

09PH  
C5975



*In vitro studies on  
HIV-1 infection of Astrocytes.*

Jennifer Clarke  
B.Sc. (Hons)

Infectious Diseases Laboratories  
Institute of Medical and Veterinary Science  
Adelaide  
South Australia

Department of Microbiology  
School of Molecular Biosciences  
University of Adelaide  
Adelaide  
South Australia

\*\*\*\*\*

A thesis submitted to the University of Adelaide in fulfilment of the  
requirements for the degree of Doctor of Philosophy

July, 2006

# Table of Contents

<b>DECLARATION.....</b>	<b>XIII</b>
<b>ACKNOWLEDGMENTS.....</b>	<b>XIV</b>
<b>ABBREVIATIONS .....</b>	<b>XV</b>
<b>PUBLICATIONS AND PRESENTATIONS ARISING FROM THIS THESIS .....</b>	<b>XVIII</b>
<b>INTRODUCTION.....</b>	<b>1</b>
1.1 BACKGROUND ON HIV .....	1
1.1.1 <i>Discovery and classification of the Human Immunodeficiency Virus</i> .....	1
1.1.2 <i>Prevalence of HIV-1 infection</i> .....	2
1.1.3 <i>Clinical course of HIV-1 infection</i> .....	4
1.1.4 <i>HIV-1 persistence</i> .....	6
1.1.5 <i>The HIV-1 Virus</i> .....	9
1.1.6 <i>HIV-1 Replication</i> .....	9
1.2 BACKGROUND TO THE CENTRAL NERVOUS SYSTEM.....	22
1.2.1 <i>Cellular Organisation of the Central Nervous System</i> .....	22
1.2.2 <i>Metabolic relationships between Neurons and Astrocytes</i> .....	28
1.2.3 <i>Barriers of the CNS</i> .....	29
1.2.4 <i>Immune System of the CNS</i> .....	32
1.3 HIV-1 INFECTION OF THE CNS AND HIV-1 INDUCED NEUROLOGICAL DISEASES .....	36
1.3.1 <i>Entry of HIV-1 into the CNS</i> .....	36
1.3.2 <i>Identification of CNS cells infected by HIV-1 in post mortem brain sections</i> . .....	39
1.3.3 <i>HIV-1 Encephalitis and neuropathology of HIV-1</i> .....	41
1.3.4 <i>HIV-1 Associated Dementia</i> .....	42
1.3.5 <i>Mechanisms underlying HAD</i> .....	44
1.4 <i>IN VITRO</i> HIV-1 INFECTION OF ASTROCYTES.....	50
1.4.1 <i>Initial release of HIV-1 core protein</i> .....	50
1.4.2 <i>Restricted infection</i> .....	51
1.4.3 <i>“Rescue” or “Reactivation” upon coculture with permissive cells</i> .....	53

1.5 MECHANISMS OF HIV-1 REPLICATION IN ASTROCYTES <i>IN VITRO</i> .....	54
1.5.1 <i>Virus Entry</i> .....	54
1.5.2 <i>Reverse transcription and integration</i> .....	56
1.5.3 <i>Transcription and Translation and mechanisms of restriction</i> .....	57
1.5.4 <i>Release of infectious virus</i> .....	59
1.6 <i>IN VITRO</i> MODELS OF ASTROCYTE INFECTION.....	59
1.6.1 <i>Consideration of the limitations of current HIV-1 astrocyte infection models</i> .....	59
1.6.2 <i>Choice of Astrocyte cell lines and HIV-1 strains for this thesis</i> .....	65
1.7 <i>IN VIVO</i> SIGNIFICANCE OF RESTRICTED HIV-1 INFECTION OF ASTROCYTES.....	66
1.8 SCOPE OF THIS THESIS .....	68
1.8.1 <i>Hypotheses</i> .....	68
1.8.2 <i>Aims</i> .....	69
1.8.3 <i>Overview of experimental approach</i> .....	70
<b>MATERIALS AND METHODS .....</b>	<b>71</b>
2.1 MATERIALS.....	71
2.1.1 <i>Cells and Cell Culture</i> .....	71
2.1.2 <i>Plasmids</i> .....	75
2.1.3 <i>Oligonucleotide Sequences</i> .....	76
2.1.4 <i>Commonly used buffers and solutions</i> .....	79
2.2 PREPARATION AND ANALYSIS OF HIV-1 VIRUS STOCKS .....	81
2.2.1 <i>Quantification of HIV-1 core protein antigen (p24)</i> .....	81
2.2.2 <i>Titration of HIV-1 virus stocks</i> .....	81
2.2.3 <i>Preparation of cell free T-cell tropic HIV-1 Stocks</i> .....	83
2.2.4 <i>Preparation of cell free macrophage tropic HIV-1 stocks</i> .....	85
2.3 INFECTION PROTOCOLS.....	86
2.3.1 <i>Cell to cell infections; persistently infected T-cell viral donors</i> .....	86
2.3.2 <i>Cell to cell infections; chronically infected macrophage donor cells</i> .....	89
2.3.3 <i>Cell-free infections for immunofluorescent analysis of viral entry</i> .....	90
2.3.4 <i>Cell-free infections for Electron Microscopy Analysis of viral entry</i> .....	91
2.3.5 <i>Cell free infections for the analysis of HIV-1 DNA, RNA and transmission of infection.</i> .....	92

2.4 IMMUNOFLUORESCENCE ASSAY AND MICROSCOPIC ANALYSIS.....	95
2.4.1 <i>Immunoflourescence Assay Protocol</i> .....	95
2.4.2 <i>Confocal Microscopic Analysis of IFA</i> .....	97
2.5 ELECTRON MICROSCOPE ANALYSIS.....	98
2.6 NUCLEIC ACID PURIFICATION / EXTRACTION .....	99
2.6.1 <i>Plasmid DNA preparations</i> .....	99
2.6.2 <i>Cell harvests for DNA and RNA extractions</i> .....	100
2.6.3 <i>HIRT Extrachromosomal and Chromosomal DNA extractions</i> .....	101
2.6.4 <i>DNA extraction from DNased virus stocks</i> .....	102
2.6.5 <i>Cellular and Viral RNA Extractions and cDNA preparation</i> .....	104
2.7 ANALYSIS OF HIV-1 DNA AND CDNA TO INVESTIGATE HIV-1 REVERSE TRANSCRIPTION, INTEGRATION AND TRANSCRIPTION. ....	105
2.7.1 <i>Copy Number Standards and Normalisation of Samples</i> .....	105
2.7.2 <i>Conventional PCR Procedures</i> .....	107
2.7.3 <i>Southern Transfer and Hybridisation Techniques</i> .....	112
2.7.4 <i>Real Time PCR Procedures</i> .....	115
2.7.5 <i>HIV-1 Reverse Transcription Analysis</i> .....	116
2.7.6 <i>HIV-1 Integration Analysis</i> .....	118
2.7.7 <i>HIV-1 RNA Analysis</i> .....	119
2.8 DETECTION OF RELEASE OF INFECTIOUS VIRUS (INFECTIVITY ASSAY).....	119
2.9 LIST OF SUPPLIERS .....	120
<b>CELL TO CELL INFECTION OF ASTROCYTES <i>IN VITRO</i>....</b>	<b>122</b>
3.1 INTRODUCTION .....	122
3.1.1 <i>Background</i> .....	122
3.2 PRELIMINARY INVESTIGATIONS ON THE CULTURE OF ASTROCYTES WITH PERSISTENTLY INFECTED T-CELL LINES .....	123
3.2.1 <i>Characterisation of the persistently infected T-cell lines</i> .....	123
3.2.2 <i>Minimising the contribution of HIV-1 replication in the virus-donor cell population</i> .....	124
3.3 CULTURE OF ASTROCYTES WITH PERSISTENTLY INFECTED T-CELL LINES .....	130
3.3.1 <i>Analysis of transmission of HIV-1 from E12 and HIIIB cells to U251-MG astrocytes</i> .....	130

3.3.2	<i>AZT treatment to distinguish between de novo reverse transcription and pre-formed HIV-1 DNA</i>	136
3.4	CULTURE OF ASTROCYTES WITH CHRONICALLY INFECTED MACROPHAGES .....	138
3.4.1	<i>Coculture of U251-MG astrocytes with HIV-1<sub>BoL</sub> infected MDMs</i> .....	139
3.4.2	<i>Minimising the contribution of HIV-1 replication in the virus-donor cell population.</i> .....	141
3.5	DISCUSSION .....	147
<b>ENTRY OF HIV-1 INTO ASTROCYTES .....</b>		<b>152</b>
4.1	INTRODUCTION .....	152
4.1.1	<i>Background</i> .....	152
4.2	IMMUNOFLUORESCENT TRACKING OF HIV-1 ENTRY INTO ASTROCYTES .....	154
4.2.1	<i>Preliminary Immunofluorescent Investigations</i> .....	154
4.2.2	<i>APS immunoreactivity in HIV-1 infected U251-MG astrocytes</i> .....	159
4.2.3	<i>Confirmation of HIV-1 immunoreactivity with an independent antibody</i> .....	168
4.2.4	<i>APS immunoreactivity in HIV-1 infected U251-MG, CCF-STTG1 and U87-MG astrocytes</i> .....	171
4.2.5	<i>Summary and consideration of the modes of endocytosis which could be involved in the uptake of virus / viral proteins by the astrocyte cells</i> .....	174
4.3	ANALYSIS OF VIRUS ENTRY INTO ASTROCYTES BY ELECTRON MICROSCOPY .....	177
4.3.1	<i>Preliminary Electron Microscopic Analysis</i> .....	177
4.3.2	<i>Identification of enveloped, mature virion-like particles within a vesicle-like structure in U251-MG astrocyte 40 mpi</i> .....	179
4.4	DISCUSSION .....	185
<b>REVERSE TRANSCRIPTION OF HIV-1 IN ASTROCYTES....</b>		<b>189</b>
5.1	INTRODUCTION .....	189
5.1.1	<i>Background</i> .....	189
5.2	PRELIMINARY INVESTIGATIONS .....	191
5.2.1	<i>Establishing a model of cell-free infection of U251-MG astrocytes</i> .....	191
5.2.2	<i>Preliminary experiments and analysis of HIV-1 infected U251-MG cultures</i> .....	193
5.3	ANALYSIS OF THE INITIAL PHASE OF VIRAL PROTEIN RELEASE BY INFECTED U251-MG ASTROCYTES .....	201
5.4	ANALYSIS OF <i>DE NOVO</i> VIRAL REVERSE TRANSCRIPTION DURING THE ACUTE PHASE OF U251-MG INFECTION .....	203
5.4.1	<i>Detection of HIV-1 DNA during acute infection of U251-MG astrocytes</i> .....	203

5.5 EXAMINATION OF THE SOURCE OF THE DETECTED HIV-1 DNA.....	206
5.5.1 <i>Assessment of the DNase I treatment of the virus inoculum.....</i>	206
5.5.2 <i>Effect of reverse transcriptase inhibitors on the level of HIV-1 DNA present during acute infection of U251-MG astrocytes.....</i>	209
5.6 ANALYSIS OF ACUTE INFECTION OF U251-MG, CCF-STTG1 AND U87-MG ASTROCYTES .....	212
5.6.1 <i>Analysis of the initial phase of viral protein release by infecte astrocytes.....</i>	213
5.6.2 <i>Analysis of the level of extrachromosomal HIV-1 DNA during astrocyte infection.....</i>	217
5.5 DISCUSSION .....	226
<b>INTEGRATION OF HIV-1 IN ASTROCYTES .....</b>	<b>232</b>
6.1 INTRODUCTION .....	232
6.1.1 <i>Background.....</i>	232
6.1.2 <i>Use of IL1<math>\beta</math> as a model of coculture stimuli for the “rescue” of infectious virus from infected astrocytes.....</i>	234
6.2 PRELIMINARY INVESTIGATIONS OF INTEGRATED HIV-1 DNA, P24 PROTEIN AND INFECTIOUS VIRUS RELEASE DURING HIV-1 INFECTION OF U251-MG CELLS WITH COCULTURE OR IL1 $\beta$ STIMULATION.....	235
6.2.1 <i>Assaying astrocyte supernatants for the release of p24 protein.....</i>	236
6.2.2 <i>Assaying astrocyte supernatants for the presence of infectious virus.....</i>	238
6.2.3 <i>Assessment of HIV-1 integration.....</i>	240
6.2.4 <i>Consideration of the lack of detectable infectious virus release and integrated HIV-1 DNA in the IL1<math>\beta</math>-stimulated cultures which had released p24 protein.....</i>	242
6.3 DETAILED ANALYSIS OF VIRAL INTEGRATION AND THE RELEASE OF INFECTIOUS VIRUS DURING THE COURSE OF U251-MG INFECTION.....	243
6.3.1 <i>Assaying astrocyte supernatants for the release of p24 protein.....</i>	245
6.3.2 <i>Assaying astrocyte supernatants for the presence of infectious virus.....</i>	247
6.3.3 <i>Assessment of HIV-1 integration.....</i>	249
6.4 DETAILED ANALYSIS OF VIRAL INTEGRATION AND THE RELEASE OF INFECTIOUS VIRUS DURING THE COURSE OF U251-MG, CCF-STTG1 AND U87-MG INFECTION.....	251
6.4.1 <i>Assaying astrocyte supernatants for the release of p24 protein.....</i>	251
6.4.2 <i>Assaying astrocyte supernatants for the presence of infectious virus.....</i>	253
6.4.3 <i>Assessment of HIV-1 integration.....</i>	257

6.5 DISCUSSION OF THE LACK OF VIRAL INTEGRATION IN THE CONTEXT OF RELEASE OF INFECTIOUS VIRUS..	262
6.5.1 <i>Characteristics of the observed infectious virus release</i> .....	262
6.5.2 <i>Hypotheses concerning the release of infectious virus in the absence of detectable provirus integration</i> .....	263
6.6 ANALYSIS OF VIRAL RNA IN INFECTED ASTROCYTES .....	267
6.6.1 <i>Background to HIV-1 RNA synthesis during virus replication</i> .....	267
6.6.2 <i>Analysis of unspliced viral RNA</i> .....	268
6.6.3 <i>Analysis of multiply-spliced viral mRNA</i> .....	270
6.7 DISCUSSION .....	273
6.7.1 <i>Summary of Integration and virus release studies</i> .....	273
<b>GENERAL DISCUSSION .....</b>	<b>278</b>
7.1 SUMMARY AND DISCUSSION.....	278
7.2 FUTURE DIRECTIONS.....	285
<b>BIBLIOGRAPHY .....</b>	<b>289</b>

## THESIS SUMMARY

HIV-1 infection of astrocytes is involved in HIV-1 induced neurological diseases and is a possible source of viral persistence. *In situ* studies of post mortem brain tissue indicate that HIV-1 infection of astrocytes does occur, but is restricted. Previous *in vitro* studies have revealed intrinsic intracellular blocks to HIV-1 transcription and translation in astrocytes. Co-culture of infected astrocytes with permissive CD4<sup>+</sup> cells has been shown to “rescue” infectious HIV-1 from the restricted infection of astrocytes, resulting in transmission of infection to the CD4<sup>+</sup> cells. However, the early viral replication steps of entry, reverse transcription and integration have not been previously characterised in detail in astrocytes, and are the focus of this study.

In this thesis, two routes of initiation of *in vitro* HIV-1 infection of astrocyte cell lines were employed; i) “cell-to-cell” infection (involving coculture of astrocytes with HIV-1 infected macrophages or T-cell lines), and ii) “cell-free” infection (direct application of cell-free virus to the astrocytes). In the cell-to-cell infection model, the process of HIV-1 reverse transcription was investigated but could not be clearly demonstrated to occur within the astrocyte cell population. The cell-free infection model permitted a more detailed analysis of the interaction between astrocytes and HIV-1, focussing on the entry and post entry HIV-1 replication steps of reverse transcription and integration. In cell-free infection, uptake of HIV-1 by astrocytes occurred within vesicles. Although infectious virus was subsequently released from these cells, there was no evidence that viral replication had occurred. Extrachromosomal viral DNA could be detected in these cells, however the level of viral DNA associated with the astrocytes declined with time and was unaffected by inhibitors of HIV-1 reverse transcription. This astrocyte-associated viral DNA was subsequently shown to be due to HIV-1 DNA in the viral inoculum. Using specific assays to detect integrated forms of HIV-1 DNA, no evidence was found for viral integration in these cells. Unspliced viral RNA was present, and declined with time post infection. Despite the striking absence of integrated HIV-1 DNA in these cells, the majority of infected astrocyte cultures sporadically released infectious virus. This demonstrated that an alternative, replication-independent pathway of HIV-1 infection and transmission, previously reported in dendritic and epithelial cells, can also occur in astrocytes.

The identification and characterisation of this replication-independent pathway of astrocyte infection in this thesis may have significant ramifications for our understanding of the entry and spread of HIV-1 through the central nervous system (a major viral reservoir). The possibility that this pathway of infection occurs in astrocytes in the CNS may also impact upon the management of viral persistence and anti-retroviral therapy evasion by the virus. The effect of this replication-independent process on astrocyte function, and the relevance of this in the pathogenesis of HIV-1 associated dementia, remains to be determined.