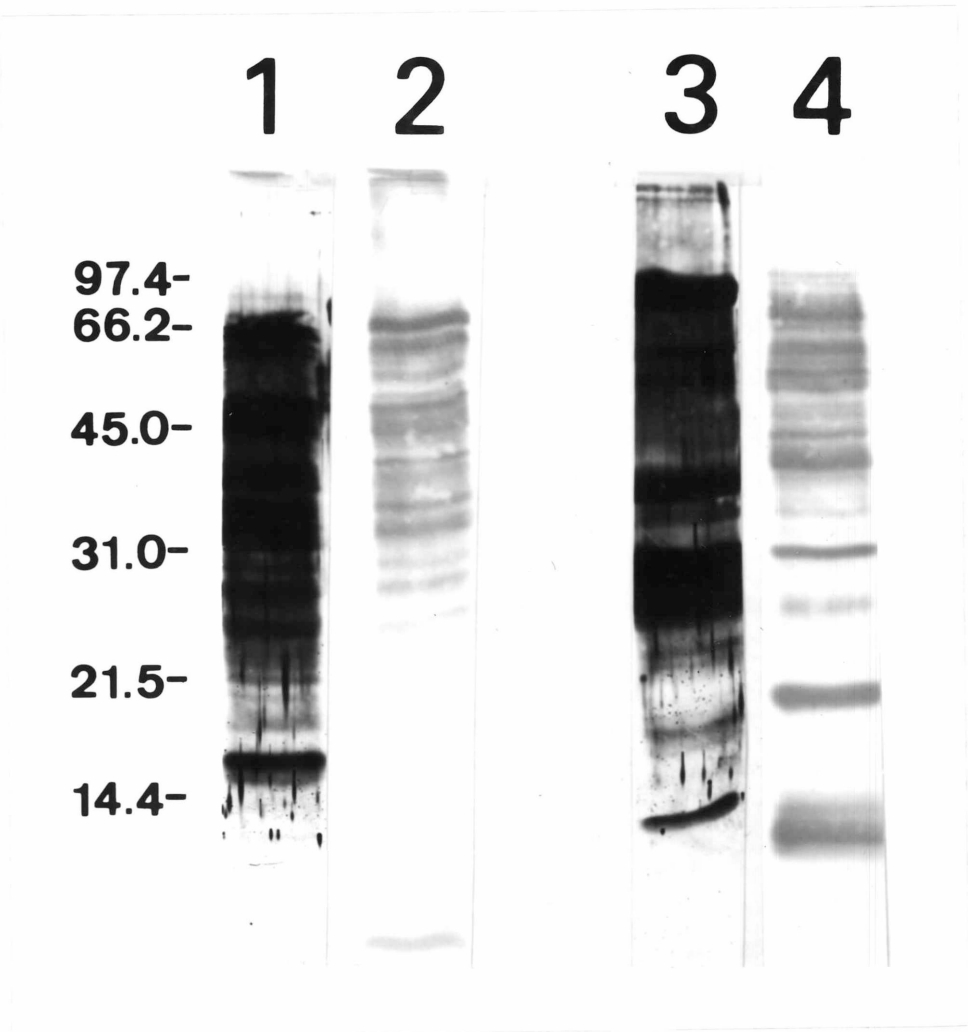
6.3.5.2 Detector antiserum. A high titre human anti-Aspergillus IgG positive serum pool was used as detector antibody for the PAb ELISA. It was obtained from multiple serum specimens taken from a 13 yr old boy with chronic granulomatous disease. This patient had persistent Aspergillus lung abscesses and subsequently developed a cavitating osteomyelitis of the spinal column from which A.fumigatus was isolated. Post-mortem cultures grew A.nidulans.

The antiserum, designated W1, reacted with a large number of antigens in both the WS2109 and WS2008 antigen extracts (Fig 6.2) and was positive in the WS2109 anti-Aspergillus IgG ELISA when diluted 1:6,400.

W1 was used in the PAb assay at a concentration which gave an antibody control A_{492} (see 6.3.5.4) of between 1.300 and 1.500. This equated to a dilution of between 1:100 and 1:125 depending on the batch of WS2109 and horseradish peroxidase conjugated anti-human IgG antibody used. This gave a final W1 dilution of between 1:1100 and 1:1375 as 10µl of diluted W1 was added to 100µl of control or test samples.

W1 was sent to the MRL for C.albicans precipitin

Figure 6.2 Immunoblots of WS2109 and WS2008 antigens.



key. lane 1, India ink stain of WS2008; lane 2, W1 stain of WS2008; lane 3, India ink stain of WS2109; lane 4, W1 stain of WS2109.
M_r standards are in the left margin.

testing and was negative.

6.3.5.3 Antigen control. BF2109 antigen was added to anti-Aspergillus antibody and Aspergillus antigen negative serum at a concentration of 6250ng/ml protein and to dialysed Aspergillus antigen negative urine at 1250ng/ml.

Spiked sera were pre-treated as in 6.3.4 and urines were used as prepared.

The BF2109 antigen was double diluted in duplicate across the microtitre plate in either heat treated antibody/antigen negative serum (diluted 1:5 in PBST) or dialysed antigen negative urine, depending on the nature of the test specimens being assayed. This gave a BF2109 antigen titration of 1250ng/ml to 0.6ng/ml.

This titration was used to determine the concentration of Aspergillus antigen in test specimens.

6.3.5.4 Inhibition ELISA. Each plate contained the following two controls: i) antibody control ie 10µl of W1 in 100µl of heat treated antibody, antigen negative serum or dialysed antigen negative urine and ii) background control ie 110µl of negative serum or urine without W1.

Both W1 and the horseradish peroxidase conjugated anti-human IgG secondary antibody were diluted in 0.1% (w/v) NFM.

100µl of test specimen, or BF2109 antigen control, was added, in duplicate, to the wells of a round bottomed

microtitre plate (Sterilin, Hounslow, Middx). 10µl of W1 was added to each well with the exception of the background control. The plates were incubated overnight at 4°C and the contents were then transferred to M129A (Dynatech) microtitre plates freshly coated with WS2109.

These were incubated at 37°C for 2h on a plate shaker (Flow Laboratories) at speed 4. The plates were washed x3 in PBST and 100µl of a 1:2000 dilution of horseradish peroxidase conjugated anti-human IgG (Dako) was added to all wells.

The plates were incubated as above for a further 2h and again washed x3 with PBST. The ELISA chromogen, o-phenylene diamine, dihydrochloride (Sigma) was dissolved in phosphate/citrate buffer pH 5.0 {3.10.10} shortly before use at 0.2% (w/v) and the enzyme substrate H_2O_2 was added to a final concentration of 0.01% (v/v). 100µl of chromogen/substrate solution was added to each well and the reaction was allowed to proceed in the dark at room temperature for 15min. The reaction was stopped by the addition of 100µl of 1M H_2SO_4 and the plates were read at 492nm in a Titertek multiscan spectrophotometer (Organon Tecnica).

Results were expressed as the percentage inhibition (%I) of a test specimen or BF2109 control A_{492} on the A_{492} of the W1 antibody control according to the formula;

$$\%I = 1 - (\text{test } A_{492} / \text{W1 control } A_{492}) \times 100.$$

6.3.6 Monoclonal antibody ELISA (MAb).

This was essentially as described for the PAb ELISA with the following modifications:

i. The coating antigen was EP1028. This was used at a concentration of 1-5µg/ml dry weight.

ii. GM was used as the control antigen. This was titrated from 1000ng/ml to 1ng/ml.

iii. The detector antibody was a monoclonal antibody to A.fumigatus GM (EBA2) (see section 6.3.7). This was used at a concentration of 1µg/ml Ig.

iv. The second antibody was an horseradish peroxidase conjugated affinity purified goat anti-Rat Ig (kappa chain specific) (Eco-Bio nv, Genk, Belgium). This was used at a concentration of 1µg/ml Ig.

6.3.7 EBA2, monoclonal antibody to A.fumigatus GM.

EBA2, a rat IgM monoclonal antibody to A.fumigatus GM, was a kind gift from Dr. D Stynen (Eco-Bio). EBA2 was specific for the galactofuranose component of GM and recognised GM prepared from all species of Aspergillus tested including A.flavus, A.niger, A.terreus and A.nidulans. No reaction was seen with culture filtrates of Geotrichum candidum, Sporothrix schenkii, Fusarium oxysporum or Mucor racemosum. However, the antibody did react with extracts of species from the closely related genus Penicillium (Debeaupuis et al, 1989).

ELISA studies with whole blastospores showed that the antibody was unreactive with C.albicans (4 strains), C.tropicalis (3 strains), C.pseudotropicalis, C.parasilosis, C.krusei, C.guillermondii and Torulopsis glabrata. However, EBA2 did react with very high levels of C.albicans type B mannan (>10µg/ml) (Debeaupuis et al, 1989).

No reactivity was seen between EBA2 and a large number of Gram positive and Gram negative bacteria.

6.3.8 Latex agglutination test (LAT).

'Pastorex Aspergillus' is a commercially available (Diagnostics Pasteur, Marnes-le-Coquette, France) LAT for the detection of Aspergillus GM. The test is based on latex particles coated with the same monoclonal antibody (EBA2) to A.fumigatus GM as described in section 6.3.7.

6.3.8.1 Sensitivity of the LAT. The LAT reagent reacted with various fungal and bacterial extracts in the same way as the EBA2 monoclonal antibody (see section 6.3.7).

The manufacturers literature stated that the LAT reagent was capable of detecting 15ng/ml GM.

6.3.8.2 Control antigen. GM was prepared as described in section 3.3.5 and used at 16.5ng/ml as the control antigen for the LAT.

6.3.8.3 Performance of the LAT. 300µl of specimen to be tested was mixed with 100µl of 4% (w/v) EDTA and heated at 100°C for 3min in a polypropylene tube. The tube was centrifuged at 13,000 x g for 5min. 40µl of the resulting supernatant was mixed with 10µl of the latex reagent on a disposable card. The mixture was rotated at 170 rpm for 5min. Visible agglutination was scored as a positive test result.

Sera which tested positive were titrated to determine the end-point titre.

6.3.8.4 Repeat testing. Because of anecdotal reports of poor reproducibility (F. Knight, MRL, personal communication) sera and treated supernatants were stored for one month after original testing at -70°C and 4°C respectively. The test was then repeated.

6.3.9 Statistical analysis.

Chi-squared analysis (incorporating Yates correction) was used to determine if the proportion of specimens which contained antigen from each patient group were significantly different. This analysis was also used to compare the different antigen detection methods.

6.4 Results.

6.4.1 PAb ELISA.

6.4.1.1 Control levels. These values were obtained by assaying each of the 100 negative control sera and 81 urines in five replicate ELISA experiments. The mean %I value for each of the individual serum and urine specimens from the five runs was calculated. The mean (and SD) %I of the 100 sera and 81 urines was then determined.

The mean (+2 SD) %I of the 100 control sera was 31%, while the same value for the 81 urines was 37%.

6.4.1.2 Inter- and intra- assay variation. These values were determined in a similar way to that described in section 4.3.6. *Aspergillus* antigen positive and negative serum or urine however, were substituted for anti-*Aspergillus* IgG positive and negative sera. Both values were <10%.

6.4.1.3 BF2109 antigen titration. 30 replicate standard curve experiments were performed with the BF2109 antigen titrated from 1250ng/ml to 0.6ng/ml in heat-treated antigen and antibody negative serum diluted 1:5 in PBST. The same number of standard curve experiments were performed with the BF2109 antigen titrated in dialysed antigen negative urine.

The mean (and 95% confidence limits) %I value for each individual BF2109 dilution was determined from the results of the replicate standard curve experiments and a graph was plotted (Fig 6.3).

As stated above the maximum %I of normal serum and urine was 31% and 37% respectively. This equated to an assay sensitivity of 14.0ng/ml of antigen in serum and 12.9ng/ml of antigen in urine.

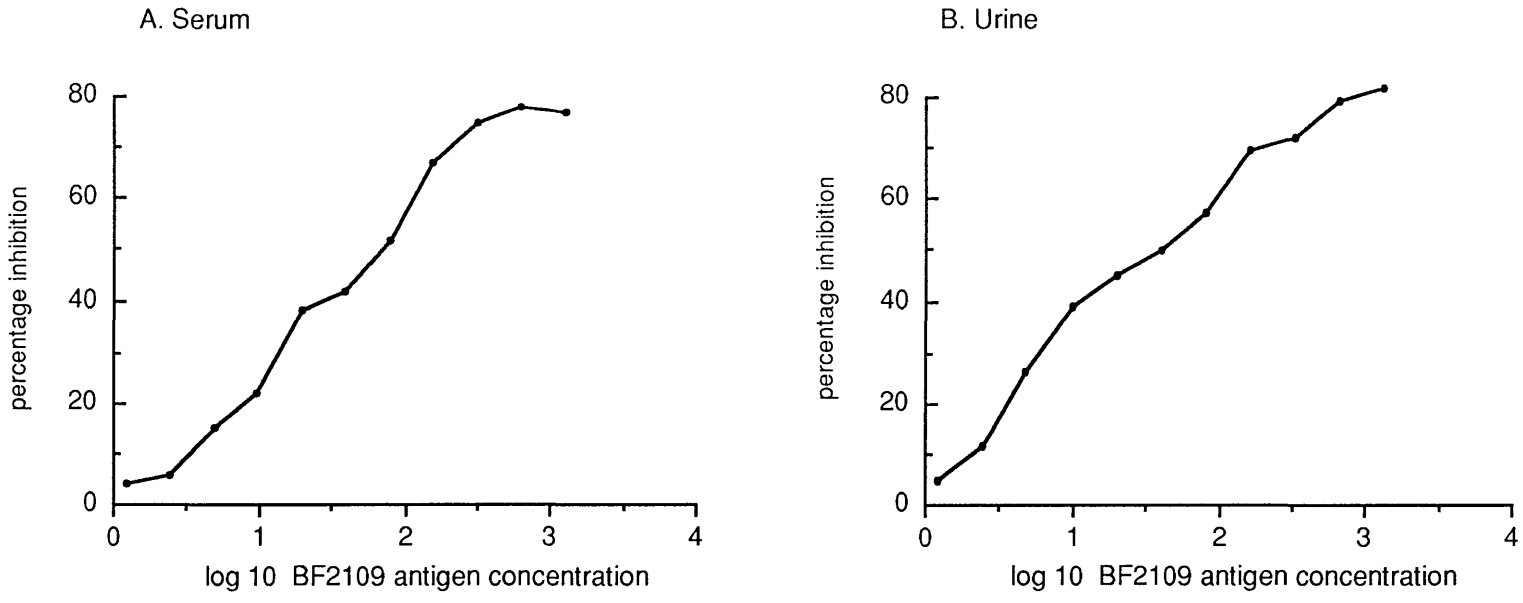
6.4.1.4 IPA patient results. A total of 482 sera (number of specimens available on each patient 5 to 55; median 23) from the 19 IPA patients and 173 urines (4 to 53; 17) from eight of these individuals (Numbers 1-3, 11, 12, 14, 17, 19) were assayed by the PAb ELISA.

18 of the 19 IPA patients had Aspergillus antigen detectable in at least one specimen (Fig 6.4; Table VI.ii). Antigenaemia was seen in 89% (17/19) and antigenuria in 75% (6/8) of cases assayed. However, antigen was detected in a significantly higher proportion of urine specimens (34/173; 20%) than sera (61/482; 13%) (Chi-squared = 5.02; $p<0.05$).

The mean concentration of circulating antigen was 91.6ng/ml (range 14ng/ml to 715ng/ml) compared to a mean level of 76.4ng/ml (14ng/ml to 562ng/ml) in urine (Fig 6.5).

A conventional clinical or laboratory diagnosis of IPA was anteceded by PAb ELISA detectable antigenaemia in 11/19 cases (range 4 to 241 days; median 13 days) and antigenuria

Figure 6.3 Titration of BF2109 antigen in serum and urine by PAb ELISA.



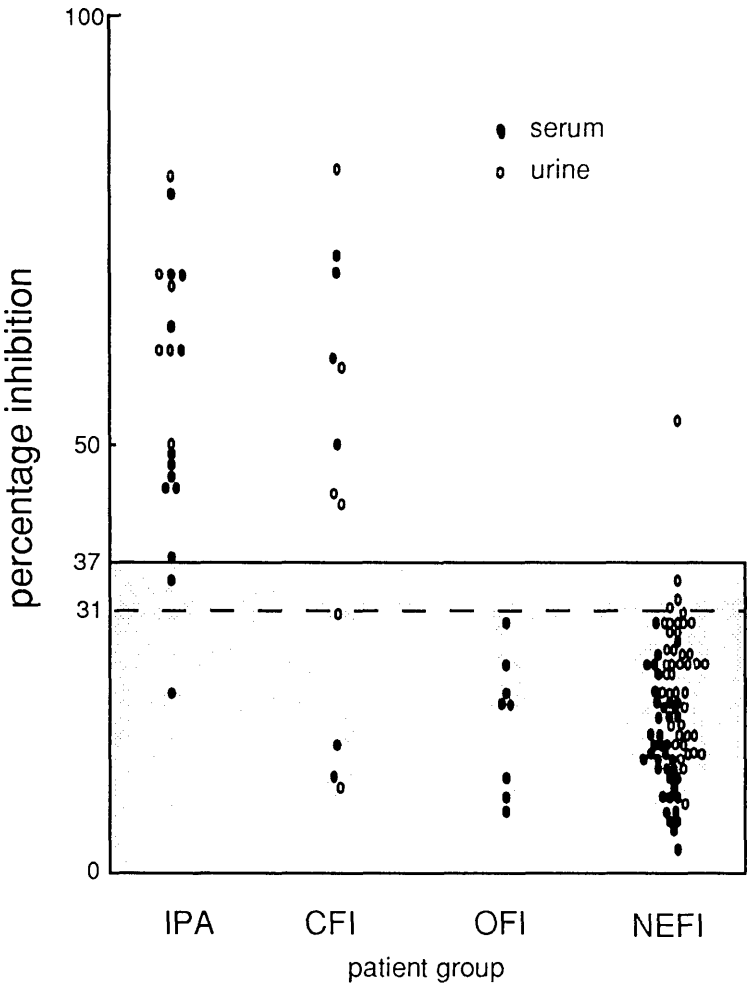
each point on the graph indicates the mean of 30 antigen titration experiments

Table VI.ii Detection of Aspergillus antigen in serum and urine of all study patients using the PAb ELISA.

	Specimen assayed					
	Serum		Urine		Combined	
Patient group	Number Patients +/t	Number Specimens +/t	Number Patients +/t	Number Specimens +/t	Number Patients +/t	Number Specimens +/t
IPA	17/19	61/482	6/8	34/173	18/19	95/655
CFI	4/12	19/310	5/9	9/84	9/12	28/394
OFI	0/8	0/148	0/2	0/39	0/8	0/187
NEFI	0/82	0/934	1/75	1/997	1/82	1/1931

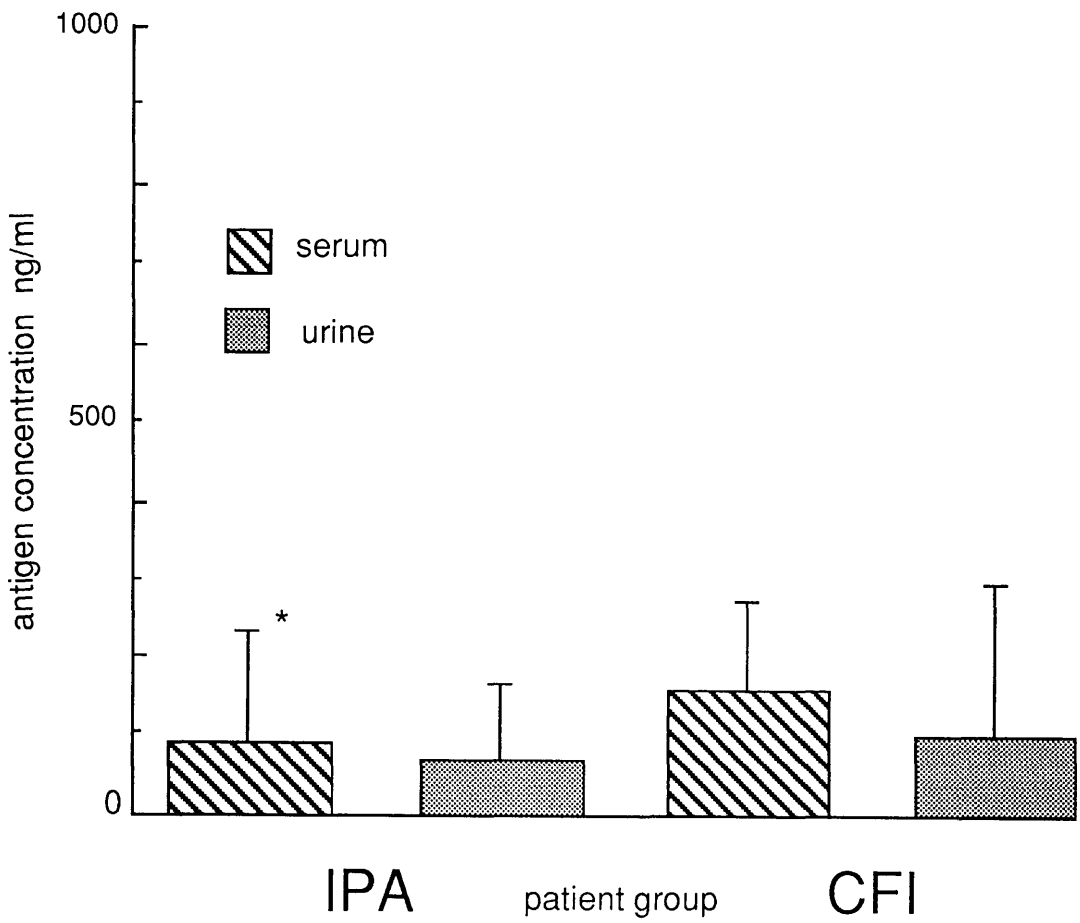
key. +, number antigen positive; t, total tested.

Figure 6.4 Maximum %I detected in all patients by PAb ELISA.



hatched area, normal range (mean plus two standard deviations) of control urines; broken line, upper limit of normal range of control sera.

Figure 6.5 Mean antigen concentration in the serum and urine of IPA and CFI patients detected by the PAb ELISA.



* bars indicate standard deviation

in 4/8 patients (1 to 14 days; 5 days) with proven IPA. When the results of serum and urinary antigen detection were combined this increased to 13/19 cases (1 to 241 days; 13 days) (Table VI.iii).

6.4.1.5 CFI patient results. 310 sera (number of specimens available on each patient 2 to 52; median 26) from the 12 CFI patients and 84 urines (4 to 20; 8) from nine of these individuals (numbers 20-25, 27, 29, 31) were assayed by the PAb ELISA. 8/12 patients had at least one positive specimen (Fig 6.4; Table VI.ii). Antigenaemia was detected in 4/12 of these patients and antigenuria in 5/9. No significant difference was seen in the proportion of urines (9/84; 11%) and sera (19/310; 6%) which were antigen positive by the PAb assay (Chi-squared = 2.1, $p>0.1$).

The mean concentration of circulating antigen was 163.3ng/ml (range 19.8ng/ml to 404ng/ml) compared with 98.6ng/ml (14.1ng/ml to 613.2ng/ml) in urine (Fig 6.5).

All four CFI patients who had detectable circulating antigen were antigenaemic before (range 9 to 65 days; median 10 days) fungal involvement was clinically suspected whereas antigenuria anteceded clinical suspicion in 3/5 (5 to 21 days; 20 days) of the individuals with detectable urinary antigen. When the results of the serum and urinary antigen assays were combined 7/8 antigen positive patients had detectable antigen prior to clinical suspicion (5 to 65

Table VI.iii Number of days by which antigen detection preceded or postdated a conventional diagnosis of IPA.

Patient number	Detection method and specimen tested				
	MAb ELISA		PAb ELISA		LAT
	Serum	Urine	Serum	Urine	Serum
1	-36	-2	-36	-14	+6
2	-7	-9	neg	-8	-7
3	-385	-65	-241	neg	-352
4	-21	NA	-13	NA	-21
5	+23	NA	+23	NA	+23
6	-24	NA	-10	NA	-24
7	-53	NA	-24	NA	-13
8	neg	NA	neg	NA	neg
9	+40	NA	+42	NA	+42
10	-15	NA	+2	NA	-15
11	neg	-3	+4	-1	-34
12	neg	-8	-8	neg	+12
13	-35	NA	-47	NA	-46
14	-20	-16	-13	-5	-20
15	-3	NA	+30	NA	-3
16	-26	NA	-24	NA	-26
17	-44	+62	+31	+31	+17
18	-24	NA	-4	NA	-23
19	-13	-10	-13	+6	-13

key. -, days prior to a conventional diagnosis of IPA being made that antigen was first detected; +, days after a conventional diagnosis of IPA was made that antigen was first detected; NA, not available; neg, antigen not detected.

Table VI.iv Number of days by which antigen detection preceded or postdated a clinical suspicion of fungal infection in CFI patients.

Patient number	Detection method and specimen tested				
	MAb ELISA Serum	MAb ELISA Urine	PAb ELISA Serum	PAb ELISA Urine	LAT Serum
19	+9	+4	neg	neg	+10
21	-11	+6	neg	+6	-8
22	-21	-28	neg	-20	-22
23	neg	-6	neg	-5	neg
24	neg	-11	neg	-21	neg
25[1]	-65	neg	-9	neg	-65
25[2]	-27	neg	neg	neg	-27
26	0	NA	neg	NA	-1
27	neg	-15	neg	neg	neg
28	-62	NA	-65	NA	-62
29	-10	+46	-12	+71	-13
30	-48	NA	neg	NA	-11
31	-22	-8	-10	neg	-22

key. -, days prior to a clinical suspicion of fungal infection that antigen was first detected; +, days after a clinical suspicion of fungal infection that antigen was first detected; NA, not available; neg, antigen not detected.
[1] & [2], this patient had two episodes of clinically suspected fungal infection.

days; 12 days) (Table VI.iv).

6.4.1.6 OFI and NEFI patient results. None of the 148 sera (number of specimens available on each patient 5 to 43; median 12) from seven patients with invasive candidosis and one with invasive mucormycosis (OFI group) assayed by the PAb ELISA were antigen positive. In addition, all 39 urine specimens available on two of these patients (numbers 37 [35 urines] and 38 [4 urines]) were antigen negative (Fig 6.4; Table VI.ii).

All 934 sera (1 to 43; 8) from the 82 patients with NEFI were antigen negative when tested by the PAb ELISA. However, a single urine specimen of the 997 (1 to 86; 11) assayed from 75 of these patients was antigen positive (Fig 6.4; Table VI.ii). This result was reproducible and the urine contained 50.6ng/ml of antigen. There was absolutely no clinical or microbiological evidence of fungal infection in this patient at the time that this urine was taken.

6.4.1.7 Value of the PAb ELISA in diagnosing IPA. The sensitivity, specificity, PPV and NPV of the PAb ELISA for the diagnosis of IPA compared to patients from the OFI and NEFI groups by: i) detection of antigenaemia; ii) detection of antigenuria and iii) detection of both serum and urinary antigen are shown in Table VI.v.

Table VI.v Sensitivity, specificity, positive and negative predictive values for the PAb ELISA, MAb ELISA and LAT in the diagnosis of IPA.

Specimen	Detection method											
	PAb ELISA				MAb ELISA				LAT			
	Sens	Spec	PPV	NPV	Sens	Spec	PPV	NPV	Sens	Spec	PPV	NPV
Serum	89	100	100	98	84	100	100	97	95	90	67	99
Urine	75	99	86	98	100	100	100	100	ND	ND	ND	ND
Both	95	99	95	99	95	100	100	99	NA	NA	NA	NA

key. Sens, sensitivity; Spec, specificity; PPV, positive predictive value; NPV, negative predictive value; ND, not done; NA, not applicable.

6.4.2 MAb ELISA.

6.4.2.1 Control levels. The mean %I of the 100 control sera and 81 control urines was calculated as described in section 6.4.1.1. The mean (+2 SD) %I of the 100 control sera was 26% and 33% for the 81 urines.

6.4.2.2 Inter- and intra- assay variation. These values were calculated as described in section 6.4.1.2 and both were <10%.

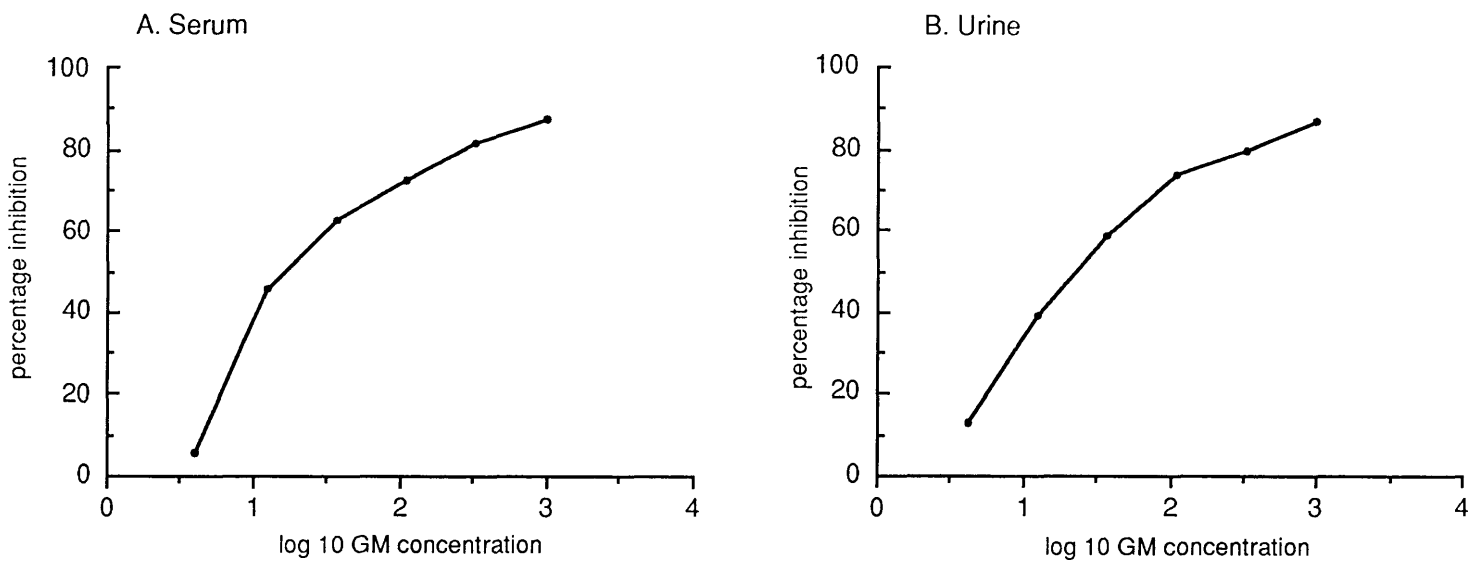
6.4.2.3 GM titration. The limit of sensitivity of the MAb ELISA was calculated as described for the PAb assay in section 6.4.1.3.

The MAb ELISA was capable of detecting 7.0ng/ml of GM in serum and 9.6ng/ml in urine (Fig 6.6).

6.4.2.4 IPA patient results. A total of 345 sera (number of specimens available on each patient 3 to 67; median 12) and 170 urines (6 to 58; 16) from the IPA patients were assayed by the MAb ELISA.

18 of the 19 IPA patients had GM detectable in at least one specimen by this assay (Fig 6.7; Table VI.vi). Circulating GM was seen in 84% (16/19) and urinary GM in 100% (8/8) of the IPA patients tested. A significantly higher proportion of urine specimens (74/170, 44%) from

Figure 6.6 Titration of GM in serum and urine by MAb ELISA.



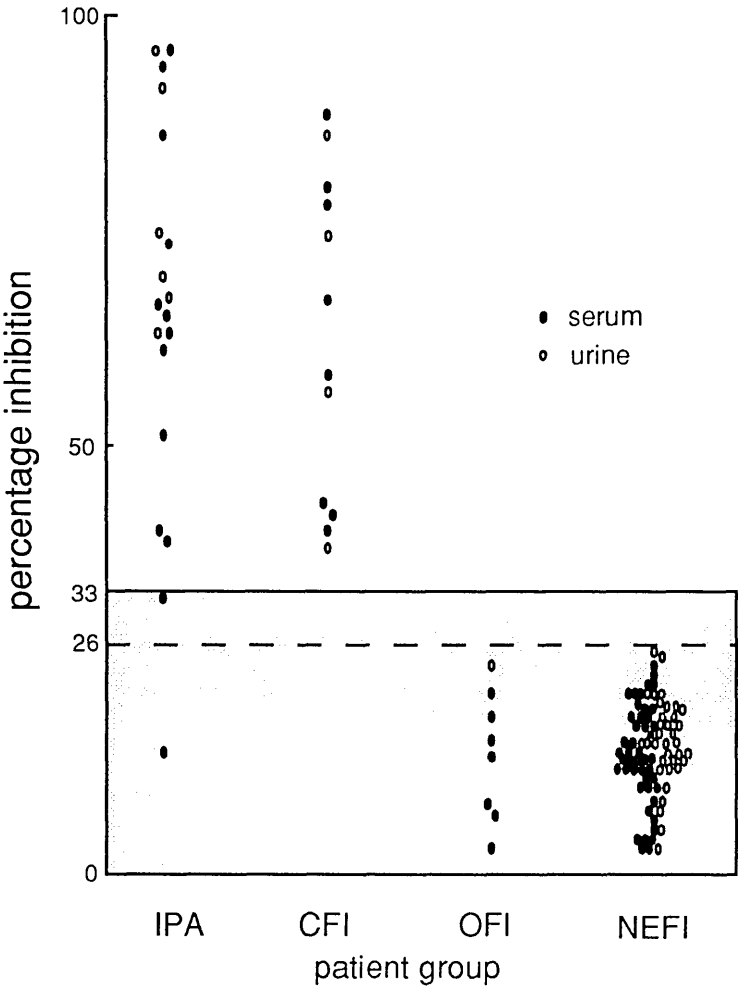
each point on the graphs indicates the mean of 30 GM titration experiments

Table VI.vi Detection of Aspergillus GM in serum and urine of all study patients using the MAb ELISA.

Patient group	Specimen assayed: Serum Number Patients +/t	Serum Number Specimens +/t	Urine Number Patients +/t	Urine Number Specimens +/t	Combined Number Patients +/t	Combined Number Specimens +/t
IPA	16/19	63/345	8/8	74/170	18/19	137/515
CFI	9/12	38/119	8/9	27/55	12/12	65/174
OFI	0/8	0/118	0/2	0/35	0/8	0/153
NEFI	0/82	0/790	0/75	0/852	0/82	0/1642

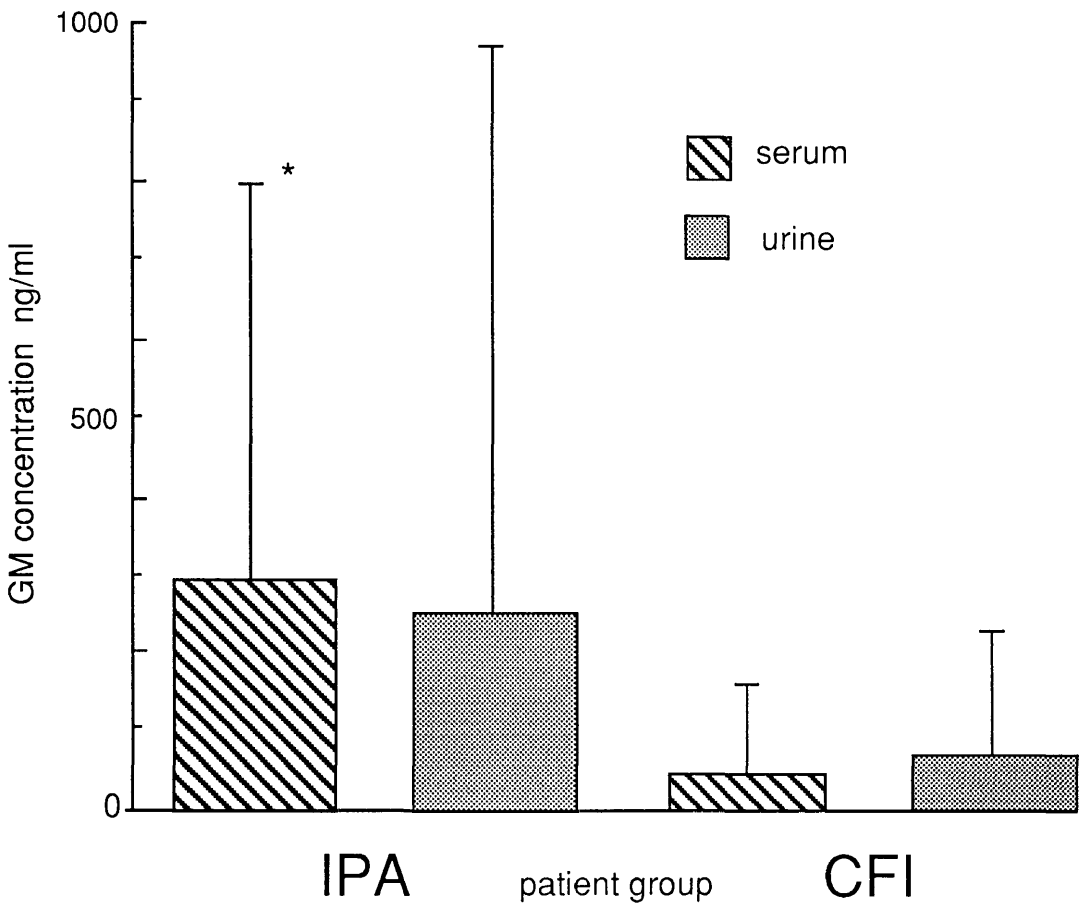
key. +, number antigen positive; t, total tested.

Figure 6.7 Maximum %I detected in all patients by MAb ELISA.



hatched area, normal range (mean plus two standard deviations) of control urines; broken line, upper limit of normal range of control sera.

Figure 6.8 Mean GM concentration in the serum and urine of IPA and CFI patients detected by the MAb ELISA.



* bars indicate standard deviation

these patients contained GM than sera (63/345, 18%) (Chi-squared = 37.31; $p<0.01$).

The mean concentration of circulating GM was 291.9ng/ml (range 7ng/ml to 1694ng/ml) compared with a mean urinary GM concentration of 244.4ng/ml (10ng/ml to 4582ng/ml) (Fig 6.8).

A clinical/laboratory diagnosis of IPA was anteceded (range 3 to 385 days; median 24 days) by the appearance of circulating GM in 14/19 cases and urinary GM in 7/8 patients (2 to 65 days; 9 days). When the results of serum and urinary GM detection were combined a diagnosis of IPA would have been made earlier (3 to 385 days; 24 days) in 16/19 patients (Table VI.iii).

6.4.2.5 CFI patient results. A total of 119 sera (number of specimens available on each patient 2 to 38; median 16) and 55 urines (4 to 12; 5) from the CFI patients were assayed by the MAb ELISA.

All 12 patients had GM detectable in at least one specimen (Fig 6.7; Table VI.vi). Circulating GM was demonstrated in 75% (9/12) of these patients and urinary GM in 89% (8/9) of those tested. A significantly higher proportion of urine specimens (27/55, 49%) than sera (38/119, 32%) from these patients contained GM (Chi-squared = 4.73; $p<0.05$).

The mean concentration of circulating GM was 47.2ng/ml

(7.1ng/ml to 667.8ng/ml) compared with a mean urinary concentration of 72.8ng/ml (9.2ng/ml to 846ng/ml) (Fig 6.8).

The appearance of circulating GM anteceded (range 10 to 65 days; median 22 days) a clinical suspicion of fungal infection in 8/13 episodes seen in the 12 patients. Urinary GM was detected earlier in 5/9 patients tested (6 to 28 days; 11 days). Combining the serum and urine assays would have given an earlier indication of fungal infection in 11/13 suspected episodes in the 12 patients (6 to 65 days; 22 days) (Table VI.iv).

6.4.2.6 OFI and NEFI patient results. No specimen from any of the 908 sera (OFI = 118, number of specimens available on each patient 5 to 27; median 8: NEFI 790, 1 to 41; 8) and 887 urines (OFI = 35, 4 to 31: NEFI = 852, 1 to 73; 11) tested from these two patient groups gave a positive result for GM in this ELISA (Fig 6.7; Table VI.vi).

6.4.2.7 Value of the MAb ELISA in diagnosing IPA. The sensitivity, specificity, PPV and NPV of the MAb ELISA for diagnosing IPA by the detection of i) circulating GM; ii) urinary GM and iii) both circulating and urinary GM are shown in Table VI.v. All values were >84%.

6.4.3 Comparison of the PAb and MAb ELISAs.

Both ELISAs had similar sensitivities, specificities,

PPVs and NPVs for the diagnosis of IPA when the results of serum and urinary antigen detection were combined (Table VI.v). However, the MAb ELISA was positive in all 12 CFI patients (cf eight with the PAb assay). Furthermore, there was a highly significant difference in the proportion of urine specimens from the IPA patients that were positive by the two ELISAs; 34/173 (20%) urines assayed by the PAb ELISA were positive compared to 74/170 (44%) by the MAb ELISA (Chi-squared = 22.73; $p<0.01$). The difference in the proportion of sera positive by each assay was not significantly different.

Notwithstanding the better overall performance of the MAb ELISA, 12.6% of sera and 7.2% of urines from the IPA patients were positive by the PAb ELISA only. The corresponding figures for the CFI group were 5.8% of sera and 8.8% of urines respectively (Table VI.vii).

6.4.4 Latex agglutination test.

6.4.4.1 Control specimens. 34 of the 81 control urines tested by the LAT demonstrated agglutination with the latex reagent. It was therefore decided not to test urines from any of the 121 patients.

Only one of the 100 control sera agglutinated with the LAT. In addition, only 1/19 patients with high levels of circulating rheumatoid factor and 1/52 sera received for

Table VI.vii Comparison of PAb and MAb ELISA results for individual serum and urine specimens, from IPA and CFI patients, tested by both assays.

Patient group	Specimen	Number of specimens (%) with ELISA results			
		PAb-,MAb-	PAb+,MAb-	PAb-,MAb+	PAb+,MAb+
IPA	Serum	249 (69.8)	45 (12.6)	47 (13.2)	16 (4.4)
IPA	Urine	81 (48.5)	12 (7.2)	52 (31.1)	22 (13.2)
CFI	Serum	173 (77.2)	13 (5.8)	32 (14.3)	6 (2.7)
CFI	Urine	25 (43.9)	5 (8.8)	23 (40.4)	4 (6.9)

key. PAb-, polyclonal ELISA negative; PAb+, polyclonal ELISA positive; MAb-, monoclonal ELISA negative; MAb+, monoclonal ELISA positive.

routine rubella antibody screening were positive. All three of these sera were only positive when tested neat.

6.4.4.2 IPA patient results. 261 sera (number of specimens available on each patient 3 to 38; median 9) from the 19 IPA patients were tested by the LAT.

Of these 19 patients 18 had at least one specimen positive for GM (Table VI.viii).

A total of 89/261 sera (34%) from the IPA patients were positive for GM. The maximum dilution of any of these sera which still caused agglutination of the LAT reagent was 1:32; equivalent to 480ng/ml of GM.

Of the 18 patients who had circulating GM 13 were antigen positive before (range 3 to 352 days; median 21 days) a conventional clinical or laboratory diagnosis of IPA was made (Table VI.iii).

6.4.4.3 CFI patient results. 105 sera (number of specimens available on each patient 2 to 17; median 8) from the 12 patients with CFI were tested by the LAT.

Nine individuals were shown to have circulating GM as detected by this test (Table VI.viii). Of the 73 sera tested 32 (31%) contained GM and the maximum end-point titre seen was 1:16; equivalent to an antigen level of 240ng/ml.

The detection of circulating GM anteceded (range 1 to 65 days; median 13 days) a clinical suspicion of fungal

Table VI.viii Detection of Aspergillus GM in sera of all patients by the latex agglutination test.

Patient group	Number of patients positive/tested	Number of sera positive/tested (%)
IPA	18/19	89/261 (34)
CFI	9/12	32/73 (31)
OFI	0/8	0/82 (0)
NEFI	8/82	13/186 (7)

involvement in 8/9 patients who were antigen positive by this LAT (Table VI.iv).

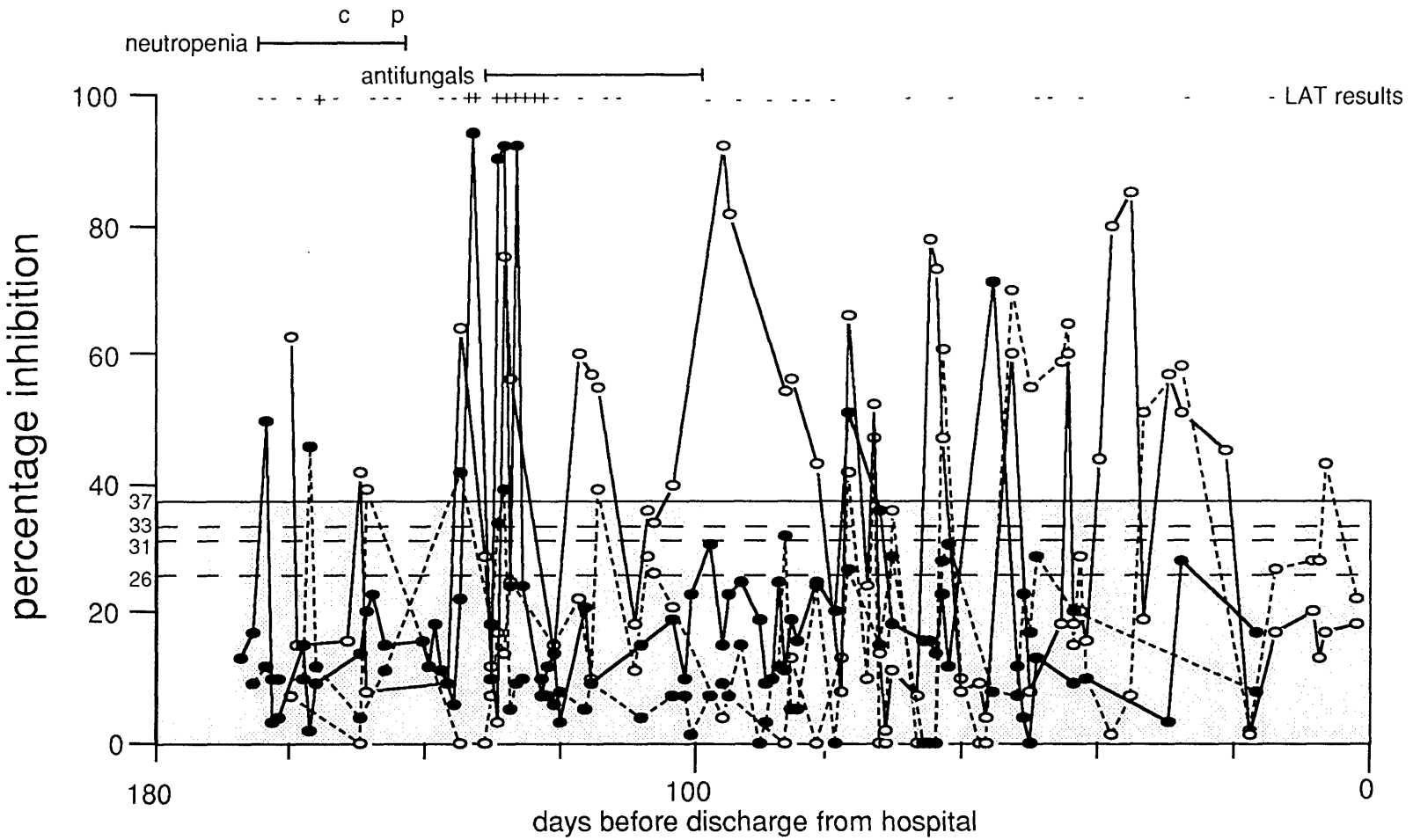
6.4.4.4 OFI and NEFI patient results. All the 82 sera (number of specimens available on each patient 5 to 27; median 7) from the eight OFI patients tested by this LAT were negative for GM (Table VI.viii).

One to three sera from each of the 82 NEFI patients were tested. Of the 186 serum samples assayed 13 (7%) were GM positive. These 13 sera came from 8/82 patients with NEFI (Table VI.viii). Only one of the patients had more than a single serum specimen positive and all 13 sera were only positive when tested neat.

6.4.4.5 Repeat testing. 50 stored supernatants (4°C) and 50 stored sera (-70°C) were repeat tested one month after original testing. No difference was seen between initial and repeat test results.

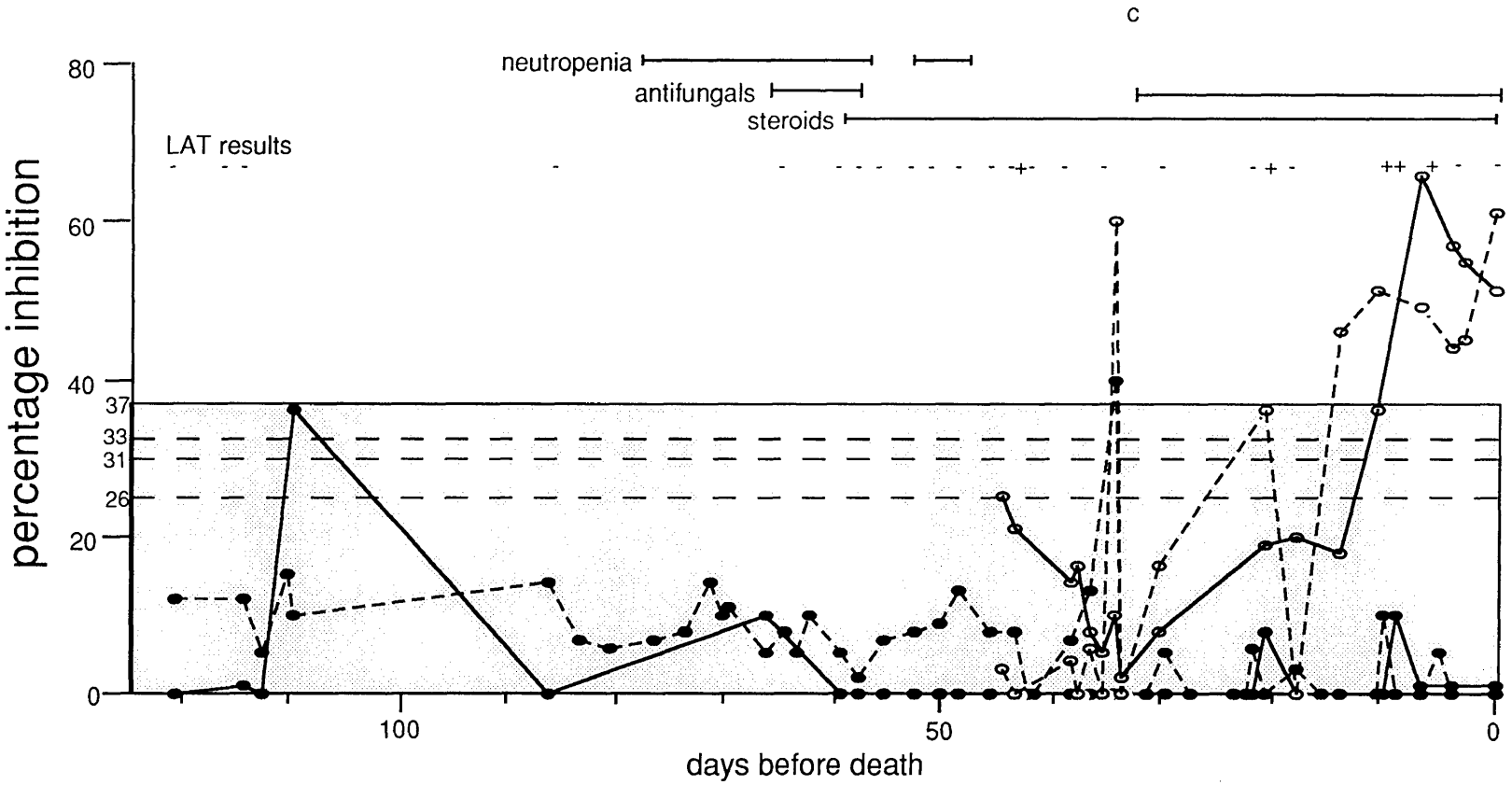
6.4.4.6 Value of the LAT in diagnosing IPA. The sensitivity, specificity, PPV and NPV of the LAT for the diagnosis of IPA compared to the OFI and NEFI groups are shown in Table VI.v.

Figure 6.9.1 Aspergillus antigen detection and clinical course of IPA patient number 14.



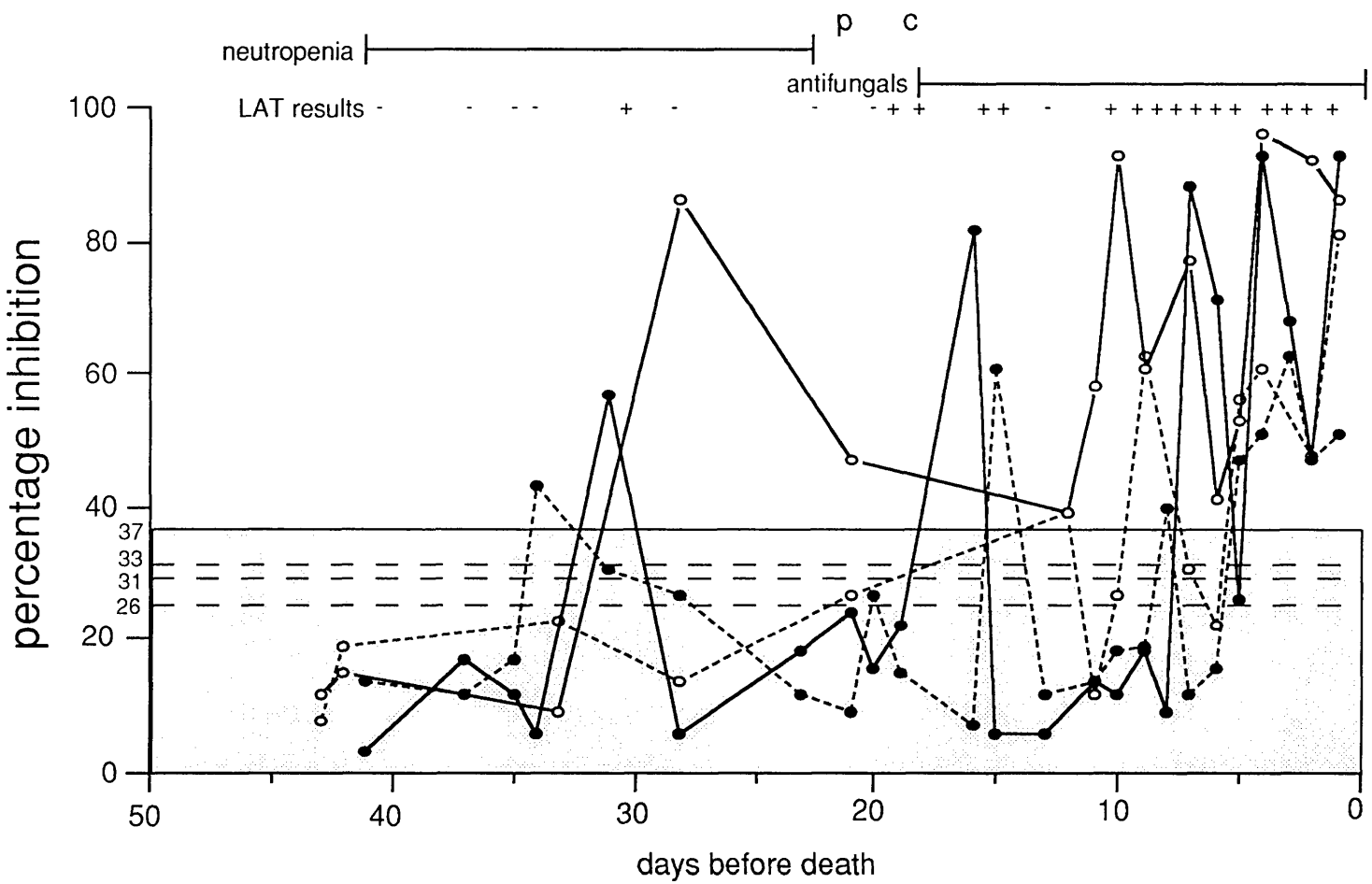
solid lines, MAb ELISA; dashed lines, PAb ELISA; closed circles, serum samples; open circles, urine samples; -, LAT negative; +, LAT positive; c, culture of A.fumigatus and A.flavus for first time; p, first appearance of pulmonary infiltrates; hatched area and broken lines, upper limit of normal ELISA values.

Figure 6.9.2 Aspergillus antigen detection and clinical course of IPA patient number 17.



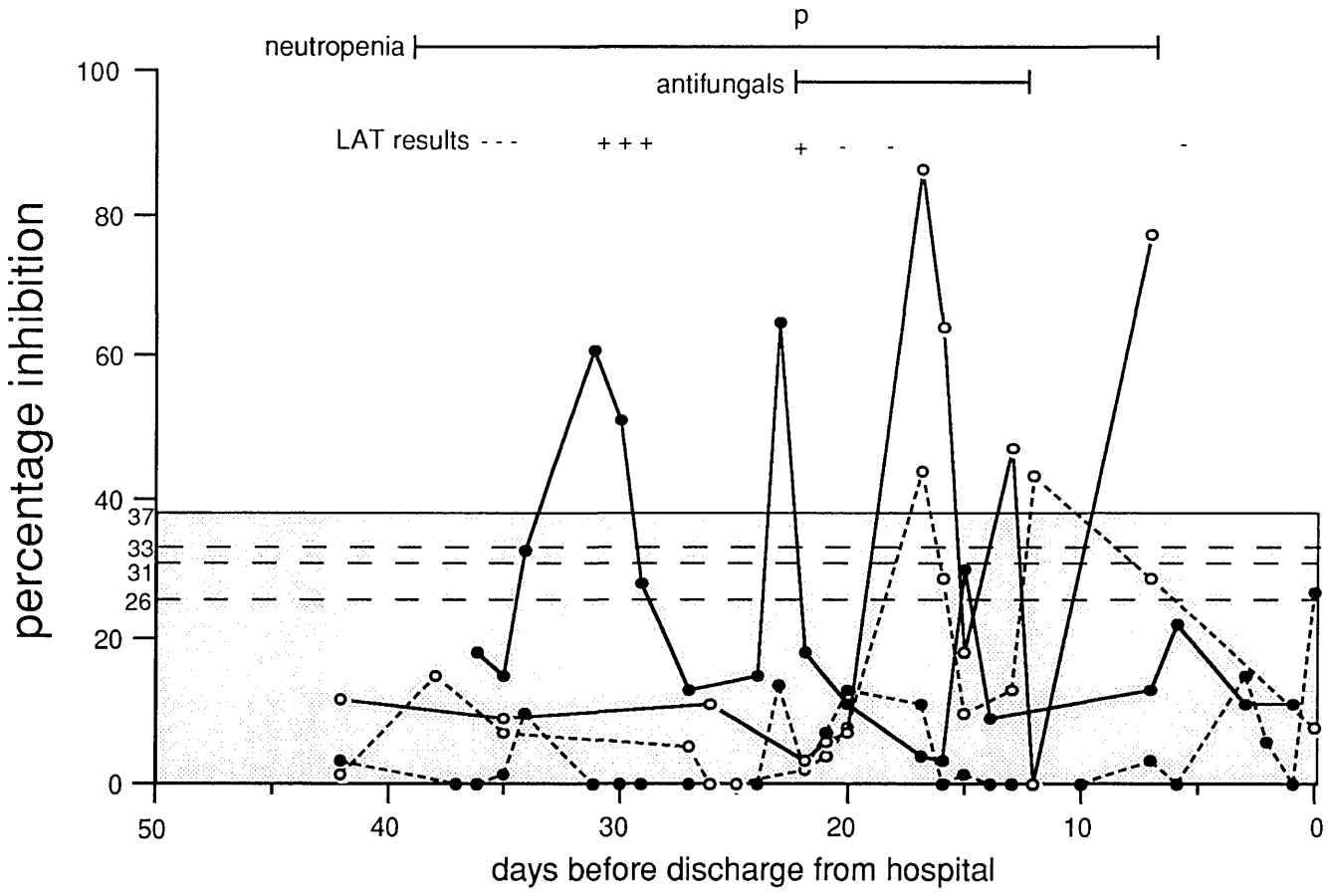
solid lines, MAb ELISA; dashed lines, PAb ELISA; closed circles, serum samples; open circles, urine samples; -, LAT negative; +, LAT positive; c, culture of A.fumigatus for first time; hatched area and broken lines, upper limit of normal ELISA values.

Figure 6.9.3 Aspergillus antigen detection and clinical course of IPA patient number 19.



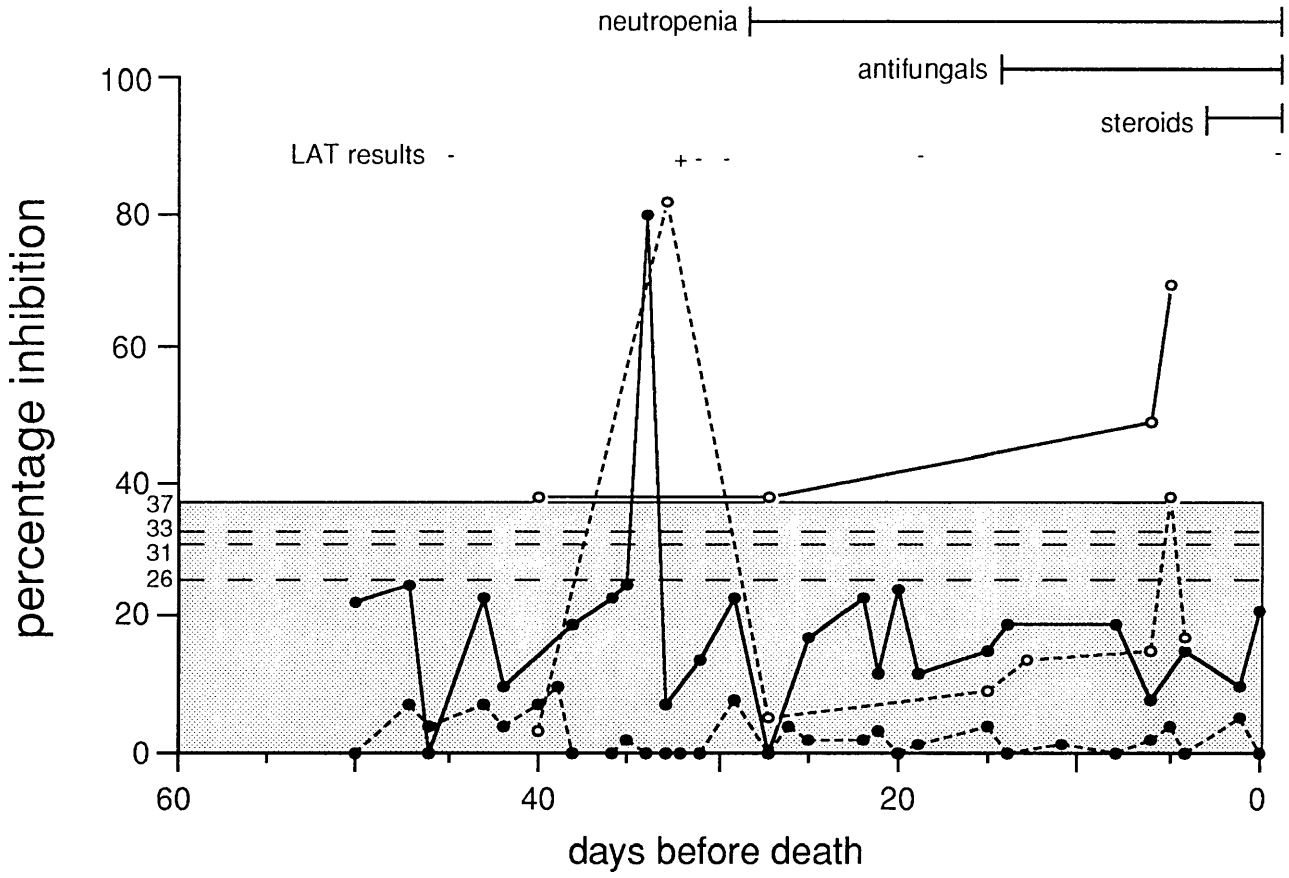
solid lines, MAb ELISA; dashed lines, PAb ELISA; closed circles, serum samples; open circles, urine samples; -, LAT negative; +, LAT positive; c, culture of A.fumigatus for first time; p, first appearance of pulmonary infiltrates; hatched area and broken lines, upper limits of normal ELISA values.

Figure 6.9.4 Aspergillus antigen detection and clinical course of CFI patient number 21.



solid lines, MAb ELISA; dashed lines, PAb ELISA; closed circles, serum samples; open circles, urine samples; -, LAT negative; +, LAT positive; p, first appearance of pulmonary infiltrates; hatched area and broken lines, upper limit of normal ELISA values.

Figure 6.9.5 Aspergillus antigen detection and clinical course of CFI patient number 22.



solid lines, MAb ELISA; dashed lines, PAb ELISA; closed circles, serum samples; open circles, urine samples; -, LAT negative; +, LAT positive; p, first appearance of pulmonary infiltrates; hatched area and broken lines, upper limit of normal ELISA values.

6.4.5 Comparison of all three antigen detection methods.

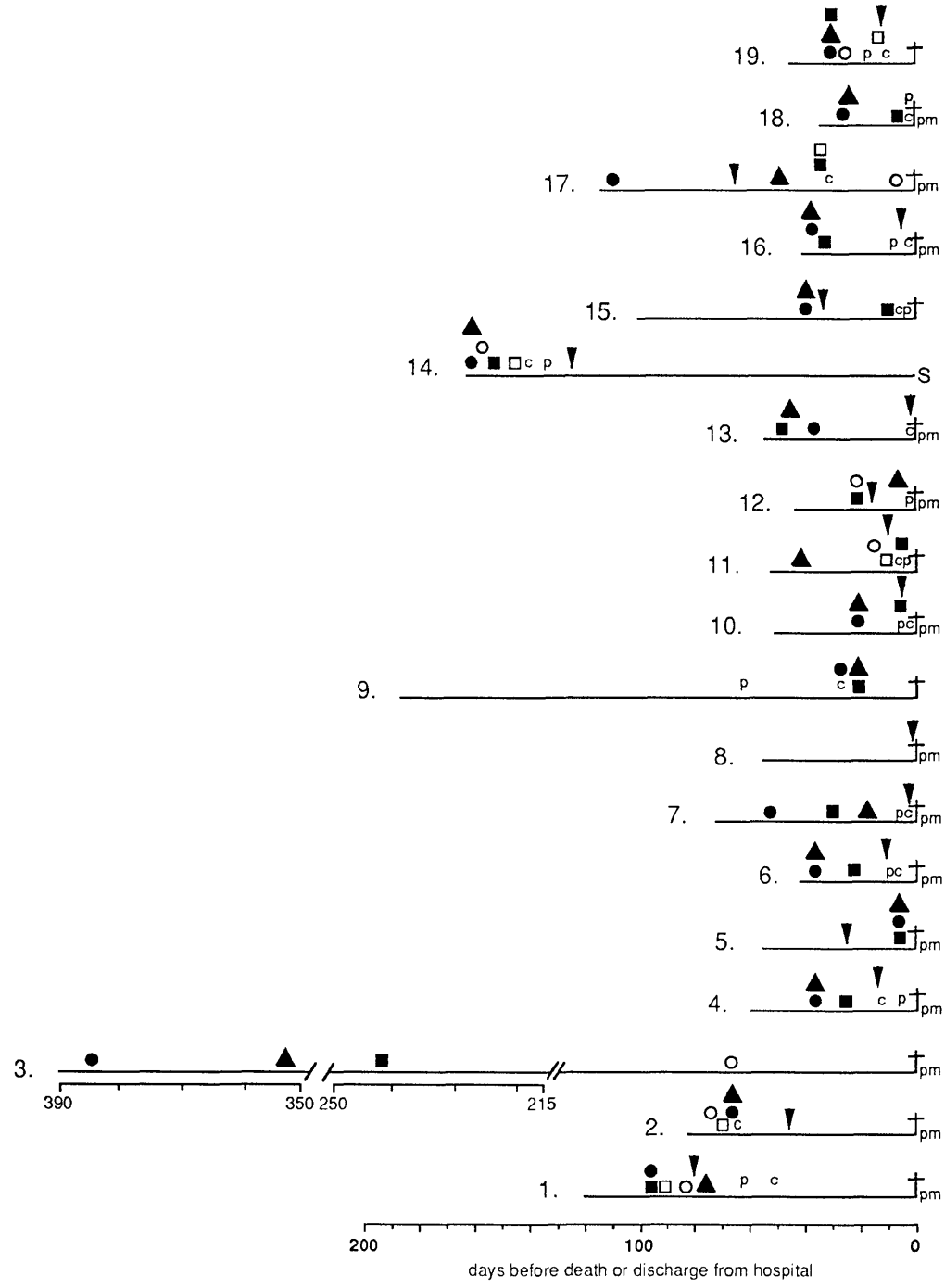
The results of the PAb and MAb ELISAs plus the LAT in relation to the clinical course of selected IPA and CFI patients are shown in Figs 6.9.1 to 6.9.5.

Results of the three methods appear similar however, when the data from individual specimens were analysed it became clear that results did not always concur. Of 241 sera from the IPA patients which were assayed by all three methods 141 (58.5%) were negative by all tests. Only 13 (5.4%) were positive by all tests. Similarly for the 105 sera assayed by all three methods from the CFI group 65 (71.9%) were completly negative and only 5 (4.8%) were always positive.

44 sera from the IPA patients and 11 from the CFI group were negative by the MAb ELISA but positive by the LAT. A smaller number of sera from the IPA (5) and CFI (6) patients were MAb ELISA positive and LAT negative. A total of 45 sera from the IPA patients and 22 from the CFI group were positive by both of these tests to detect GM.

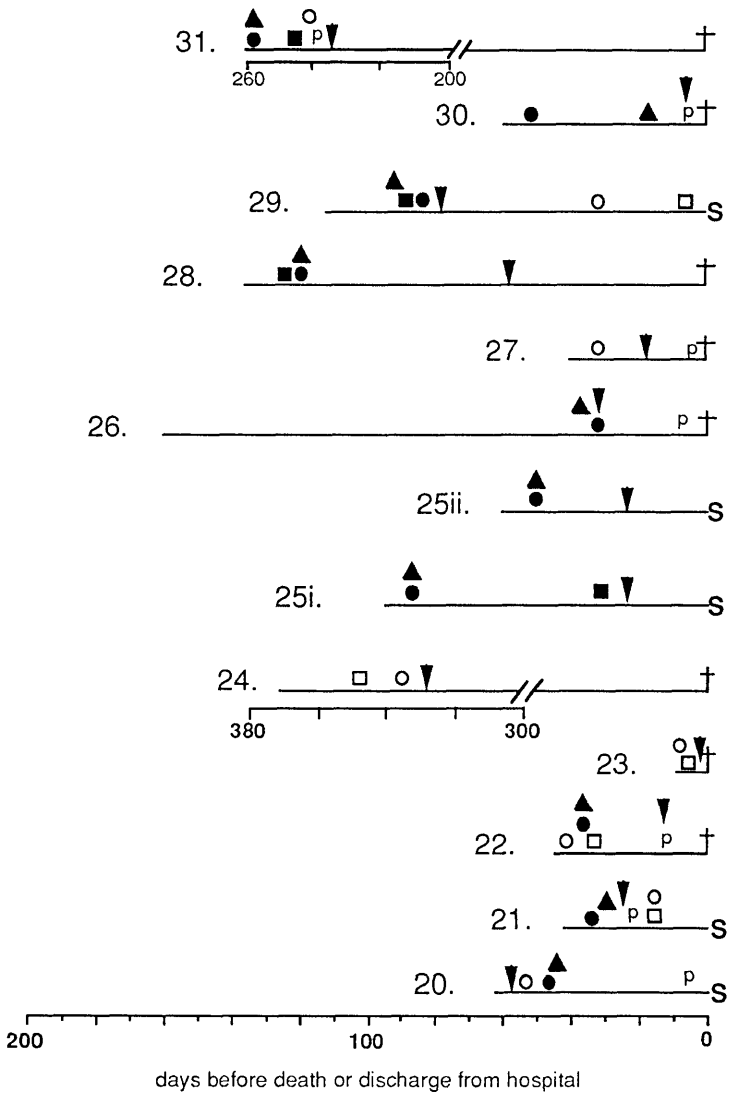
If the results from all three methods were combined antigen would have been detected prior to a clinical or laboratory diagnosis in 16/19 (84%) proven cases and in 12/13 (92%) episodes of clinically suspected fungal infection. Figs 6.10 and 6.11 indicate which of the three antigen detection methods were positive first in each of the IPA and CFI patients.

Figure 6.10 Comparison of Aspergillus antigen detection and conventional methods in the early diagnosis of IPA.



p, first appearance of pulmonary infiltrates; c, first culture of Aspergillus spp.; ▼ ,earliest administration of antifungal therapy; antigen detection tests: first positive; ■, PAb ELISA (serum); □ ,PAb ELISA (urine); ● , MAb ELISA (serum); ○ , MAb ELISA (urine); ▲, LAT; †, death; pm, post mortem; S, survived.

Figure 6.11 Comparison of Aspergillus antigen detection and conventional methods as early indicators of CFI.



p, first appearance of pulmonary infiltrates; ▼ ,earliest administration of antifungal therapy; antigen detection tests: first positive; ■ , PAb ELISA (serum); □ ,PAb ELISA (urine); ● , MAb ELISA (serum); ○ , MAb ELISA (urine); ▲, LAT; † , death; S, survived.

6.4.6 Fluctuation of antigen levels.

In all IPA and CFI patients the level of antigen detected by all three tests fluctuated over time (Fig 6.9.1 to 6.9.5). The maximum variation in antigen levels detected by the PAb ELISA in IPA patients was 548ng/ml (minimum detected 14ng/ml; maximum detected 562ng/ml) [patient 19] and 599ng/ml (14ng/ml; 613ng/ml) [patient 22] in the CFI patients. The corresponding levels for the MAb ELISA were 4574ng/ml (8ng/ml; 4582ng/ml) [patient 19] in the IPA patients and 838ng/ml (8ng/ml; 846ng/ml) [patient 21] in the CFI group. Titres of GM detected by the LAT also fluctuated.

No correlataion was seen between: administration of amphotericin B; administration of corticosteroids; culture of aspergilli and the detection of Aspergillus antigen. A terminal increase in antigen levels was seen in 7/18 fatal cases of proven IPA.

6.5 Discussion.

The sensitivity and specificity of all three antigen detection assays for the diagnosis IPA evaluated in this thesis was >90%. These values compare favourably with previous experience (Table VI.i) confirming that antigen detection can effectively diagnose this infection.

All assays were capable of diagnosing IPA caused by species of Aspergillus other than A.fumigatus. Two patients; one with an A.flavus infection and another with an A.glaucus group infection were both antigen positive by all three tests. This is not surprising as aspergilli are known to share antigenic determinants (Kim and Chaparas, 1979; Mishra, 1984).

The specificity of the two ELISAs was confirmed by the fact that only a single positive result was seen in all the OFI and NEFI patients who had a variety of fungal, bacterial and viral infections at the time of testing.

Overall the best performing test was the MAb ELISA with sensitivity, specificity, PPV and NPV all in excess of 95%. This confirms previous findings that GM was the most common Aspergillus antigen present in body fluids during IPA (Reiss and Lehmann, 1979; deRepentigny et al, 1987; Dupont et al, 1987). However, 12.6% of sera and 7.2% of urines from IPA patients were positive by the PAb ELISA only, thereby indicating the presence of non-GM antigens. It would seem

prudent therefore, in order to ensure maximum antigen detection, to employ both assays. This was also recommended by Wilson et al (1987) who demonstrated 4μg/ml of antigen in a single serum sample with an ELISA inhibition assay but were unable to demonstrate any antigen in the same specimen when using a double antibody sandwich method.

There was no significant difference in the proportion of sera and urines which were positive when assayed by the PAb ELISA. However, a much higher percentage of urines (44%) than sera (18%) from the IPA patients contained GM. This result was in agreement with the finding of Dupont et al (1987) who were able to detect circulating GM in only 2/12 patients with documented IPA compared to urinary antigen in 7/13.

It is known that Aspergillus antigens are rapidly cleared from the circulation in mice (Richardson et al, 1979) and it has also been suggested that GM can be cleared by glomerular filtration in humans (Dupont et al, 1987). This may help to explain why antigen was found more often in urine. A further possible explanation is that as sera were diluted 1:5 in PBST prior to assay, this may have reduced the sensitivity of the assay for specimens which contained very low levels of GM.

However, although GM was detected in a higher proportion of IPA urines by the MAb ELISA the mean concentration of circulating GM (291.9ng/ml) was slightly

higher than that observed in urine (244.4ng/ml). The level of GM detected in serum was consistent with previous reports where concentrations up to 356ng/ml have been demonstrated (deRepentigny et al, 1987; Dupont et al, 1987). However, the concentration of GM in urine was much higher than has previously been reported; Dupont et al (1987) detected a maximum of 83ng/ml of GM in urine compared to a maximum concentration of 4582ng/ml in the IPA patients studied here. It was possible that the use of a monoclonal antibody to detect GM, as in this work, was responsible for the increased sensitivity. Dupont and co-workers used a polyclonal rabbit antiserum to A.fumigatus which presumably contained Ig directed to antigenic determinants other than GM which may have reduced the sensitivity of their assay.

The mean antigen concentration of 91.6ng/ml in serum and 76.4ng/ml in urine detected by the PAb ELISA was similar to reported experience (Shaffer et al, 1979b; Weiner, 1980; Sabetta et al, 1985; LePape and Deunff 1987; Dupont et al, 1987; Wilson et al, 1987; Fujita et al, 1988; Johnson et al, 1989).

In all patients who were antigen positive by either ELISA, levels of both circulating and urinary antigen fluctuated widely. This was consistent with previous findings (Wilson et al, 1987; Johnston et al, 1989). However, unlike a mouse model of IPA (Patterson et al, 1988) where administration of amphotericin B resulted in a

reduction of circulating carbohydrate antigen, the level of antigen detected in our patients could not be related to administration of antifungal drugs. Also the presence of antigen did not relate to other treatments or to the isolation of Aspergillus sp (Fig 6.9.1 to 6.9.5). Furthermore, an increase in antigen titre was seen in only 7/18 fatal cases of IPA as death approached. This was inconsistent with previous studies which have suggested that antigen concentration increased as infection progressed (Dupont et al, 1987; Fujita et al, 1988; Johnson et al, 1989).

It is possible to speculate on the reason for the fluctuation in antigen concentrations. The majority of these patients received large volumes of intravenous fluids which can result in both haemo- and urinary dilution: this may have affected antigen levels. In addition, if the clearance of Aspergillus antigen from the circulation of mice (Richardson et al, 1979) also occurs in humans, antigen titres would be affected. A possible mechanism for the clearance of GM from the circulation has been elucidated by Bennett et al (1987) who demonstrated that radiolabelled GM was taken up by the mannosyl receptor on rat kupffer cells. The authors speculated that macrophage mannose receptors could constitute a general method for the clearance of fungal mannans. The patients' burden of Aspergillus could affect antigen concentration; however, it is extremely

difficult, at present, to determine with accuracy the fungal load of an individual patient and therefore to determine what effect it may have on antigen levels. The majority of IPA patients in this study had aspergillosis restricted to a pulmonary focus. It has been demonstrated in experimental infection that animals with greater extra-pulmonary involvement have more circulating antigen than those with localised infection (Phillips and Radigan, 1989). This may help to explain why a rise in antigen titre with progress of the infection was not the rule in the patients studied here.

The maximum percentage of specimens from IPA patients which were antigen positive was 44% which lies between the level of 28% reported by Johnson et al (1989) and 76% by Talbot and co-workers (Talbot et al, 1987). Concominant with this finding was the observation that a minimum of 56% of specimens from IPA patients did not contain antigen detectable by any of these assays. There are three possible explanations for these 'false negative' results: i) there was no antigen present. If this were the case nothing can be done to improve sensitivity; ii) the concentration of antigen was too low to detect. It is known that Aspergillus antigens can be present at very low concentrations in sera (Sabetta et al, 1985; Fujita et al, 1988) and urine (Dupont et al, 1987) during IPA. However, the sensitivity of the assays evaluated here, and previously, are very high with a minimum detectable antigen level of 1.6ng/ml reported

(Fujita et al, 1988). Improvement in assay sensitivity may increase diagnostic capability; or iii) the circulating and excreted antigen(s) are not recognised by current assays. This is perhaps the most probable explanation; many workers have used different antigen and antibody preparations (see Table VII.iii) to successfully detect Aspergillus antigens, thereby suggesting that a large variety of antigens are present in body fluids during IPA. Furthermore, the results presented in this thesis suggest that non-GM antigens are present during infection. Identification of these antigens should allow an improvement in assay sensitivity. This will be dealt with in the next chapter.

Whatever the reason, the low proportion of positive specimens from patients with proven IPA indicates that both serum and urine must be tested as often as possible in order to minimise the number of false negative results (Wilson et al, 1987).

The sensitivity, specificity and NPV of the LAT were similar to those of the two ELISAs, but the PPV of this test was much lower (67%) due to a number of positive results obtained in the NEFI group of patients. These sera were not positive by the MAb ELISA. It is difficult to explain these results and the same reaction (ie MAb negative, LAT positive) seen in 55 sera from the IPA and CFI groups. Both of these tests were based on the same monoclonal antibody to A.fumigatus GM and therefore similar results would have been

expected. Two explanations are possible for these observations; i) the LAT reagent is reacting non-specifically with components in the serum or ii) the NEFI patients had transient Aspergillus infections which did not manifest clinically. In other evaluations of this LAT test (Dupont et al, 1990; Van Cutsem et al, 1990) a high level of positive results in control subjects was not found. This suggests that the second explanation is the more feasible; however, the sensitivity of the MAb ELISA was greater than that of the LAT and these sera were MAb negative. The reason for the discrepancy remains unclear.

A smaller number of IPA and CFI sera (11) were positive by the MAb assay and negative by the LAT. They all had antigen concentrations below 15ng/ml ie the limit of sensitivity of the latex test.

Notwithstanding the problems of the LAT it has two major advantages. Firstly, it is commercially available through Diagnostics Pasteur which would allow it to be evaluated in a large multi-centre trial. Secondly, it is quick and easy to perform and does not require any specialised laboratory equipment which would allow its use in a routine diagnostic laboratory.

It has been suggested that the early diagnosis and treatment of IPA results in an improved outcome (Aisner et al, 1977; Burch et al, 1987) whereas failure to treat is almost uniformly fatal.

Of the 31 IPA and CFI patients in this study, 28 received empirical antifungal therapy on the basis of clinical and laboratory evidence of fungal infection alone. Despite this only 5 (1 IPA and 4 CFI) of the 31 patients survived (all received amphotericin B). If antigen detection had been used as a criterion on which to begin empirical antifungal therapy the majority of patients would have been treated earlier irrespective of the test employed (Tables VI.iii and VI.iv). Empirical antifungal therapy would have been initiated a median of 13 days earlier (range 1 to 241) in 13/19 IPA patients based on the results of the PAb assay, 24 days earlier (3 to 352) in 16/19 if the MAb assay had been used and 21 days earlier (3 to 385) in 13/19 for the LAT. The respective figures for the CFI patients were a median of 12 days (5 to 65) in 7/12 patients by the PAb ELISA, 22 days (6 to 65) in 11/13 episodes by the MAb assay and 13 days (1 to 65) in 8/12 patients for the LAT. Combining all antigen detection results would have resulted in earlier antifungal therapy in 16/19 patients with proven infection (median 24 days; range 3 to 385) and 11/12 CFI patients (21 days; 1 to 65).

GM was detected earlier than the non-GM antigens recognised by the polyclonal ELISA in 14/18 antigen positive IPA patients. In a further two patients, GM was detected on the same day and in only two patients did antigen detection by the PAb ELISA precede the appearance of GM. This

indicates that GM was released early in the course of IPA and that the antigens detected by the PAb ELISA were not found until later in infection. However, as a human serum was used in the PAb ELISA subtle differences in the temporal expression of Aspergillus antigens may have been masked (due to the polyclonal nature of the human serum). Identification of the antigens which reacted with the human antiserum and the production of specific antibodies to them would allow a better analysis of the pattern of antigen expression over time. It is not surprising that GM was detected early in the course of infection as it is a constituent of the fungal cell wall and is easily sloughed off into the enviroment during growth in vitro (J-P. Latge, personal communication).

Of the eight IPA patients from whom urine specimens were available, urinary antigen was detected earlier than circulating antigen in two by the PAb ELISA and three by the MAb assay (Table VI.iii). This finding suggests that early antigen can be found in both types of specimen and that both serum and urine should be tested in order to maximise the detection of IPA early in the course of infection.

In conclusion this work confirms that the detection of Aspergillus antigen predicts the development of IPA and that testing of patients 'at risk' should improve diagnosis of this infection. Urine was more likely to contain GM, but both serum and urine should be tested. Antigen detection was

positive earlier than conventional clinical and microbiological methods of diagnosis and could be used as a criterion on which to initiate prompt antifungal therapy. Finally, it has been demonstrated that 12.6% of sera and 7.2% of urines from patients with IPA were PAb+,MAb-; this indicated that Aspergillus antigens other than GM were present in body fluids during IPA.

CHAPTER SEVEN: ASPERGILLUS ANTIGEN DETECTION BY IMMUNOBLOTTING.

7.1 Introduction.

The previous chapter has confirmed and extended the findings of other studies (Reiss and Lehmann, 1979; DeRepentigny et al, 1987; Dupont et al, 1987) showing that GM was present in the serum and urine of humans with IPA. GM is a constituent of the *Aspergillus* cell wall (Bardalaye and Nordin, 1977) and has been demonstrated in a large number of *Aspergillus* spp (Debeaupuis et al, 1989). It is not surprising that this antigen has been detected in IPA considering that when *Aspergillus* spp are grown *in vitro* there is release of large amounts of this polysaccharide into the culture medium (J.P. Latge, personal communication) (Table VII.i).

The chemical structure of GM has been studied by many workers after *in vitro* cultivation of the fungus. This work has produced conflicting results with regard to the exact nature of the molecule (Table VII.ii). However, most groups agree that the basic structure consists of a backbone composed of repeating mannose residues substituted with oligogalactose side chains. Some of the galactose must be in the furanose configuration since mild acid hydrolysis (0.1N HCl at 100°C for 24h) results in destruction of the sugars. Mild acid hydrolysis also causes reduction in the immunogenicity of GM, suggesting that the galactofuranose

Table VII.i Composition of a culture filtrate antigen prepared from a 48 hour fermenter culture of A.fumigatus 1028.

Component	% (w/w) dry weight of CF antigen	Comment
hexose	14	Gal:Man 1.5:1
hexosamines	29	100% galactosamine glucosamine trace only
free hexosamines	21	of total 29%
amino acids	40	

key. Gal:Man, galactose:mannose ratio.
I am grateful to J-P Latge for this information.

Table VII.ii Proposed structures of Aspergillus GM.

Reference	GM source	Gal:Man	Backbone	Side chains
Sakaguchi et al 1967	CF/ME	1:1	?	?
Azuma et al 1971	ME	1:1.5	1->2 1->6 Man	6<-(Galf)$_n$
Reiss & Lehmann 1979*	ME	1:1.2	1->6 Man	1->2 Man 1 Man (1->2 or 1->4 Gal)$_2$ -Galf
Barreto-Bergter et al 1981	ME	1-1.6:1	1->6 Man	2<-(1->2 Man)$_{1-3}$ 6<-(1->5 Galf)$_6$
Bennett et al 1985	ME	1:1.9	1->2 Man	Galp or Galf-Galf (Man)$_{1-?}$
DeRepentigny et al 1987;	ME	1:1.2-1.6	?	?
J-P Latge personal communication	CF	1.5:1	1->2 1->6 Man ?? Galp	Galf(1->5)-(1->2 Man)$_2$?? Galp

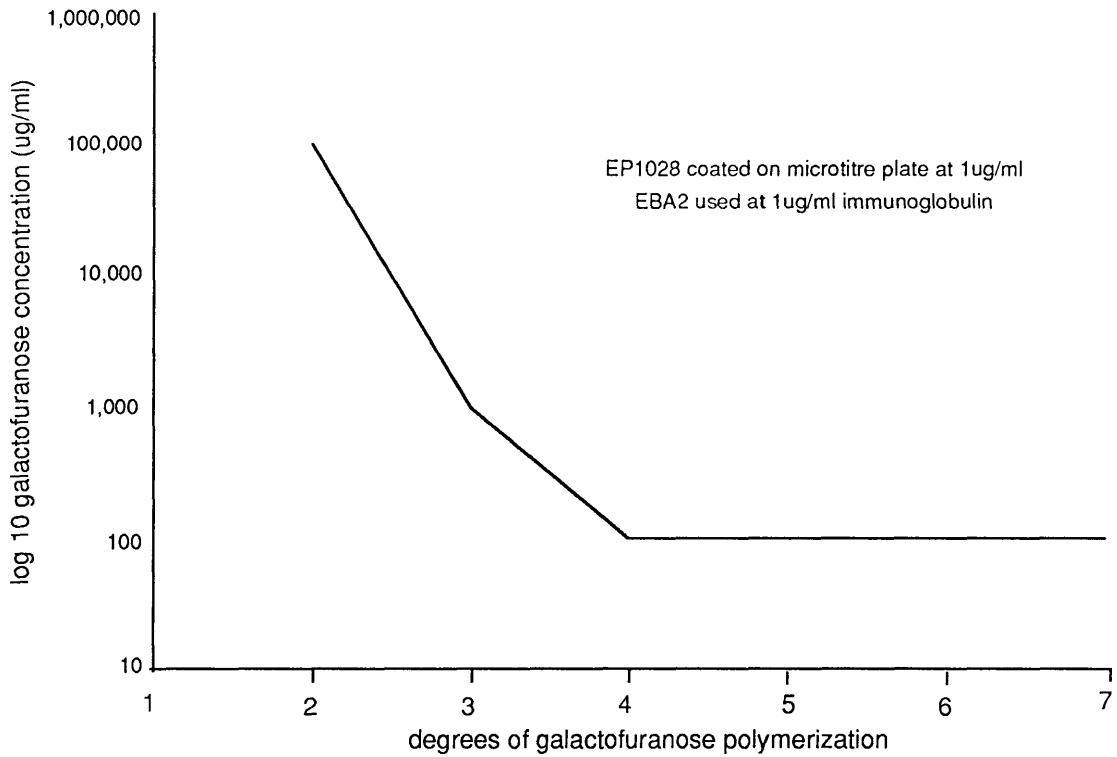
key. GM, galactomannan; Gal:Man, galactose:mannose ratio; CF, culture filtrate; ME, mycelial extract; Man, mannose; Gal, galactose; Gal f, galactofuranose; Gal p, galactopyranose; ?, unknown structure; ??, possible component.
*structure stated as 1->6 Man but data in paper suggests a 1->2 Man.

residues are the immunodominant portion of the polysaccharide (Bennett at al, 1985). Further evidence for this hypothesis comes from the finding that a synthesized galactofuranose tetramer can inhibit the binding of anti-GM monoclonal antibody (EBA1) to EP1028 (Fig 7.1; J-P. Latge, personal communication).

In vivo studies have suggested that GM isolated from body fluids was different to the polysaccharides prepared in vitro. GM isolated from the serum of rabbits with IPA had a M_r of 125 kDa whereas when prepared from a somatic extract of A.fumigatus the antigen had an apparent M_r of 25-75 kDa (Lehmann and Reiss, 1978; Reiss and Lehmann, 1979). These authors speculated that the increased M_r was due to binding of the GM to protein in vivo and that the reduced M_r of the in vitro extract was a result of the cleavage of the protein from the polysaccharide during the alkali extraction procedure required to dislodge GM from the cell wall. Similarly GM isolated from the urine of rabbits with IPA had a wide M_r range of 10 kDa to 70 kDa (Dupont et al, 1987). The control GM purified by these workers had a M_r of 38 kDa (Bennett et al, 1985).

Notwithstanding these discrepancies between GM isolated from in vivo and in vitro sources it remains the only characterized antigen of Aspergillus which has been detected in body fluids during IPA (Reiss and Lehmann, 1979; de Repentigny et al, 1987; Dupont et al, 1987).

Figure 7.1 Concentration of beta (1->5) galactofuranose polymers of various lengths required to give 50% inhibition of binding of EBA2 monoclonal antibody to EP1028 in ELISA.



I am grateful to Dr. J-P. Latge for allowing me to include this figure.

There is however, a certain amount of evidence that antigens of Aspergillus other than GM are present during IPA. A large number of different fractions have been used in various assays for the detection of Aspergillus antigens in body fluids (Table VII.iii). While many of these fractions undoubtedly contain GM they also probably contain a number of undefined antigens (Shaffer et al, 1979a; Richardson et al, 1979; Sabetta et al, 1985; Wilson et al, 1987; LePape and Deunff, 1987). In addition, the antisera used to detect Aspergillus antigens have generally been prepared using crude antigen extracts as immunogens (Sabetta et al, 1985; Dupont et al, 1987; Wilson et al, 1987; Fujita et al, 1988) (Table VII.iii). Presumably therefore a large amount of the Ig in these antisera will not be directed against GM but to other antigens that are known to be present in these extracts. Hence a positive test result, in these assays, could indicate the presence of any antigen, in the complex mixtures, to which the antisera was directed and does not neccessarily mean the detection of GM.

More direct evidence for the presence of non-GM antigens in IPA comes from the observation that some circulating Aspergillus components, from experimentally infected rabbits, do not bind to conA but can inhibit binding of anti-Aspergillus antibody in ELISA (DeRepentigny et al, 1987).

The most compelling evidence for the presence of non-GM

Table VII.iii Antigen fractions and antisera which have been employed in the detection of Aspergillus antigen in experimental and human IPA.

Reference	Antigen	Antiserum raised to
Lehmann & Reiss 1979	GM + <1% aa M_r = 50 +/- 25	125 kDa circulating antigen
Weiner & Coats Stephens 1979	M_r = 150-180 kDa undefined fraction from mycelium	total mycelium
Shaffer et al 1979a	78% CHO, 10% protein fraction chromatographically isolated from mycelium	total mycelium
Richardson et al 1979	crude total mycelial extract	total mycelial extract
Sabetta et al 1985	Supernatant of 10% TCA precipitation of crude mycelial extract	conidia
Wilson et al 1987	conA binding portion of a water soluble extract of mycelium	high titre human anti-aspergillus and cell walls
LePape & Deunff 1987	conA binding portion of a water soluble extract of mycelium	conA binding fraction
Dupont et al 1987	GM + 0.7% N and 0.17% P M_r = 20-50 kDa.	live conidia
Fujita et al 1988	Undefined fraction chromatographically isolated from an ethanol precipitate of broken mycelium	cell walls
Johnson et al 1989	crude culture filtrate antigen	human ABPA serum

key. GM, galactomannan; aa, amino acids; M_r, molecular mass; kDa, kilodaltons; CHO, carbohydrate; TCA, trichloroacetic acid; conA, concanavalin-A.

antigens in body fluids during IPA however, comes from the results precented in the preceding chapter; 12.6% of sera and 7.2% of urines, from IPA patients, were positive by the PAb ELISA but negative by the MAb assay. As the MAb ELISA was specific for GM the positive results obtained with the PAb assay must have been due to antigens other than GM.

The obvious question to ask therefore is, what are these antigens? It is known that aspergilli are antigenically very complex when grown _in vitro_ with up to 150 spots being demonstrated by 2-dimensional immunoelectrophoresis (Piechura et al, 1983).

This also appears to be the case _in vivo_ as evidenced by the large number of specific antibodies to _Aspergillus_ that can be demonstrated in both the normal individual (Bardana et al, 1972b; Schonheyder and Andersen, 1982; 1984b; Richardson et al, 1984) and patients with varying forms of aspergillosis (see Tables IV.i and V.i).

In addition to the inherent antigenic complexity of the aspergilli it is known that the composition of antigen fractions can vary due to culture conditions (Kauffman and deVries, 1980a), strain or species of _Aspergillus_ cultured (Hearn et al, 1980b; Wallenbeck et al, 1984) and the source of antigenic material, ie whether cell walls, metabolic or somatic extracts (Kim et al, 1978). However, aspergilli also share antigenic determinants. Mishra (1984), using the criteria of percent qualitative sharing showed that various

strains of A.fumigatus shared between 80 and 100% of antigens. In addition Kim and Chaparas (1979) demonstrated that A.fumigatus shared upto 48% of antigens with A.flavus and 32% with A.niger. Furthermore, specific antigens of Aspergillus have been isolated which appear to be common antigenic determinants as demonstrated by their ability to bind specific antibodies from large numbers of ABPA and aspergilloma patients who presumably did not have the same infecting strain or even species. These individual antigens have been described in the introduction to chapter 5 and are summarised in Table V.i.

Two recent reports however, have furthered our understanding of the nature of Aspergillus antigens present in experimental IPA. Phillips and Radigan (1989) isolated circulating Aspergillus antigens from 15 rabbits with experimental infection by affinity chromatography. When the column eluates were probed with a guinea pig hyperimmune antiserum to Aspergillus, a reactive band with an apparent M_r of 80 kDa was seen in six animals. This antigen was heat stable but was not further characterized. It was detectable in the circulation 48h after the onset of infection.

In the second study (Yu et al, 1990) four antigens of Aspergillus with apparent M_rs of 88 kDa, 40 kDa, 27 kDa and 20 kDa were identified in the serum and urine of experimentally infected rats. The 27 kDa antigen was the most reactive. All four antigens were sensitive to protease

treatment and bound Coomassie blue, suggesting that they were at least partly proteinaceous. In rats which were infected intravenously antigen was detected in urine 24-48h post-infection whereas when the conidia were given intratracheally, antigenuria was not seen until 96-120h post- infection. Antigenaemia always appeared after antigenuria. Antigens of similar M_r to those seen in serum and urine were present in a CF antigen prepared from A.fumigatus strain H11-20.

7.2 Aims

The aims of this section of the thesis were twofold: i) to characterize any non-GM Aspergillus antigens which may be present in the serum or urine of patients with IPA and ii) to identify antigens containing GM which were present.

Two approaches were adopted. Firstly, serum and urine specimens from IPA patients were pooled on the basis of their results in the MAb and PAb inhibition ELISAs. This was done in order to determine if specific antigens were responsible for giving a positive test result in each ELISA. Secondly, serum and urine specimens from individual IPA patients were examined in order to determine which, if any, antigen(s) was present in a majority of infected individuals.

Immunoblotting was chosen as the detection method because it has been used successfully to demonstrate

antigenaemia and antigenuria in a number of infections (Huskinson et al, 1989; Hyde et al, 1989).

Affinity chromatography was used initially in an attempt to extract the Aspergillus antigens from specimens. However, this approach did not prove successful.

As a consequence direct examination of the unfractionated specimens was attempted. As the antigenic mosaic of Aspergillus is very complex and different extracts of the fungus do not neccessarily share antigenic determinants (Bardana, 1978; Hearn and Mackenzie, 1981) it was considered prudent to evaluate a large number of anti-Aspergillus antisera, directed against a number of antigenic fractions (Table VII.iv), in order to maximise the number of antigens which could be detected. Dot blot experiments were therefore performed to screen the antisera and determine which was the most reactive. The most reactive antiserum was then used to probe immunoblots of patients' specimens.

In addition to these antisera a monoclonal antibody to A.fumigatus GM was used to probe the blots in order to reveal GM containing antigens.

7.3 Patients, materials and methods.

7.3.1 Patient specimens.

7.3.1.1 Serum. Serum samples were available on all 19 IPA patients. Four pools of sera from these patients were made based on results obtained with each individual specimen in the PAb and MAb inhibition ELISAs, ie PAb negative (-),MAb-; PAb positive (+),MAb-; PAb-,MAb+; PAb+,MAb+. These pools were made in an attempt to see if specific antigens were responsible for the different antigen ELISA results observed.

7.3.1.2 Urine. Urine samples were available on eight of the 19 IPA patients as described in section 6.3.1. Urines from each of these eight patients were pooled separately and given an identification number as follows; patient number 1 (U5), 2 (U1), 3 (U2), 11 (U7), 12 (U3), 14 (U10), 17 (U4) and 19 (U6).

In addition, urine specimens from two children with IPA, confirmed at post-mortem, were received from Dr. H. Holzell, Gt. Ormond Street Hospital for Sick Children, London. These children had undergone bone marrow transplantation for acute lymphocytic leukaemia and severe combined immunodeficiency respectively. Their ages were 3 and 6 years and the causative organism of IPA was

A.fumigatus in each case. These urines were designated U8 and U9 respectively.

PAb-,MAb-; PAb+,MAb-; PAb-,MAb+; PAb+,MAb+ urine pools were prepared from individual specimens with the same aim as described for the serum pools.

7.3.2 Control specimens.

Serum and urine specimens from 23 NEFI patients which were all PAb-,MAb- were pooled separately and used as the controls.

At the time of collection 4 patients had positive blood cultures containing coagulase negative staphylococci.

7.3.3 Urine preparation.

As the affinity chromatography extracts (see section 7.3.7 below) from urine were not very reactive (see section 7.4.1.2) it was decided to concentrate these specimens and to electrophorese them directly in an attempt to detect specific Aspergillus antigens associated with IPA.

Individual patient urines were filtered (0.5µm, Flow Labs) dialysed overnight at 4°C against distilled water and concentrated ten-fold under vacuum in a Rotavapor. Concentrated urines were centrifuged at 13,500 x g for 20min and the supernatants stored at -70°C until analysed.

7.3.4 Antigens.

WS antigen from A.fumigatus NCPF 2109 was prepared as described in section 3.3.2.

GM was prepared from EP1028 as described previously (section 3.3.5).

A CF antigen was prepared from the culture medium of a 48h fermenter culture of A.fumigatus 1028 as described in section 3.3.1. This was designated CF1028.

7.3.5 Antisera and monoclonal antibody.

A wide range of rabbit antisera to different fractions of A.fumigatus NCPF 2109 (Table VII.iv) were used in this section of the thesis.

The majority of these antisera were gifts from Dr. V. Hearn at the MRL, but the antisera to the EP of A.fumigatus 1028 was a gift from Dr. J-P. Latge, Insitut Pasteur.

A rat IgM monoclonal antibody to A.fumigatus GM was a kind gift from Dr. D. Stynen (Eco-Bio). This antibody, designated EBA1, was produced at the same time as EBA2. This has been described in section 6.3.7. The specificity of EBA1 was the same as EBA2.

EBA1 was initially isotyped as an IgG antibody. Therefore the secondary antibody used in this blotting work was a horseradish peroxidase conjugated anti-rat IgG (whole molecule) (Sigma Chemical Co.). Subsequently Eco-Bio informed me that this antibody was in fact an IgM molecule.

Table VII.iv Rabbit antisera used in this study.

Rabbit antiserum	Immunogen	Reference
anti-WS	total WS extract	Wilson & Hearn 1983a
anti-BF	conA binding portion of WS extract	Wilson & Hearn 1983a
anti-UBF	conA non bound fraction of WS extract	Wilson & Hearn 1983a
anti-CW	cell walls	Hearn & Mackenzie 1979
anti-PP	protoplasts	Wilson & Hearn 1982
anti-EP	ethanol precipitate of ferementer CF	Lab method J-P Latge*
pre-immunization	bleed from anti-CW rabbit prior to immunization	NA

key. WS, water soluble; conA, concanavalin-A; CF, culture filtrate; NA, not applicable.
* Unite de Mycologie, Institut Pasteur, Paris, France.
anti-EP was prepared from A.fumigatus 1028.
All other antisera were prepared with fractions from A.fumigatus NCPF 2109.

Insufficient clinical material was available to repeat all the experiments with an anti-rat Ig (kappa chain specific) (Eco-Bio) secondary antibody. However, probing of the WS2109 extract and an antigen positive urine (U10) with EBA1 followed by anti-rat IgG (whole molecule) (Sigma Chemical Co.) or anti-rat Ig (kappa chain specific) (Eco-Bio) as secondary antibodies revealed identical band patterns. The results obtained with EBA1 and the anti-rat IgG (whole molecule) (Sigma Chemical Co.) have therefore been included.

7.3.6 Preparation of IgG fraction from high titre human anti-Aspergillus IgG sera.

0.5ml of each of the 13 human anti-Aspergillus IgG positive control sera described in section 4.3.2 were pooled and the IgG fraction was extracted according to the method of McKinney and Parkinson (1987).

The serum pool was mixed with 4 volumes (26ml) of 60mM $CH_3COO.Na$, pH 4.0, and the pH was adjusted to 4.5 with 0.1N NaOH. A total of 650µl (ie 25µl/ml) of caprylic acid was added very slowly while the mixture was being stirred at 50rpm. This was left mixing for 30min and then centrifuged at 10,000 x g for 30min. The supernatant was removed and mixed with a one tenth volume of x10 PBS. The pH was adjusted to 7.4 with 5.0N NaOH. The mixture was coooled to 4°C and fractionated with 0.277g/ml (45% (w/v)) of $(NH_4)_2SO_4$ by stirring at 4°C for 30min. The supernatant was

discarded after centrifugation at 5,000 x g for 30min and the precipitate was redissolved in 650µl of PBS. This was dialysed overnight against PBS and passed through a 0.2µm filter (Flow Laboratories).

7.3.7 Affinity chromatography.

Three affinity columns were prepared by ligating the following onto CNBr-Sepharose 4B (Pharmacia, Milton Keynes, Bucks): i) WS2109 antigen extract; ii) CF1028 antigen extract or, iii) purified human anti-*Aspergillus* IgG prepared from antibody positive control sera.

7.3.7.1 Preparation of affinity columns. CNBr-Sepharose 4B was swollen in 1mM HCl (5ml per g of dry gel) overnight at 4°C and washed with 1mM HCl (200ml per g of dry gel).

Ligand to be bound to the gel (ie 50mg (protein) of WS2109, CF1028 or purified human anti-*Aspergillus* IgG) was dissolved in 10ml coupling buffer {3.10.12} and incubated with the gel at 4°C overnight with end-over-end turning. Non bound molecules were washed off with coupling buffer and the gel was blocked with 0.1M Tris-HCl, pH 8.0 containing 0.1% (v/v) Tween 20 for 16h at 4°C. The gel was then washed with three cycles of alternating pH buffers; 0.1M acetate buffer, pH 4.0 {3.10.13} and 0.1M tris buffer, pH 8.0 {3.10.14}.

7.3.7.2 Use of columns. All three affinity columns were used in the same way. The general protocol for use of the columns is described below.

Samples to be used in affinity chromatography were either dissolved, diluted or dialysed against PBS (see below) prior to binding to the columns. All binding was done overnight at 4°C with end-over-end turning of the gel/sample mixture. The mixture was then placed in a disposable column (Bio-Rad) and the non-bound fraction eluted with PBS at a flow rate of 1ml/min. Eluate from the column was monitored at 280nm and all fractions with an absorbance greater than background levels were pooled. When the A_{280} reached base line levels the mobile phase was changed to 0.1M glycine-HCl pH 2.2. 2ml fractions were collected into three drops of 1M K_2HPO_4 and the absorbance monitored as above. Again all fractions showing absorbance levels above background were pooled.

An anti cell wall antiserum (anti-CW; see Table VII.iv) was diluted 1:10 in PBS and incubated with either the WS2109 or CF1028 affinity columns as described. The non-bound fraction was collected, dialysed extensively against distilled water and then concentrated back to its original volume. These fractions were designated non-bound anti-CW (WS) or non-bound anti-CW (CF) depending on the column used.

The unconcentrated urine pools described above (section 7.3.1.2) were dialysed against PBS overnight and

then incubated with the anti-Aspergillus IgG column. The non-bound and bound fractions were collected, dialysed against distilled water and concentrated back to their original volume.

The serum pools were mixed with an equal volume of x2 PBS and then treated as the urines.

After use columns were washed with a large volume of PBS and stored at 4°C in PBS containing 0.01% (w/v) Thimerosal (Sigma Chemical Co.).

The rationale behind the use of the two antigen ligated columns and the anti-Aspergillus IgG column was different. The two antigen columns were used to prepare anti-CW that had had all the specific anti-Aspergillus antibodies removed whereas the human anti-Aspergillus IgG column was used in an attempt to extract specific Aspergillus antigens from patients' serum and urine.

7.3.8 Dot-blotting.

Dot blotting of the original unfractionated serum and urine pools and the bound and non-bound fractions from the anti-Aspergillus IgG affinity column was done to determine the most reactive antibody of those listed in Table VII.iv.

The specimens were double diluted in PBS to 1:160 and then 1µl of each dilution was placed onto a previously wetted nitrocellulose sheet (Hybond C, Amersham International, Amersham, Bucks) and allowed to dry. Free

binding sites were blocked with 5% (w/v) NFM for 45min at 25°C. The sheets were rinsed with 0.1% (w/v) NFM and incubated with one of the antisera (diluted 1:750), or EBA1 (1µg/ml of Ig) in 0.1% (w/v) NFM for 4h at 25°C. The sheets were washed with 0.1% (w/v) NFM and incubated with secondary antibody conjugated to horseradish peroxidase [either anti-rabbit IgG (whole molecule) (Dako) or anti-rat IgG (whole molecule) (Sigma Chemical Co.)] diluted 1:1000 in 0.1% (w/v) NFM) for 60min at 25°C. Colour was revealed with a diaminobenzidine chromogen, H_2O_2 substrate system as described in section 5.3.5.2.

The antiserum showing the most reactivity with the lowest specimen dilution was determined by eye. Two antisera ie anti-CW and anti-PP were equally reactive (Fig 7.2) but the anti-CW was used for the bulk of the work as it was available in a much larger quantity.

7.3.9 SDS-PAGE.

WS2109 antigen, the original unfractionated urine pools, individual IPA patient urines, the NEFI urine control plus the bound and non-bound fractions from the anti-*Aspergillus* IgG column were separated by SDS-PAGE as described in section 3.6.

7.3.10 Electroblotting of SDS-PAGE gels.

The electrophoresed antigens and patient specimens

were transferred onto nylon membranes as described in section 3.7.

7.3.11 Immunostaining of blots.

Anti-CW and EBA1 were used to immunostain the antigen and specimen blots. Both these and the secondary antibodies were used at the same concentrations as detailed in the dot-blotting section (7.3.8). Bands were revealed as described in section 5.3.5.2.

7.3.12 Estimation of M_r.

M_r standards were run on each gel. These standards were the same as described in section 3.9. Estimation of the M_r of unkown antigens was also determined as described in that section.

7.3.13 Preparation of monospecific antibodies from polyclonal antisera.

The WS2109 antigen was electrophoresed and blotted as described above except that a preparative comb was used instead of the normal ten well comb and the percentage of acrylamide was increased to 20% {3.10.15}. Each gel was loaded with approximately 450µg of protein.

After transfer a single vertical strip was cut from the middle of the blot and stained with anti-CW as described above. In the meantime the other portion of the blot was

blocked with 5% (w/v) NFM in 10mM KH_2PO_4 pH 7.5, containing 150mM NaCl. After blocking, the unstained blots were stored between wet sheets of filter paper in a moist chamber at 4°C.

On completion of staining, the single strip was aligned with the rest of the blot and the position of major antigen bands were noted on the unstained portion. Horizontal sections equating to these bands were then cut from the unstained blot and labelled for identification. Each strip was then incubated with 2ml of anti-CW diluted 1:40 in 0.1% (w/v) NFM for 4h at 25°C.

Non-bound anti-CW was discarded and the strips were washed for 2min, x3, in 0.1% (w/v) NFM. Bound antibody was eluted by adding 2ml of eluting buffer B {3.10.16} to each strip and vortexing vigourously for 30sec. The eluate was collected into 10µl of 1M Tris base to restore pH balance and the procedure was repeated twice more. This resulted in 6ml of specific antibody at an approximate dilution of 1:120 when compared with the original anti-CW.

These monospecific antibodies were then used to stain patient urine blots.

7.4 RESULTS.

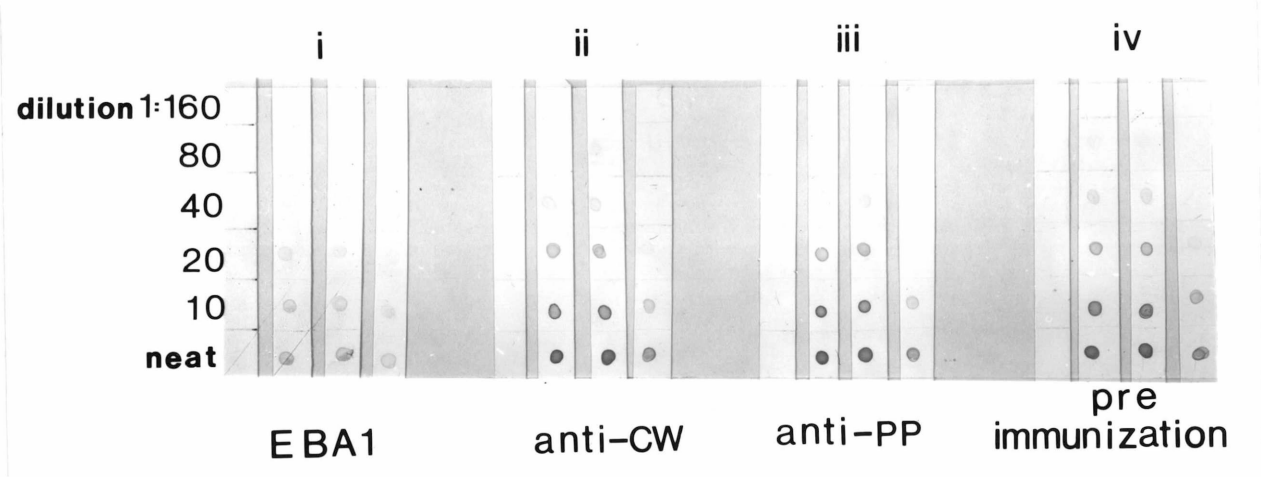
7.4.1 Dot-blotting experiments.

7.4.1.1 Serum. Measurement of the A_{280} of the eluate from the anti-Aspergillus IgG column showed that there were components of all serum pools which bound to the column. However, when these fractions were probed with the antisera to the different Aspergillus fractions no reaction was seen (Fig 7.2, lanes 1; Table VII.v.i).

The anti-Aspergillus IgG column non-bound fractions of all serum pools (including the PAb-,MAb-) reacted with all the antisera tested including the pre-immunization serum (Fig 7.2, lanes 2; Table VII.v.i). This suggested that the binding of the rabbit antisera was non-specific. Confirmation of this non-specific binding was provided by the observation that all rabbit antisera reacted in the same manner with unfractionated human serum that was either antigen negative (PAb-,MAb-) or antigen positive (PAb+,MAb+) (Fig 7.2, lanes 3 and 4; Table VII.v.i). Because of this non-specific binding patients' sera were not studied further in these experiments.

7.4.1.2 Urine. As with the serum pools monitoring of the eluates from the anti-Aspergillus IgG column at 280nm showed that components of all IPA patient urine pools, except the

Figure 7.2 Reactivity of various anti-Aspergillus antisera and EBA1 monoclonal antibody to GM with anti-Aspergillus IgG affinity column serum bound and non-bound fractions plus unfractionated Aspergillus antigen positive and negative sera.



strip 1 in each set of four, anti-Aspergillus IgG affinity column bound serum; strip 2, non-bound serum fraction; strip 3, unfractionated Aspergillus antigen positive serum; strip 4, unfractionated antigen negative serum.

Table VII.v.i Reactivity of anti-Aspergillus IgG affinity chromatography fractions and unfractionated antigen positive and negative sera with antisera to different Aspergillus extracts and EBA1.

	Affinity chromotography extracts								Unfractionated Sera	
Antisera	P-,M- B	P-,M- NB	P+,M- B	P+,M- NB	P-,M+ B	P-,M+ NB	P+,M+ B	P+,M+ NB	P-,M-	P+,M+
pre immun	-	+	-	+	-	+	-	+	+	+
anti-WS	-	+	-	+	-	+	-	+	+	+
anti-BF	-	+	-	+	-	+	-	+	+	+
anti-UBF	-	+	-	+	-	+	-	+	+	+
anti-CW	-	+	-	+	-	+	-	+	+	+
anti-PP	-	+	-	+	-	+	-	+	+	+
anti-EP	-	+	-	+	-	+	-	+	+	+
EBA1	-	+	-	+	-	+	-	+	+	+

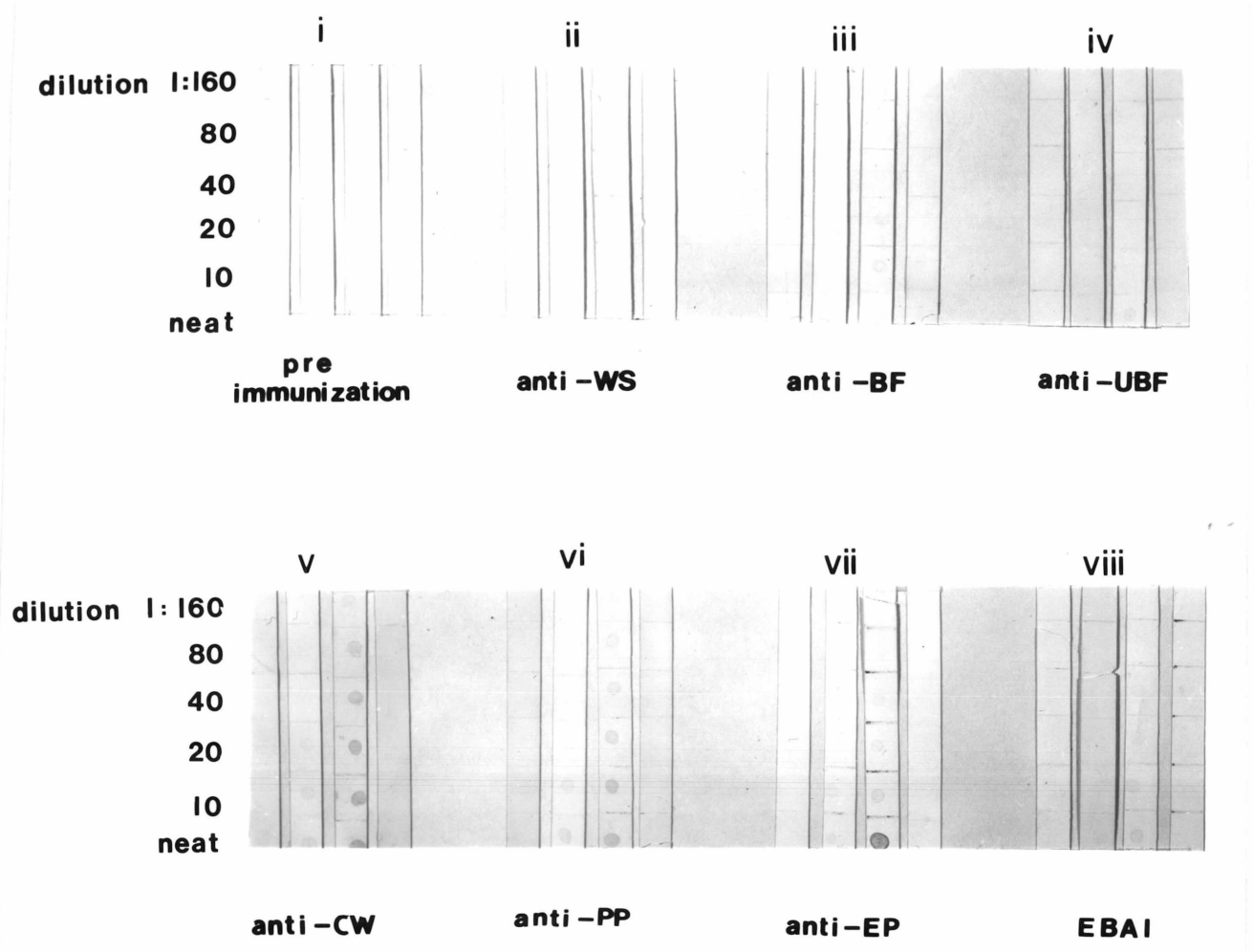
key. P-, polyclonal ELISA negative; P+, polyclonal ELISA positive; M-, monoclonal ELISA negative; M+, monoclonal ELISA positive; B, fraction bound to anti-Aspergillus IgG column; NB, fraction not bound to anti-Aspergillus IgG column; -, no reaction with antiserum; +, reaction with antiserum.
See Table VII.iv for description of antisera.

PAb-,MAb-, were bound by the column. However, none of these fractions reacted with any of the rabbit antisera when tested in dot blotting experiments (Fig 7.3, lanes 1; Table VII.v.ii).

When the urine non-bound fractions were probed with the various antisera, a weak reaction was seen to all the antigen positive pools (i.e. PAb+,MAb-; PAb-,MAb+; PAb+,MAb+) with at least four antisera (Fig 7.3, lanes 2; Table VII.v.ii). No reaction was seen with any antisera against the antigen negative (PAb-,MAb-) pool. In addition, the pre-immunization rabbit antisera did not react with any non-bound fraction (Fig 7.3.i; Table VII.v.ii). The specificity of binding to IPA patient urine components was confirmed when antigen negative (PAb-,MAb-) and positive (PAb+,MAb+) concentrated, unfractionated urine pools were probed with the same antisera. The antigen negative urine pool did not react with any of the antisera (Fig 7.3, lanes 4; Table VII.v.ii) whereas the antigen positive pool reacted strongly with all but the pre-immunization serum (Fig 7.3, lanes 3; Table VII.v.ii). The least reactive antiserum was anti-UBF (Fig 7.3.iv, lane 3) and the most reactive were anti-CW and anti-PP (Fig 7.3.v and vi, lanes 3). Anti-CW was chosen for the majority of the experiments as it was available in a much larger quantity.

EBA1 also reacted specifically with the concentrated PAb+,MAb+ unfractionated urine and the non-bound fractions

Figure 7.3 Reactivity of various anti-Aspergillus antisera and EBA1 monoclonal antibody to GM with anti-Aspergillus IgG affinity column urine bound and non-bound fractions plus unfractionated Aspergillus antigen positive and negative urines.



strip 1 in each set of four, anti-Aspergillus IgG affinity column bound urine; strip 2, non-bound urine fraction; strip 3, unfractionated Aspergillus antigen positive urine; strip 4, unfractionated antigen negative urine.

Table VII.v.ii Reactivity of anti-Aspergillus IgG affinity chromatography fractions and unfractionated antigen positive and negative concentrated urine with antisera to different Aspergillus extracts and EBA1.

	Affinity chromotography extracts								Unfractionated concentrated urine	
Antisera	P-,M- B	P-,M- NB	P+,M- B	P+,M- NB	P-,M+ B	P-,M+ NB	P+,M+ B	P+,M+ NB	P-,M-	P+,M+
pre immun	-	-	-	-	-	-	-	-	-	-
anti-WS	-	-	-	-	-	-	-	-	-	+
anti-BF	-	-	-	-	-	+	-	+	-	+
anti-UBF	-	-	-	+	-	+	-	+	-	+
anti-CW	-	-	-	+	-	+	-	+	-	+
anti-PP	-	-	-	+	-	+	-	+	-	+
anti-EP	-	-	-	+	-	+	-	+	-	+
EBA1	-	-	-	-	-	+	-	+	-	+

key. P-, polyclonal ELISA negative; P+, polyclonal ELISA positive; M-, monoclonal ELISA negative; M+, monoclonal ELISA positive; B, fraction bound to anti-Aspergillus IgG column; NB, fraction not bound to anti-Aspergillus IgG column; -, no reaction with antiserum; +, reaction with antiserum.
See Table VII.iv for description of antisera.

from the PAb-,MAb+ and PAb+,MAb+ urine pools (Fig 7.3.viii; Table VII.v.ii).

7.4.2 Immunostaining of WS2109 extract.

When the electrophoresed and blotted WS2109 extract was probed with anti-CW a large number of bands were present (Fig 7.6, lane A). The most intense staining was seen with antigens of apparent M_rs 11 kDa, 18 kDa and 29 kDa.

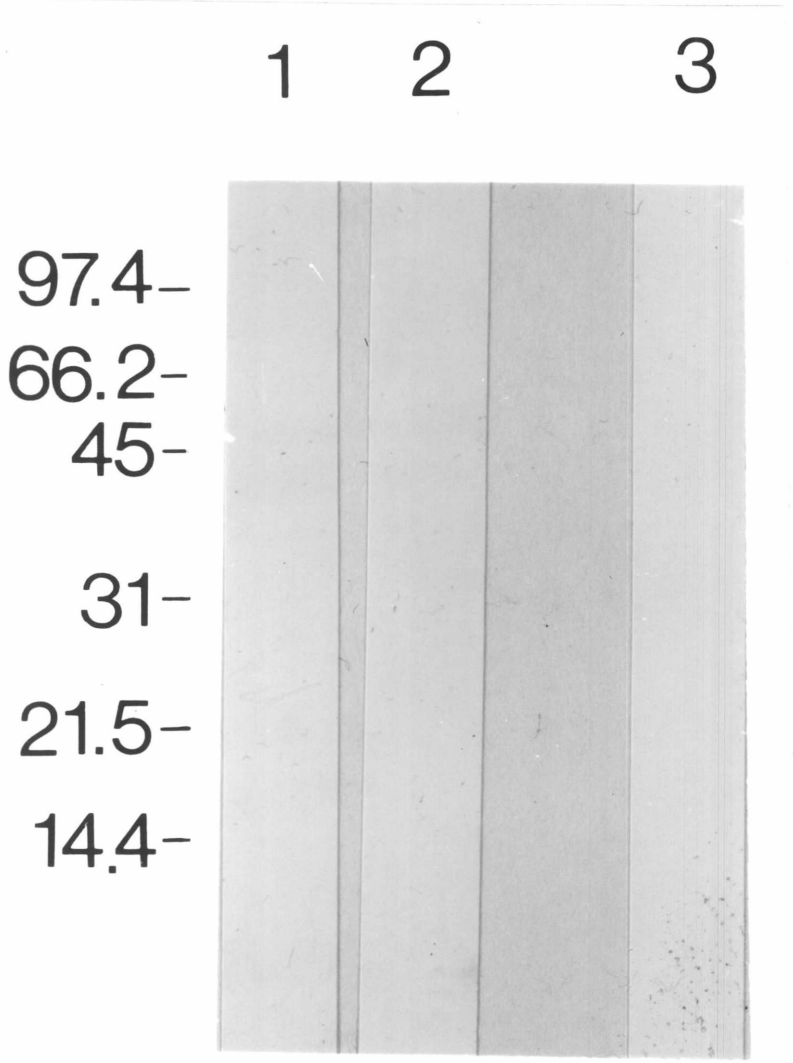
EBA1 produced a completely different pattern with bands of apparent M_rs 45 kDa, 55 kDa, 63 kDa, 65 kDa, 98kDa and >100 kDa visible. In addition these bands were separated by a large amount of diffuse staining (Fig 7.7, lane 1).

7.4.3 Immunostaining of NEFI control urine.

Although the dot-blotting experiments showed the concentrated antigen negative (PAb-,MAb-) urine from the IPA patients to be unreactive with the various antisera it seemed prudent to subject the negative urine pool from the NEFI patients to electrophoresis and blotting, to see if any individual bands were reactive with either anti-CW or EBA1.

This urine pool showed staining patterns which were the same when incubated with either anti-CW or pre-immunization serum. Diffuse, lightly stained bands were seen at approximately 16 kDa, 25 kDa, 40 kDa and 50 kDa (Fig 7.4,

Figure 7.4 Immunoblots of NEFI urine pool stained with pre-immunization serum, anti-CW and EBA1 monoclonal antibody to GM.



key. lane 1, pre-immunization serum; lane 2, anti-CW; lane 3, EBA1.
M_r standards are in the left margin.

lanes 1 and 2). EBA1 did not stain any component of this urine (Fig. 7.4, lane 3).

7.4.4 Immunostaining of IPA patient urines.

Dot-blotting demonstrated that both anti-CW and EBA1 bound to unfractionated, concentrated antigen positive (PAb+,MAb+) urine much more than to the antigen positive eluates from the anti-Aspergillus IgG column (Fig 7.3.v and 7.3.viii). The four urine pools, obtained by combining specimens with the same ELISA results, and the urines from the 10 individual IPA patients were therefore concentrated (x10), electrophoresed, blotted and probed in order to identify both GM and non-GM containing antigens.

7.4.4.1 PAb-,MAb- urine pool. This IPA patient urine pool produced the same pattern when incubated with pre-immunization serum and anti-CW (Fig 7.5, lanes 1a and 1b). Weak non-specific staining was seen at apparent M_rs of 25 kDa and 65 kDa. No staining was seen when the pool was incubated with EBA1 (Fig 7.5, lane 1c).

7.4.4.2 PAb+,MAb- urine pool. When incubated with anti-CW this urine pool revealed 5 bands with apparent M_rs of 11 kDa, 13 kDa, 14 kDa, 18 kDa and 29 kDa (Fig 7.5, lane 2b). Only weak staining was seen with the pre-immunization serum at approximately 20 kDa (Fig 7.5, lane 2a).

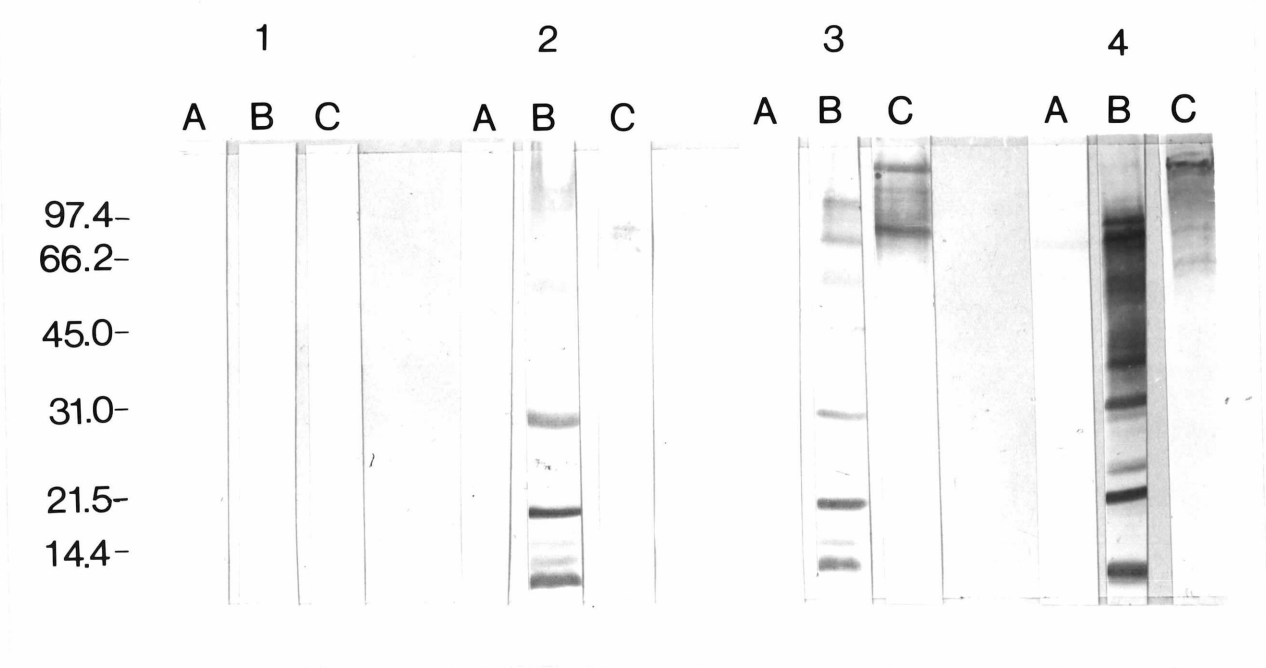
EBA1 produced a small amount of diffuse staining between approximately 100 kDa and 66 kDa (Fig 7.5, lane 2c).

7.4.4.3 PAb-,MAb+ urine pool. This pool produced a similar band pattern to the PAb+,MAb- urine pool when stained with anti-CW (Fig 7.5, lane 3b) However, there were additional bands with apparent M_rs of 38 kDa, 44 kDa and >100 kDA. In addition a small amount of diffuse staining was seen between 55 kDa and >100 kDa. Only weak non-specific staining at approximately 60 kDa and 100 kDa was seen with the pre-immunization sera (Fig 7.5, lane 3a).

When this pool was stained with EBA1 it was much more reactive than the PAb+,MAb- pool (Fig 7.5, lane 3c). Two defined bands with apparent M_rs of 70 kDa and >100 kDa were present. The rest of the staining between 60 kDa and >100 kDa was diffuse.

7.4.4.4 PAb+,MAb+ urine pool. When this pool was probed with anti-CW the band pattern produced was essentially the same as the PAb-,MAb+ pool except that the band at 44 kDa was not present while an additional band at 21 kDa was seen. In addition a large amount of diffuse staining was seen between 30 kDa and >100 kDa (Fig 7.5, lane 4b). The staining of this pool with the anti-CW antisera was more intense than the other two antigen positive pools. As before only very weak staining was seen with the pre-immunization serum (Fig

Figure 7.5 Immunoblots of IPA patient urine pools stained with pre-immunization serum, anti-CW and EBA1 monoclonal antibody to GM.



lanes 1a-1c, PAb-,MAb- pool; lanes 2a-2c, PAb+,MAb- pool; lanes 3a-3c, PAb-MAb+ pool; lanes 4a-4c, PAb+,MAb+ pool; lanes 1a-4a stained with pre-immunization serum; lanes 1b-4b stained with anti-CW; lanes 1c-4c stained with EBA1
M_r standards are shown in the left margin.

7.5, lane 4a).

EBA1 produced a pattern identical to that seen with the PAb-,MAb+ urine (Fig 7.5, lane 4c).

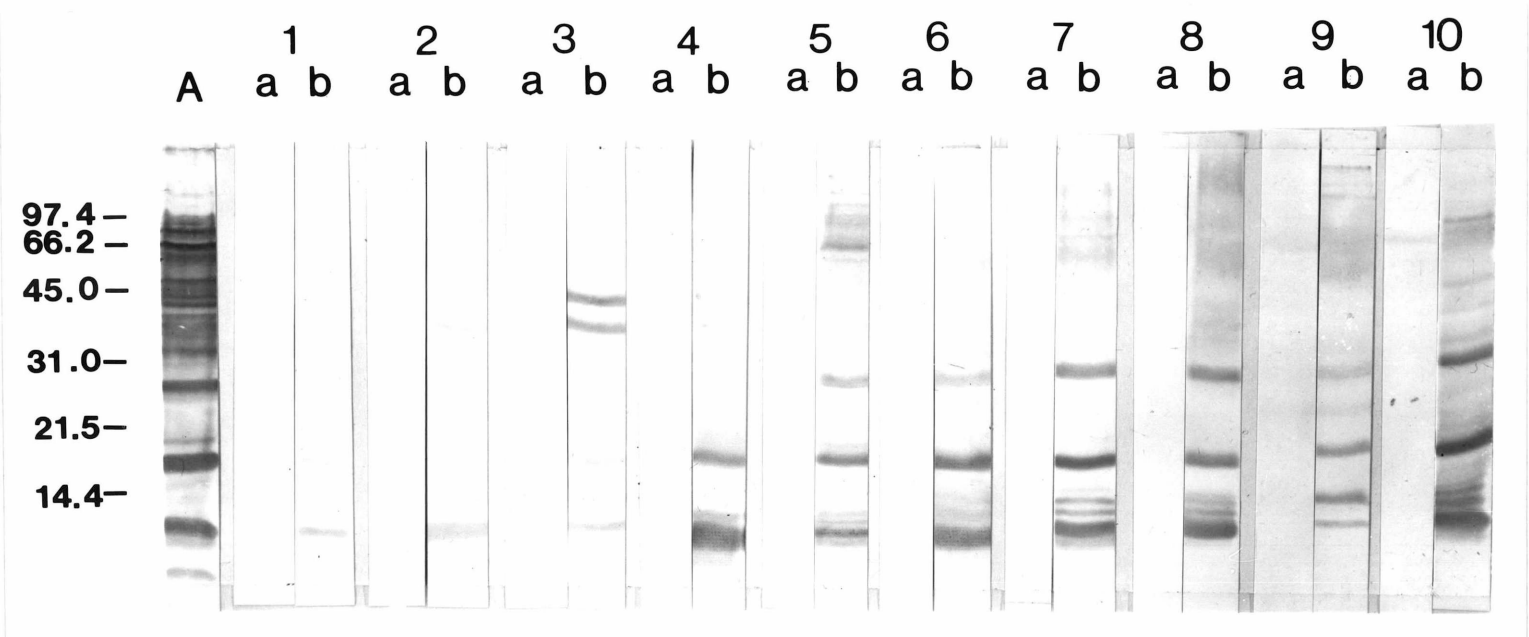
7.4.4.5 Individual IPA patient urines. When the IPA patients' urines were probed, with anti-CW, seven antigens were detected with apparent M_rs of 11 kDa, 13 kDa, 14 kDa, 18 kDa, 29 kDa, 38 kDa and 44 kDa (Fig 7.6, lanes 1b-10b; Table VII.vi). Each urine showed a different antigen band pattern. However, two bands at 11 kDa and 18 kDa were 'major' antigens as they were found in ten and nine of the IPA patient urines respectively. Three bands (13 kDa, 14 kDa and 29 kDa) were 'intermediate' antigens occurring in up to six urines and the remaining two bands at 38 kDa and 44 kDa were 'minor' antigens found in only three and two urines respectively.

Diffuse bands with M_rs greater than 60 kDa were also present in six urines (Fig 7.6, lanes 5b-10b).

Similar antigen patterns were seen when ^{125}I-Protein A or ^{125}I-anti-rabbit IgG were used instead of HRP conjugated anti-rabbit IgG thereby demonstrating the specificity of the binding.

None of the seven antigen bands could be detected with pre-immunization serum, but weak non-specific staining was seen at 23 kDa, 59 kDa, 67 kDa and 100 kDa (Fig. 7.6, lanes 1a-10a).

Figure 7.6 Immunoblots of individual IPA patient urines stained with pre-immunization serum and anti-CW.



lane A, WS2109 stained with anti-CW; lanes 1a-10a, U1-U10 stained with pre-immunization serum; lanes 1b-10b, U1-U10 stained with anti-CW

M_r standards are shown in the left margin.

Table VII.vi Antigen content of the PAb-,MAb-; PAb+,MAb-; PAb-MAb+; PAb+,MAb+ urine pools, NEFI control urine pool and the 10 individual IPA patient urines revealed with anti-CW.

Antigen M_r kDa	Urine pools				NEFI	Individual urines (U1-U10)									
	P-M-	P+M-	P-M+	P+M+	pool	1	2	3	4	5	6	7	8	9	10
>100	-	-	+	-	-	-	-	-	-	+	-	-	-	-	+
60	-	-	+	+	-	-	-	-	-	+	-	+	-	-	+
44	-	-	+	-	-	-	-	+	+	-	-	-	-	-	-
38	-	-	+	+	-	-	+	+	+	-	-	-	-	-	-
29	-	+	+	+	-	-	-	-	-	+	+	+	+	+	+
21	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-
18	-	+	+	+	-	+	-	+	+	+	+	+	+	+	+
14	-	+	+	-	-	-	-	-	-	+	-	+	+	+	+
13	-	+	+	-	-	-	-	-	+	+	+	+	+	-	+
11	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+

key. P-M-, PAb-,MAb-; P+M-, PAb+,MAb-; P-M+, PAb-,MAb+; P+M+, PAb+, MAb+; -, absence of antigen; +, presence of antigen.

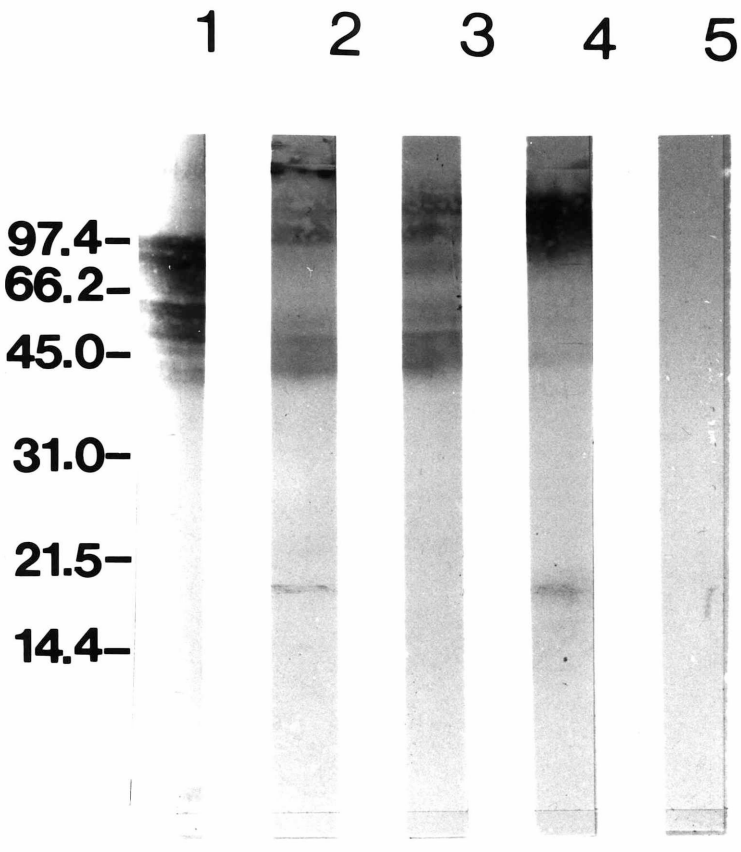
Sufficient urine was available from three patients (U5, U7, U10) to allow probing with the EBA1 monoclonal antibody to A.fumigatus GM. A totally different band pattern was seen with this antibody when compared with anti-CW (Fig. 7.7, lanes 2-4). Staining was weaker and bands were more diffuse. The majority of staining occurred at M_rs >45 kDa (Fig. 7.7, lanes 1-4). However, two urines (U5 and U10) showed a weak band at approximately 21 kDa (Fig. 7.7, lanes 2 and 4). None of the antigens revealed with anti-CW could be demonstrated when the urines were probed with EBA1 confirming that they do not contain GM.

7.4.5 Homology between Aspergillus antigens detected in urine and components of the WS2109 and CF1028 extracts.

Because similar M_r antigens were seen in urines and the WS2109 extract (i.e. 11 kDa, 18 kDa and 29 kDa) it was decided to see if these antigens were homologous. Firstly by absorbtion experiments and then by purification of monospecific antibodies.

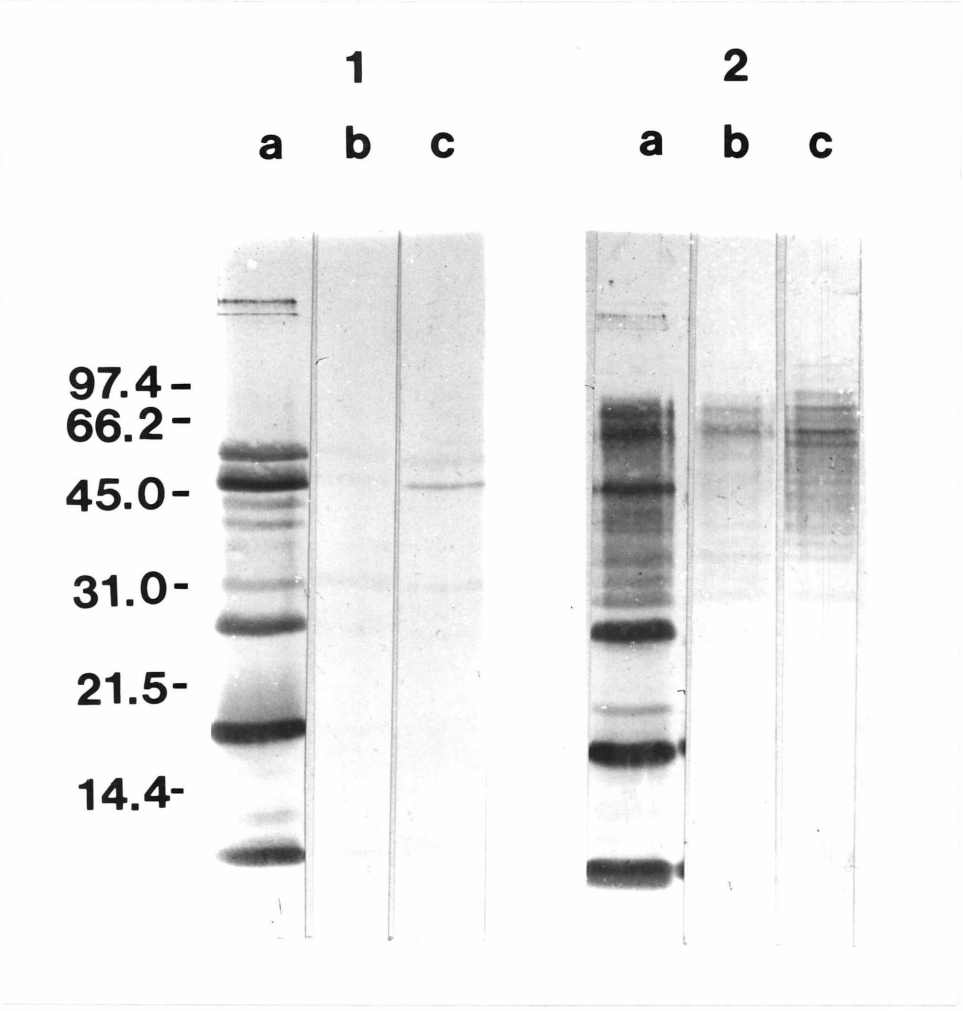
7.4.5.1 Absorbtion experiments. Probing of WS2109 and U10 with non-bound anti-CW (WS) and non-bound anti-CW (CF) led to a large reduction in binding to the majority of antigen bands when compared to blots which had been stained with unfractionated anti-CW. The bands at 11 kDa, 18 kDa and 29 kDa in U10 were completely removed (Fig 7.8, lanes 2b 2c) as

Figure 7.7 Immunoblots of WS2109 antigen, individual IPA patient urines and NEFI urine pool stained with EBA1 monoclonal antibody to GM.



key. lane 1, WS2109; lane 2, U5; lane 3, U7; lane 4, U10; lane 5, NEFI pool.
M_r standards are in the left margin.

Figure 7.8 Immunoblots of IPA patient urine (U10) and WS2109 antigen stained with anti-CW, non-bound anti-CW (WS) and non-bound anti-CW (CF).



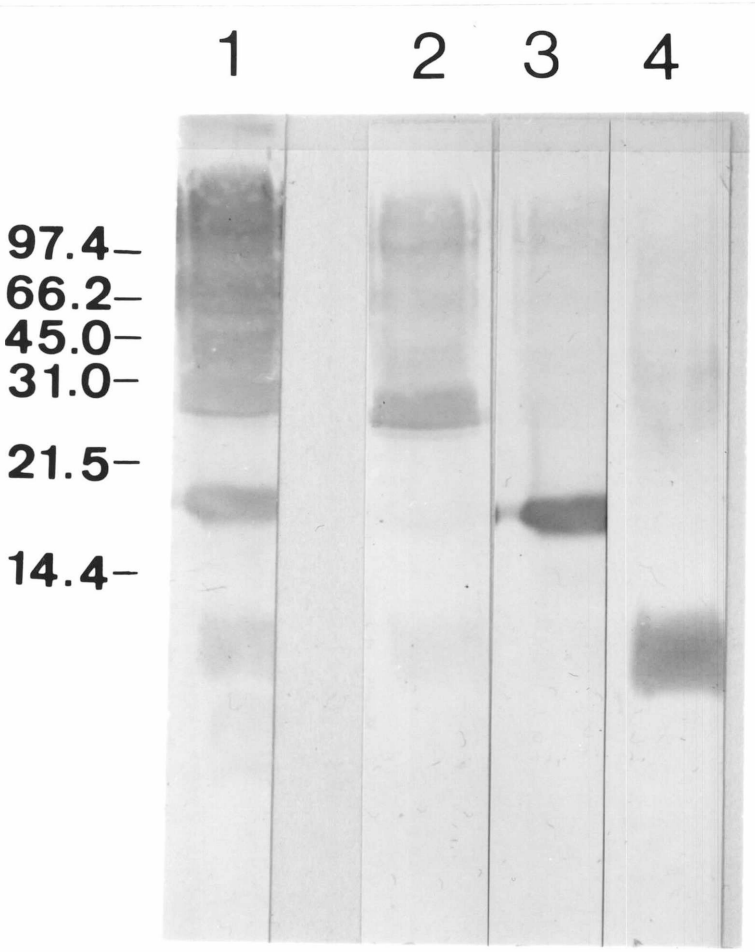
key. lanes 1a-1c, WS2109; lanes 2a-2c, U10; lanes 1a and 2a stained with anti-CW; lanes 1b and 2b stained non-bound anti-CW (WS); lanes 1c and 2c stained with non-bound anti-CW (CF).
M_r standards are in the left margin.

were similar M_r antigens in the WS2109 extract (Fig 7.8, lanes 1b, 1c). This suggests that homology exists between components of these in vitro preparations and the antigens present in patients' urine. Furthermore, bands at 11 kDa, 18 kDa and 29 kDa were clearly visible in the WS2109 extract when it was stained with anti-CW (Fig. 7.6, lane A; Fig. 7.8, lane 1a).

7.4.5.2 Monospecific antibody experiments. In order to confirm the homology between the in vitro and in vivo Aspergillus antigens monospecifc antibodies to the 11 kDa (anti-11), 18 kDa (anti-18) and 29 kDa (anti-29) antigens of the WS2109 extract were prepared as described in section 7.3.13.

U10 was electrophoresed in a 20% acrylamide gel (the higher percentage of acrylamide was used in order to acheive greater resolution in the lower M_r range) and then blotted onto nylon. Anti-11, anti-18 and anti-29 were then used to probe the blots. As can be seen in Fig 7.9 (lanes 2 to 4) all three monospecific antibodies bound only to antigens of similar M_r in U10 to those in the WS2109 extract from which they had been eluted.

Figure 7.9 Immunoblots of IPA patient urine (U10) probed with monospecific antibodies to the 11 kDa, 18 kDa and 29 kDa antigens of WS2109.



key. lane 1, WS2109 stained with anti-CW; lane 2, U10 stained with monospecific antibody to the 29 kDa antigen (anti-29); U10 stained with anti-18; lane 4, U10 stained with anti-11.
M_r standards are in the left margin.

7.5 DISCUSSION.

Most of the methods described to detect circulating Aspergillus antigens during IPA have employed ill defined reagents. For example the conA binding fractions often used in ELISAs (Wilson et al, 1987; LePape and Deunff, 1987) have been shown to be complex mixtures of at least 14 antigens (Wilson and Hearn, 1983a). A positive test result could therefore indicate the presence of any one of these molecules.

Currently the only defined antigen known to circulate during IPA is GM (Lehmann and Reiss, 1979; Dupont et al, 1987; deRepentigny et al, 1987). GM has been characterized chemically after extraction from mycelium produced in vitro by many workers (see Table VII.ii) and was shown to have a M_r of between 18 kDa and 50 kDa. The purified GM used in the experiments presented here had a M_r of 21 kDa as determined by gel filtration (J-P. Latge, personal communication). However, the circulating GM-containing antigen detected by Reiss and Lehmann (1979) had a M_r of greater than 125 kDa, while that detected by Dupont et al (1987) had a wide M_r ranging from 10 kDa to 70 kDa and the GM antigen detected by deRepentigny et al (1987) in experimental infection had a M_r of 50 kDa to 100 kDa. It has been suggested that the increased size of GM detected in vivo was due to complexing of the polysaccharide to protein. The results presented here

demonstrate that the majority of GM-containing antigens have apparent M_rs >45 kDa (Fig. 7.7, lanes 1-4) which confirms the findings of other groups.

The diffuse staining seen in these blots was characteristic of a highly glycosylated moiety. The finding of specific GM antigens with apparent M_rs of >100 kDa and 60 kDa was also consistent with previous reports. The >100 kDa antigen had a similar M_r to the 125 kDa GM reported by Lehmann and Reiss (1979), while the 60 kDa band was within the range described by Dupont et al (1987) and deRepentigny et al (1987).

Probing of the PAb-,MAb+ and PAb+,MAb+ urine pools, with EBA1, showed similar staining patterns to that seen in the individual IPA patient urines; whereas a much lower level of staining was seen with the PAb+,MAb- pool. This suggests that the higher M_r components of the two MAb+ pools were responsible for the positive MAb ELISA results. The decreased intensity of staining observed with EBA1 in the PAb+,MAb- urine pool probably reflects the lower concentration of GM containing antigens in these urines which were undetectable by ELISA.

The similar band patterns found in patient urines when probed with EBA1 demonstrated that the GM-containing antigens released by different Aspergillus spp in vivo were similar since the blots shown in Fig 7.7 (lanes 2 to 4) are of urines from patients with IPA due to A.fumigatus,

A.glaucus and a mixed A.fumigatus/A.flavus infection. This finding was expected as it has been shown that in vitro polysaccharide extracts from a large numebr of Aspergillus spp reacted with monoclonal antibodies to A.fumigatus GM (Debeaupuis et al, 1989).

In the introduction to this chapter evidence was presented for the possible presence of non-GM antigens in body fluids during IPA. This work confirms these observations and demonstrates the non-GM antigens that were present during IPA.

A different antigen pattern was seen in all 10 IPA patient urines and the three antigen positive pools when stained with anti-CW. However, antigens with apparent M_rs of 11 kDa and 18 kDa appeared to be major antigens as they were detected in the urine of ten and nine IPA patients respectively and in all three antigen positive urine pools. Intermeadiate antigens with M_rs of 13 kDa, 14 kDa and 29 kDa were detected in upto six individual urines and two or three of the positive pools. Four other minor antigens with apparent M_rs of 38 kDa, 44 kDa, 60 kDa and >100 kDa were detected in two or three individual urines and up to two of the antigen positive pools while a single antigen (M_r 21 kDa) was detected in one urine pool only.

It would appear likely that these antigens were responsible for the positive results obtained with the PAb ELISA as W1 reacted strongly with antigens of similar M_rs in

the WS2109 extract (Fig 6.2). The fact that some of these antigens were detected in the PAb-,MAb+ urine pool was surprising. It may be that the negative PAb ELISA result obtained was due to a lower concentration of these antigens in this pool. Evidence for this was provided by the observation that x10 concentration of the unfractionated urines resulted in increased binding of anti-CW.

The observation that 12.7% of IPA patients' sera tested by the PAb ELISA were positive suggests that the antigens detected in urine were also circulating during IPA. The inability to demonstrate these antigens in sera was due to the non-specific binding of both primary (anti-CW and EBA1) and secondary antibodies to components of human serum plus the non-reactivity of fractions bound to the anti-Aspergillus IgG column. This failure to demonstrate Aspergillus antigens in affinity column bound fractions prepared from sera (cf Phillips and Radigan, 1989), and urines (cf Yu et al, 1990), was probably a consequence of the IgG used to produce the column. A total human IgG fraction was used which presumably contained a large proportion of antibodies directed to both Aspergillus antigens which were not present in body fluids and totally irrelevant antigens. Therefore only a small proportion of the IgG ligated onto the gel would have been directed against circulating Aspergillus antigens. This could have resulted in the majority of circulating Aspergillus antigen

passing directly through the column. Use of specific antibodies directed against the antigens detected in urine should allow demonstration of the same antigens in serum.

Seven of the 10 IPA patients studied here had IPA due to strains of A.fumigatus. The other three were due to A.flavus, A.glaucus and mixed A.fumigatus/A.flavus. All three urines from these patients (U1, U5 and U10) contained the 11 kDa and 18 kDa antigens suggesting that they are shared among the different species of Aspergillus. This finding has the fortunate practical consequence that if tests based on these antigens were used for the diagnosis of IPA, then cases caused by species other than A.fumigatus would be detected.

Further evidence that the aspergilli share antigenic determinants comes from the observation that U5 and U10 were the most reactive individual IPA patient urines with anti-CW, an antiserum raised to a cell wall fraction of A.fumigatus NCPF 2109. These results also demonstrate that different species of Aspergillus express shared antigenic determinants in vivo, just as has been observed previously in vitro (Kim et al, 1979; Mishra et al, 1984).

The only other reports of Aspergillus antigen detection in IPA by immunoblotting have been from experimental models. Phillips and Radigan (1989) identified a heat stable antigen with an apparent M_r of 80 kDa in the sera of 6/15 rabbits with IPA. No antigen of this M_r was

detected in the urine of the IPA patients studied here. It was possible however, that this antigen was present only in the sera of IPA patients as its M_r would have prevented removal from the circulation by glomerular filtration. This explanation was unlikely though as the majority of IPA patients had sustained renal dysfunction which would have rendered the kidney permeable to this antigen.

In another report Yu et al (1990) demonstrated four antigens in the serum and urine of infected rats with apparent M_rs of 20 kDa, 27 kDa 40 kDa and 88 kDa. The 27 kDa antigen was detected most often. This antigen had a similar M_r to the 29 kDa detected here. In addition the M_r of the 20 kDa antigen was similar to that of the 18 kDa in the IPA patient urines. The blots presented by these workers in their paper were striking in their similarity to those presented here and it is possible that the antigens were the same. However, confirmation of this hypothesis will have to wait until both sets of antigens have been characterized.

In addition to this immunoblotting work on Aspergillus antigens, the detection of specific antibodies to characterized antigens has increased our knowledge on the nature of circulating and excreted components of the fungus (see Table V.i). The majority of this work has been done on patients with non-invasive forms of aspergillosis. Brouwer (1988) demonstrated antibodies to antigens with apparent M_rs of 18 kDa, 24-26 kDa and 36-38 kDa in an immunoblot study of

precipitin positive sera while, Kurup et al (1989) demonstrated that the major antibody response of ABPA patients was to Aspergillus antigens with a M_r <14 kDa. The work presented in this chapter extends these findings to IPA and demonstrates that antigens of similar M_rs (ie 11 kDa, 13 kDa, 14 kDa, 18 kDa and 38 kDa) were present.

Matthews et al (1985) showed that the predominant antibody response in IPA was to an antigen of 40 kDa. No antigen of this M_r was seen in the IPA patients although it was similar in size to the 38 kDa antigen.

The limited supply of IPA patient urine neccessitated the pooling of specimens from individuals. This precluded the study of the temporal release of Aspergillus antigens in IPA. However, adsorption of anti-CW, with the 48h CF1028 extract, led to a reduction in binding to the urinary antigens. This suggests that these antigens were produced and released early in the course of infection.

The examination of serial IPA patient specimens with specific antibodies to the excreted antigens should allow the detection of Aspergillus components which are released early in the infection.

The lack of urines also prevented the further characterization of both the GM and non-GM antigens. If material had been available then the effect of heat treatment, proteases, exo-polysaccharidases on the antigens plus their ability to bind to conA would have given valuable

information on their nature. However, the homology observed between the 11 kDa, 18 kDa and 29 kDa antigens in patients' urine and the WS2109 extract suggests that the fungus produces these antigens both *in vivo* and *in vitro*. This finding should allow information on the nature of the urinary antigens to be derived from antigens purified from *in vitro* extracts.

The results of this chapter show that both GM and non-GM antigens are present during IPA. This pattern is repeated in other fungal infections where both polysaccharide (Cherniak et al, 1980) and protein (Reiss and Bragg, 1986; Reiss et al, 1986b) antigens can be demonstrated in body fluids. For example in systemic candidosis it has been shown that both a mannan-containing glycoprotein (Kahn and Jones, 1986b) and a 47/48 kDa protein (Matthews and Burnie, 1988) antigen were circulating.

In conclusion it can be said that the purification and characterization of the non-GM antigens described here should lead to the development of novel reagents which could be used in conjunction with tests for GM, and hopefully allow the rapid detection of *Aspergillus* infection.

CHAPTER EIGHT. FINAL DISCUSSION.

Progress in medical science, while undoubtedly beneficial to the majority of the population, has sometimes brought with it unwanted and unexpected complications. The recent increase in the number of opportunistic fungal infections reported in severely immunosuppressed patients (Fraser et al, 1979) is one of these problems. To paraphrase Dr. M. Rinaldi, in a lecture given to the annual meeting of the British Society for Mycopathology at York in the spring of 1989 ''there ain't no such thing as a fungus that can't kill; given the right predisposing factors''.

One of the major groups of fungi involved in this devastating onslaught on the immunosuppressed patient population are members of the genus Aspergillus (Pirsch and Maki, 1986). These fungi are normally benign, saprophytic soil fungi which have little impact on the immunocompetent host. However, immune depression due to naturally occurring disease states, such as haematological malignacy, and the use of high dose chemotherapy, irradiation and corticosteroids has resulted in a large population of patients 'at risk' of developing invasive infection with these fungi.

The prompt initiation of antifungal therapy can improve on the generally poor prognosis documented in these patients (Aisner et al, 1977). However, the definitive

diagnosis of IPA remains difficult. Clinical signs are non-specific, culture of the fungus is only possible in a small percentage of patients, even with widely disseminated infection, and furthermore may represent colonization rather than invasion. Consequently a large number of cases are only confirmed at post-mortem.

The diagnosis of IPA by serological means was the topic of this thesis. An 'at risk' population of 121 patients undergoing bone marrow transplantation or remission induction therapy for haematological malignancy, inborn errors of metabolism, soft tissue malignancies or congenital immunodeficiency were studied. 19 of the patients developed proven IPA.

The detection of specific anti-Aspergillus IgG antibodies was evaluated as a method of diagnosing IPA because of several encouraging reports in the literature (Holmberg et al, 1980; Matthews et al, 1985; Trull et al, 1985). No correlation could be found between the presence of anti-Aspergillus IgG antibodies and the development of IPA in the patients studied here. This finding was not surprising given the immune dysfunction seen in this and similar patient populations (Gerson et al, 1984).

The presence of anti-Aspergillus IgG antibodies appeared to be uniform in both the patients and healthy blood donors used as controls. The ubiquitous exposure of individuals to conidia of Aspergillus spp seems to result in

a low but detectable level of specific antibody (Bardana et al, 1972b). An elevated level of specific IgG was seen in patients with ABPA as has been previously reported (Table IV.i).

Conversely the detection of Aspergillus antigen could be used to predict the development of IPA. This finding was in agreement with previous reports of this approach (Table VI.i).

GM appeared to be the predominant antigen of Aspergillus present during IPA as it was detected in up to 44% of specimens from patients with proven infection. However, at least seven other antigens, which did not contain GM, were also present in the urine of patients with IPA. The purification, characterization and devlopment of specific antibodies to these antigens should lead to the introduction of novel assays which may improve diagnostic capabilities. This work should be facilitated by the observation that antigens with M_rs of 11 kDa, 18 kDa and 29 kDa found in IPA patients' urine were homologous with antigens in extracts of A.fumigatus NCPF 2109; therefore these extracts could be used as a source of antigens which are present in vivo.

Lack of clinical material did not allow the temporal expression of these antigens to be elucidated. The identification of Aspergillus antigens released early in the course of infection should be a priority as this would allow

the development of tests which were specifically directed at the detection of 'early' antigens.

If antigen detection is to become widely accepted a simple to perform, reliable and completely characterized assay will be required. In common with other reports, the two ELISAs evaluated in this thesis do not achieve all these goals. They are simple and reliable; however, the use of polyclonal antisera and uncharacterized antigen fractions precludes their use in the routine diagnostic laboratory. They remain in the preserve of reference centres and laboratories with a special interest in IPA. The use of a monoclonal antibody to GM in the MAb ELISA was a step in the direction of having completely characterized assays. However, the complexity of Aspergillus antigen release reported in this thesis suggests that it may be neccessary to perform a battery of antigen detection tests on each specimen in order not to miss a positive result; at least until the elucidation of an 'early' antigen can be achieved.

The LAT is completely characterized and is also commercially available. However, the PPV of this test was only 67% which indicates a problem with specificity. If this could be overcome by the manufacturers, this simple test may find a place in the routine diagnostic laboratory. Other workers (Dupont et al, 1990; van Cutsem et al, 1990) experienced no specificity problems when evaluating this test. Currently this LAT is the only completely defined

commercially available test for the detection of Aspergillus antigen. A large scale clinical evaluation of this test would enable its value in the diagnosis of IPA to be assessed.

The fluctuation in antigen titres that we observed will necessitate regular specimens being obtained from all 'at risk' patients. Specimens should not just be taken when clinical evidence suggests a possible diagnosis of IPA as initiation of antifungal therapy on clinical criteria alone, in the individuals studied here, resulted in the successful treatment of only 1/19 patients with proven IPA and only 4/12 with suspected fungal infection. The testing of specimens should also be done regularly as IPA progresses rapidly and the storage of specimens for a long period could delay initiation of appropriate therapy.

If the detection of Aspergillus antigen by all three tests had been used as the criterion on which to initiate antifungal therapy 27/31 of the IPA and CFI patients would have been treated earlier than on the basis of conventional diagnosis. It is likely that this earlier initiation of antifungal therapy based on a positive antigen test would have resulted in increased survival among these patients, since improved response rates have been reported when empirical treatment is started early in the course of suspected infections (Aisner et al, 1977; Burch et al, 1987). The findings presented in this thesis suggest that a

controlled trial should be undertaken comparing early treatment based on antigen detection with conventional management.

Research into the development of new antifungal agents to treat IPA should be a priority. A safe, effective and non-toxic antifungal agent would greatly reduce the impact of IPA and the other invasive mycoses which afflict the immunosuppressed patient population.

Currently the drug of choice for IPA is amphotericin B which can have severe side effects. A new liposomal formulation of this drug appears to be just as effective against a range of fungi but with markedly reduced side effects (Szoka et al, 1987). The new azole itraconazole also offers hope as an effective agent against Aspergillus (Prentice and Bradford, 1989). However, there appear to be problems with absorbtion of this compound which may affect its usefulness (Boogaerts et al, 1989).

Prevention though is better than cure. Aspergillus conidia are acquired from the air and if these propagules can be removed there will be a substantial reduction in this extrinsic risk factor. The use of high efficiency particulate air filters (HEPA) which can change the air in a room a large number of times per hour offers hope. However, the installation of these filters is extremely costly and may not be possible for all patients. The pre-selection of patients for nursing in HEPA filtered rooms, from an already

high risk population, who are particularly 'at risk' for IPA such as individuals undergoing T-cell depleted bone marrow transplants may be beneficial.

Chemoprophylaxis may also reduce the incidence of IPA (Tricot et al, 1987). A large scale controlled trial of itraconazole for this purpose in neutropenic patients is currently underway and the results are awaited with interest. The administration of prophylactic reagents via the respiratory route merits serious consideration. The use of prophylactic nebulised amphotericin B has recently been reported to be of some use in the prevention of IPA (Conneally et al, 1990). The use of enilconazole in the agricultural and horse racing industries offers an example of what could be achieved. The antigen detection tests described above could be used to monitor the efficacy of prophylactic antifungal therapy for the prevention of IPA.

The encouraging results obtained with Aspergillus antigen detection tests should not discourage research into alternative methods of diagnosis which could also bring about earlier administration of empirical antifungal therapy. Recent advances in imaging techniques such as computed tomography (CT) have produced encouraging results without the need for invasive intervention techniques (Kuhlman et al, 1987). Specific double halo shadowing followed by the development of an air crescent has been seen on serial CT scans from patients with IPA when conventional

chest x-rays have appeared normal.

Molecular biology may also have a role to play in the future diagnosis of IPA. The polymerase chain reaction technique can detect very low amounts of specific DNA and an evaluation of this method for the detection of Aspergillus DNA in the BALF of 'at risk' patients is in its early stages at the Royal Postgraduate Medical School, Hammersmith Hospital, London.

Detection of fungal metabolites may also prove a fruitful area for research. It has been reported that oxalic acid was present in BALF from patients with IPA. The finding that 29% (w/w) of the CF antigen of a 48h culture of A.fumigatus 1028 was galactosamine suggests that this aminosugar may be present in body fluids during IPA.

It is hoped that this work has contributed towards improving the diagnostic capability for IPA; indeed I feel that the demonstration of non-GM containing antigens in the urine of infected patients has opened up an exciting new area for research. However, perhaps the final remark of this thesis should be that while steps have been taken towards the goal of establishing a universally accepted test for the diagnosis of IPA an immense amount of work has still to be done. The opportunity to be involved in this challenge is very exciting.

Bibliography.

Aisner, J., Schimpff, S.C. and Wiernik, P.H. Treatment of invasive aspergillosis: Relation of early diagnosis and treatment to response. Ann. Int. Med. 86: 539-543. (1977).

Andrews, C.P. and Weiner, M.H. Immunodiagnosis of invasive pulmonary aspergillosis in rabbits. Fungal antigen detected by radioimmunoassay in bronchoalveolar lavage fluid. Am. Rev. Resp. Dis. 124: 60-64. (1981).

Azuma, I., Kimura, H., Hirao, F., Tsubura, E., Yamamura, Y. and Misaki, A. Biochemical and immunological studies on Aspergillus III. Chemical and immunological properties of glycopeptide obtained from Aspergillus fumigatus. Jap. J. Microbiol. 15: 237-246. (1971).

Bardalaye, P.C. and Nordin, J.H. Chemical structure of the galactomannan from the cell wall of Aspergillus niger. J. Biol. Chem. 252: 2584-2591. (1977).

Bardana, E.J., McClatchy, J.K., Farr, R.S. and Minden, P. The primary interaction of antibody to components of aspergilli. I. Immunologic and chemical characteristics of a non-precipitating antigen. J. Allergy Clin. Immunol. 50: 208-221. (1972a).

Bardana, E.J., McClatchy, J.K., Farr, R.S. and Minden, P. The primary interaction of antibody to components of aspergilli. II. Antibodies in sera from from normal persons and patients with aspergillosis. J. Allergy Clin. Immunol. 50: 222-234. (1972b).

Bardana, E.J., Gerber, J.D., Craig, S. and Cianciulli, F.D. The general and specific humoral immune response to pulmonary aspergillosis. Am. Rev. Resp. Dis. 112: 799-805. (1975).

Bardana, E.J. Culture and antigen variants of Aspergillus. J. Allergy Clin. Immunol. 61: 225-227. (1978).

Bardana, E.J. The clinical spectrum of aspergillosis. Part 1: epidemiology, pathogenicity, infection in animals and immunology of Aspergillus. Crit. Rev. Clin. Lab. Sci. 13: 21-83. (1981).

Barnes, R.A. An investigation of the problem of invasive aspergillosis in neutropenic patients. M.D. Thesis, University of London. (1988).

Barnes, R.A. and Rogers, T.R. Response rates to a staged antibiotic regimen in febrile neutropenic patients. J. Antimicrob. Chemother. 22: 759-763. (1988).

Barreto-Bergter, E.M. and Travassos, L.R. Chemical structure of the D-galacto-D-mannan component from hyphae of Aspergillus niger and other Aspergillus spp. Carb. Res. 86: 273-285. (1981).

Bennett, J.E., Bhattacharjee, A.K. and Glaudemans, C.P.J. Galactofuranosyl groups are immunodominant in Aspergillus fumigatus galactomannan. Mol. Immunol. 22: 251-254. (1985).

Bennett, J.E., Freidman, M.M. and Dupont, B. Receptor mediated clearance of Aspergillus galactomannan. J. Infect. Dis. 155: 1005-1010. (1987).

Bennett, J.H. On the parasitic vegetable structure found growing in living animals. Trans. R. Soc. Edinburgh 15: 277. (1844).

Benoit, G., Feuilhade De Chauvin, M., Cardonnier, C., Astier, A. and Bernaudin, J.F. Oxalic acid level in bronchoalveolar lavage fluid from patients with invasive pulmonary aspergillosis. Am. Rev. Resp. Dis. 132: 748-751. (1985).

Boogaerts, M.A., Verhoef, G.E., Zachee, P., Demuynck, H., Verbist, L. and De Beule, K. Antifungal prophylaxis with itraconazole in prolonged neutropenia: correlation with plasma levels. Mycoses 32: Suppl 1: 103-108. (1989).

Brouwer, J. Detection of antibodies against Aspergillus fumigatus: Comparison between double immunodiffusion, ELISA and immunoblot analysis. Int. Arch. Allergy Appl. Immunol. 85: 244-249. (1988).

Brummond, W., Resnick, A., Fink, J.N. and Kurup, V.P. Aspergillus fumigatus specific antibodies in allergic bronchopulmonary aspergillosis and aspergilloma: evidence for a polyclonal antibody response. J. Clin. Microbiol. 25: 5-9. (1987).

Burch, P.A., Karp, J.E., Merz, W.G., Kuhlman, J.E. and Fishman, E.K. Favourable outcome of invasive aspergillosis in patients with acute leukaemia. J. Clin. Oncol. 5: 1985-1993. (1987).

Burton, J.R., Zachery, J.B., Bessin, R., Rathbun, H.K., Greenough, W.B., Sterioff, S., Wright, J.R., Slavin, R.E. and Williams, G.M. Aspergillosis in four renal transplant recipients; diagnosis and effective treatment with amphotericin B. Ann. Int. Med. 77: 383-388. (1972).

Calvo, M.A., Abarca, L., Bruguera, T., Casanes, J. and Calvo, R.M. Contribution to the study of Aspergillus fumigatus Fresenius. Mycopathologia 89: 49-50. (1985).

Calvanico, N.J., Du Pont, B.L., Huang, C.J., Patterson, R., Fink, J.N. and Kurup, V.P. Antigens of Aspergillus fumigatus. I. Purification of a cytoplasmic antigen reactive with sera of patients with Aspergillus related disease. Clin. Exp. Immunol. 45: 662-671. (1981).

Cherniak, R., Reiss, E., Slodki, M.E., Plattner, R.D. and Blumer, S.O. Structure and antigenic activity of the capsular polysaccharide of Cryptococcus neoformans serotype A. Mol. Immunol. 17: 1025-1032. (1980).

Cohen, M.S., Isturiz, R.E., Malech, H.L., Root, R.K., Wilfert, C.M., Gutman, L. and Buckley, R.H. Fungal infection in chronic granulomatous disease. The importance of the phagocyte in defence against fungi. Am. J. Med. 71: 59-66. (1981).

Cole, G.T., Sun, S.H. and Huppert, M. Isolation and ultrastructural examination of conidial wall components of Coccidioides and Aspergillus. Scan. Elec. Micro. IV: 1677. (1982).

Conneally, E., Cafferkey, M.T., Daly, P.A., Keane, C.T. and McCann, S.R. Nebulized amphotericin B as prophylaxis against invasive aspergillosis in granulocytopenic patients. Bone Marr. Transpl. 5: 403-406. (1990).

Corbel, M.J. and Eades, S.M. Examination of the effect of age and acquired immunity on the susceptibility of mice to infection with Aspergillus fumigatus. Mycopathologia 60: 79-85. (1977).

Debeaupuis, J.P., Sarfati, J., Goris, A., Stynen, D., Diaquin, M. and Latge, J.P. Exocellular polysaccharides from Aspergillus fumigatus and related taxa. In R.A. Samson and J.I. Pitt (eds.), Modern concepts in Penicillium and Aspergillus classification. Plenum Publishing Corp., London. (1989).

DeGregorio, M.W., Lee, M.W.F., Linker, C.A., Jacobs, R.A. and Ries, C.A. Fungal infections in patients with acute leukaemia. Am. J. Med. 73: 543-548. (1982).

Dewair, M. and Baur, X. RAST for measurement of IgG antibodies to A.fumigatus in sera of patients with different lung diseases. J. Immunol. Meths. 75: 117-128. (1984).

Diamond, R.D., Krzesicki, R., Epstein, B. and Wellington, J. Damage to hyphal forms of fungi by human leukocytes "in-vitro". A possible host defence mechanism in aspergillosis and mucormycosis. Am. J. Pathol. 91: 313-323. (1978).

Diamond, R.D. and Clark, R.A. Damage to Aspergillus fumigatus and Rhizopus oryzae hyphae by oxidative and non-oxidative microbiocidal products of human neutrophils in vitro. Infect. Immun. 38: 487-495. (1982).

Diamond, R.D., Huber, E. and Haudenschild, C.C. Mechanisms of destruction of Aspergillus fumigatus hyphae mediated by human monocytes. J. Infect. Dis. 147: 474-483. (1983).

Dochez, A.R. and Avery, O.T. The elaboration of specific soluble substance by Pneumococcus during growth. J. Exper. Med. 26: 477. (1917).

Drutz, D.J. and Catanzaro, A. Coccidioidomycosis. State of the art. Am. Rev. Resp. Dis. 117: 727 (1978).

Dube, H.C. An introduction to fungi, p. 189-194. Vikas Publishing House Pvt. Ltd., New Delhi, India. (1983).

Dupont, B., Huber, M., Kim, S.J. and Bennett, J.E. Galactomannan antigenaemia and antigenuria in aspergillosis: Studies in patients and experimentally infected rabbits. J. Infect. Dis. 155: 1-11. (1987).

Dupont, B., Improvisi, L. and Prevot, F. Detection de galactomannane dans les aspergilloses invasives humaines avec un test au latex. Bull. Soc. Fr. Mycol. Med. 19:35-41. (1990).

Fietta, A., Sacchi, F., Mangiarotti, P., Manara, G. and Grassi, G.G. Defective phagocyte Aspergillus killing associated with recurrent pulmonary Aspergillus infection. Infection 12: 10-13. (1984).

Fisher, B.D., Armstrong, D., Yu, B. and Gold, J.W.M. Invasive aspergillosis. Progress in early diagnosis and treatment. Am. J. Med. 71: 571-577. (1981).

Fraser, D.W., Ward, J.I., Ajello, L. and Plikaytis, B.D. Aspergillosis and other systemic mycoses. The growing problem. J.A.M.A. 242: 1631-1635. (1979).

Frisvad, J.C. High performance liquid chromatographic determination of profiles of mycotoxins and other secondary metabolites. J. Chromatography 392: 333-347. (1987).

Froudist, J.H., Harnett, G.B. and McAleer, R. Comaprison of immunodiffusion and enzyme linked immunosorbent assay for antibodies to four Aspergillus species. J. Clin. Pathol. 42: 1215-1221. (1989).

Fujita, S.I., Matsubara, F. and Matsuda, T. Demonstration of antigenaemia in patients with invasive aspergillosis by biotin-streptavidin enzyme-linked immunosorbent assay. J. Lab. Clin. Med. 112: 464-470. (1988).

Galgiani, J.N., Dugger, K.O., Ito, J.I. and Weiden, M.A. Antigenaemia in primary coccidioidomycosis. Am. J. Trop. Med. Hyg. 33: 645-649. (1984).

Gerber, J.D. and Jones, R.D. Immunologic significance of aspergillin antigens of six species of Aspergillus in the serodiganosis of aspergillosis. Am. Rev. Resp. Dis. 108: 1124-1129. (1973).

Gerson, S.L., Talbot, G.H., Hurwitz, S., Strom, B.L., Lusk, E.J. and Cassileth, P.A. Prolonged granulocytopenia: The major risk factor for invasive pulmonary aspergillosis in patients with acute leukaemia. Ann. Int. Med. 100: 345-351. (1984).

Gerson, S.L., Talbot, G.H., Lusk, E., Hurwitz, S., Strom, B.L. and Cassileth, P.A. Invasive pulmonary aspergillosis in adult leukaemia: clinical clues to its diagnosis. J. Clin. Oncol. 3: 1109-1116. (1985).

Glimp, R.A. and Bayer, A.S. Pulmonary aspergilloma: diagnostic and therapeutic considerations. Arch. Inter. Med. 143: 303-308. (1983).

Gold, J.W.M., Fisher, B., Yu, B., Chein, N. and Armstrong, D. Diagnosis of invasive aspergillosis by passive hemagglutination assay of antibody. J. Infect. Dis. 142: 87-94. (1980).

Gordon, M.A., Lapa, E.W. and Kane, J. Modified indirect fluorescent-antibody test for aspergillosis. J. Clin. Microbiol. 6: 161-165. (1977).

Greenberger, P.A. and Patterson, R. Application of enzyme-linked immunosorbent assay (ELISA) in diagnosis of allergic bronchopulmonary aspergillosis. J. Lab. Clin. Med. 99; 288-293. (1982).

Greenberger, P.A. Aspergillosis - clinical aspects. Zbl. Bakt. Hyg. A 261: 487-495. (1986).

Harvey, C. and Longbottom, J.L. Characterization of a major antigenic component of Aspergillus fumigatus. Clin. Exp. Immunol. 65: 206-214. (1986a).

Harvey, C. and Longbottom, J.L. Development of a sandwich ELISA to detect IgG and IgG sub-class antibodies specific for a major antigen (Ag 7) of Aspergillus fumigatus. Clin. Allergy 16: 323-330. (1986b).

Harvey, C. and Longbottom, J.L. Characterization of a second major antigen Ag 13 (antigen C) of Aspergillus fumigatus and investigation of its immunological reactivity. Clin. Exp. Immunol. 70; 247-254. (1987).

Hearn, V.M. and Mackenzie, D.W.R. The preperation and chemical composition of fractions from Aspergillus fumigatus wall and protoplasts possessing antigenic activity. J. Gen. Microbiol. 112: 35-44. (1979).

Hearn V.M. and Mackenzie, D.W.R. Mycelial antigens from two strains of Aspergillus fumigatus: An analysis by two-dimensional immunoelectrophoresis. Mykosen 23: 549-562. (1980).

Hearn, V.M., Wilson, E.V., Prctor, A.G. and Mackenzie, D.W.R. Preperation of Aspergillus fumigatus antigens and their analysis by two-dimensional immunoelectrophoresis. J. Med. Microbiol. 13: 451-458. (1980a).

Hearn, V.M., Proctor, A.G. and Mackenzie, D.W.R. The preperation and partial characterization of antigenic fractions obtained from the mycelial walls of several Aspergillus species. J. Gen. Microbiol. 119: 41-49. (1980b).

Hearn V.M. and Mackenzie, D.W.R. Analysis of wall antigens of Aspergillus fumigatus by two-dimensional immunoelectrophoresis. J. Med. Microbiol. 14: 119-129. (1981).

Hearn, V.M., Donaldson, G.C. and Healy, M.J.R. A method to determine significant levels of Immunoglobulin G to Aspergillus fumigatus antigens in an ELISA system and a comparison with counterimmunoelectrophoresis and double diffusion techniques. J. Immunoassay 6: 165-187. (1985).

Hearn, V.M. Serodiagnosis of aspergillosis. In H., van den Bossche, D.W.R., Mackenzie and G., Cauwenbergh (eds). Aspergillus and aspergillosis. Plenum Press, New York. (1988).

Hearn, V.M., Griffiths, B.C. and Gorin, P.A.J. Structural analysis of water soluble fractions obtained from Aspergillus fumigatus mycelium. Glycoconjugate J. 6:85-100. (1990).

Herbert, P.A. and Bayer, A.S. Fungal pneumonia (part 4). Invasive pulmonary aspergillosis. Chest 80: 220-225. (1981).

Holmberg, K., Berdischewsky, M. and Young, L.S. Serologic immunodiagnosis of invasive aspergillosis. J. Infect. Dis. 141: 656-664. (1980).

Huskinson, J., Stepick-Biek, P. and Remington, J.S. Detection of antigens in urine during acute toxoplasmosis. J. Clin. Microbiol. 27: 1099-1101. (1989).

Hyde, F.W., Johnson, R.C., White, T.J. and Shelburne, C.E. Detection of antigens in urine of mice and humans infected with Borrelia burgdorferi, etiologic agent of Lyme disease. J. Clin. Microbiol. 27: 58-61. (1989).

Johnson, T.M., Kurup, V.P., Resnick, A., Ash, R.C., Fink, J.N. and Kalbfleisch, J. Detection of circulating Aspergillus fumigatus antigen in bone marrow transplant patients. J. Lab. Clin. Med. 114: 700-707. (1989).

Johnston, R.B. and Baehner, R.L. Chronic granulomatous disease. Correlation between pathogenesis and clinical findings. Paediatrics 48: 730. (1971).

Jonsson, A.G. and Martin, S.M. Protease production by Aspergillus fumigatus. Agric. Biol. Chem. 28: 734-739. (1964).

Kahn, F.W. and Jones J.M. The role of bronchoalveolar lavage in the diagnosis of invasive pulmonary aspergillosis. Am. J. Clin. Pathol. 86: 519-523. (1986a).

Kahn, F.W. and Jones, J.M. Latex agglutination test for the detection of Candida antigens in sera of patients with invasive candidiasis. J. Infect. Dis. 153: 579-585. (1986b).

Kan, V.L. and Bennett, J.E. Lectin-like attachment sites on murine pulmonary alveolar macrophages bind Aspergillus fumigatus conidia. J. Infect. Dis. 158: 407-414. (1988).

Karam, G.H. and Griffin, F.M. Invasive pulmonary aspergillosis in nonimmunocompromised nonneutropenic hosts. Rev. Infect. Dis. 8: 357-363. (1986).

Kauffmann, H.F. and de Vries, K. Antibodies against Aspergillus fumigatus. I. Standardization of the antigenic composition. Int. Archs Allergy Appl. Immunol. 62: 252-264. (1980a).

Kauffmann, H.F. and de Vries, K. Antibodies against Aspergillus fumigatus II. Identification and quantification by means of crossed immunoelectrophoresis. Int. Archs. Allergy Appl. Immunol. 62: 265-275. (1980b).

Kaufman, L. and Blumer, S. Diagnostic value of the cryptococcal antigen test. J. Infect. Dis. 147: 173-177. (1983).

Kauffmann, H.F., Beaumont, F., Meurs, H., van der Heide, S. and de Vries, K. Comparison of antibody measurements against Aspergillus fumigatus by means of double diffusion and enzyme-linked immunosorbent assay (ELISA). J. Allergy Clin. Immunol. 72: 255-261. (1983).

Kauffmann, H.F., van der Heide, S., van der Laan, S., Hovenga, H., Beaumont, F. and de Vries, K. Standardization of allergenic extracts of Aspergillus fumigatus. Liberation of IgE binding components during cultivation. Int. Archs. Allergy Appl. Immunol. 76: 168-173. (1985).

Kauffmann, H.F., van der Heide, S., Beaumont, F., Blok, H. and de Vries, K. Class specific antibody determination against Aspergillus fumigatus by means of the enzyme-linked immunosorbent assay III. Comparative study: IgG, IgA, IgM, ELISA titres, precipitating antibodies and IgE binding after fractionation of the antigen. Int. Archs. Allergy Appl. Immunol. 80: 300-306. (1986).

Kenny, G.E., Principles, problems and strategies in the use of antigenic mixtures for the enzyme-linked immunosorbent assay. J. Clin. Microbiol. 17: 655-665. (1983).

Khan, Z.U., Richardson, M.D. and Warnock, D.W. Evaluation of a rapid enzyme-linked immunosorbent assay for IgG antibodies to Aspergillus fumigatus in the serological diagnosis of allergic aspergillosis. Int. Archs. Allergy Appl. Immunol. 73: 205-211. (1984a).

Khan, Z.U., Richardson, M.D., Crutcher D.L. and Warnock D.W. Use of Aspergillus fumigatus culture filtrate fractions in ELISA for the serological diagnosis of allergic aspergillosis. Mykosen 27: 327-339. (1984b).

Kim, S.J. and Chaparas S.D. Characteization of antigens from Aspergillus fumigatus I. Preperation of antigens from organisms grown in completely synthetic medium. Am. Rev. Resp. Dis. 118: 547-551. (1978).

Kim, S.J., Chaparas, S.D., Brown, T.M. and Anderson, M.C. Characterization of antigens from Aspergillus fumigatus II. Fractionation and electrophoretic, immunologic and biologic activity. Am. Rev. Resp. Dis. 118: 553-560. (1978).

Kim, S.J. and Chaparas, S.D. Characterization of antigens from Aspergillus fumigatus. III. Comparison of antigenic relationships of clinically important aspergilli. Am. Rev. Resp. Dis. 120: 1297-1303. (1979).

Kim, S.J., Chaparas, S.D. and Buckley, H.R. Characterization of antigens from Aspergillus fumigatus. IV. Evaluation of commercial and experimental preperations and fractions in the detection of antibody in aspergillosis. Am. Rev. Resp. Dis. 120: 1305-1311. (1979).

Kothary, M.H., Chase, T. Jnr. and Macmillan, J.D. Correlation of elastase production by some strains of Aspergillus fumigatus with ability to cause pulmonary invasive aspergillosis in mice. Infect. Immun. 43: 320-325. (1984).

Kricka, L.J., Carter, T.J., Burt, S.M., Kennedy, J.H., Holder, R.L., Halliday, M.I., Telford, M.E. and Wisdom, G.B. Variability in the absorption properties of microtitre plates used as solid supports in enzyme immunoassay. Clin. Chem. 26: 741-744. (1980).

Kuhlman, J.E., Fishman, E.K., Burch, P.A., Karp, J.E. and Zerhouni, E.A. Invasive pulmonary aspergillosis in acute leukaemia. The contribution of CT to early diagnosis and aggressive management. Chest 92: 95-99. (1987).

Kurup, V.P., Fink, J.N., Scribner, G.H. and Falk, M.J. Antigenic variability of Aspergillus fumigatus strains. Microbios 19: 191-204. (1977).

Kurup. V.P., Ting, E.Y. and Fink, J.N. Immunochemical characterization of Aspergillus fumigatus antigens. Infect. Immun. 41: 698-701. (1983).

Kurup. V.P. Interaction of A.fumigatus spores with pulmonary alveolar macrophages of rabbits. Immunobiology 166: 53-61. (1984).

Kurup. V.P. Enzyme-linked immunosorbent assay in the detection of specific antibodies against Aspergillus in patient sera. Zbl. Bakt. Hyg. A. 261: 509-516. (1986).

Kurup, V.P., Resnick, A., Scribner, G.H., Gunasekaran, M. and Fink, J.N. Enzyme profile and immunochemical characterization of Aspergillus fumigatus antigens. J. Allergy Clin. Immunol. 78: 1166-1173. (1986a).

Kurup. V.P. John, K.V., Resnick, A. and Fink, J.N. A partially purified glycoprotein antigen from Aspergillus fumigatus. Int. Archs. Allergy Appl. Immunol. 79: 263-269. (1986b).

Kurup, V.P., Greenberger, P.A. and Fink, J.N. Antibody response to low molecular weight antigens of Aspergillus fumigatus in allergic bronchopulmonary aspergillosis. J. Clin. Microbiol. 27: 1312-1316. (1989).

Kwon-Chung, K.J. Aspergillus: Diagnosis and description of the genus. In H., Vanden Bossche, D.W.R., Mackenzie, and G., Cauwenbergh (eds). Aspergillus and Aspergillosis. Plenum Press, New York. (1988).

Laemmli, U.K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680-685. (1970).

Lehmann, P.F. and White, L.O. Acquired immunity to Aspergillus fumigatus. Infect. Immun. 13: 1296-1298. (1976).

Lehmann, P.F. and Reiss, E. Invasive aspergillosis: antiserum for circulating antigen produced after immunization with serum from infected rabbits. Infect. Immun. 20: 570-572. (1978).

Lehrer, R.I. and Jan, R.G. Interaction of Aspergillus fumigatus spores with human leukocytes and serum. Infect. Immun. 1: 345-350. (1970).

LePape, P. and Deunff, J. Detection of Aspergillus glycoprotein antigen in sera of patients with invasive aspergillosis by an enyme-linked immunosorbent assay inhibition technique. In E., Drouhet, G.T., Cole, L., de Repentigny, J.P., Latge and B., Dupont, (eds.). Fungal Antigens. Isolation, purification and detection. Plenum Publishing Corp., New York. (1986).

LePape, P. and Deunff, J. Antigene glycoproteique circulant d'Aspergillus fumigatus detection dans le serum de souris par une technique ELISA. Bull. Soc. Fr. Mycol. Med. 16: 169-172. (1987).

Leung, P.S.C., Gershwin, M.E., Coppel, R., Halpern, G., Novey, H. and Castles, J.J. Localization, molecular weight and immunoglobulin subclass response to Aspergillus fumigatus allergens in acute bronchopulmonary aspergillosis. Int. Archs. Allergy Appl. Immunol. 85: 416-421. (1988).

Levitz, S.M. and Diamond, R.D. Killing of Aspergillus fumigatus and Candida albicans yeast phase by iron-hydrogen peroxide-iodide cytotoxic system: comparison with the myeloperoxidase-hydrogen peroxide-halide system. Infect. Immun. 43: 1100-1102. (1984).

Levitz, S.M. and Diamond, R.D. Mechanisms of resisteance of Aspergillus fumigatus conidia to killing by neutrophils "in-vitro". J. Infect. Dis. 152: 33-42. (1985).

Lew, A.M. The effect of epitope density and antibody affinity on the ELISA as analysed by monoclonal antibodies. J. Immunol. Meths. 72: 171-176. (1984).

Libansky, J. The investigation of the cellular type of immunity in patients with lymphoproliferative and myeloproliferative diseases. Int. J. Cancer 4: 288-294. (1969).

Longbottom, J.L. and Pepys, J. Pulmonary aspergillosis: diagnostic and immunologic significance of antigens and C-substance in Aspergillus fumigatus. J. Path. Bact. 88: 141-152. (1964).

Longbottom, J.L. Allergens of Aspergillus fumigatus: Identification and characterization of components combining with specific IgE antibody. Zbl. Bakt. Hyg. A 261: 503-508. (1986a).

Longbottom, J.L. Antigens and allergens of Aspergillus fumigatus, II. Their further identification and partial characterization of a major allergen (Ag 3). J. Allergy Clin. Immunol. 78: 18-24. (1986b).

de Magaldi, S.W. and Mackenzie, D.W.R. Specificity of antigens from pathogenic Aspergillus species. I. Studies with ELISA and immunofluoresence. Sabouraudia 22: 381-394. (1984).

Malo, J.L., Longbottom, J., Mitchell, J., Hawkins, R. and Pepys, J. Studies in chronic allergic bronchopulmonary aspergillosis. 3. Immunological findings. Thorax 32: 269-274. (1977).

Marier, R., Smith, W., Jansen, M. and Andriole, V.T. A solid-phase radioimmunoassay for the measurement of antibody to Aspergillus in invasive aspergillosis. J. Infect. Dis. 140: 771-779. (1979).

Matthews, R., Burnie, J.P., Fox, A. and Tabaqchali, S. Immunoblot analysis of serological responses in invasive aspergillosis. J. Clin. Pathol. 38: 1300-1303. (1985).

Matthews, R.C., Burnie, J.P. and Tabaqchali, S. Isolation of immunodominant antigens from sera of patients with systemic candidiasis and characterization of serological response to Candida albicans. J. Clin. Microbiol. 25: 230-237. (1987).

Matthews, R. and Burnie J.P. Diagnosis of systemic candidiasis by an enzyme-linked dot immunobinding assay for a circulating immunodominant 47-kilodalton antigen. J. Clin. Microbiol. 26: 459-463. (1988).

McKinney, M. and Parkinson, A. A simple, non-chromatographic procedure to purify immunoglobulins from serum and ascites fluid. J. Immunol. Meths. 96: 271-278. (1987).

Merkow, L.P., Pardo, M., Epstein, S.M., Verney, E. and Sidransky, H. Lysosomal stability during phagocytosis of Aspergillus flavus spores by alveolar macrophages of cortisone treated mice. Science 160: 79-81. (1968).

Merkow, L.P., Epstein, S.M., Sidransky, H., Verney, E. and Pardo, M. The pathogenesis of experimental and pulmonary aspergillosis: An ultrastructural study of alveolar macrophages after phagocytosis of A. flavus spores in vivo. Am. J. Pathol. 62: 57-74. (1971).

Meunier, F. Prevention of mycoses in immunocompromised patients. Rev. Infect. Dis. 9: 408-416. (1987).

Meyer, R.D., Young, L.S., Armstrong, D. and Yu, B. Aspergillosis complicating neoplastic disease. Am. J. Med. 54: 6-15. (1973).

Micheli, P.A. "Nova Plantarum Genera" Florentiae. (1729).
Mishra, S.K., Falkenberg, S. and Masihi, K.N. Efficacy of enzyme-linked immunosorbent assay in serodiagnosis of aspergillosis. J. Clin. Microbiol. 17: 708-710. (1983).

Mishra, S.K. Antigenic profile of some typical and septate phialide-strains of Aspergillus fumigatus. Sabouraudia 22: 91-100. (1984).

Miyaji, M. and Nishimura, K. Relationship between proteolytic activity of Aspergillus fumigatus and invasiveness of mouse brain. Mycopathologica 62: 161-166. (1979).

Mohan, S.B., Warren, R.C. and Richardson, M.D. Sero-diagnosis of invasive aspergillosis: attempts to determine antigen and antibody relevance to infection. Mycopathologia 70: 37-41. (1980).

Mullbacher, A., Waring, P. and Eichner, R.D. Identification of an agent in cultures of Aspergillus fumigatus displaying anti-phagocytic and immunomodulating activity in vitro. J. Gen. Microbiol. 131: 1251-1258. 1985).

Myers, J.D. and Atkinson, K. Infection in bone marrow transplantation. Clin. Haematol. 12: 791-811. (1983).

Neill, J.M., Sugg, J.Y. and McCauley, D.W. Serologically reactive material in spinal fluid, blood and urine from a human case of cryptococcosis (torulosis). Proc. Soc. Exp. Biol. Med. 77: 775-778. (1951).

Noble, W.C. and Clayton, Y.M. Fungi in the air of hospital wards. J. Gen. Microbiol. 32: 397-402. (1963).

Pagani, A., Spalla, R., Ferrari, F.A., Duse, M., Lenzi, L., Bretz, U., Baggiolini, M. and Siccardi, A.G. Defective Aspergillus killing by neutrophil leucocytes in a case of systemic aspergillosis. Clin. Exp. Immunol. 43: 201-207. (1981).

Patterson, R., Wang, J.L.F., Roberts, M. and Zeiss, C.R. Comparison of radioimmunoassay techniques in the detection of IgE and IgG antibody activity against Aspergillus fumigatus antigens. J. Immunol. 120: 66-71. (1978).

Patterson, T.F., Miniter, P., Ryan, J.L. and Andriole, V.T. Effect of immunosuppression and amphotericin B on Aspergillus antigenaemia in an experimental model. J. Infect. Dis. 158: 415-422. (1988).

Phillips, P. and Radigan, G. Antigenaemia in a rabbit model of invasive aspergillosis. J. Infect. Dis. 159: 1147-1150. (1989).

Piechura, J.E., Huang, C., Cohen, S.H., Kidd, J.M., Kurup, V.P. and Calvanico, N.J. Antigens of Aspergillus fumigatus II. Electrophoretic and clinical stuidies. Immunology 49: 657-665. (1983).

Piechura, J.E., Kurup, V.P., Fink, J.N. and Calvanico, N.J. Antigens of Aspergillus fumigatus III. Comparative immunochemical analyses of clinically relevant aspergilli and related fungal taxa. Clin. Exp. Immunol. 59: 716-724. (1985).

Pirsch, J.D. and Maki, D.G. Infectious complications in adults with bone marrow transplants and T-cell depletion of donor marrow. Ann. Intern. Med. 104: 619-631. (1986).

Prentice, A.G. and Bradford, G.R. Prophylaxis of fungal infections with itraconazole during remission-induction therapy. Mycoses 32: Suppl 1: 96-102. (1989).

Rapper, K.B. and Fennell, D.I. "The genus Aspergillus". Williams and Wilkins, Baltimore, USA. (1965).

Reed, C. Variability of antigenicity of Aspergillus fumigatus. J. Allergy Clin. Immunol. 61: 227-229. (1978).

Reiss, E. and Lehmann, P.F. Galactomannan antigenaemia in invasive aspergillosis. Infect. Immun. 25: 357-365. (1979).

Reiss, E. Aspergillus fumigatus. In E., Reiss (ed). Molecular Immunology of Mycotic and Actinomycotic Infections. Elsevier, New York. (1986).

Reiss E. and Bragg, S.L. Immunochemical analysis of histoplasmin proteins and polysaccharides, p. 417-430. In E., Drouhet, G.T., Cole, L., deRepentigny, J.P., Latge and B., Dupont (eds.). Fungal antigens. Isolation, purification and detection. Plenum Publishing Corp., New York. (1986).

Reiss, E., Kuykendall, R.J. and Kaufman, L. Antigenaemia in rabbits infected with Candida albicans serotype B: detection by enzyme immunoassay and preliminary characterization of the antigen. J. Med. Vet. Mycol. 24: 259-269. (1986a)

Reiss, E., Knowles, J.B., Bragg, S.L. and Kaufman, L. Monoclonal antibodies against the M-protein and carbohydrate antigens of histoplasmin characterised by the enzyme-linked immunoelectrotransfer blot. Infect. Immun. 53: 540-546. (1986b).

de Repentigny, L., Boushira, M., Ste-Marie, L. and Bosisio, G. Detection of galactomannan antigenaemia by enzyme immunoassay in experimental invasive aspergillosis. J. Clin. Microbiol. 25: 863-867. (1987).

Richards, M. Atmospheric mould spores in and out of doors. J. Allergy 25: 429. (1954).

Richardson, M.D., White, L.O. and Warren R.C. Detection of circulating antigen of Aspergillus fumigatus in sera of mice and rabbits by enzyme-linked immunosorbent assay. Mycopathologia 67: 83-88. (1979).

Richardson, M.D., Stubbins, J.M. and Warnock, D.W. Rapid enzyme-linked immunosorbent assay (ELISA) for Aspergillus fumigatus antibodies. J. Clin. Pathol. 35: 1134-1137. (1982).

Richardson, M.D., Turner, A., Warnock, D.W. and Llewellyn, P.A. Computer assissted rapid enzyme-linked immunosorbent asssay (ELISA) in the serological diagnosis of aspergillosis. J. Immunol. Meths. 56: 201-207. (1983).

Richardson, M.D. and Warnock, D.W. Antigen and antibody attachment in ELISA for Aspergillus fumigatus IgG antibodies. J. Immunol. Methods 66: 119-132. (1984).

Richardson, M.D., Llewellyn, P.A. and Warnock, D.W. Antibody to Aspergillus fumigatus antigens in normal sera: influence on positive-negative discrimination in ELISA. J. Immunoassay 5: 205-220. (1984).

Rinaldi, M.G. Invasive aspergillosis. Rev. Infect. Dis. 5: 1061-1077. (1983).

Robertson, M.D., Seaton, A., Milne, L.J.R. and Raeburn, J.A. Suppression of host defences by Aspergillus fumigatus. Thorax 42: 19-25. (1987a).

Robertson, M.D., Seaton, A., Milne, L.J.R. and Raeburn, J.A. Resistance of spores of Aspergillus fumigatus to ingestion by phagocytic cells. Thorax 42: 466-472. (1987b).

Robertson, M.D., Seaton, A., Raeburn, J.A. and Milne, L.J.R. Inhibition of phagocyte migration and spreding by spore diffusates of Aspergillus fumigatus. J. Med. Vet. Mycol. 25: 389-396. (1987c).

Sabetta, J.R., Minter, P. and Andriole, V.T. The diagnosis of invasive aspergillosis by an enzyme-linked immunosorbent assay for circulating antigen. J. Infect. Dis. 152: 946-953. (1985).

Sakaguchi, O., Suzuki, M. and Yokota, K. Effect of partial acid hydrolysis on precipitin activity of Aspergillus fumigatus galctomannan. Jap. J. Microbiol. 12: 123-124. (1968).

Sakaguchi, O., Yokota, K. and Suzuki, M. Immunochemical and biochemical studies on fungi XIII. On the galactomannan isolated from mycelia and culture filtrates of several filamentous fungi. Jap. J. Microbiol. 13: 1-9. (1969).

Sandhu, D.K., Sandhu, R.S., Damodaran, V.N. and Randhawa, H.S. Effect of cortisone on bronchopulmonary aspergillosis in mice exposed to spores of various Aspergillus species. Sabouraudia 8: 32-38. (1970).

Sathapatayavongs, B., Batteiger, B.E., Wheat, J., Slama, T.G. and Wass, J.L. Clinical and laboratory features of disseminated histoplasmosis during two large urban outbreaks. Medicine 62: 263-270. (1983).

Schaefer, J.C., Yu, B. and Armstrong, D. An Aspergillus immunodiffusion test in the early diagnosis of aspergillosis in adult leukeamia patients. Am. Rev. Resp. Dis. 113: 325-329. (1976).

Schaffner, A., Douglas, H. and Braude, A.I. Selective protection against conidia by mononuclear and against mycelia by polymorphonuclear phagocytes in resistance to Aspergillus. J. Clin. Invest. 69: 617-631. (1982).

Schaffner, A., Douglas, H., Braude, A.I. and Davis, C.E. Killing of Aspergillus spores depends on the anatomical source of the macrophage. Infect. Immun. 42: 1109-1115. (1983).

Schaffner, A. Acquired immune deficiency syndrome: Is disseminated aspergillosis predictive of underlying cellular immune deficiency. J. Infect. Dis. 149: 828-829. (1984).

Schaffner, A., Davis, C.E., Schaffner, T., Markert, M., Douglas, H. and Braude, A.I. In vitro susceptibility of fungi to killing by neutrohil granulocytes discriminates between primary pathogenicity and oppurtunism. J. Clin. Invest. 78: 511-524. (1986).

Schonheyder, H. and Andersen, P. An indirect immunofluoresence study of antibodies to Aspergillus fumigatus in sera from children and adults without aspergillosis. Sabouraudia 20: 41-50. (1982).

Schonheyder, H., Andersen, P. and Stenderup, A. Serum antibodies to Aspergillus fumigatus in patients with pulmonary aspergillosis detected by immunfluoeresence. Acta Pathol. Microbiol. Immunol. Scand. Sect. B 90: 273-279. (1982).

Schonheyder, H. and Andersen, P. Determination of antibodies to partially purified Aspergillus antigens by an enzyme-linked immunosorbent assay. Int. Archs. Allergy Appl. Immunol. 70: 108-111. (1983).

Schonheyder, H. and Andersen, P. Fractionation of Aspergillus fumigatus antigens by hydrophobic interaction chromatography and gel filtration. Int. Archs. Allergy Appl. Immunol. 73: 231-236. (1984a).

Schonheyder, H. and Andersen, P. IgG antibodies to purified Aspergillus fumigatus antigens determined by enzyme-linked immunosorbent assay. Int Archs. Allergy Appl. Immunol. 74: 262-269. (1984b).

Schonheyder, H., Storgaard, L. and Andersen, P. Variation of a 470,000 daltons antigen complex and catalase antigen in clinical isolates of Aspergillus fumigatus. Sabouraudia 23: 339-349. (1985).

Sepulveda, R. Longbottom, J.L. and Pepys, J. Enzyme-linked immunosorbent assay (ELISA) for IgG and IgE antibodies to protein and polysaccharide antigens of Aspergillus fumigatus. Clin. Allergy 9: 359-371. (1979).

Shaffer, P.J., Medoff, G. and Kobayashi, G.S. Demonstration of antigenaemia by radioimmunoassay in rabbits experimentally infected with Aspergillus. J. Infect. Dis. 139: 313-319.(1979a).

Shaffer, P.J., Kobayashi, G.S. and Medoff, G. Demonstration of antigenaemia in patients with invasive aspergillosis by solid phase (Protein A-rich Staphylococcus aureus) radioimmunoassay. Am. J. Med. 67: 627-630. (1979b).

Shale, D.J. and Faux, J.A. The evaluation of a quantitative enzyme-linked immunosorbent assay (ELISA) for anti-Aspergillus fumigatus IgG. J. Immunol. Meths. 77: 197-205. (1985).

Slavin, R.G., Hutcheson, P.S. and Knutsen, A.P. Participation of cell-mediated immunity in allergic bronchopulmonary aspergillosis. Int. Archs. Allergy Appl. Immun. 83: 337-340. (1987).

Slutyer, T. De vegetabilibus organismi animalis parasites ac de novo epiphyto in pityriasi versicolore obvio. Dissertation, Berlin. (1847).

Spector, T. Refinement of the coomassie blue method of protein quantification. Anal. Biochem. 86: 142-146. (1978).

Staib, F. Vorsclaege zur Bekaempfung aerogener tiefer Mykosen bei immungeschwaechten Personen. Bundesqesundheitsblatt 28: 132. (1985).

Stix, E. Pilzsporengehalt der luft in Meunchen. Meunch. Med. Wschr. 119: 79. (1977).

Suzuki, S., Suzuki, M., Yokota, K., Sunayama, H. and Sakaguchi, O. On the immunochemical and biochemical studies of fungi. XI. Cross reaction of the polysaccharides of Aspergillus fumigatus, Candida albicans, Saccharomyces cerivissiae and Tricophyton rubrum against Candida albicans and Saccharomyces cerivissiae antisera. Jap. J. Microbiol. 11: 269-273. (1967).

Szoka, F.C., Millholland, D. and Barza, M. Effect of lipid composition and liposome size on toxicity and in vitro fungicidal activity on liposome-intercalated amphotericin B. Antimicrob. Agents Chemother. 31: 421-429. (1987).

Talbot, G.H., Weiner, M.H., Gerson, S.L., Provencher, M. and Hurwitz, S. Serodiagnosis of invasive aspergillosis in patients with hematologic malignancy: Validation of the Aspergillus fumigatus antigen radioimmunoassay. J. Infect. Dis. 155: 12-27. (1987).

Taylor, M.L. and Longbottom J.L. Partial charcterization of a rapidly released antigenic/allergenic component (Ag5) of Aspergillus fumigatus. J. Allergy Clin. Immunol. 81: 548-557. (1988).

Tee, R.D., Gordon, D.J. and Newman Taylor, A.J. Cross-reactivity between antigens of fungal extracts studied by RAST inhibition and immunoblot technique. J. Allergy Clin. Immunol. 79: 627-633. (1987).

Tilden, E.B., Hatten, E.H., Freeman, S., Williamson, W.M. and Koenig, V.L. Preparattion and properties of the endotoxins of Aspergillus fumigatus and A.flavus. Mycopathologica 14: 325-346. (1961).

Towbin, H., Staehelin, T. and Gordon, J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. Proc. Natl. Acad. Sci. U.S.A. 76: 4350-4354. (1979).

Tran Van Ky, P., Biguet, J. and Fruit, J. Loaclisation et frequence des arcs des immunoelectrophoregrammes produits par le serum des malades atteints de mycetomes aspergillaires applique contre l'antigene Aspergillus fumigatus. Rev. Immunol. 30: 13-20. (1966).

Tran Van Ky, P., Biguet, J. and Vaucelle, T. Etude d'une fraction antigenique d'Aspergillus fumigatus support d'une activite catalasique. Consequence sur le diagnostic immunologique de l'aspergillose. Rev. Immunol. 32: 37-52. (1968).

Tricot, G., Joosten, E., Boogaerts, M.A., van de Pitte, J. and Cauwenberg, G. Ketoconazole versus itraconazole for antifungal prophylaxis in patients with severe granulocytopenia: preliminary results of two nonreandomized studies. Rev. Infect. Dis. 9: Suppl: 594-599. (1987).

Trull, A., Parker, J. and Warren, R.E. IgG enzyme-linked immunosorbent assay for diagnosis of invasive aspergillosis: retrospective study over 15 years of transplant recipients. J. Clin. Pathol. 38: 1045-1051. (1985).

van Cutsem, J., Meulemans, L., Van Gerven, F. and Stynen, D. Detection of circulating galactomannan by Pastorex Aspergillus in experimental invasive aspergillosis. Mycoses 33: 61-69. (1990).

van der Heide, S., Kauffmann, H.F. and de Vries, K. Measurement of specific IgE and IgG antibodies against Aspergillus fumigatus antigen in patient sera by use of enzyme immunoassays: Influence of different procedures of antigen immobilization. J. Allergy Clin. Immunol.73: 813-818. (1984).

van der Heide, S., Kauffmann, H.F. and de Vries, K. Cultivation of fungi in synthetic and semi-synthetic liquid medium. II. Immunochemical properties of the antigenic and allergenic extracts. Allergy 40: 592-598. (1985).

Virchow, R. Beitrage zur Lehre von den Beim Menschen vorkommenden pflanzlichen Parasiten. Arch Path. Anat. 9: 557. (1856).

Waldorf, A.R., Levitz, S.M. and Diamond, R.D. In vivo bronchoalveolar macrophage defence against Rhizopus oryzae and Aspergillus fumigatus. J. Infect. Dis. 150: 752-760. (1984).

Waldorf, A.R. and Diamond, R.D. Neutrophil chemotactic responses induced by fresh ans swollen Rhizopus oryzae spores and Aspergillus fumigatus conidia. Infect. Immun. 48: 458-463. (1985).

Wallenbeck, I. Ankrust, L. and Einarsson, R. Antigenic variability of different strains of Aspergillus fumigatus. Int. Archs. Allergy Appl. Immunol. 73: 166-172. (1984).

Warnock, D.W. Indirect immunofluorescence test for the detection of Aspergillus fumigatus antibodies. J. Clin. Pathol. 27: 911-912. (1974).

Washburn, R.G., Hammer, C.H. and Bennett, J.E. Inhibtion of complement by culture supernatants of Aspergillus fumigatus. J. Infect. Dis. 154: 944-951. (1986).

Watson, J.G. Problems of infection after bone marrow transplantation. J. Clin. Pathol. 36: 683-692. (1983).

Weiner, M.H. and Coats-Stephens, M. Immunodiagnosis of systemic aspergillosis. I. Antigenaemia detected by radioimmunoassay in experimental infection. J. Lab. Clin. Med. 93: 111-119. (1979).

Weiner, M.H. Antigenaemia detected by radioimmunoassay in systemic aspergillosis. Ann. Int. Med. 92: 793-796. (1980).

Weiner, M.H. Antigenaemia detected in human coccidioidomycosis. J. Clin. Microbiol. 18:136. (1983).

Weiner, M.H., Talbot, G.H., Gerson, S.L., Filice, G. and Cassileth, P. Antigen detection in the diagnosis of invasive aspergillosis. Utility in controlled, blinded trials. Ann. Int. Med. 99: 777-782. (1983).

Weiner, M.H., Talbot, G.H., Gerson, S.L., Fetchick, R., Andrews, C., Peacock, J.E., Filice, G., Cohen, M., Provencher, M. and Cassileth, P. Detection of fungal antigen in body fluids for diagnosis of invasive aspergillosis. Zbl. Bakt. Hyg. A 261: 517-522. (1986).

Wheat, L.J., Kohler, R.B. and Tewari, R.P. Diagnosis of disseminated histoplasmosis by detection of Histoplasma capsulatum antigen in serum and urine specimens. N. Engl. J. Med. 314: 83-88. (1986).

White, L.O., Richardson, M.D., Newham, H.C., Gibb, E. and Warren, R.C. Circulating antigen of Aspergillus fumigatus in cortisone treated mice challenged with conidia: Detection by counterimmuno-electrophoresis. FEMS Microbiol. Letts. 2: 153-156. (1977).

Williams, D.M., Weiner, M.H. and Drutz, D.J. Immunologic studies of disseminated infection with Aspergillus fumigatus in the nude mouse. J. Infect. Dis. 143: 726-733. (1981).

Wilson, E.V. and Hearn, V.M. A comparison of surface and cytoplasmic antigens of Aspergillus fumigatus in an enzyme-linked immunosorbent assay (ELISA). Mykosen 25: 653-661. (1982).

Wilson, E.V. and Hearn, V.M. Use of Aspergillus fumigatus mycelial antigens in enzyme-linked immunosorbent assay and counterimmunoelectrophoresis. J. Med. Microbiol. 16: 97-105. (1983a).

Wilson, E.V. and Hearn, V.M. Comparison of partially purified mycelial and culture filtrate antigens of Aspergillus fumigatus by enzyme-linked immunosorbent assay. Sabouraudia 21: 195-203. (1983b).

Wilson, E.V., Hearn, V.M. and Mackenzie, D.W.R. Evaluation of a test to detect circulating Aspergillus fumigatus antigen in a survey of immunocompromised patients with proven or suspected invasive disease. J. Med. Vet. Mycol. 25: 365-374. (1987).

Winston, D.J., Territo, M.C., Ho, W.G., Miller, M.J., Gale, R.P. and Golde, D.W. Alveolar macrophage dysfunction in human bone marrow transplant recipients. Am. J. Med. 73: 859-866. (1982).

Yamazaki, M., Suzuki, S. and Miyaki, K. Tremorgenic toxins from Aspergillus fumigatus fresnius. Chem. Pharm. Bull. 19: 1739-1740. (1971).

Young, R.C., Bennett, J.E., Vogel, C.L., Carbone, P.P. and De Vita, V.T. Aspergillosis. The spectrum of disease in 98 patients. Medicine 49: 147-173. (1970).

Young, R.C. and Bennett, J.E. Invasive aspergillosis. Absence of detectable antibody response. Am. Rev. Resp. Dis. 104: 710-716. (1971).

Young, R.C., Bennett, J.E., Geelhoed, G.W. and Levine, A.S. Fungaemia with compromised host resistance: a study of 70 cases. Ann. Inter. Med. 80: 605-612. (1974).

Yu, B., Niki, Y. and Armstrong, D. Use of immunoblotting to detect Aspergillus fumigatus antigens in sera and urines of rats with experimental invasive aspergillosis. J. Clin. Microbiol. 28: 1575-1579. (1990).

Yu, V.L., Mudder, R.R. and Poorsatter, A. Significance of isolation of Aspergillus from the respiratory tract in diagnosis of invasive pulmonary aspergillosis. Results from a three year prospective study. Am. J. Med. 81: 249-254. (1986).

Publications arising from this work.

Haynes, K.A., Barnes, R.A., Wilson, E.V., Hearn, V.M., Mackenzie, D.W.R. and Rogers, T.R. Aspergillosis in immunocompromised patients (part II): Evaluation of an enzyme-linked immunosorbent assay for detection of Aspergillus antigen. pp377. In E., Drouhet, G.T., Cole, L., deRepentigny, J.P., Latge and B., Dupont (eds). Fungal antigens. Isolation, purification and detection. Plenum Publishing Corp., New York. (1986).

Barnes, R.A., Haynes, K.A. and Rogers, T.R. Aspergillus antigen detection in marrow transplant patients. Bone Marrow Transpl. 2: suppl. 1: 271. (1987).

Haynes, K.A. and Rogers, T.R. Improved detection of Aspergillus antigens in body fluids with a monoclonal antibody to galactomannan. Program Abstr. 29th Intersci. Conf. Antimicrob. Agents Chemother. Abstr. No. 1146. (1989).

Haynes, K.A., Latge, J.P. and Rogers, T.R. Detection of Aspergillus antigens associated with invasive infection. J. Clin. Microbiol. 28: 2040-2044. (1990).

Rogers, T.R., Haynes, K.A. and Barnes, R.A. Value of antigen detection in predicting invasive pulmonary aspergillosis. Lancet 336: 1210-1213. (1990).