

**A molecular study of the immunopathogenesis of TB
spondylitis in HIV-infected and –uninfected patients**

By

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Declaration

The experimental work described in this thesis was carried out at the Africa Centre Laboratory, Doris Duke Medical Research Institute, Nelson R Mandela School of Medicine, under the supervision of Prof S.Cassol, Prof T.Ndung'u and co-supervision of Prof S.Govender.

All work presented in this study was original research conducted by the author, unless otherwise stated in the text or acknowledgements. This work has not been submitted previously in any other form to this or any other University. Due acknowledgement was given to work by others when used in this dissertation.



S.Danaviah

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Dedication

This work is dedicated to my greatest blessing, my Family whose sustained support, encouragement and love motivate me daily. None of this would be possible without you and I cannot express how grateful I am to, and for you! I thank God each day for blessing me with the three of you!

Publications

Peer Reviewed Publications

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Presentations

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Danaviah S, Chelule P, Gordon M, Naicker A, Dorsamy V, Kumar KPS, Govender S, Cassol S. Divergent HIV-1 evolution in spinal tuberculosis granulomas: association with immunolocalised CD68⁺ macrophages. 3rd IAS Conference on HIV Pathogenesis and Treatment. Rio de Janeiro, Brazil; 24-27 July 2005.

Danaviah S, De Oliveira T, Chelule P, Gordon M, Graham N, Kumar KPS, Govender S, Cassol S. Genetic differences in plasma and granuloma-derived HIV-1 of patients with spinal tuberculosis: evidence for compartmentalization and independent evolution. International Meeting on Emerging Trends in TB research, New Delhi, India, November 2004.

Danaviah S, Naicker A, Govender S, Reddy R, Kumar K, Bishop K, Cassol S. Histopathology of spinal TB granulomas in HIV-1 positive and negative patients and localized macrophage immunoreactivity. 14th International AIDS Conference. 7-14 July 2002, Barcelona, Spain. Abstract #A10050

National peer reviewed presentations

Danaviah S, Naicker T, Govender S, De Oliveira T, Gordon M, Dorsamy V, Cassol S. An evaluation of HIV⁺ and HIV⁻ spinal tuberculous granulomas – relevance to viral compartmentalisation. 44th Annual Microscopy Society of South Africa Conference. Pietermaritzburg, SA, 5-7 December 2005.

Danaviah S, De Oliveira T, Chelule P, Gordon M, Naicker T, Dorsamy V, Graham N, Cassol S Govender S. HIV-1 in spinal tuberculosis granulomas and association with viral diversity. AstraZeneca College of Health Sciences Research Symposium. 28 September 2005

Danaviah S, Govender S, Kumar KPS, Naicker A, Bishop K, Cassol S. Histopathology and macrophage immunoreactivity of spinal TB granulomas in HIV-1 positive and negative patients. 15th International Congress on Electron Microscopy. September 2002, Durban, South Africa. Abstract #1167

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Ethical Approval

The Institutional Biomedical Ethics Research Board of the University of KwaZulu Natal approved the study. The reference number for the study was: H112/02

Abstract

Human immunodeficiency virus (HIV) and *Mycobacterium tuberculosis* (Mtb) infections are two of the most devastating pandemics of the modern era. Recent escalations in the incidence of all forms of tuberculosis (TB) have paralleled the worldwide expansion of the HIV epidemic, making HIV the single most important underlying promoter of the current TB epidemic. Approximately 2 million South Africans are currently HIV/TB co-infected, a large proportion of whom develop skeletal disease often involving the spine. Spinal TB, a devastating disease which may lead to severe deformity and/or paralysis, has escalated by 30% in the local setting over a 5 year period to 2005. Fibrous encapsulation of spinal granulomas forms isolated compartments dually-infected with HIV-1/TB, and heavily infiltrated with macrophages. Due to rapid viral turnover, dynamic site-specific fluctuations in HIV quasi-species are common with unhindered migration between anatomical compartments. Understanding the origins and genetic evolution of HIV-1 at these sites is important to developing effective and targeted treatment strategies. Moreover, the South African HIV epidemic is characterized by an escalating incidence of atypical opportunistic and non-tuberculous mycobacterial (NTM) infections, both posing significant diagnostic and therapeutic challenges. Previous reports of the spinal TB have, to date made no association between HIV infection and susceptibility to opportunistic spinal infections.

This study therefore, classified the infective organisms by genotyping in order to establish the occurrence of Mtb as well as atypical mycobacterial infections in this cohort. In addition, this study investigated viral heterogeneity in the coinfecting lesion by analysing the HIV-1 *env* (C2V5 region) sequences isolated from plasma and granuloma compartments. The occurrence of viral heterogeneity was correlated with the level and pattern of macrophage infiltration and ultrastructural changes in granulomas. .

Tissue biopsies of epidural granulomas were collected at surgery, from 60 patients (38 HIV⁻; 22 HIV⁺); clinically and radiologically diagnosed with TB spondylitis. All

patients received a mean of 8 weeks anti-TB therapy (range 5-24 weeks). A separate biopsy, collected simultaneously, was submitted for routine TB diagnostic assays (microscopy; culture). Blood collected at surgery from all patients, was submitted for routine haematological investigations including CD3+, CD4+ and CD8+ T-cells counts, HIV-1 serology and viral loads, in the case of HIV-positive patients (n=22; 100µl plasma; 10-12mg tissue).

A portion of tissue was processed for histopathology (H&E, ZN, PAS) (20 HIV⁻; 15 HIV⁺), immuno-localisation of CD68+ (macrophage/monocyte marker), CD4+ and CD8+ cells (20 HIV⁻; 15 HIV⁺), and transmission electron microscopy (TEM) (8 HIV⁻ and 5 HIV⁺). TEM sections were viewed using a Jeol JEM-1011 and image analysis for histopathology, IHC and TEM used the SIS software. Bacterial DNA was extracted from heat inactivated positive cultures; a 500bp region of the 16S rDNA gene was PCR amplified and sequenced in order to genotype the infective bacterial organism. Viral RNA, extracted from plasma and granulation were RT-PCR amplified, cloned and sequenced from co-infected patients. Paired PCR-amplified products and sequences were available in a subset of 7 of the co-infected population. All phylogenetic analyses were performed using PAUP, PAML, MEGA and prosite. Modified Swofford-Maddison and Slatkin-Maddison tested compartmentalization; LAMARC determined migration and coalescence.

The overall mean age was 32 years (range = 2-71 years), 34 years in the HIV⁻ and 29 years in the HIV⁺ groups. Forty-one (41/60) specimens were culture negative (68%). Of these organisms, 16 were identified as Mtb (8/38 HIV⁻; 8/22 HIV⁺) and 3 as NTM (3/38 HIV⁻). In 7/38 HIV⁻ and 8/22 HIV⁺ patients, CD4+ T-cell counts were <500 cells/µl while in 4/22 HIV⁺ adult patients CD4+ T-cell counts were <200 cells/µl. All patients were ARV naïve despite fulfilling WHO guidelines for treatment initiation since this study preceded initiation of the national roll-out. Histopathological features were diagnostic of TB in 91.4% of specimens while the remaining 8.6% displayed features atypical of a TB infection. Although not statistically significant, a trend towards greater intensity and distribution of CD68 immuno-reactivity in the HIV⁻ group was noted. CD4 reactivity was sparse to absent and was independent of HIV status while CD8 reactivity was greater in the HIV-

infected group. Ultra-structural features were consistent with an active immune response and healing in HIV⁻, in contrast with the predominantly degenerative and necrotic HIV⁺ specimens. Neutrophils, macrophages and immune cells were detected in the former group but were rare in the HIV⁺ group. Multi-nucleated giant cells were present both HIV-uninfected and infected specimens. The predominance of grossly dilated rough endoplasmic reticulum (rER) seen to be infiltrated with an amorphous, granular material was a unique finding. This phenomenon was evident largely in the HIV-infected group. Lysosomal/phagosomal inclusions resembled degenerative material, intact bacilli and virus-like particles.

There was a 40% concordance between routine TB culture identification and 16S rDNA genotyping. Three Mtb (2 HIV⁻; 1 HIV⁺); 2 NTMs (2 HIV⁻) and 5 environmental bacilli (3 HIV⁻; 2 HIV⁺) were identified. NTMs included *M. intracellulare* and *M. fortuitum*, and environmental bacilli included *S. maltophilia*, *S. marscens* and *B. sanguinis*. These NTMs, although typically associated with immune-suppressed hosts, were more common to the HIV⁻ group. Moreover evidence of CD4⁺ lymphopenia was noted in two HIV-uninfected patients; one with an Mtb and the other with an environmental bacterial infection.

Granulation-specific compartmentalization of HIV-1 was observed in 1 paediatric and 3 adult patients. The remaining 3 patients (2 paediatric, 1 adult), showed evidence of viral migration between plasma and tissue compartments. The mean inter-patient diversity among plasma-derived sequences was 15.3 ± 1.1 , compared to 12.5 ± 1.0 for granuloma-derived sequences. Strong coalescence forces were present in the tissue sequences of 3 patients (2 paediatric, 1 adult). All 3 showed evidence of virus migration from tissue to plasma; while plasma to tissue migration was evident in remaining 4 patients. The intensity of CD68⁺ immunoreactivity correlated with diversity between tissue and plasma viruses ($r=-0.867$; $p<0.05$).

This study illustrated that TB spondylitis in combination with HIV infection is a serious health hazard in KZN. The genotypes of the infective organisms were variable and independent of HIV-status, highlighting the need for a broad-range diagnostic tool in this setting. CD68 immuno-localisation demonstrated similar distribution patterns of macrophage/macrophage-derived cells in HIV-infected and

un-infected specimens. CD4 and CD8 immunolocalisation confirmed the chronic nature of spinal TB. In addition, this study demonstrated divergent viral evolution in spinal granulomas. Ultra-structural evidence of intra-lysosomal virus-like particles indicated phagocytic cells and macrophages as a possible cellular reservoir. The role of macrophages and undifferentiated phagosomes in disease progression warrants further investigation.

Abbreviations

Ab	Antibody
ADCC	Antibody dependant cell- mediated cytotoxicity
AFB	Acid fast bacilli
Ag	Antigen
AIDS	Acquired immunodeficiency syndrome
AM	Alveolar macrophages
APC	Antigen presenting cells
ART	Antiretroviral therapy
ARV	Antiretrovirals
ASP	Anti-sense protein
BAL	Bronchoalveolar lavage
CK2	Casein kinase II phosphorylation site
CMI	Cell mediated immunity
CTL	Cytotoxic T-lymphocytes
CRF	Circulating recombinant forms of HIV-1
DAB	Diamino-benzidine
DC	Dendritic cell
DDBJ	DNA Data Bank of Japan
DOTS	Directly Observed Treatment Strategy
DPX	Distyrene tricresyl phosphate xylene
EC	Eastern Cape province
EDTA	Ethylenediaminetetra-acetic acid
ELISA	Enzyme linked immunosorbent assay
<i>env</i>	Envelope
ER	Endoplasmic reticulum
ESAT-6	Early secretory antigenic target-6
ESR	Erythrocyte sedimentation rate
FS	Free State province
G	Gauteng province
H&E	Haematoxylin and Eosin
H ₂ O ₂	Hydrogen peroxide

HAART	Highly active anti-retroviral therapy
HIV	Human immunodeficiency virus
HIV ⁻	HIV-uninfected
HIV ⁺	HIV-infected
Hsp65	Heat shock protein 65
IFN γ	Interferon gamma
IHC	Immunohistochemistry
IL	Interleukin
ITS	Internaly transcribed spacer
IVD	Intravenous drug
KGV	King George V Hospital
KZN	KwaZulu-Natal province
LAM	Lipoarabinomannan
LAMP	Lysosomal/endosomal associated trans-membrane proteins
LG	Langerhans giant cells
LM	Light microscopy
LTR	Long terminal repeat
M	HIV-1 Main Group
MAC	<i>Mycobacterium avium</i> complex
MDR-TB	Multi-drug resistant tuberculosis
MGIT	Mycobacteria growth indicator tube
MHC	Major histocompatibility complex
ML	Maximum likelihood
MP	Mpumalanga
MR	Mannose receptor
mRNA	Mitochondrial ribosomal nucleic acids
Mtb	<i>Mycobacterium tuberculosis</i>
MTCT	Mother-to-child-transmission
xDR-TB	Extensively / Extreme drug resistant TB
N	HIV-1 Non-M/non-O group
nAb	Neutralising antibodies
NCBI	National Centre of Biotechnology Information
NF- κ B	Nuclear Factor- κ B

NK	Natural killer
NNRTI	Non-nucleoside reverse transcriptase inhibitors
NO	Nitric oxide
NOS	Nitric oxide synthase
NRAMP1	Natural resistance associated macrophage protein
NTM	Non-tuberculous mycobacteria
O	HIV-1 Outlier group
OI	Opportunistic infections
PAS	Periodic acid-schiff technique
PBS	Phosphate buffered saline
PDGF	Platelet derived growth factor
PI	Protease inhibitors
PKC	Protein kinase C phosphorylation site
PPD	Purified protein derivative
R5	CCR5 macrophage tropic HIV-1 variants
rDNA	Ribosomal deoxyribose nucleic acid
rER	Rough endoplasmic reticulum
RGD	Gram positive anchoring nuclar RGD (Arg-Gly-Asp) binding site; a cross-lined tripeptide constituting the major binding site for a number of integrins ligands
RIDOMM	Ribosomal Differentiation of Medical Microorganisms
RNI	Reactive nitrogen intermediates
ROI	Region of interest
RT	Reverse transcriptase
RT-PCR	Reverse transcriptase-polymerase chain reaction
SIV	Simian immunodeficiency virus
SR	Scavenger receptor
TB	Tuberculosis
TEM	Transmission electron microscopy
Th (1/2)	T-helper (1/2)
TLR	Toll-like receptors
TNF	Tumour necrosis factor
UN	United Nations

WHO	World Health Organisation
XMDR-TB	Extreme multi-drug resistant tuberculosis
ZN	Ziehl-Neelsen

Chapter 1 – Introduction and Literature Review

1.1 Introduction

1.1.1 Statement of the problem

As a direct result of human immunodeficiency virus (HIV) and tuberculosis (TB) co-infection, indications are that the HIV epidemic in Kwa-Zulu Natal (KZN) is two years ahead of the rest of South Africa (SA) (Colvin et al., 2001). Furthermore, records place TB/HIV fatality rates from a treatment center in SA at 24.7% in 1995, an increase of 135% from 1991/1992 (Connolly et al., 1998). Dual infection, unfortunately, results in poor outcomes accounting for approximately a quarter of a million annual deaths worldwide (Gayle and Hill, 2001, WHO, 2004). These reports relate to pulmonary TB. The incidence of spinal TB at King George V Referral Hospital (KGV), KZN, SA, has increased by 30% from 2000-2005 (Govender, 2006).

Favourable outcomes were observed in spinal TB infected patients even among HIV co-infected individuals following surgery and standard anti-TB chemotherapy (Govender, 2006). This is despite receiving no anti-retroviral therapy (ART). Unlike the plethora of literature characterizing pulmonary TB at an immunological and molecular level, related knowledge of spinal disease remains poorly defined (Alothman et al., 2001, Govender et al., 2000a, Govender et al., 2001a, Ho et al., 1995, Niu et al., 2000).

1.1.2 Rationale behind study

Clinical presentation forms the basis of spinal TB diagnosis and is often, not substantiated by laboratory testing. Therefore there is no indication of the nature of the infective organism, its relative virulence or drug resistance patterns. As a result, individualized pathogen-specific chemotherapy regimes cannot be implemented. Even the most advanced culture methods currently available e.g. Mycobacteria Growth Indicator Tube (MGIT), have a low recovery rate (~10%) from clinical samples and

require extended incubation times for a positive result (Jayakumar et al., 2001). But with molecular technology, organisms can be identified in a single assay (Beggs et al., 2000, Cloud et al., 2002, de Baar et al., 2001, Martin et al., 1993, Mikhailovich et al., 2001, Patel et al., 2000, Rhee et al., 1999, Tortoli et al., 2001). The 16S rDNA gene is a favourable genotyping target as it is highly conserved across all Eubacteria (Cloud et al., 2002, Edwards et al., 1989, Mondragon-Barreto et al., 2000, Raghava et al., 2000, Ruiz et al., 2002).

Prior to the national ART rollout co-infected patients were solely reliant on anti-TB chemotherapy, enhanced nutritional support and surgery for disease resolution. Yet patients improved clinically, incorporated allograft and instrumentation and recovered neurologically (Govender et al., 2001b, Govender et al., 2001a). In addition, bringing clarity to the pathogenesis of spinal disease particularly in HIV co-infected patients will enhance current understanding of both diseases and may steer the development of vaccines, individualized interventions and diagnostic tools. The cost of anti-TB chemotherapy and therapeutic drug monitoring is an added economic burden to emerging nations. A comprehensive understanding of the disease complex, its progression and the related host response is necessary to determine whether short-term therapy is practical and therapeutically sound for infective spondylitis.

In contrast to TB, the HIV genome is highly heterogeneous, particularly in the nucleotide (NT) sequence of the envelope (*env*) gene (Collins et al., 2000). Genetic variability is most often a result of extremely variable viral replication rates (Collins et al., 2000) within compartmentalized microcosms in different organ systems. TB is known to increase HIV viral loads and consequently viral heterogeneity in co-infected patients (Stenger and Rollinghoff, 2001). It is proposed that viral infectivity and pathogenesis is genome linked and is primarily a product of transcriptional regulation of the long terminal repeats (LTRs). The rapid dissemination of subtypes E and C in SE Asia and sub-Saharan Africa (Montano et al., 1997) and, interestingly, parallels the explosion of the TB epidemic in these regions. Identifying the circulating subtype in this cohort may be an important indicator of the evolutionary and/or replicative pressures exerted on the HIV genome in the absence of ARVs.

Bearing in mind that HIV and TB are both macrophage tropic intracellular pathogens and have exerted a notable public health impact in the South African setting in recent years, appropriate preventative and therapeutic interventions are crucial. Although it is known that the cascade of events characterizing the immune response to TB is dominated by macrophages and lymphocytes, their specific phenotypes and relative distribution within the granulomatous spinal lesion remains unclear (Gonzalez-Juarrero et al., 2001). The distinct interactions between macrophages and antigen-specific T cells play an important role in disease development and progression (Hoshino et al., 2002, Stenger and Rollinghoff, 2001). In addition, discrete and uniform aggregates of immune cells, such as CD4⁺ and CD8⁺ cells occur in pulmonary granulomas (Gonzalez-Juarrero et al., 2001). Since, few reports of the immune response to spinal TB (Govender et al., 2000a, Govender et al., 2001a) are presently available it is unclear whether the same applies to this disease. There is no single determinant of susceptibility or resistance but a number of synergistic factors related to the host and pathogen. The success of the invading mycobacterium may, in part, be due to their ability to invade, replicate and persist within mononuclear cells (Beatty et al., 2000). Exploring tissue morphology of spinal granulomas will yield important insights into diseases pathogenesis and latency.

1.1.3 Objectives of the study

This study aimed to:

1. Compare and contrast the histopathology of spinal TB granulomas of HIV-infected and –uninfected patients. (Chapter 3)
2. Compare the phagocytic response by CD68 immuno-localisation in HIV-uninfected with HIV co-infected specimens Chapter 3)
3. Describe the ultra-structural changes induced by HIV co-infection (Chapter 3)
4. Genotype the bacterial pathogen from clinical and/or culture specimens. (Chapter 4)
5. Compare plasma- with tissue-derived HIV-1 variants as a measure of viral heterogeneity in HIV/TB co-infected granulomas (Chapter 5)

1.1.4 Hypothesis

Fibrous encapsulation of spinal granulomas creates a unique microenvironment infiltrated with immune cells that is permissive for divergent HIV evolution.

1.1.5 Experimental approach



A total of 60 patients (38 HIV-uninfected; 22 HIV-infected) were recruited into the study based on the rate of intake at a primary referral hospital in the KZN region. Blood and tissue specimens were subjected to the following:

1. Routine HIV and TB diagnostic assays
2. Quantification of haematological markers i.e. FBCs
3. Histopathological and ultrastructural evaluation
4. Immunolocalisation of CD68+ macrophages/macrophage derived cells
5. Bacterial genotyping
6. HIV cloning and sequencing

Each method will be discussed in a stand-alone chapter comprising of a brief introduction, a summary of related materials and methods, results and a discussion of the results including the limitations. A random sub-sampling of the study population (N=60) was undertaken for each investigation, while ensuring that, each sub-population included representative HIV-infected and –uninfected specimens. The final sample size for histo-pathological, immuno-histology and ultra-structural investigations were dependant on the quality of the processed tissue specimens. Bacterial genotyping and HIV-heterogeneity analysis was based on obtaining analysable amplification products.

1.2 Literature Review

1.2.1 Tuberculosis

1.2.1.1 Historical perspective

Tuberculosis is one of the oldest human pathogenic diseases known to man dating as far back as 5000BC (Wellons et al., 2004). Disease involving the spine and bone and joint has also been reported following radiological and molecular analyses of paleopathological specimens (Conlogue, 2004). The origin of human TB infections remains controversial. It is hypothesised that human disease stemmed from zoonotic transfection to Indo-European cattle herders (Smith, 2003a), however the absence of *M. bovis* in early paleo-pathological specimens refutes this hypothesis (Zink et al., 2003). Current theories, supported by phylogenetic evidence showing independent evolution of *M. tuberculosis* (Mtb) and *M. bovis* from a common ancestor, allude to a precursor complex related to *M. africanum* or *M. canetti* (Brosch et al., 2002).

TB spread throughout Europe during the 16th and 17th centuries, becoming the leading cause of death on the continent for nearly two centuries (18th and 19th centuries). The subsequent decline in both incidence and mortality was a consequence of adaptive Darwinian evolution and/or natural selection (Smith, 2003a). Spread to Asia, Eastern Europe and Africa, regions where TB was virtually unheard of, was doubtless, initiated by colonisation and contact with European travellers having a devastating effect in these regions (Jackson et al., 2006, Mhalu, 2005).

The mid-19th century heralded the first in a long history of interventions directed against TB with the first sanatorium strategy initiated by Herman Brehmer (Europe) and Dr EL Trudeau (America) (Bloom and Murray, 1992). At the time nutritional support, bed rest and isolation was the only treatment strategy. The discovery of rifampicin in 1943 revolutionized TB treatment. It is still used today. Later, the advent of isoniazid and streptomycin saw a 5% per annum decline in TB incidence rates from 1953 to 1984 (Zajac and Melcher, 1991). This downward trend was not sustainable and, after a levelling off in 1985, was reversed (Zajac and Melcher, 1991) to the overwhelming number of current infections due, largely, to the human immunodeficiency virus (HIV)

(Health_Protection_Agency_Tuberculosis, 2004). Such that TB has once again reached epidemic proportions, approximately 5000 years after its first infections.

1.2.1.2 Epidemiology

1.2.1.2.1 Patient demographics

Worldwide TB commonly occurs in people between the ages of 15-60 (WHO, 2002) although children as young as 24 months have been diagnosed with spinal TB (Govender and Parbhoo, 1999). Country, age, race, gender and socio-economic status impact case rates (Beers and Berkow, 1999). Since childhood TB is commonly contracted from adults, paediatric epidemiology parallels that of adults and accounts for 8-20% of TB-related deaths (Kabra et al., 2004). Children stand a 2-5% annual risk of infection and a 5% risk of progressing to active TB in developing countries (Kabra et al., 2004). These rates are 6 times greater among HIV-infected children. It is generalised that TB is an adult disease in developed nations but a disease of individuals in their first 3 decades of life in other regions, particularly impoverished nations (Kumar, 2005).

1.2.1.2.2 The global epidemic

TB is now recognised as the leading cause of death due to a single pathogen second only to HIV (Dye et al., 2006). New infection rates projected to reach 1 billion by 2020 (WHO, 2004) have already been exceeded twice over. At present, developing nations bear the greatest disease burden with half of all TB cases concentrated in Asia and 29% in Africa (WHO, 2006, Narain and Lo, 2004). The African epidemic is 6 times greater than the European epidemic but SE Asia far exceeds both at 33% of all incident cases (WHO, 2006). Ninety nine percent of resultant deaths occur in developing countries (WHO, 2003). Increased incidence rates in developed countries such occur predominantly (63% of all TB cases) among the immigrant populations (Health_Protection_Agency_Tuberculosis, 2004). A concomitant increase in extra-pulmonary TB has also been observed worldwide (Houshian et al., 2000, WHO, 2006).

In addition, patients in Eastern Europe and Central Asia are 10 times more likely to contract multi-drug resistant (MDR) TB. In South Asia, and most developing nations, weak public health infrastructure, inadequate resources and education, and MDR-TB infections impede control of the TB epidemic (Zaidi et al., 2004). As a result, MDR-TB infection rates parallel the upward trend in overall TB incidence (Cho et al., 2000). More recently, the emergence of extensively or extreme drug resistant TB (xDR-TB) in rural KwaZulu-Natal, SA, raised the alarm of a new and deadly epidemic (Sidley, 2006). Whether drug resistance is a consequence of patient non-compliance or the natural evolution of the bacilli is presently unclear. Regardless, the unfortunate and alarming consequence remains increased therapeutic failure and mortality rates.

1.2.1.2.3 The South African epidemic

South Africa (SA) has the highest number of TB cases in Africa and features strongly among the top 10 TB incidence nations worldwide (WHO, 2002). From a national figure of 526 cases /100 000 people in 2000, TB prevalence rates increased to 556/100 000 in 2001 (WHO, 2002). There were 339 078 new infections in 2004 alone. Currently, in excess of 500 000 South Africans are TB-infected, approximately 6 000 of whom have MDR-TB (Sidley, 2006). Of its nine provinces KwaZulu-Natal (KZN), Eastern Cape (EC) and Gauteng (G), in descending order, are the three highest incidence regions (DOH, 2002). Proportionally, the number of HIV/TB infected individuals is highest in KZN. Clearly, KZN is the epicentre of both epidemics in SA (Table 1.1). Both HIV and TB are also included in the 5 leading causes of death in SA according to government surveys (DOH, 2002). The highest prevalence of TB was detected among males and was the most common cause of death in the 40-49 years age group. Ethnicity also exerted an impact on HIV- and TB-related mortality rates. Current trends indicate a rise in TB incidence rates in developing nations particularly in sub-Saharan Africa which parallels the explosive HIV epidemic; and a decline or plateau in other countries where TB and HIV chemotherapies are readily available. These findings further underscore the urgent need to expand existing anti-retroviral (ARV) and anti-TB treatment programs in regions where these epidemics converge.

Table 1.1: Estimates of TB infection and HIV/TB co-infection rates by province in 2002

Province	Estimated no. of TB cases	Proportion of HIV ⁺ /TB ⁺ (%)
KwaZulu-Natal (KZN)	39 650	49.8
Eastern Cape (EC)	38 783	25.2
Gauteng (G)	30 176	30.0
W. Cape (WC)	22 942	16.8
N. Province (NP)	15 667	21.5
N. West (NW)	10 821	30.7
Mpumalanga (MP)	10 052	44.3
Free State (FS)	9 429	36.9
N. Cape (NC)	298	18.5
National Total	180 507	32.8

HIV/AIDS and TB- the deadly pair. Health information, evaluation and research (DOH, 2002).

1.2.1.2.4 Epidemiology of extra-pulmonary TB

The figures quoted thus far refer solely to the pulmonary TB epidemic and does not account for infections at other anatomical sites such as the pleura, bones and joints. TB at these extra-pulmonary sites accounts for approximately 15-20% of TB cases among HIV-uninfected patients, but in the wake of the explosive HIV epidemic this figure has risen to 50% of all TB cases (Sharma and Mohan, 2004). In developed countries, related incidence rates have increased significantly in recent years among immigrant populations. In some instances, an increase of 50 times that of local inhabitants as well as an increase in the number of sites involved, was noted (Houshian et al., 2000).

Osteoarticular TB occurs in approximately 3-4% of all TB cases worldwide, (Tuli, 2003). Affected sites include the knee and elbow joints, and the bones of the feet and hands (Sharma and Mohan, 2004). Mycobacterial infections of the upper extremities and non-weight bearing bones are as uncommon as skull and intra-cranial infections

(Allison et al., 1999). Often misdiagnosis considerably underestimates related incidence rates. In excess of 50% of osteoarticular TB cases involve the spine. Currently, an estimated 2 million individuals are afflicted with infections of the spine (Rajaskeran, 2003). Mortality rates among these patients range from 2 to 30% (Cuffe et al., 1994).

1.2.1.3 The bacterium

The etiologic agents of TB, belonging to the genus *Mycobacterium*, are slow growing, obligatory aerobic, gram-positive, intracellular pathogens (Cole et al., 1998). Of the more than 70 species currently characterized, approximately 30% are known human pathogens (Cloud et al., 2004). Mycobacteria are one of a few pathogens capable of infecting any organ in the body but prefer the oxygen-rich pulmonary tissue (Raja et al., 2004, Sharma and Mohan, 2004). Infection ensues following anatomic invasion by *Mycobacterium tuberculosis* (Mtb), occasionally by *M.avium* complex (MAC) or *M.kansasii* (Smith, 1991).

1.2.1.3.1 The bacterial genome

The Mycobacterial genome was largely a mystery until recent advances in molecular technology enabled the sequencing of the full 4.4 mega base long Mtb (H37RV bacterial stain) genome encoding 4 000 genes (Smith, 2003a)..

The greater proportion of the genome is dedicated to genes of, as yet, unknown function (38.2%), followed by genes responsible for intermediary metabolism and respiration (22.0%) (Smith, 2003a) presumably activated during latency. The third most prominent genes are those producing proteins for the cell wall and cell processes. Approximately 30% of its genome or 200 genes are dedicated to lipid synthesis and enzymes involved in fatty acid metabolism, which enable the organism to utilise fatty acids as a carbon source (Cole et al., 1998, Smith, 2003a). The bacterial cell wall is its most complex cellular component (Russell, 2001a). In response to oxygen (O₂) depletion (reduced O₂

levels) studies have demonstrated cell wall thickening indicative of protective adaptation (Cunningham and Spreadbury, 1998).

The TB genome is distinguished by limited sequence diversity, an extended generation time and slow adaptive evolution (Tanaka, 2004). Consequently, the genome is highly conserved. However, the contention that TB has a stable conserved genome is refuted by the rapid emergence of drug resistance in treated populations (Tanaka, 2004). In an analysis of Mtb IS6110 sequences, Tanaka (Tanaka, 2004) demonstrated the influence of positive selection on the TB genome in infected hosts but admits that the data is not conclusive for various reasons (Tanaka, 2004). Among them is that bacterial populations may have been removed from the mutational-drift equilibrium, the actual bacterial heterogeneity may not have been reflected in the sampling and the small sample size may account for the low rate of polymorphisms (Tanaka, 2004). Host immune responses and anti-microbial interventions exert strong selection pressures (Tanaka, 2004) on evolutionary changes but these factors do not act independently. It is likely that selected regions of the genome remain highly conserved.

1.2.1.4 Spinal Tuberculosis

Although pulmonary tuberculosis is the most common form of human Mycobacterial infections extra-pulmonary diseases involving various anatomical sites also occur (Das et al., 2003). These include the tonsils, lymph nodes, abdominal organs, and bones and joints (Beers and Berkow, 1999). Spread of bacilli from a primary focus of infection to remote anatomical sites, initiates extra-pulmonary disease. Despite the influx of a number of organisms, few survive since the large majority of bacilli are unable to initiate infection while others become dormant (Beers and Berkow, 1999). These remote infection loci may therefore provide microenvironments for adaptive bacterial evolution (Tanaka, 2004), which may generate tissue specific isolates. One such site is the spine and the vertebrae, intervertebral discs and epidural tissue associated with the spinal column.

1.2.1.4.1 Disease pathology

The spinal cord is the inter-digitating centre of motor, sensory and visceral activity in the central grey matter (Manz, 1983). Maintaining the integrity of the spinal cord is imperative as even small lesions cause significant neurological deficits (Manz, 1983). It is for this reason that spinal pathologies are so debilitating.

Infection of the spine is commonly through haematogenous (Wellons et al., 2004) or lymphatic spread from a pulmonary or, less commonly, a renal or gastrointestinal reservoir (Pertuiset et al., 1999, Rajaskeran, 2003). A direct blood supply to the inter-vertebral disc, via an extensive network of para-vertebral and intra-osseous collateral arteries, enables direct haematogenous spread of infection (Allison et al., 1999, Pertuiset et al., 1999). This secondary spread may be either a reactivation event or leaching from an active primary site. Direct infection from a local site occurs rarely (Cheung and Leong, 2003). Evidence for re-activation events was inferred following genotyping of multi-lesional TB-strains from pulmonary and extra-pulmonary sites (du Plessis et al., 2001). Exogenous re-infection is an additional route of disease acquisition in patients experiencing a second disease occurrence (Sharma and Mohan, 2004). Moreover, spinal TB patients often (55%) report previous contact with pulmonary TB-infected patients (Govender et al., 2000a). Two types of osteoarticular TB are described namely; a caseous, exudative abscess-forming type common to children and a granular type associated with adults (Kumar, 2005).

The infection initially involves the adjoining metaphyses and the vertebral disc but can spread to multiple, often successive, vertebral bodies (Rajaskeran, 2003). Infection of a single vertebral body may also occur (Allison et al., 1999, Sharma and Mohan, 2004). The thoraco-lumbar region is commonly involved however lumbo-sacral TB, although rare, occurs in 2-3% of all spinal TB cases (Govender et al., 2001b, Govender and Parbhoo, 1999, Govender et al., 2001a, Sharma and Mohan, 2004, Wellons et al., 2004) while discrete sacral infection is even more exceptional. Also uncommon is infection at multiple vertebral levels with just one such report involving the cervical, thoracic and lumbar spine in current literature (Turgut, 2001).

1.2.1.4.2 Clinical pathology of spinal TB

As with pulmonary TB, spinal infection is typified by granuloma and Langerhans giant cell formation (Cheung and Leong, 2003). Clinical features are also similar. However, the primary differences include a lack of pulmonary manifestation (cough), and localised back pain becoming increasingly incapacitating.

Recent infection manifests as extensive granuloma formation with accompanying fluid pus. As the disease progresses an abscess with caseous white pus forms. Abscess formation, a characteristic of advanced disease, presents as a para-vertebral swelling at the site of infection which may advance down the psoas muscle to the thigh with advancing disease (Beers and Berkow, 1999), (Lifeso, 1990). The abscess can take many forms, such as a retropharyngeal (cervical spine), fusiform/bulbous para-vertebral (thoracic spine), mediastinal, or psoas (lumbar spine) abscess (Sharma and Mohan, 2004). Extradural granulation tissue and extensive abscess formation are characteristic of both HIV-infected and -uninfected patients (Govender et al., 2001a). An abscess wall forms around the infected tissue in the large majority of cases (Govender et al., 2001a). Thus fibrosis effectively contains the infection forming a discrete granuloma.

However, with disseminated disease, a more diffuse pathology results. Mechanical back pain often accompanied by abscess formation, is followed by bone destruction (Cheung and Leong, 2003). Infected bone becomes softened and compressed due to pressure from adjacent uninfected vertebral bodies (Sharma and Mohan, 2004). The disease then infiltrates the bony cortex and in so doing, impedes the inter-vertebral blood supply resulting in necrosis and eventual collapse of the disc ensues. The most debilitating and unfortunate clinical presentation of spinal TB is paraplegia (Pott's paraplegia), which occurs in approximately a third of all spinal TB-infected patients (Sharma and Mohan, 2004). Bone destruction and collapse, is the primary cause of cord compression, neurological deficit and ultimately, paraplegia. This develops during the active phase of disease pathology (Sharma and Mohan, 2004). Therefore, progressive disease, inevitably, leads to collapse of the vertebral bodies and debilitating deformity (15% of cases), or severe deformity (3-5% of cases). Clearly, the clinical impact is devastating and disease resolution may not be as simple as administering conventional anti-TB chemotherapy (Govender, 2005).

Bone destruction and TB spondylitis is often seen in children (Govender and Parbhoo, 1999). Poor nutrition, delayed treatment and/or an impaired immune response, such as in HIV-infected patients, exacerbates bone destruction (Govender et al., 2001b, Govender et al., 2001a). Spinal compression and neurology occurs when pus, bone and disc fragments infiltrate the spinal canal and exert pressure on the spinal cord. Consequently, granulomatous inflammation coupled with TB spondylitis leads to secondary cord (Rajaskeran, 2003) angulations, compression (Manz, 1983) and deformity. Exudates from the granuloma may penetrate the ligaments, travelling along fascial planes, blood vessels and nerves to manifest as a cold abscess at remote sites (Sharma and Mohan, 2004).

Overall, children experience more disseminated pathology compared with adults (Lifeso et al., 1985), which is commonly associated with and exacerbated by HIV co-infection (Kabra et al., 2004). The distinctive physiology and immature immune systems of children make paediatric TB vastly different from that of adults. Paediatric disease is characteristically pauci-bacillary, and is often difficult to diagnose (Lodha and Kabra, 2004). It is also more aggressive with greater deformity and neurological deficit as compared with adults (Bailey et al., 1972, Fountain et al., 1975).

1.2.1.5 The immune response to infection

1.2.1.5.1 The stages of infection

Although primary infection can become active at any site in the body, it usually occurs in pulmonary apices. If untreated, an actively infected individual is capable of infecting from 10 to 15 others per year (WHO, 2004). Most intriguing is that only 5-10% of infected individuals develop active TB, and is usually associated with a compromised immune system (Davies and Grange, 2001a). Thus of the millions of individuals exposed to TB approximately 90% mount an effective immune response against the pathogen.

TB pathogenesis, as described in this section, refers to pulmonary TB. Infection is multiphased and is described as either a three (primary/initial, a latent, and a reactivation) (Beers and Berkow, 1999) or four (Iseman, 2000) phased model. The latter model will be discussed in this review of the literature. Briefly, Phase I refers to the transmission of infection and is dependant on functional alveolar macrophages, the virulence of the bacilli and their robustness. Phase II is when the infection initiates, bacilli proliferate culminating in the spread of infection locally, to the hilar lymph nodes and systemically. Dissemination of infective bacteria occurs via several mechanisms such as lysis of infected macrophages (Ehlers et al., 2003) through either necrosis or apoptosis (Russell, 2001a). This is also the phase during which the immune response is activated, specifically the humoral immune response, and a burst of activity to phagocytose the bacteria as well as attract immune-effector cells to the site of infection ensues. Unfortunately, the process of phagocytosis also provides a breeding ground for the bacilli within macrophages. The cellular immune response or cell-mediated immunity (CMI) is initiated during Phase III. Antigen presenting cells (APC) play a vital role at this stage, not only attracting immune cells to the site of infection but also activating T-cells to release cytokines such as interferon-gamma (IFN- γ) that stimulate, attract and activate a host of other immune cells important to the anti-TB immune response. If the immune response is robust, the bacterial load plateaus, eventually declines and the primary infection resolves (Raja, 2004, Tanaka, 2004). The infection, in the immuno-competent host, is effectively halted at this point and is characterised by a dermal delayed-type hypersensitivity reaction but an absence of clinical manifestation (Fenhalls et al., 2000). The infecting bacilli are not eliminated, rather become sequestered in discrete lesions or granulomas (Kaufmann, 2002, Raja, 2004). Bacilli may remain latent within these granulomas for an extended period or may be reactivated or released into the airways. Since bacilli are still present within the host, the risk of disease persists (Kaufmann, 2002). Clinical disease may develop within months or years once infection is established (Beers and Berkow, 1999). In contrast, the immuno-compromised host develops primary disease immediately following exposure (Kaufmann, 2002). This constitutes Phase IV. For a more detailed description the reader is referred to the publication by Iseman (2000).

1.2.1.5.2 Granuloma formation

Granuloma formation is the hallmark of an effective anti-TB immune response. Granulomas are widely associated with tuberculous, pathogenic, meningial or opportunistic infections, such as mycoses (coccidiomycosis and histoplasmosis) (Williams, 1991). Other causative agents include organic or inorganic particulates, foods and chemical agents.

Granulomas are multi-cellular structured masses (Mornex et al., 1994) and form unique microenvironments, each with their own unique cytokine expression profiles (Fenhalls et al., 2000). The granuloma is a layered structure, consisting of a centre of necrosis, and radiating outwards, epithelioid histiocytes, giant cells, lymphocytes and finally a fibrotic cuff. Poly-morphonuclear leukocytes may line the cavity wall (Fig. 1.1). Histologically, the central core of necrosis appears caseous (Roitt et al., 2001). Langerhans Giant cells (LGs), collagen fibrils, lymphocytes and a sparse distribution of plasma cells occur commonly within these structures while epithelioid cells, macrophages and giant cells occur towards the granuloma core of necrosis. Granulomas are not restricted to pulmonary tissue, as they develop in every organ system of the body (Ioachim, 1983, Mornex et al., 1994). Regardless of their location, the mechanisms that govern granuloma development are the same (Ioachim, 1983). A granuloma will persist until the pathogenic or irritant particle is destroyed, or resolved, as much as possible, after which the granuloma resolves (Adams, 1983).

Granuloma formation, which is induced and maintained by selected cytokines, is essential to disease containment (Kaufmann, 2002). The process is a complex spatial-temporal interaction between the TB bacilli, representative host immune cells (CD4+, CD8+ T-cells, macrophages) and effector molecules (cytokines and chemokines) (Ioachim, 1983, Sergovia-Juarez et al., 2004). This phenomenon is dependant on the migration to and aggregation of macrophages at the site of infection, occurring as rapidly as 24-hours post-infection (DesJardin et al., 2002b). Factors essential to modulating granuloma formation include chemokine diffusion, prevention of macrophage overcrowding, temporal/ spatial distribution and density of lymphocytes, and macrophage activation (Sergovia-Juarez et al., 2004). Finally, fibrous encapsulation of the necrotic focus is the last step in the immune response towards disease containment

(Raja, 2004) and occurs when fibroblast and extra-cellular matrix deposits around and within the granuloma (Mornex et al., 1994). This lymphocytic layer surrounding the granuloma is comprised predominantly of CD4+ T-cells but may also contain dendritic cells (DC), and epithelioid cells (Raja, 2004).

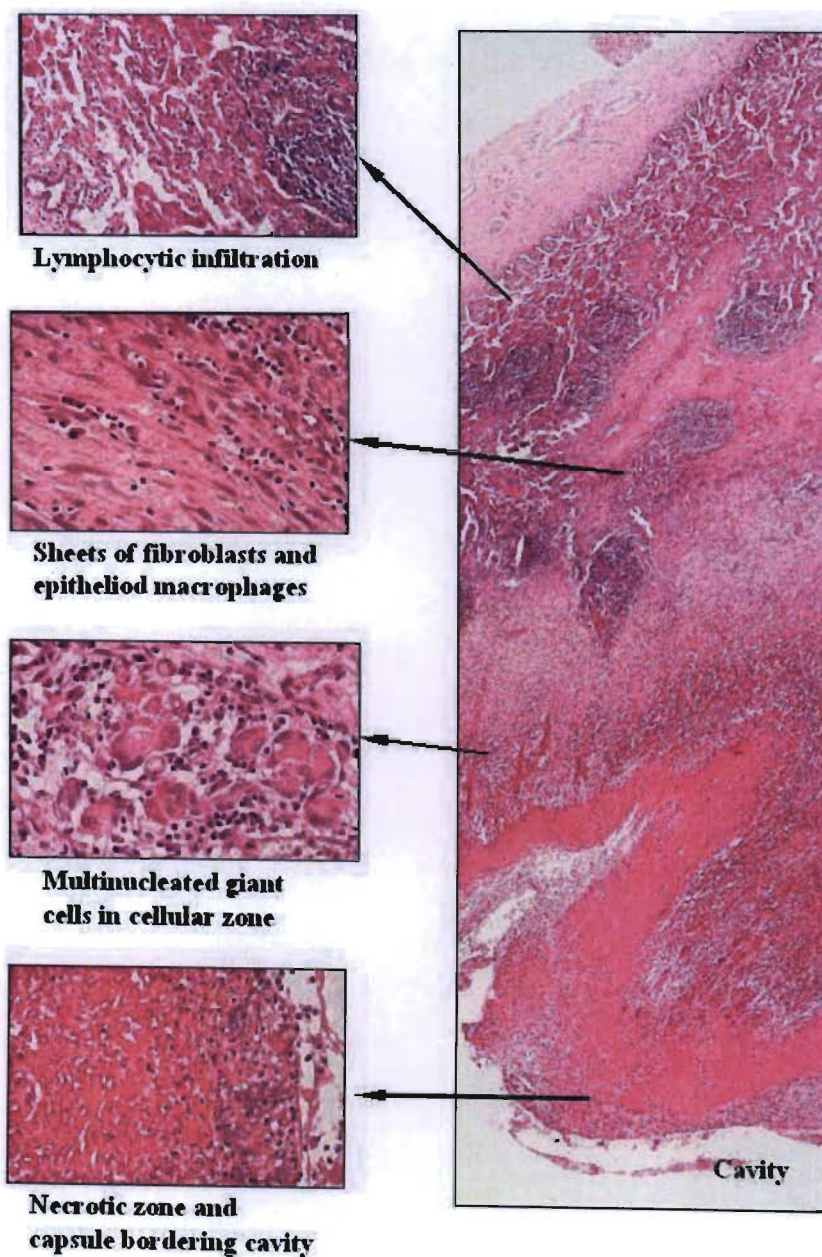


Figure 1.1: Light micrograph of a typical pulmonary TB granuloma illustrating distinct cellular and a-cellular zones (with kind permission Prof J. McKinney (McKinney, 2004))

1.2.1.5.3 Immune cells involved in the anti-TB response

Granulomas centre on the activity of monocytes or mononuclear phagocytes as well as lymphocytes, plasma cells, myofibroblasts, B-cells and eosinophils, which migrate to the site of inflammation (Qiu et al., 2001).

Macrophages are the scavengers of the immune system, and have evolved to phagocytose and dispose of pathogens and other foreign particles (Roitt et al., 2001). The unique and varied functions of macrophages, from phagocytosis to antigen presentation, are determined by their local microenvironment. Macrophages display extensive morphological heterogeneity and differentiate into epithelioid cells, for example, following stimulation (Holness and Simmons, 1993). Macrophages may also fuse together to form a multi-nucleated giant cell (Adams, 1976). Macrophages are highly motile but, may be tissue or organ specific (Holness and Simmons, 1993). They display effective microbicidal activity if activated by $\text{IFN}\gamma$, prior to a TB infection (Russell, 2001a). Tissue macrophages are long-lived cells that are encountered on infection by the TB bacillus, therefore, are in constant communication with various cellular populations involved in the immune response (DesJardin et al., 2002b). Functional differences between adherent and non-adherent cultured monocytes are major determinants of infectivity and intra-cellular growth (Barker et al., 1996).

A primary function of macrophages remains their phagocytic activity and the CD68 antibody has been used to localise phagocytic/endocytic cells such as macrophages of the germinal centre, tonsils, splenic red pulp, dermal connective tissue, Kupffer cells from the liver, alveolar macrophages and blood monocytes (Pulford et al., 1989, Roitt et al., 2001). The molecule belongs to the family of lysosomal/endosomal associated trans-membrane proteins (LAMP) (Holness and Simmons, 1993). Several studies have attempted to define CD68 function. It is proposed that this protein aids in macrophage localisation to tissue and organ specific sites by binding to selected lectins and selectins (Holness and Simmons, 1993). Others have associated CD68 proteins with class-A scavenger receptor (SR-A) protein expression in macrophages (Fenhalls et al., 2000, Gough et al., 2001). Furthermore, it has been proposed that CD68+ cells secrete $\text{IFN}\gamma$, within TB granulomas to enable macrophages to auto-regulate their localised activation (Fenhalls et al., 2000). However, $\text{IFN}\gamma$ has no significant up-regulatory effect on

CD68 expression (Holness and Simmons, 1993).

Although the initial phase of infection is characterised by macrophages phagocytosing the TB bacilli, they are not activated and thus cannot destroy the pathogen but rather play an effector role by initiating the immune response and presenting the TB antigens to T-cells (Iseman, 2000). Activated T-cells rapidly migrate to the site of infection to interact with antigen presenting cells (APCs) and effectively contain the spread of infection (Raja, 2004) (Table 1.2). Following phagocytosis by macrophages, MHC class II molecules present the bacterial Ags to CD4⁺ T-cells (Raja, 2004). The T-cell populations, involved in the immune response, are predominantly T-helper type1 (Th1) cells (Table 1.2) (Kaufmann, 2002). Both Th1 and Th2 responses have been detected in TB-infected human and in animal models (Raja, 2004). Th1-secreted cytokines (IL-2, IFN- γ) play a protective role, while that of Th2-cytokines (IL-4, IL-5, IL-10) may be extraneous or negative to the immune response (Raja, 2004). Moreover, the intensity of the Th1 response can be correlated with clinical disease pathogenesis (Raja, 2004). Favourable clinical outcomes were shown in patients with detectible granuloma IL-4 levels by immunohistochemistry (IHC) (Fenhalls et al., 2000).

CD4 cells (Table 1.2) are vital to the anti-TB immune response (Fenhalls et al., 2000). In CD4-depleted patients, reactivation of latent TB often produces a fatal pathology with increased bacterial numbers. CD8⁺ T-cells are also involved in the anti-TB immune response as early as 12 hours post-infection. These cells are initiated sooner than the CD4⁺ cells, and have been implicated in the lyses of infected macrophages and dendritic cells (Raja, 2004, Stenger et al., 1997). Their presence correlates with slower disease progression (Yu et al., 1995).

Table 1.2: T-cell populations involved in the immune response against TB

T-cell	Mode of action
CD4	Recognise Ags processed & presented by MHC class II Produce lymphotoxin α (LT α), cytokines eg. IFN- γ
CD8	Recognise Ags processed & presented by MHC class I Secrete perforin, granulysin and cytokines eg. IFN- γ
Gamma/delta T-cells	Recognise unusual Ags not processed/presented by MHCs Targets infected macrophages
CD1	Recognise glycolipids presented by CD1 molecules Secrete perforin and granulysin – targets infected macrophages

Adapted from Kauffman (Kaufmann, 2002)

1.2.1.5.4 Chemokines and cytokines

Cytokine and chemokine expression profiles have been established for pulmonary TB in various animal models and human studies (Collins, 2001, Collins and Kaufmann, 2001b, Davies and Grange, 2001a, Davies and Grange, 2001b, Rhoades et al., 1995, Shigenaga et al., 2001) have established cytokine and chemokine expression profiles for pulmonary TB. These profiles differ between the early and late phases of disease (Rhoades et al., 1995). However, to our knowledge, only two reports of the immune response to spinal TB (Govender et al., 2000a, Govender et al., 2001a) are presently available.

Chemokines and cytokines are essential to granuloma formation and maintenance (Adams, 1983, Kaufmann, 2002, Qiu et al., 2001) serving either a synergistic or antagonistic function by fulfilling one of three roles namely as simulators of hematopoiesis, regulators of the innate or adaptive immune responses. A cascade of cytokines and chemokines characterize the immune response against TB (Table 1.3). Interferon gamma (IFN γ), an important component of the immune response, is produced by macrophages (Table 1.3 and 1.4), activated T-, B- and NK cells (Ehlers, 2003). Fenhalls et al, (Fenhalls et al., 2000) demonstrated TNF- α , IFN- γ and IL-4 expression in CD68⁺ myeloid cells. These were novel findings, since IFN- γ and IL-4 are usually

expressed by T-cells. Tumour necrosis factor (TNF) plays a role in apoptosis, cell activation, recruitment and differentiation serving a protective role against disease (Kaufmann, 2002). Sustained disease containment is also mediated by TNF α since it is involved in continuous recruitment of inflammatory cells (Ehlers et al., 2003). Any disruption in TNF α production, at any stage of disease progression, would severely retard the immune response and result in disseminated, uncontrolled infection (Fenhalls et al., 2000). Moreover, latent TB is reactivated and primary infection is aggravated in its absence (Ehlers et al., 2003). Conversely, TNF has been shown to aggravate the immuno-pathology of TB (Roach et al., 2002) while anti-TNF therapy (thalidomide) resulted in clinical improvement (Tramontana et al., 1995). In addition, IL-10 influences a decrease in the lytic activity of CD4 and CD8 cells and inhibits MHC cytotoxicity against infected cells (de la Barrera et al., 2004). Finally, varying expression profiles of other cytokines associated with lymphocyte-mediated cytotoxicity such as IFN- γ can be demonstrated between healthy and TB infected ($p < 0.03$) individuals (Toossi et al., 2004b). For a more detailed description of cytokines, chemokines and their role in the anti-TB immune response, please consult the publications of Iseman (2000) and Raja (2004a).

1.2.1.5.5 Antimicrobial response

Included among the antimicrobial arsenal of macrophages is the production of reactive oxygen intermediates (ROI) e.g. hydrogen peroxide (H₂O₂) and reactive nitrogen intermediates (RNI). Darwinian evolution of Mtb to combat the effect of NO toxicity (Chan et al., 2001) has produced currently circulating TB isolates. Nitric oxide synthase (NOS) and reactive nitrogen intermediates (RNI) are potent, and arguably the best studied mechanisms of anti-microbial activity in macrophages (Chan et al., 2001). The primary mediator NOS₂, is essential for Mycobacterial control in the acute and chronic phases of disease. A human control mechanism involving RNIs is more ambiguous requiring further elucidation, unfortunately, related investigations are complicated by a lack of an appropriate *in vitro* model (Chan et al., 2001).

Table 1.2: Immune modulators important to the host immune response against TB

Immune modulator	Function	Secretory cell / organ
IFN- γ	Activate macrophages, regulate Th1/Th2 balance, protects against T-cell apoptosis, intracellular infection, enhance Ag presentation, correlated with stage of disease and nutritional status	CD4 ⁺ , CD8 ⁺ and NK cells
TNF- α	Promotes migration of cells to infection, modulates expression of adhesion, cytokine and chemokine molecules, mediates granuloma formation, contains/ sequesters infection	Macrophages, DCs, T-cells
IL-1	Important for biological defence mechanisms. In conjunction with TNF- α modulates acute phase of disease (fever, cachexia), enhances IL-2 and IL-2 receptor expression	Monocytes Macrophages
IL2	Protects T-cells against apoptosis, intracellular infection, mediates expansion of Ag specific lymphocytes	CD4 ⁺ T-lymphocytes
IL-6	Involved in inflammation, hematopoiesis and T-cell differentiation, may be important to initial immune response	
IL10	Negative or no effect on immune response, deactivates macrophages by down-regulating IL-12 and thereby IFN- γ , inhibits CD4 ⁺ T-cells	Macrophages, T-cells
IL12	Possible role in Th1/Th2 cellular differentiation; induce Th1 response in undifferentiated cD4 cells	
TGF- β	Important anti-inflammatory, deactivates macrophage production of ROI and RNI; inhibits T-cell proliferation, impedes NK and CTL function, down regulates IFN- γ , IL-1 and TNF- α ,	Monocytes
IL-8	Recruits neutrophils, T lymphocytes and basophils,	Monocytes / macrophages, uncommonly - fibroblasts, keratinocytes
MCP-1	Decrease during convalescent phase of disease	
RANTES	Decrease during convalescent phase of disease	Macrophages
MIP1 α , 2, MCP-1, -3, -5, IP10	Formation and maintenance of granuloma	Macrophages

Adapted from Raja, 2004 (Raja, 2004)

Phagosome-lysosome fusion and intra-lysosomal acidic hydrolase degradation is a primary mechanism of TB elimination (Raja, 2004). Yet another response to TB infection is apoptosis. Apoptosis, or programmed cell death, was demonstrated in TB-infected monocyte/macrophages, particularly from HIV-infected patients (Mustafa et al., 2005, Placido et al., 1997). The induction of apoptosis is dose dependant and 3-fold greater in TB-infected patients compared with normal controls. Markers of apoptosis such as caspase 1 and 8 are strongly associated with levels of caspases and with HIV-infection (Wanchu et al., 2004). Others have correlated levels of caseation with macrophage and T-cell apoptosis (Fayyazi et al., 2000). They showed that regressive granulomas lack apoptotic cells, while active granulomas with caseous necrosis, contain numerous apoptotic CD68+ macrophages and T-cells (Fayyazi et al., 2000).

1.2.1.5.6 Host genetic and environmental factors

With the successful deciphering of the human genome, a number of genomic regions conferring resistance/susceptibility to a range of infections have been described (Kwiatkowski, 2000). Mounting evidence implicates host genetic factors as primary determinants of susceptibility to infection and risk of developing acute pathology (Kwiatkowski, 2000). In relation to TB, these factors include diabetes, age, gender, ethnicity and integrity of immune response (Bellamy, 2003, Bellamy et al., 1999, Bellamy et al., 1998, Selvaraj et al., 2000, Selvaraj et al., 1999, Selvaraj et al., 1998). Other contributors include stress, poor living conditions, co-infections, malnutrition, vitamin deficiency and natural immunity (Zajac and Melcher, 1991). In addition the human leukocyte antigen (HLA) (Raja, 2004) (Table 1.4) plays an important role in host susceptibility to infection. Associations between TB infection and HLA types have been demonstrated (Table 1.4) (de Vries, 1989, Dubaniewicz et al., 2000, Dubaniewicz et al., 2005, Lombard et al., 2006), (Ravikumar et al., 1999, Selvaraj et al., 1998, Shanmugalakshmi and Pitchappan, 2002).

Table 1.3: Summary of the HLA alleles implicated in the host response to TB infection

Reference	Nationality of cohort	HLA types conferring susceptibility	HLA types conferring resistance
(Lombard et al., 2006)	South Africa	HLA-DRB1 HLA-DQB1	VDR1
(Dubaniewicz et al., 2003)	Polish	HLA-B62(15) HLA-Cw5	-
(Vejbaesya et al., 2002)	Thai	HLA-DQB1*0502	HLA-DQA1*0601 HLA-DQB1*0301
(Shanmugalakshmi and Pitchappan, 2002)	Indian	HLA-DRB1*02 HLA-DRB1*1501	-
(Dubaniewicz et al., 2000)	Polish	HLA-DRB1*16	HLA-DRB1*13
(Ravikumar et al., 1999)	Indian	HLA-DRB1*1501 HLA-DQB1*0601	HLA-DPB1*04

1.2.1.5.7 Latency and immune evasion

As previously stated, macrophages are essential to containing and eliminating a TB infection. However, selected bacilli are able to evade the host's natural defence mechanisms and survive within intracellular phago-lysosomes (Fossati et al., 2003). Determining how this is achieved remains a challenge to researchers. This is because the bacillus has to avoid, not only the complex and varied immune responses directed against it, but also the antagonistic intra-lysosomal environment, and possible concurrent chemotherapy (Fenhalls et al., 2002b).

Granulomas may also represent a significant reservoir of latent infection (Karakousis et al., 2004). During latency, the live bacilli exist in a non-replicating, hypo-metabolic state, which may persist for several years (Karakousis et al., 2004). Mycobacteria either evade or diminish immune induction and response once it is activated (Russell, 2001b, Russell, 2001a). The bacilli have devised various mechanisms (Table 1.5) to survive within macrophages (Chan and Flynn, 2004, Russell, 2001b, Russell, 2001a). In addition, mechanisms invoked during this process prevent the activation of a localised host immune response and consequently Mycobacterial killing. Reports have described

bacterial induction of a basic pH within lysosomes that is also associated with lower vacuolar ATPase levels (Sturgill-Koszycki et al., 1994). Others have characterised genes that enhance intracellular survival in macrophages (Dahl et al., 2001, Wei et al., 2000) or have implicated various factors such as calmodulin dependent Ca^{2+} signalling, Ag processing and lipoarabinomannan (LAM) (Chan and Flynn, 2004, Karakousis et al., 2004) but the actual mechanism of induction of latency remains ill-defined.

Table 1.4: Strategies of immune evasion employed by TB bacilli

Immune evasion	TB mechanism	Reference
Alter antigen presentation by macrophages / APCs	Induce cells to produce TGF- β , IL-10, IL-6	(Hmama et al., 2004)
Modulate antigen presentation		(Raja, 2004)
Scavenge oxygen radicals	Sulphatides, LAM, pholic-glycolipid-I	(Chan et al., 2001)
Inhibition of neutrophil migration	Disruption of glycolytic enzyme phosphofructokinase	(Anderson et al., 1991)

1.2.1.6 Treatment of TB

Current interventions comprise a 4 drug-cocktail (ethambutol hydrochloride, isoniazid, pyrazinamid and rifampicin) for 2 to 9 months, typically over a short course of 6 months (Cunningham and Spreadbury, 1998, Lodha and Kabra, 2004). The patient is rendered non-infectious within 10-14 days following initiation of treatment (Beers and Berkow, 1999). Prophylactic interventions are administered to individuals with a recent TB infection who are most likely to develop active disease, such as children, young adults (<25 year old) and the elderly (Beers and Berkow, 1999) but is strongly indicated in HIV-infected individuals. However, adherence to protracted regimes and misuse or abuse of drugs is a persistent concern, as is the resultant emergence of drug resistant bacterial variants. Delays in accessing treatment impacts disease progression cure rates, mortality and morbidity. The primary reasons for delayed interventions include

patient stigmatisation and a propensity of many in developing countries to seek traditional rather than conventional medicine. The emergence of drug resistant strains is thus inevitable in these instances. In developed countries multi-drug resistant TB (MDR-TB) accounts for more than 15% of all infections while 6 000 of the 500 000 current TB infections (1.2%) in South Africa, are drug resistant (Sidley, 2006).

1.2.1.6.1 Treatment, surgical interventions and complications of spinal TB

The preferred treatment regimes for TB spondylitis are usually conservative (rifampicin, isoniazid, pyrazinamide and ethambutol) for up to 18 months (Beers and Berkow, 1999, Govender et al., 2001b, Govender and Parbhoo, 1999). However, in certain instances, surgical intervention is necessitated, and is only undertaken once patients achieve clinically acceptable haemoglobin (>100g/l) and albumin (>300g/l) levels, usually within 5-9 weeks of sound nutritional support (Govender et al., 2001a). The indications for surgery vary but is not influenced by HIV status and/or CD4 count, treatment response, or severity of neurological deficit (Kumar, 2005). Surgery is often necessary in paediatric cases since the deformity usually does not resolve after anti-TB chemotherapy but advances with growth (Rajaskeran, 2003). During surgery, diseased bone and tissue is removed, and a femoral allograft containing morsellised intramedullary rib harvested from the patient is inserted (Govender and Parbhoo, 1999, Govender et al., 2001a). Fixation or insertion of instrumentation is only indicated in patients with advanced deformity (Beers and Berkow, 1999).

Outcomes following surgery are favourable regardless of HIV status and may relate to the immune response stimulated following allograft insertion (Govender et al., 2007, Govender et al., 2001b, Govender et al., 2001a). Paediatric patients show allograft incorporation within 18-24 months compared to 3-5 years in adults (Govender et al., 2001b). The accelerated incorporation of the allograft among children can be attributed to a greater osteogenic potential stimulated by the intra-medullary rib material. Neurological recovery within 6 months post-surgery has been reported in a cohort of South African children (Govender and Parbhoo, 1999). Unfortunately no indication of the patient's HIV status was presented therefore the impact of HIV co-infection on post-surgical recovery rates could not be assessed. In general, outcomes depend on the

degree and duration of cord compression as well as the age and general clinical status of patients (Kumar, 2005). Again, this report (Kumar, 2005) did not describe the impact of HIV co-infection on neurological and clinical recovery rates. Few postoperative complications associated with malnutrition, diabetes or neutropenia have been reported in patients treated at the King George V Hospital, KZN independent of HIV status (Govender et al., 2001a).

1.2.1.7 Diagnosis of TB

Diagnostic assays are significant to clinical outcomes by directing treatment initiation or conclusion (Sharma and Mohan, 2004). It is only through cautious and appropriate diagnostic procedures, coupled with early initiation of anti-TB chemotherapy, that TB can be treated with any degree of success (Sharma and Mohan, 2004).

Presumptive diagnosis of infectious spondylitis based on clinical and radiological presentation rather than conventional histopathology often directs treatment, since laboratory results are indeterminate, in most instances. Diagnosis is often based on presentation of backache, fever and malaise (Sinan et al., 2004). Magnetic resonance (MR) imaging, a common diagnostic tool (Beers and Berkow, 1999, Sinan et al., 2004) also has limitations particularly in drug-resistant cases (Sharma et al., 2003), but still offers exceptional visualisation of the TB lesion and the best likelihood of an early diagnosis (Sinan et al., 2004).

In terms of its laboratory diagnosis, there still remains a dependency on traditional diagnostic methods such as the Mantoux skin test, microscopy (Ziehl-Neelsen and/or fluorochrome staining), radiography, liquid/solid culture identification and gastric lavage (Garg et al., 2003). Unfortunately, the findings of these laboratory assays are often inconclusive or negative (Garg et al., 2003). In addition these assays are protracted often yielding results several weeks after patients have first presented, further exacerbating patient morbidity and mortality. The most common TB diagnostic assay, the tuberculin skin test has been utilized for more than 85 years (Garg et al., 2003), despite its associated interpretive difficulties in BCG vaccinated individuals. In addition, false negatives in a non-Mtb infection (Garg et al., 2003) pose further

challenges. This is further complicated by regional differences in host genetic factors and exposure profiles to endemic bacteria (de O Liverato et al., 2004). Moreover, childhood TB is diagnostically challenging due to difficulties in obtaining an adequate clinical sample (Kabra et al., 2004).

1.2.1.8 Mycobacterial identification

Apart from clinical features, the most common diagnostic tools presently employed include microscopy (Haematoxylin & Eosin, Ziehl Neelsen techniques), TB culture, enzyme linked immunosorbent assay (ELISA) and molecular techniques (Mukherjee et al., 2002). The current diagnostic gold standard is demonstration of Mycobacteria in body fluids such as blood, urine and broncho-alveolar lavage (BAL) fluid (Lodha and Kabra, 2004) by microscopy or culture. Often additional assays are performed to identify organisms that are indeterminate by conventional means. As Scarparo et al (Scarparo et al., 2002), demonstrated only a fraction of the total number of clinical samples is successfully grown in culture with the result that a large proportion (>60%) of infections remain undiagnosed. Improved culture techniques include the BACTEC radiometric assay, Septicheck AFB system and the Mycobacterial growth indicator tube system (MGIT) (Kabra et al., 2004). The MGIT, which uses a fluorescence based detection system, relies on Mycobacterial growth for a positive result (Kabra et al., 2004). However, it carries a high potential for false negative findings.

Newer diagnostic approaches exploit molecular techniques, such as the polymerase chain reaction (PCR) and include restriction fragment length polymorphisms (RFLP), fluorescence amplified fragment length polymorphisms (FAFLP) (Ahmed et al., 2003), molecular beacon assays and sequencing (Cloud et al., 2002). Single base differences using molecular beacon technology enables the pathogen identification (de Baar et al., 2001, Martin et al., 1993, Patel et al., 2000, Tortoli et al., 2001), provides an understanding of their infectivity, pathogenicity (Rhee et al., 1999) and drug resistance patterns. It is cautioned, however, that a negative PCR diagnosis can never completely discount a TB infection nor can a positive result be conclusive for a positive TB infection (Lodha and Kabra, 2004). Despite these advances, identification is still dependant on bacterial cultures. It may, therefore, prove useful to genotype the

infective organism directly from the clinical samples, as this translates to obvious and significant benefits to the patient.

Comparative studies have consistently demonstrated the superiority of sequencing over conventional identification techniques where the former showed improved specificity and sensitivity (Cloud et al., 2002, Martin et al., 1993, Patel et al., 2000, Tortoli et al., 2001). Several genomic sites have been examined including the hsp65, and 16-23S internally transcribed spacer (ITS), but the 16S rDNA region (Cloud et al., 2002) provides the most favourable target. This region is highly conserved across the genus, but still retains sufficient base differences to be phylogenetically relevant. In addition, the 16S genome is common to all Eubacteria, (Zumarraga et al., 1999), therefore may be applied as a general diagnostic tool. Unfortunately, the foremost restriction to implementing newer diagnostic technologies, particularly in developing nations, is their high cost. In addition, improved sensitivity is not always complemented by greater specificity, necessitating the expansion of more attractive alternative diagnostic tools.

1.2.2 Human Immunodeficiency Virus (HIV)

1.2.2.1 Historical perspective

The Human Immunodeficiency Virus (HIV) is undoubtedly, *the* definitive disease condition of the present generation, the likes of which had not been seen since the advent of modern medical practices. The first cases of HIV were documented in 1981 among homosexual males, exhibiting severely compromised immune responses (Fauci, 2003, Gottlieb et al., 1983). Their point of convergence was severely depleted CD4+ T lymphocyte counts. Later, recognition of similar symptoms among the greater population implied a common infectant subsequently identified as HIV.

1.2.2.1.1 The origins of HIV

Phylogenetic evidence places the origin of the virus in Central West Africa in 1950 while epidemiological data established HIV in the US as early as 1978 (CDC, 1987).

Much controversy however, still persists regarding the origins of the virus. Subsequent studies comparing the HI virus with the simian immunodeficiency virus (SIV) confirmed two zoonotic transfection events (Fauci, 2003, Van Heuverswyn et al., 2006) from the *Pan troglodytes troglodytes* (chimpanzees) to humans. Recently, *P.t.troglodytes* was identified as the natural reservoir of HIV-1 Group M and N subtypes (Keele et al., 2006) while viruses resembling Group O variants were isolated from gorillas in Cameroon (Van Heuverswyn et al., 2006). The origin of HIV-1 group M was estimated at between the 1920's and 1930's, while, according to phylogenetic and mathematical modelling, the zoonotic transfection to humans is purported to have occurred approximately 300 years ago (Korber et al., 2000, Salemi et al., 2001). However, it is argued that this estimate represents the coalescence of the simian ancestor of HIV-1 group M and its closest SIV_{cpz} ancestor (Salemi et al., 2001). The actual infancy of the HIV epidemic may have effectively been shrouded by the remote setting of its origins in Central and West Africa, and the lack of infrastructure and communication in this region. Similarly, molecular modelling places the origins of Group O isolates at a similar time frame as group M, at around 1920 (1890-1940) (Lemey et al., 2004).

1.2.2.1.2 Circulating forms of HIV

Two main forms currently circulate namely, HIV-1 and HIV-2. HIV-1 is, further, classified into 3 main groups; M (main), O (outliers) and N (non-M/ non-O) viruses. Group M viruses account for almost all of the HIV infections worldwide and are divided, based on variation in the envelope (*env*) and *gag* genes, into subtypes ranging from A-J. Several recombinant forms that merge 2 or more subtypes (Collins et al., 2000), (Robertson et al., 1991) presently circulate worldwide. Until the late 1980's HIV-1 subtype B was predominant worldwide, but HIV-1 subtype C began an explosive outbreak in southern Africa and Ethiopia (Gordon et al., 2003) that has yet to subside. Many factors have been proposed to account for this explosive increase in subtype C infections including greater diversity at the protease cleavage sites (De Oliveira et al., 2003), an extra NF- κ B site in the long terminal repeat (LTR), greater stability of C viruses, viral attenuation (Ball et al., 2003) and a 5 amino acid insertion in the *vpu* region (Alimonti et al., 2003). These factors remain speculative, as no conclusive

account for the advance in the HIV subtype C epidemic, through Africa, Asia and Eastern Europe, has been proven. While HIV-1 has swept throughout the world, HIV-2 remains concentrated in West Africa (Shanmugam et al., 2000). HIV-2 is less virulent and infective compared with HIV-1 and has even been shown to reduce the infectivity of the latter (Kanki et al., 1996), (Nkengasong et al., 2000). In addition, survival rates are significantly greater in HIV-2 compared with HIV-1 infected individuals (Marlink et al., 1994).

1.2.2.2 HIV epidemiology

1.2.2.2.1 The global epidemic

At the end of 2001, approximately 40 million people globally were living with HIV (Fig 1.3) (UNAIDS, 2006). Worldwide, a cumulative total of more than 60 million people up to 2003, have been infected with HIV since it was first described in 1989, almost a third of whom have since died (Fauci, 2003).

Initially the epidemic was concentrated in North America, Western Europe and sub-Saharan Africa, but has spread to encompass all nations of the world (Shankarappa et al., 2001). Of the current infections, 95% occur in developing nations (Chakraborty, 2005). Presently sub-Saharan Africa has, by far, the highest incidence and prevalence rates globally (Karim, 2000, UNAIDS, 2006). India has the world's second largest HIV-infected population of 4.58 million people following an introduction estimated to have occurred in the mid-1980's (Shankarappa et al., 2001). The Indian epidemic is further compounded by TB co-infection (Zaidi et al., 2004). Approximately a third of people living with HIV/AIDS are between the age of 15-24 years constituting an economically important demographic that includes women of childbearing age (Chakraborty, 2005). Most infected individuals do not know their status!

Meeting the millennium goal of a two third reduction in child mortality by 2015 cannot be achieved if the present rate of paediatric disease is not stemmed or controlled (Zaidi et al., 2004). Mother-to-child transmission (MTCT) rates ranged from 15-20% in Europe, 15-30% in the USA and 25-40% in Africa in 1999 (McIntyre and Brocklehurst,

1999). Globally, approximately 600 000 new infections occur annually, 1500 occurring daily in sub-Saharan Africa, equating to a 25-42% vertical transmission rate in the region (Chakraborty, 2005). The Indian epidemic is heterogenous with regional differences in patterns of spread (Zaidi et al., 2004). It is said that HIV and AIDS 'increasingly has a woman's face' (Pope and Haase, 2003) since heterosexual transmission has increased such that half the infected adults are female of childbearing age (Chakraborty, 2005). Of these women, 77% reside in sub-Saharan Africa (UNAIDS, 2006).

Despite the fact that the majority of HIV-infected persons worldwide are infected with HIV-1 subtype C (Shankarappa et al., 2001) investigation of HIV immuno-pathogenesis and molecular evolution has centred on subtype B (Mani et al., 2002). It is thus primary to the development of vaccines and appropriate therapies that studies be conducted in subtype C endemic regions such as the local KZN setting. The alarming and unfortunate truth is that the magnitude of the HIV/AIDS pandemic has yet to be realised (Fauci, 2003).



Figure 1.3: Global map illustrating estimates of HIV infection in adults and children as of 2003.

1.2.2.2.2 The South African epidemic

The South African HIV epidemic began much later than others in the region but has since exploded to constitute one of the highest infection rates in the world. From less

than 1% prevalence among pregnant women attending antenatal clinics in 1990 the number of pregnant women infected with HIV expanded to 25% in 2001 (DOH, 2002). In 2004 the prevalence rates in South Africa ranged from 15.4% in the Western Cape Province to 40.7% in KwaZulu-Natal (DOH, 2004). The national average increased from 26.5% (95% CI = 25.5-27.6%) in 2002, and 27.9% (95% CI = 26.8-28.9%) in 2003 to 29.5% (95% CI = 28.5-30.5%) in 2004 (DOH, 2004). By the year 2000, HIV-related deaths were the leading cause of mortality among young adults in the country (Dorrington et al., 2002). Current figures indicate 5.5 million South Africans were living with HIV/AIDS in 2005, which is a prevalence rate of 18.8% (Global Health, 2006).

1.2.2.3 The HIV genome

HIV comprises a 9kb genome, which codes for 9 gene products divided into 3 structural and 6 regulatory/accessory genes. These genes encode more than 15 gene products (Fauci, 2003). HIV-1 produces three transcripts of 9kb, 4-5kb and 2kb in length, which following splicing produces 30 different mRNA's (Fauci, 2003, Kuiken et al., 2002) and associated gene products. The structural (*gag*, *pol*, *env*), regulatory (*tat*, *rev*) and accessory genes (*vif*, *vpr*, *vpu*, *vpx*, *nef*) (Kuiken et al., 2002) can be viewed in greater detail in Appendix A. The *env* gene, codes for the viral glycoprotein, the external glycoprotein gp120 and the transmembrane protein gp41 (Kuiken et al., 2002). This gene product is important since it contains the binding site for the CD4 receptor and the amino acids that determine the co-receptor usage for viral entry (Kuiken et al., 2002). In addition, viral tropism is determined by specific amino acid profiles of the V3 region of the *env* gene.

1.2.2.3.1 HIV clades

HIV evolution is dynamic, typified by the rapid radiation of HIV-1 M strains into individual subtypes or clades (Robertson et al., 1991, Weiss, 2003). Indeed clades differ by 10 to 25% of their genetic material (McMichael and Hanke, 2003).

Regional and country differences within clades have been demonstrated (Shankarappa et al., 2001). The analysis of HIV-1 subtype C sequences from 23 countries including India, South Africa, Zimbabwe, Burundi and Botswana by Shankarappa et al (Shankarappa et al., 2001) revealed a significant ($p < 0.0001$) country-dependant distribution. The sequences from South Africa and Zimbabwe, furthermore, display scattered phylogenetic distributions, indicative of introductions from numerous lineages (Gordon et al., 2003, Shankarappa et al., 2001). The Indian epidemic, on the other hand, exemplifies either a founder effect or limited spread of new introductions through the population. Of the HIV-1 subtypes, subtype C isolates infect more individuals than any other clades and predominate in marginalized countries such as India, sub-Saharan Africa and Brazil (Shankarappa et al., 2001).

1.2.2.3.2 HIV replication and heterogeneity

The replication rate of HIV is high with 10^{10} virions produced daily and 10^6 - 10^{10} cells infected at any one time (Rodrigo et al., 1999). HIV regeneration time is estimated at 1–2 days/generation, resulting in highly diverse sequence populations within an individual (Collins et al., 2002a), (Rodrigo et al., 1999). Various factors increase viral replication rates including cytokines such as TNF- α , and co-infections such as TB. Higher viral loads in turn result in a more rapid disease progression (Toossi et al., 2004b, Toossi et al., 2004a). Viral set-point defined as the level at which viral load remains stable is also predictive of clinical outcomes and disease progression (Mani et al., 2002).

The high heterogeneity of HIV is due primarily to the error-prone reverse transcriptase and is manifest primarily in the nucleotide (NT) sequence of the envelope glycoprotein (*env*) and the *gag* genes (Collins et al., 2000). Variability most often results from high viral replication rates that in turn, differ (Collins et al., 2000) within compartmentalized microcosms of different organ systems. Despite a high turnover rate and high genomic variability, amino acid substitutions rarely influence function (Weiss, 2003), infectivity or route of transmission. Greater variability leads to rapid development of drug resistance, and immune escape with the added implication of complicating drug development (Peleg et al., 2002). It is thus expected that the future of the epidemic will

not conform to current mono-clade geographic distributions with an emergence of circulating recombinant forms (CRF) of multiple clades and even of Group M and O forms (Weiss, 2003).

The HIV genome is interspersed with conserved as well as variable regions. Conserved regions at the gene level relate to functional RNA secondary structure, while conservation at the protein level is a result of functional and structural protein constraints (Peleg et al., 2002). Important protein secondary structures include the transactivation-responsive (TAR) and the rev-responsive elements (RRE). More recently, a previously undescribed secondary structure was reported in the C1 region of the *env* gene (Peleg et al., 2002). This finding revealed the importance of the C1 region of the *env* gene compared with the less conserved C2-C5 region of the same gene (Peleg et al., 2002) implying the presence of a functionally important protein-coding region in the former. The functional role of these conserved regions has not been fully elucidated and illustrates the importance of evaluating nucleotide polymorphisms in conjunction with disease pathology to be relevant to the clinical setting.

1.2.2.4 HIV entry and pathogenesis

1.2.2.4.1 Transmission

The terms ‘immunological synapse’ (Weiss, 2003) and ‘genetic bottleneck’ (Leitner and Albert, 1999) have been applied to the process of viral transmission. HIV enters the host via one of 4 main routes; sexual, parenteral (IVD, blood and blood products), exposure in health care settings and mother to child transmission (Chakraborty, 2005, Weiss, 2003). No evidence for transmission by insects has as yet been verified.

More than 80% of infections are via sexual contact; 70% of which are vaginal and 10% anal (Roitt et al., 2001). HIV transmission is dose dependant with a probability of approximately 0.001 (95% CI 0.0008-0/0015) per sexual encounter (Gray et al., 2001). High viral loads, genital ulcers (Gray et al., 2001) and localised inflammation are primary determinants of transmission (Bhoopat et al., 2001). Langerhans and dendritic-type cells are, likely, the first to be infected (Bhoopat et al., 2001). Increased

inflammation, characterized by elevated Langerhans and associated dendritic cells, was more common to HIV-infected (83%) compared with -uninfected (21%) patients (Bhoopat et al., 2001). A 0.4–2.0% diversity separates the average genetic distance between the donor and transmitted viral variants (Leitner and Albert, 1999). Any ruptures in the mucosal barrier which expose susceptible cells in the sub-mucosa allows the entry and dissemination of viral particles following sexual exposure (Bhoopat et al., 2001, Pope and Haase, 2003). On the other hand, intact mucosal surfaces are no barrier to transmission in high-risk individuals.

MTCT transmission is primarily vertical, during labour and delivery (66%), but may also occur perinatally *in-utero* or postnatal via breast-milk (BM) (Chakraborty, 2005). Perinatal materno-foetal transfusion or compromise of the placental barrier, which allows virions to migrate trans-placentally to the foetus, promotes *in-utero* transmission. The most common means of MTCT occurs during labour when the infant skin or mucous membranes are exposed to maternal blood and other secretions (Chakraborty, 2005). Finally, continued exposure of the infant to HIV-infected BM results in variable transmission frequencies over the first year of life with the highest rates of 84% occurring by 12 months of age (Nduati et al., 2000). Recent findings have demonstrated exclusive breastfeeding for the first 6 months of life significantly reduces transmission rates when compared with mixed feeding in a South African population (Coovadia et al., 2007).

1.2.2.4.2 Early events following transmission

The virus rapidly targets dendritic (DC), Langerhans (LC) and CD4⁺ T cells found in profusion in vaginal mucosa (Bhoopat et al., 2001, Collins et al., 2002b). Of the infected cellular population in the vaginal mucosal, 40- 60% are LCs compared to only 30% T-cells (Bhoopat et al., 2001). Two models have been proposed to describe the immediate events following sexual exposure to HIV (Fig. 1.4). The first contends that HIV replicates at the site of infection for several days as demonstrated in SIV models (Zhang et al., 1999) while the second proposes that HIV is transported almost immediately to the lymph nodes where it is disseminated. Again, the latter contention was demonstrated in SIV models where HIV was noted in the lymph nodes within 48

hours of exposure (Joag et al., 1997) suggesting that a pre-existing inflammatory condition may have assisted in the rapid transport of the virus (Pope and Haase, 2003).

Establishing HIV infection is complex and multi-factorial (Fauci, 2003) but occurs rapidly (Pope and Haase, 2003). Infection is initiated in the CD4⁺ T-lymphocytes at the site of exposure (Pope and Haase, 2003). The virus rapidly installs itself in the lymph nodes where prolific replication, dissemination to lymphoid organs and establishment of disease ensues (Collins et al., 2002b). Despite dissemination to possibly all organ systems, the foremost site of viral replication, storage and persistence remains the lymphoid tissue (Fauci, 2003, Pope and Haase, 2003). Macrophages and DCs appear to aid viral pathogenesis by recruiting and enhancing viral replication in T-cells without activating cellular defence mechanisms (Pope and Haase, 2003).

The HIV-1 molecule enters the target cell via one of two mechanisms; namely by fusing with the cell membrane at the cell surface and releasing its contents into the cytoplasm, or by endocytosis (Weiss, 2003). These mechanisms of entry occur with equal frequency in T-cells. They can occur independently, since fusion requires both CD4 and related co-receptors, while endocytosis is facilitated by CD4 receptors only (Schaeffer et al., 2004). The authors (Schaeffer et al., 2004) demonstrated that restriction of one mode of entry results in exploitation of the other. Most notable is the *nef* region which facilitates the endocytic trafficking of HIV (Geyer et al., 2002). Earlier studies have also shown membrane and receptor mediated endocytosis and the occurrence of viral particles within endocytic vacuoles (Dezutter-Dambuyant et al., 1991, Pudney and Song, 1994, Tacchetti et al., 1997).

1.2.2.4.3 Cellular entry

HIV entry into cells occurs when a tri-molecular complex forms between the gp120 region of the *env* gene, the CD4 receptor and either the chemokine co-receptor CCR5 or CXCR4 (Fauci, 2003, Murphy, 2001). Stimulation by Ab additionally, enhances viral entry using Fc receptors indicating an alternative route of infection should CD4⁺ T-cells be depleted (Roitt et al., 2001).

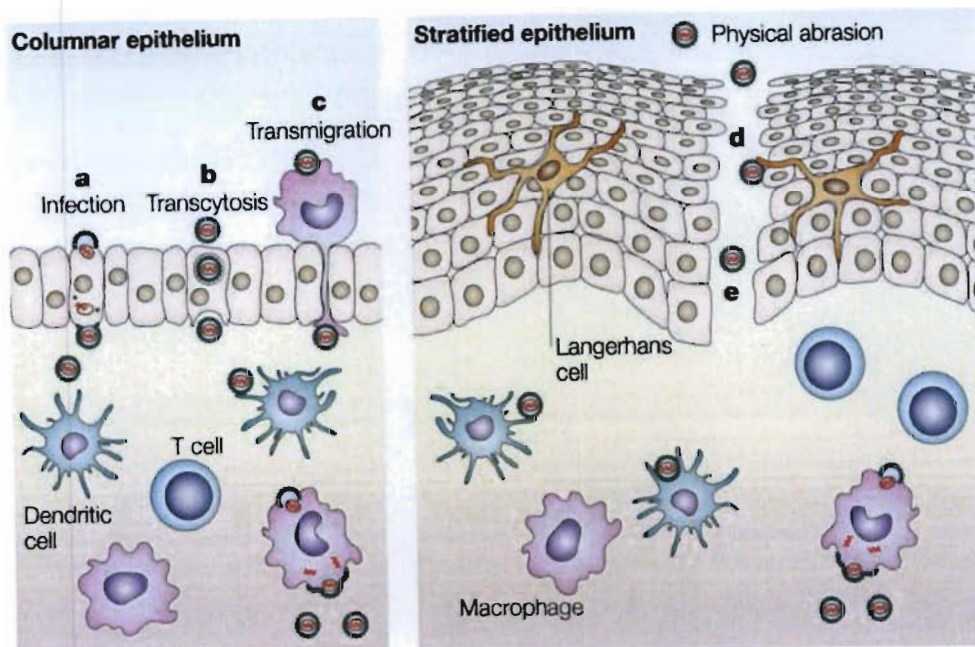


Figure 1.4: Illustration of the hypothesized cellular events following sexual transmission of HIV-1. Adapted from Shattock & Moore, (Shattock and Moore, 2003)

DCs, macrophages and T-cells expressing the CCR5 molecule are initially targeted to establish disease (Pope and Haase, 2003) while viral gene products promote macrophage and DC association with T-cells in order to advance the infection. For the most part, the type of cells infected is determined by substrate availability. Isolates may be dual- (R5X4) or monotropic (R5 or X4) and will be elaborated on in Section 1.2. The R5 receptor molecule is imperative to viral entry such that individuals with a 32 base pair deletion in the CCR5 gene are highly resistant to infection despite repeated exposures (Fauci, 2003, O'Brien and Moore, 2000). These findings also illustrate the transmissibility of R5 viruses compared with X4 variants which emerge later (Pope and Haase, 2003). Therefore, in addition to the normal loss of CD4⁺ T-cells, HIV preferentially infects and destroys this cellular sub-population (Pope and Haase, 2003). In instances where the cells are infected with multiple provirus particles development of recombinant forms, greater viral heterogeneity, drug resistance and escape mutations may ensue (Weiss, 2003).

The greatest paradox of HIV infection is the atypical stimulation of the immune response and the simultaneous depletion of immune cells (Fauci, 2003). The specific mechanisms by which HIV achieves cellular killing remain ambiguous but several alternatives seem likely. They include RNA and unintegrated DNA accumulation

within cells and intracellular binding of CD4 and gp120; infected binding uninfected cells leading to syncytium formation; gp12-specific CTL responses; surface-bound gp120 rendering cells vulnerable to Ab dependant cell-mediated cytotoxicity (ADCC); HIV peptides mimicking super-Ags resulting in expansion and eventual killing of large numbers of cells; apoptosis, and finally, budding weakening the cell wall (Roitt et al., 2001).

1.2.2.4.4 Viral tropism

The *env* gene codes for two glycoproteins; the gp120 and gp41 the former responsible for host cell receptor binding and cellular tropism (Williams and Dye, 2003). Viral tropism determines the cellular sub-population that the virus will infect and is dependant on the HIV subtype, species of the host and the specific tissue that is infected (Cullen, 2001, Huff, 2003). Entry and successful completion of the viral life cycle is determined by host cellular factors and co-factors (Cullen, 2001).

HIV-1 tropism is extremely narrow both with respect to host species and tissue targets. Earlier studies in primate models identified the *gag* gene as the major determinant of HIV-1 species tropism (Cullen, 2001). Viral isolates can be categorised into either T-cell or macrophage tropic phenotypes. The former refers to isolates able to replicate in T-cells, utilize the CXCR4 co-receptor, are highly sensitive to neutralisation by soluble CD4 and monoclonal Abs and their inability to induce syncytium formation in T-cell cultures. These viral isolates are referred to as non-syncytium inducing (NSI) variants. On the other hand, viral isolates that replicate in primary CD4 T cells,, utilizes CXCR4 co-receptor, and induce syncytia under similar conditions (Cullen, 2001) are referred to X-tropic or syncytium inducing (SI) variants (Huff, 2003). You have this paragraph mixed up. Macrophage-tropic variants also infect primary CD4 T cells (but not immortalized T cells) and are NSI. SI variants can infect CD4 cells (both primary and immortalized) but they do not infect macrophages.

Factors conferring tropism were later localised to the V3 (third variable region) or the gp120 region of the *env* gene (Hwang et al., 1991, O'Brien et al., 1990). HIV-1 replication *in vivo* and *in vitro* is limited by its affinity for CD4 and the availability of cellular co-receptors (Cullen, 2001). Disease progression and end-stage disease is now

widely associated with a switch from an R5- to an X4-tropic variant (Jensen et al., 2003) while early disease is characterised by isolates almost exclusively of R5-utilising phenotype (Cullen, 2001, Jensen et al., 2003). It is theorised that a switch in tropism is a result of either selection pressure (drug or immune) or a reflection of the natural evolution of the virus.

HIV-1 subtypes also differ in their co-receptor usage, for example biological assays as well as bioinformatics analysis demonstrated that subtype B variants utilise either R5 or X4 co-receptors while subtype C variants are largely R5-tropic (Cullen, 2001, De Oliveira et al., 2003, Gordon et al., 2003, Johnston et al., 2003). However, X4-tropic viruses have been reported in treated subtype C infected patients, in contrast to untreated patients with exclusively R5 (NSI)-tropic variants (Johnston et al., 2003). The syncytium inducing variants isolated from treated patients also displayed a propensity for the R5-coreceptor indicating circulation of dual-tropic variants in treated patients (Johnston et al., 2003). This has obvious bearing on vaccine and drug development. It has been suggested that characterising the mediators of syncytium formation would be an important step towards controlling infection since syncytium inhibition (SI) antibodies correlate directly with clinical outcomes in children (Brenner et al., 1991). Several studies have described various mechanisms by which syncytium formation is inhibited and they relate to CD4 expression (LaBonte et al., 2003), DC-SIGN (Noble et al., 2003) and antibodies targeting gp41 fusion intermediates (Golding et al., 2002).

1.2.2.5 Viral kinetics and disease progression

Viral kinetics studies have shown that viral replication is constant and drives CD4+ T-cell turnover (Ho et al., 1995). However, a limited number of virions infect non-dividing cells, or a subset of infected cells reverts to an inactive state giving rise to a population of latently infected cells (Fauci, 2003). This pool of latently infected cells are capable of, and do migrate to various parts of the body. In this way, both cellular and anatomical reservoirs are formed. Latently infected patients display undetectable viral loads (< 50-100 copies/ml), but quiescent infected cells produce a minute quantity of virions or transcripts (Pope and Haase, 2003). Activation of a latent reservoir and/or reactivation of virions that is complexed with follicular DCs, leads to the production of

infective virus (Pope and Haase, 2003). This inevitably culminates in viral rebound which may be delayed by months. Latently infected cells may, furthermore, play a prominent role in HIV dissemination since they feature significantly at transmission (Pope and Haase, 2003), while active cells expand the infection.

Viral set-point defines the course of clinical disease. A higher set-point is associated with rapid immune-suppression, disease progression and a shorter period of latency (Hatano et al., 2001). Due to the rapid disease progression in paediatric patients, viral set point is short-lived while in adults is maintained through latency (Lifson et al., 1997). High viral loads that persist for the first 2 years of life in paediatric patients (Newell and Peckham, 1994) result in poor outcomes. This elevated viral load may be attributed to viral kinetics, the magnitude of HIV-permissive cellular population and the lack of appropriate and effective immune responses against the virus (Chakraborty, 2005).

1.2.2.6 Clinical features of infection

Clinically, primary HIV infection manifests as malaise, muscle ache, swollen lymph nodes, sore throat and rashes (Roitt et al., 2001). Chronic infection without clinical manifestation persists for variable time periods but averages 10 years (Lama and Planelles, 2007). Delayed or long-term non-progression, where patients remain asymptomatic with no significant immune changes for up to and in excess of 10 years, occurs both in adults and children and in the absence of ARVs (Warszawski et al., 2007). Progressive and late stage disease is characterised by fevers, night sweats, diarrhoea, weight loss and disorders affecting the mucosa and skin such as oral candidiasis, shingles, anogenital HSV and other dermatological infections (Roitt et al., 2001). The impact of opportunistic infections (OI) is felt throughout HIV pathogenesis due to reactivation of latent infection or to atypical opportunistic pathogens (Roitt et al., 2001). OIs are characteristic of late stage disease and the onset of AIDS. Disease progression in perinatally-infected infants is more rapid such that the number of HIV-related deaths among children is 5-times the infection rates in this demographic (Barnhart et al., 1996, Chakraborty, 2005). Paediatric disease manifestation is varied and includes hepatosplenomegaly, hepatitis, nephropathy, developmental delay,

recurrent bacterial infections and malignancies (Chakraborty, 2005). A primary co-factor is tuberculosis particularly in developing nations where the bacilli are endemic.

1.2.2.7 Other elements of the HIV immune response

The primary characteristic of the HIV immune response is that it is depleted since the breadth of the immune response is diminished (Roitt et al., 2001). Both cell mediated and humoral immune factors are recruited against an HIV infection (Fauci, 2003, Letvin and Walker, 2003).

1.2.2.7.1 Cytokines and chemokines

Since chemokines and cytokines are integral to an effective immune response against infections (Murphy, 2001), viruses have devised means of corrupting their role through molecular mimicry of these peptides and their receptors (Murphy, 2001). These mimics can be categorized into 5 classes; anti-chemokines, leukocyte chemo-attractants, cell entry, cell growth and angiogenic factors. Cytokine and chemokine profiles of SIV infected primates during chronic infection, revealed a negative correlation between CD4⁺ T-cell counts and mRNA expression of, IL10, MIP-1 α , -1 β and Rantes (Hofmann-Lehmann et al., 2002). Interestingly, cytokine and β -chemokine production was not correlated with VL but with CD4⁺ cell counts. In this context, elevated IL-10 levels and rapid disease progression was believed to result from increased immune activation and a shift from a Th1 (IL12, IFN γ) to Th2 immune responses. Hofmann-Lehmann et al, (Hofmann-Lehmann et al., 2002) propose that the discrepancy between Th1 and Th2 cytokines stems from increased Th2 cytokine production rather than insufficiency in their Th1 counterparts.

Viral homologues include members of the CC and CR gene families, most notably, the CCR5 receptor molecule of HIV (Murphy, 2001). HIV *tat*, additionally, acts as a chemokine mimic in its extra-cellular state, where it may display chemotactic properties towards neutrophil, basophils, mast cells and monocytes and antagonistic properties towards CXCR4 (Murphy, 2001). The biochemical properties of X4 is a likely rationale

for the blockade reaction of tat towards these molecules, as compared to the more basic R5 molecule, that is induced to evolve over X4 or remains unaltered.

1.2.2.7.2 Antibody responses

There have been conflicting views on the role of antibodies (Ab) in the host's defence against HIV infection. In fact, related research has centred exclusively on the cellular immune responses. While it is known that neutralizing antibodies (nAb) recognise the V3 loop, CD4 binding site and gp41 transmembrane protein of the *env* gene (Letvin and Walker, 2003), their role in the control of viremia remains contentious. The highly immunogenic properties of the *env* have thus generated much interest with regards a vaccine candidate. Studies have demonstrated the production of nAb, specifically, 5 monoclonal Ab's, in the sera of HIV-infected patients (Moore et al., 2001). It is expected, unfortunately, that nAb's may not prove viable since extremely high titres are required to generate a response (McMichael and Hanke, 2003). Furthermore, HIV is easily and rapidly able to generate polymorphisms or antigenic variants to escape these nAb.

1.2.2.7.3 Cytotoxic T-lymphocytes and MHCs

In contrast, virus specific CTLs pose a strong immune response and have been observed at all stages of infection (Letvin and Walker, 2003). More importantly, CTLs are found in all anatomic compartments including blood, pulmonary spaces, lymph nodes, spleen, skin, CSF, semen, vaginal and GI mucosa (Letvin and Walker, 2003). Control of viremia particularly at the early stages of infection is positively correlated with the emergence of a CTL response giving rise to the current widely-held assumption that a strong T-cell response will initiate abortive infection or control viremia (McMichael and Hanke, 2003). Animal models show that vaccinated individuals display significantly lower viral loads than unvaccinated controls, even up to 1000 fold less (McMichael and Hanke, 2003). Within 6 hours memory T-cells mature and are activated by Ag to produce cytokines (TNF α , IFN γ), chemokines (RANTES, MIP-1 α , -1 β) and cytolytins (granzymes, perforin) (McMichael and Hanke, 2003). Among the HIV regions targeted

by vaccine design are the *nef*, reverse transcriptase (RT) and *env* genes (McMichael and Hanke, 2003). It must be remembered, that while CTLs seem the answer to the HIV epidemic and unquestionably do stimulate a strong immune response, they cannot prevent HIV infection as nAb can (McMichael and Hanke, 2003). This is due to the fact that CTLs only react to peptides presented by cells already infected by HIV. Since full anti-viral activity takes days to develop they serve to control disease, not protect against infection.

The MHC Class I alleles additionally, are predictive of the rate at which disease progresses, are the major determinant of the specific viral peptides presented and direct the scope of the immune response (Letvin and Walker, 2003). The inherent variability of these alleles in a population confers variable responses as exemplified by diverse outcomes and rates of disease progression in HIV-infected individuals. Selected HLA alleles, for instance HLA-B5701, are associated with long-term non-progression to AIDS (Migueles et al., 2000). MHC molecules and the CTL response are purported to present the most promising answer to effectively containing and/or eliminating HIV infection. In MTCT, the commonality between infant and maternal HLA-class 1 molecules is high, allowing the virus to evade the host immune response in the infant and culminating in accelerated rates of disease progression in this demographic (Goulder et al., 1996).

Although HIV targets CD4+ T-cells, these cells do play a significant role in the immune response against HIV. The focus of research efforts from the onset has, thus, been the lymphocyte population but it is now hypothesised that the T-cell infection is the observable pathology obscuring the more insidious macrophage disease (Weiss, 2003).

1.2.2.8 HIV immune escape

Despite evidence for both cellular and humoral immune responses against HIV, the host still fails to stem disease progression (Letvin and Walker, 2003). The most likely rationalization is the emergence of genetic mutants in selected epitopes that allow these isolates to escape the immune response. These mutants, stimulated by immune or drug selection pressure, can out-compete wild-type virus (Letvin and Walker, 2003). It is for

this reason, amongst others, that a lasting vaccination, and the development of an appropriate vaccine, has proven so complex. Susceptibility and resistance to viral infection is complex and made more difficult by the diversity in viral and HLA-genes (Letvin and Walker, 2003).

1.2.2.9 HIV treatment

Current approaches to treatment include adaptive therapy, cytokine therapy, therapeutic immunization, combination highly active anti-retroviral therapy (HAART) and treatment interruption (Letvin and Walker, 2003). The drug azidovudine (zidovudine, AZT) targeting the HIV reverse transcriptase was the first effective drug to be implemented in 1987 (Fauci, 2003), resulting in short-term outcomes of reduced mortality rates and delayed progression to AIDS (Roitt et al., 2001). Targeted drug design yielded more effective drugs aimed at disrupting susceptible sites in the viral replication cycle. These include the protease, fusion and entry inhibitors (Fauci, 2003).

Reducing HIV viral loads, to levels below 1500 copies/ml or lower, to undetectable levels, is the aim of ART therapy (Pope and Haase, 2003). In addition to the obvious clinical benefits, slowing the replication rate of HIV assists in reducing the rate of mutation and consequently, the rate at which immune-escape and drug resistant mutants emerge (Roitt et al., 2001). Greater understanding of the cellular and humoral immune responses has yielded promising targets for future drug interventions and vaccines (Letvin and Walker, 2003). The most encouraging is a combination of a T-cell epitope and a strong Ab-stimulating vaccine (McMichael and Hanke, 2003). Whether co-operation between these tenets will yield a vaccine remains to be seen.

Nucleotide and amino acid polymorphisms resulting from drug selection pressure have been described (Gordon et al., 2003) but the greater danger is the emergence of multi-drug resistant strains (Weiss, 2003) in the wake of large-scale ARV interventions. While it is important to treat every infected individual, it is equally important to treat these patients optimally, since sub-optimal drug levels result in rapid generation of resistance mutations. It is, thus, critical that compliance be monitored and sharing of drugs is prevented (Weiss, 2003). Unfortunately, the high costs of ARV treatment and

monitoring are prohibitive in developing countries. Despite setbacks, the SA-ARV rollout was implemented in 2003 and has gained momentum in recent years (AFSA, 2005). In January 2005, approximately 29 000 HIV-infected persons were on treatment nationally; 8467 of whom were from KZN and the majority were adults (AFSA, 2005). The significant discrepancy between infected patients requiring treatment and those receiving treatment, persists but the inequity between them, is closing.

1.2.2.10 HIV-1 Compartmentalisation

HIV populations are homogenous only at primary and end-stage disease (Leitner and Albert, 1999). The high rate of viral replication and accumulation of mutations typical of HIV generates highly diverse or heterogeneous sequence populations at other stages of disease (Collins et al., 2002a). HIV-1 disseminates to various organ systems leading to compartmentalization and divergent evolution of subpopulations or quasi-species (Collins et al., 2002a). Migrations of these variants may proceed unrestrained from discrete locations into circulation and vice-versa encouraging greater systemic heterogeneity and divergent evolution at these isolated sites (Collins et al., 2002a, Delwart et al., 1997). Quasi-species are, thus, discrete, subpopulations of those in circulation or phylogenetically related to virions evolved independently in tissue compartments such as the pleura and lungs in TB co-infected patients (Collins et al., 2002a). Both spatial and temporal intra-patient phylogenetic fluctuations occur commonly as a consequence (Grenfell et al., 2004). Of the many remote compartments studied thus far, the brain (Korber et al., 1994, Sanjuan et al., 2004), semen (Alimonti et al., 2003, Choudhury et al., 2002, Delwart et al., 1998) and lymph nodes are the only confirmed discrete HIV compartments (Korber et al., 1994). Other organ systems that may form compartments include the lungs (Collins et al., 2000, Collins et al., 2002a), genital tract (Poss et al., 1998) and blood (Zhu et al., 1996). Autopsy specimens confirm viral sequestration and heterogeneity in the brain, lung and testis (Collins et al., 2002a, van't Wout et al., 1998). Van't Wout, et al (467) reported viral quasispecies in blood that are phylogenetically related to virions evolved independently in tissue compartments such as the pleura and lungs. Whether the same is true about the spine has not been investigated.

Bearing in mind that viral diversity, which is influenced directly by mutation rate, is positively correlated and proportional to the number of rounds of replication, it follows that viral heterogeneity will be compounded temporally (Mani et al., 2002). In addition, Mani et al, (Mani et al., 2002) correlated greater divergence with viral load, but could not determine whether differences in diversity and viral load were gender dependant.

At transmission, HIV populations are homogenous or less diverse, but change temporally, becoming progressively more heterogeneous (Learn et al., 2002, Meyerhans et al., 1989). It has been argued that the stage of disease and occurrence of quasispecies are not correlated, and that vast differences are detected between different isolates (Meyerhans et al., 1989). The assumption that a single variant is transmitted to establish disease (Leitner and Albert, 1999) has generated much controversy. It is argued that an early selection process that targets functionally important sites in the *env* and *gag* genes produces a more homogenous viral population early in infection (Learn et al., 2002). On the other hand, co-infections, particularly TB, greatly influence HIV evolution and diversity, and is mediated by constant localised immune stimulation (Collins et al., 2002a).

TB co-infection increases HIV viral loads by up to 160 fold, which may be reversible following anti-TB therapy (CIPLADOC, 2001/2002). Immune stimulation, especially at low levels, impacts viral adaptation, due to the high selective pressure exerted on the viral genome (Grenfell et al., 2004). Higher viral diversity in actively infected TB patients, is promoted by the microenvironment (granulation tissue) created by the bacillus itself (Collins et al., 2002b, Garrait et al., 1997). Hence, TB acts to increase viral replication and subsequent viral diversity in two ways - it induces HIV replication in activated, infected immune cells (Toossi et al., 2001) and encourages secretion of stimulatory cytokines and chemokines, such as TNF- α , a recognised promoter of HIV replication (Collins et al., 2002b, Garrait et al., 1997, Raja, 2004). Collins, et al (Collins et al., 2002b) reported an association between tissue-localized immune stimulation and greater viral heterogeneity, higher viral loads and altered cytokine profiles.

Although the increase in viral replication rates and sequence heterogeneity is reversible in patients with acute immune stimulation (Ostrowski et al., 1998), the same has not been verified in chronically infected patients such as those with TB (Collins et al., 2000). In some instances, co-infection paradoxically results in slower disease progression, high CD4 counts and low viral loads but a more rapid accumulation of HIV sequence heterogeneity (Collins et al., 2000, Meyerhans et al., 1989).

1.2.2.10.1 HIV heterogeneity and the immune response

It has become increasingly evident that identifying and analysing viral reservoirs is important to present understanding of intra- and inter-patient HIV evolution and phylodynamics. Not only do these compartments impact on treatment, but also on patient outcomes, as escape mutants generated at these remote sites may become dominant with progressive disease or following treatment. Adaptive events that generate escape mutants occur every 25 generations (Williams and Dye, 2003). This is the most rapid adaptation rate ever recorded producing a sizeable viral population that is inaccessible to the immune response (Williamson, 2003). Treatment and patient outcomes are thus greatly impacted since CTL's control viremia, and persistence of infection (Borrow et al., 1997).

HIV genetic variants differ in their replication efficiency, cellular tropism and their sensitivity to immune selection pressures (Korber et al., 1994, van't Wout et al., 1998). Effective treatment of HIV, with current and future drug regimes, can only be achieved if treatment reaches all infected cells (macrophages or T-cells) which, van't Wout et al, (van't Wout et al., 1998) suggest is a likely reservoir within non-lymphoid tissue. Cosenza et al, (Cosenza et al., 2004) illustrated the potential of macrophages and microglial cells as viral reservoirs and demonstrated their resilience against apoptosis. Macrophage tropic isolates (CCR5/R5 exploiting) are the predominant cell population in TB-infected individuals (Santucci et al., 2004). HIV-1 replication and consequent increased R5 expression was demonstrated in *M. avium*-infected cells (Wahl et al., 1998). In contrast increased CXCR4 (X4) expression was demonstrated in AMs of pulmonary Mtb/HIV-infected patients (Hoshino et al., 2002). Differences between these findings may be a result of an *in vitro* versus an *ex vivo* assay, and an *M. avium*

versus an *Mtb* infection. It may, additionally, reflect an expansion of the initial infecting variant rather than a switch from one co-receptor usage to another. Still others report expansion of R5 tropic variants (Morris et al., 2001, Santucci et al., 2004). Morris et al (Morris et al., 2001), in a South African cohort of HIV subtype C/TB infected individuals, demonstrated that, despite advanced disease, no change in co-receptor usage occurred. Their findings would suggest that R5-utilising macrophage-tropic variants were preferentially amplified. This is also consistent with other reports of limited prevalence of X4 variants in subtype C populations (Gordon et al., 2003, Tien et al., 1999) with few exceptions (Shankarappa et al., 2001).

When disease is established the tropism of virions is restricted to the particular cell population that carried the virus into the compartment therefore a distinct “clade” with corresponding tropism emerges (Korber et al., 1994). Indeed, immunohistological staining of brain tissue correlated a greater population of macrophages and microglial cells with distinct separation / compartmentalisation of brain and blood viral variants while lymphocyte infiltration of the tissue was correlated with intermingling or migration between the compartments (Korber et al., 1994). Similarly, macrophage tropic variants form clear subsets in the nervous system and testicles of isolates from autopsied individuals (Collins and Kaufmann, Collins et al., 2002a, Sanjuan et al., 2004) were unable to conclusively demonstrate compartmentalisation of HIV in pleural tissue, a site that is infiltrated by activated leukocytes in response to TB infection. Spinal tissue on the other hand is infiltrated by macrophages that mature to giant cells and epithelioid cells. It is thus most likely that the predominant cell type infiltrating the infected tissue is one of the primary determinants of tissue-localised divergent HIV evolution. It was noted that the cells infiltrating the TB-infected tissue were activated leukocytes, a large proportion of which would be HIV-infected and would not have been present prior to TB infection (Collins et al., 2002a). Furthermore, the close contact between HIV-infected lymphocytes and TB-infected macrophages at the site of infection invariably leads to interaction between them and ultimately to HIV transmission, activation and up-regulation (Collins et al., 2002b).

Sequence analysis largely ignores the structural implications of amino acid polymorphisms and incorrectly assumes site-specific amino acid substitution models to be uniform or homogeneous (Yang, 2000). Codons can undergo positive, negative or

neutral selection. Codon substitution models measuring the selective constraint on a protein, calculates the ratio of nonsynonymous / synonymous (d_n/d_s) mutation. A nonsynonymous mutation is neutral if $\omega = 1$, positive or advantageous if $\omega > 1$ and purifying or deleterious if $\omega < 1$ (Yang, 2000). Positive selection refers to amino acid polymorphisms that confer a fitness and/or virulence advantage to the isolate. In addition, chemical properties of an amino acid, may be more informative than the rate of substitution itself (Yang, 2000), since amino acids with similar chemical properties seem more inclined to replace each other in order to preserve the functional integrity and fold conformation of the peptide. Chemotherapy and immune selection pressures are most commonly implicated as strong inducers of positive selection and viral evolution. Due to its importance to the immune response, changes in receptor fit and evasion of the immune response are potential sources of positive selection (Williamson, 2003). It has been shown that transmission is a neutral event where selection pressure has no influence on the overall substitution rate (Leitner and Albert, 1999). Fixed substitutions therefore survive transmission. However, it is deceptive to assume that the transmitted isolate is the dominant donor variant (Leitner and Albert, 1999). Leitner and Albert, (Leitner and Albert, 1999) also contend that viral evolution is a neutral event where nucleotide substitutions are a stochastic rather than a deterministic phenomenon.

The phenomenon of variable positive selection and evolutionary rates is reiterated at different protein coding regions over the entire HIV genome (De Oliveira et al., 2003). Studies have demonstrated that different regions of the *env* gene experience varying rates of positive selection (Mani et al., 2002, Williamson, 2003). Surprisingly, the V1-V2 region has the highest adaptive rate (1 event/2.5 months) followed by the C2-V3 (1 event/5.9 months.) and V4-V5 regions (1 event/12.2 months.) (Williamson, 2003). Accordingly, calculating the rate of positive selection over the entire HIV genome, or a substantial portion thereof, may not fully illustrate selection pressures exerted at specific sites. There are strong associations between the stage of disease and the frequency and strength of positive selection (Ross and Rodrigo, 2002). Computational analysis of HIV subtype B and C sequences revealed that *tat* and *rev*, rather than the *env* gene is under positive selection (De Oliveira et al., 2003). The findings of these studies have illustrated viral diversity in, predominantly subtype B isolates and from patients in the

acute or late stage of disease. The dynamic, is expected, will be vastly different in patients in the disease-free stage of HIV infection (Mani et al., 2002).

1.2.3 HIV and TB co-infection

1.2.3.1 Historical Perspective

Opportunistic infections (OI) are, by definition, exploitative organisms and the explosive HIV epidemic has provided, potentially, more than 40 million immune-compromised hosts (Weiss, 2003). The OI most often associated with HIV is TB (Narain and Lo, 2004). Mammals susceptible to lentiviruses such as HIV are also vulnerable to Mycobacterial infection (Collins et al., 2002b). Early in the HIV/AIDS epidemic, co-infection with atypical Mycobacteria such as *M. avium-intracellulare* and the *M. avium* complex were reported, but it was only in recent years that HIV and Mtb has been significantly associated (Smith, 1991). The risk of developing active disease increases with progressive immune-suppression.

1.2.3.2 Epidemiology of co-infection

At present 2.5 million of the 1 billion individuals infected with TB are co-infected with HIV (Narain and Lo, 2004). Sub-Saharan Africa is severely affected, with 40% of all TB cases in the region associated with HIV. The equivalent global figure lies at a mere 8% (WHO, 2002). A Nigerian study placed the HIV-prevalence among TB-infected patients between 1990 and 2002 at 28% (Seleye-Fubara and Etebu, 2004). Overall, in excess of two-thirds of the 15 million co-infected patients worldwide, reside in sub-Saharan Africa (WHO, 2006). Global estimates places the percentage of all new TB cases attributed to HIV at 9% while in African countries this may be as high as 31%.

HIV is recognised as the single most important determinant of the escalation in TB incidence in Africa (WHO, 2004). There has been a 7-fold increase of TB cases in the worst affected countries such as Vietnam (Nguyen et al., 2004) a burden against which current treatment strategies are proving ineffective. Add to this the fact that 15% of all

AIDS deaths are due to TB and one is able to appreciate the devastating impact of HIV/TB co-infection. In fact, HIV increases the risk of developing active TB by a factor of 20 (Collins et al., 2002b). While HIV-uninfected patients are at a 10% lifetime risk of developing TB, HIV-infected individuals stand a 10% chance per year and a 60% lifetime risk of progressing to active disease (Nguyen and Pieters, 2005, CIPLADOC, 2001/2002). Interestingly, the Eastern Mediterranean, despite acknowledging TB as a serious health hazard in the region does not acknowledge any impact of HIV on TB epidemiology (Gillini and Seita, 2002). However, concerns are currently mounting, that the impact of co-infection, particularly in light of the explosive subtype C epidemics in Asia and sub-Saharan Africa will become increasingly significant (Collins et al., 2002a, Montano et al., 1997). Indeed, the UK cites TB as the leading cause of HIV related deaths and the most common AIDS defining condition of 2002 (Health_Protection_Agency_Tuberculosis, 2004). It is an unfortunate reality that the marginalized populations of the world that are most affected by the HIV and TB epidemics. Limited access to education and treatment strategies is the primary impediments (Narain and Lo, 2004) to making an effective impression on these epidemics. However, interventions can be successful and to date, Kerala (India) and Sri Lanka have reduced their HIV and TB burdens (Zaidi et al., 2004).

HIV has impacted the balance between TB-infected and uninfected individuals, as well as the epidemiology, natural history and clinical evolution of Mycobacterial infections (de O Liverato et al., 2004). Highest rates of co-infection occur in younger individuals (25-44 year age group) while TB alone seems confined to older individuals (de O Liverato et al., 2004). In view of the fact that the 24-44 year age group represents the most economically viable demographic of any country, depletion of their numbers would result in significant social, economic and political consequences. Most alarming is that the spread of infectious and highly virulent strains of, both HIV and TB, may parallel the increase in human migration and travel (Ahmed et al., 2003). In Africa, where the HIV pandemic is rampant, infectious diseases are on the increase. The dire prediction of an explosion in TB infections, appearing as and when HIV becomes established, has already manifested in Southern Africa and South East Asia. The impact on the young is equally alarming. In Jamaica, significant increases in the incidence of active TB were noted from 1999 to 2002 among children, all of whom received the BCG vaccine (Geoghagen et al., 2004). Of these, 46% were HIV-infected, experienced

more severe disease pathology and a higher mortality rate (Geoghagen et al., 2004). Similarly, HIV/TB co-infection occurred in 48% of a paediatric cohort from Durban, South Africa (Jeena et al., 2002) illustrating that co-infection was more common than previous estimates. The authors reaffirm that the disease pathology among dually infected children is often acute (43%) and misdiagnosed (21.2%) requiring extreme diligence in order to reduce patient morbidity and mortality in the HIV-infected patients (Jeena et al., 2002). Collectively, these studies suggest that a region with an explosive HIV epidemic is likely to experience an elevated TB incidence (Narain and Lo, 2004).

1.2.3.3 Pathology of co-infection

On acquiring HIV, progression to active TB occurs rapidly (Narain and Lo, 2004). The profile of opportunistic infections (OI) among HIV infected children differs from that of HIV-infected adults (Mofenson et al., 2004). Adults acquire OI's prior to HIV infection whereas children manifest a primary infection when their immune response is greatly depleted from an established HIV disease. Children are liable to manifest non-pulmonary diseases particularly in reference to TB (Mofenson et al., 2004). In contrast to other OIs, TB occurs at all stages of HIV infection (Toossi, 2000). Recent findings from HIV/*M. leprae* infected patients suggests that each disease progresses as a single infection (Pereira et al., 2004). Cell mediated immunity is impaired resulting in both qualitative and quantitative defects in the CD4+ T-cell population (Seligmann et al., 1987).

Recently it was shown that HIV-1 replication was inhibited in monocyte-derived-macrophages that were stimulated by heat-inactivated Mtb and culture filtrates (Goletti et al., 2004). The authors established that inhibition of replication occurred soon after viral entry and hypothesise that HIV replication is suppressed by Mtb-induced soluble factors. In an earlier publication, Goletti et al (Goletti et al., 1998) investigated the influence of cytokines on TB-induced HIV replication. Their findings indicated that inhibition of IL-2, IL-1 β and TNF- α resulted in a decrease in TB-associated HIV-1 replication. IL-10 and TGF- β neutralisation on the other hand, enhanced HIV replication (Goletti et al., 1998). These findings contradict conventional understanding that co-infection manifests as a more severe pathology with enhanced viral replication.

Indeed, 5-160 fold increases in viral replication rates were recorded in co-infected patients during the acute phase of TB pathogenesis (Goletti et al., 1996). The rate of replication in these patients was influenced by previous TB purified protein derivative (PPD) sensitisation and level of cellular activation (Goletti et al., 1996).

In adults, co-infection, most probably arises from a reactivation of latent TB while in new infections, disease progression is much more rapid than in HIV-uninfected patients (Ahmed and Hasnain, 2004) (Kabra et al., 2004). Similarly, co-infected children experience a much more severe, more disseminated disease particularly (Swaminathan, 2004). While pulmonary TB has a non-specific presentation (Raja, 2004), it is contended that HIV-infection does not influence clinical manifestation (Mofenson et al., 2004). This is indeed debatable. The clinical profile of TB is said to be changing (Kabra et al., 2004) particularly among children who experience a more rapid progression to death, compared with HIV-uninfected paediatric or adult patients (Swaminathan, 2004). In both adults and children, the clinical manifestation of a TB infection is directly related to the degree of immune-suppression (Smith, 1991).

Moreover, delayed diagnosis and/or diagnostic errors delay treatment interventions (Kabra et al., 2004) (Narain and Lo, 2004). Included among the primary reasons for diagnostic difficulties are greater incidence of sputum negatives among the co-infected, non-specific X-ray findings, non-specific clinical presentation (Smith, 1991) and other pulmonary infections (Narain and Lo, 2004). Diagnosis is dependant on bacterial isolation from clinical samples, but clinical specimens are often AFB negative in co-infected patients.

Clinical outcomes are very poor in dually infected patients since HIV and TB each advance the progression of the other (Gayle and Hill, 2001). HIV co-infection has been cited as the most important risk factor associated with reactivation of latent TB infection (de O Liverato et al., 2004). The specific manifestations of HIV-associated TB and non-HIV-associated disease, remains contentious. By and large, early features in co-infected individuals resemble that of TB⁺/HIV⁻ patients, while a more advanced phase is typified by unusual clinical findings stemming from a depletion of CD4 cells (de O Liverato et al., 2004).

Approximately 50-72% of patients with extreme immune suppression manifest extra-pulmonary TB infections consequently these infections are included among AIDS-defining conditions (Smith, 1991). The most common site of extra-pulmonary TB associated with HIV is the lymph node (27-33%) (Sharma and Mohan, 2004, Smith, 1991) and other sites include neurological tissue, pleura, pericardia and abdominal systems. Despite the parallel emergence of the HIV and pulmonary TB pandemics globally, similar trends could not be established with spinal TB and HIV in developed nations (Pertuiset et al., 1999). In a retrospective study (Pertuiset et al., 1999) of spinal TB over the period 1980-1994 none of the patients were HIV positive.

In light of their extreme immune-suppression, HIV-infected patients at late-stage disease, become susceptible to virtually any opportunistic pathogen (Smith, 1991) even the most seemingly innocuous organisms. Just as HIV heterogeneity is increased by TB co-infection, distinct TB genotypes have been demonstrated in HIV infected and uninfected patients (Ahmed et al., 2003) and were correlated with host immune status. It is, therefore, proposed that a favourable microcosm for TB evolution, particularly of less virulent strains, is provided in an HIV-infected host; where immune selection pressure is weakened or absent entirely (Ahmed and Hasnain, 2004). Infection with isolates belonging to the *Mycobacterium avium* complex (MAC); include *M. avium* and *M. intracellulare*; occur at greater frequency with increasing age and decreasing CD4+ T-cell counts (Mofenson et al., 2004). Organisms such as *M. kansasii*, *M. gordonae*, *M. xenopi*, *M. fortuitum*, *M. chelonae*, *M. haemophilum* and *M. bovis* are all non-pathogenic environmental Mycobacteria, not commonly associated with human disease. However, their association with disseminated TB pathogenesis in HIV-infected individuals is escalating (Smith, 1991). The spread of these less virulent strains can be rapid and extensive particularly with strong international economic, social and travel links of modern society. The South African setting, with its explosive HIV epidemic and high index of exposure for Mycobacteria and opportunistic pathogens, is likely to mirror this emergence of less virulent or atypical Mycobacterial infections.

1.2.3.4 HIV/TB co-infection and the immune response

It has been demonstrated that HIV/pulmonary TB co-infection produces overlapping

and distinctive immune responses (Hertoghe et al., 2000). Commonality between the HIV and TB immune responses and pathogenesises include a significant reliance on CMI (Narain and Lo, 2004). Moreover, CD4⁺ cells are an absolute requirement to an efficient immune response to TB (Scanga et al., 2000). However, HIV decimates this cellular subpopulation (Narain and Lo, 2004, Raja, 2004). Consequently, disseminated disease cannot be prevented.

It is hypothesised that the CD4⁺ T cell population is responsible for maintaining TB latency in infected individuals and preventing disease reactivation (Scanga et al., 2000). This contention was investigated in a murine model of latency, where it was demonstrated that IFN γ , iNOS and macrophage activation were unaffected by CD4⁺ depletion but disease pathology was advanced and fatal. The apparent conclusion is that CD4⁺ T-cells are required to maintain latency, the mechanisms of which surpass mere IFN γ production and macrophage activation (Scanga et al., 2000). The failure of the immune response is postulated to encompass several factors such as CD4⁺ T cell depletion effecting up-regulation of macrophage deactivating cytokines and possible loss of CD8 cytolytic function (Scanga et al., 2000).

Recent findings have shed light on the persistence of TB in macrophages and contributed significantly to present understanding of the host effector mechanisms of TB and TB/HIV co-infection (Cassol et al., 2005, Toossi et al., 2004a). Macrophages are known primary reservoirs of TB infection (Russell, 2001a) and have been recently implicated as cellular reservoirs of HIV-1 infection (Cassol et al., 2005). *Mycobacterium tuberculosis* infected monocytes and alveolar macrophages (AM) are able to activate the HIV-LTR region in the presence or absence of HIV tat which is mediated by TNF- α (Toossi et al., 2004a). Thus, Mtb infected monocytes and macrophages can transactivate HIV in latently infected cells (Toossi et al., 2004a). On the other hand, TNF- α , is correlated with NO production (Sharma and Mohan, 2004) and plays an important immune-modulatory role in TB clearance and control of disease progression. Interestingly, TB/HIV co-infection stimulates greater cytokine responses, as measured by IFN γ and TNF α than HIV-infection alone and this response is reconstituted following ARV therapy (Oh et al., 2005). This was furthermore, demonstrated in patients with CD4 cell counts of <200 cells/mm³. Moreover, TB increases the susceptibility of lymphocytes to HIV infection (Santucci et al., 2004).

Similarly, a depressed Th1 immune response, characteristic of HIV infection, results in increased susceptibility to TB (Sharma and Mohan, 2004).

It has been shown that CD4⁺ and CD8⁺ T-cell counts are lower in dually infected, compared with HIV or TB infected individuals (Rodrigues et al., 2003). This is an expected finding. However, the authors report varying degrees of decline in CD4 levels in naïve, memory and effector cellular populations, which was independent of single (HIV or TB) or dual (HIV/TB) infection. The depletion of CD4⁺ cells is reflected in a failed immune response and poor granuloma formation (Raja, 2004). Despite considerable decline in CD8⁺ T-cell numbers activation status is elevated in HIV⁺/TB⁺ patients even in excess of HIV-infected patients, which was attributed to the co-circulation of HIV and TB in the patients studied (Rodrigues et al., 2003). It was recently demonstrated that the activity levels of NK and CD8⁺ cells rather than the number of cells, is more significant to the immune response (Raja, 2004). CD4 depletion without concomitant reduction in IFN γ levels alludes to other possible producers of IFN γ including macrophages, CD8⁺, NK and CD4⁻CD8⁻ T-cells (Fremont et al., 2004). As a consequence of gp120 protein of HIV *env* gene binding to the CD4⁺ receptors, Ag presentation of TB peptides by MHC Class II molecules, is impaired in co-infected patients (Raja, 2004).

In HIV infected spinal TB patients, CD4⁺/CD8⁺ lymphocyte ratios were reversed, with significantly lower CD8⁺ counts ($p < 0.05$), which was strongly associated with mortality rates (Govender et al., 2001a). Thus, CD4⁺/CD8⁺ cell counts remain the primary determinant of outcomes and mortality rates (Narain and Lo, 2004). In direct contrast, it was demonstrated, in a South African cohort, that higher mortality rates were associated with CD4⁺ T-cell counts of >200 cells/ μ l and those not at advanced disease/AIDS at baseline (Badri et al., 2001). In another South African cohort, IFN- γ was inversely correlated with CD4 counts (Mayanja-Kizza et al., 2001).

1.2.3.5 Combining Anti-retroviral therapy (ARV) and TB chemotherapy

Of the 25 countries with the highest HIV prevalence, only 4 have achieved their TB treatment targets as outlined by the WHO (Maher et al., 2005). Response to anti-TB

chemotherapy is similar in HIV-infected and uninfected persons and its efficacy is not dependant on degree of immuno-suppression (Narain and Lo, 2004). However, adverse effects arising from TB treatment are common to HIV-infected patients (Narain and Lo, 2004, Pereira et al., 2004). This is believed to be a consequence of immune reconstitution triggered by ARVs.

It has been proposed that combining anti-retrovirals (ARV) and TB chemotherapy could save up to 500 000 dually infected African people (WHO/UN, 2004). Fewer than half the global HIV/TB co-infected patients are receiving anti-TB therapy. This is despite a much lower cost compared with ARV therapy (WHO/UN, 2004). It is only with collaboration between TB and HIV treatment strategies that any impact can be made on this dual epidemic. Interventions that may prove useful include simultaneous ART, increased rates of TB case finding, and preventative therapy in the form of isoniazid and cotrimoxazole (Maher et al., 2005, Mwaungulu et al., 2004). Cotrimoxazole is, in fact, a potent anti-TB chemotherapy, implementation of which resulted in notable reductions in mortality rates and dramatically improved outcomes, in co-infected patients in a Malawian cohort (Mwaungulu et al., 2004). One of the primary constraints to treatment delivery remains delayed diagnosis. Therefore, in order to prevent transmission to patient contacts including health workers, it is recommended that TB treatment be initiated in the interim, based on a high index of suspicion rather than conclusive culture or microscopy (Smith, 1991).

Unfortunately, drug therapy is more complex in the HIV-infected, since protease inhibitors (PI) and non-nucleoside RT inhibitors (NNRTI), prescribed as part of antiretroviral therapy (ART) interacts adversely with rifampicin (Narain and Lo, 2004). Combining these drugs results in increased anti-TB drug-related toxicity and delivery of ARTs at sub-therapeutic levels (Small and Fujiwara, 2001). While complex, combining therapies is not impossible but necessitates a complete understanding of the related drug interactions. It is however, recommended that protease inhibitors be administered after completion of the TB chemotherapy (Narain and Lo, 2004) and that HAART is initiated early in the treatment of co-infected patients with CD4 counts ≤ 100 cells/ μ l (Kwara et al., 2004). Of course this study was conducted in a developed nation with an HIV subtype B epidemic and outcomes may differ when compared to those in the KZN subtype C endemic region. The phenomenon of the paradoxical reaction,

where patients receiving HAART become non-responsive to anti-TB treatment, results in a more disseminated and severe pathology (Breen et al., 2004).

1.2.4 Microscopy and disease pathology

The utility of microscopy over other more widely applied methods such as flow cytometry, has been demonstrated, displaying the ability to discern specific cellular morphologic features, otherwise indistinguishable by any other means (Giuliani et al., 2001).

1.2.4.1 Light microscopy

Diagnosis of TB remains problematic since current methods may be time-consuming, non-specific, of low sensitivity and almost wholly reliant on culture (Zajac and Melcher, 1991). Assessment of clinical samples by direct staining and microscopy is widely used and purported to be one of the more economical and rapid techniques presently available to disadvantaged nations (Kivihya-Ndugga et al., 2004). The Ziehl-Neelsen (ZN) technique, is a routine diagnostic assay for acid-fast bacilli such as *Mycobacteria*, and localises lipids in the bacterial capsule.

A specialised division of microscopy, immunohistochemistry (IHC) had its beginnings in the early 20th century with great strides in sensitivity and specificity taken towards the end of the century (Bunea and Zarnescu, 2001). IHC localises a range of tissue-associated antibodies (Ab) or antigens (Ag) via in situ targeting of antigen-antibody interactions. The value of IHC, in the clinical as well as the research setting, has been demonstrated (Bunea and Zarnescu, 2001). However, one of its primary restrictions is the lack of appropriate and specific markers for various cells and their products. Monoclonal antibodies are preferable for a number of reasons including greater specificity and reproducibility despite their lack of avidity and the chances of cross-reactivity when more than one epitope is present (Bunea and Zarnescu, 2001). The alternative is a polyclonal antibody that recognises several epitopes, and is easy to prepare but Ag and Ab purification is required, it may not be reproducible, production is

limited and a high rate of non-specificity and background staining may arise (Bunea and Zarnescu, 2001). The choice of Ab is thus very subjective and dependant on the specific requirements of the research enquiry.

1.2.4.2 Electron microscopy

Electron microscopy (EM) affords the opportunity to examine the ultra-structural features of a cell in response to various chemical and physiological changes in the localised tissue.

Ultra-structural investigation of Mtb illustrated, among other features, widely distributed ribosomal particles, electron-dense polyphosphate granules and DNA fibres (Dahl, 2004, Dahl et al., 2001, Takade, 2003). The cellular envelope consists of a plasma membrane (PM), a thinner inner layer and a thicker outer layer termed an asymmetric PM compared with a symmetrical PM characteristic of a degenerative cell (Takade, 2003). The cell wall has three covalently bonded macromolecules namely, peptidglycans, arabinogalactans and mycolic acids with glycolipids and mycolic acids forming the outer-most layer of the cell wall. Despite differences in their relative virulence and infectivity, Mtb and *M. leprae* do not differ in their cell wall architecture (Takade, 2003) indicating factors other than the cell wall as determinants of pathogenesis. Mehta et al, (Mehta et al., 1996), in comparing murine and human macrophages, used EM to demonstrate similar patterns of infection and bacterial multiplication in the initial stages of disease, but vastly different rates of clearance in response to drug therapy. The most notable ultra-structural finding was that cell type, together with temperature, promoted intracellular bacterial replication, even in the presence of appropriate antibiotics (Mehta et al., 1996). It must be remembered that tissue culture environments will differ from the *in vitro* physiology and biochemistry; therefore, differences between *in vivo* and *in vitro* tissue and cellular morphology must be expected.

Development of mononuclear phagosomes parallels the progression of granuloma formation and manifests as distinct histological changes. Mononuclear phagocytes contain profuse peroxidase positive granules, which are lost as the cells mature to

macrophages and epithelioid cells (Adams, 1983). Immunoelectron localisation studies of the HIV-1 *env* antisense protein (ASP) coded by the minus strand isolated the protein to the cellular membrane and in the released viral particle (Briquet and Vaquero, 2002). This transcript of the minus strand, while unusual, appears to play a role in viral structure and packaging.

Chapter 2 – Clinical and laboratory findings contrasting HIV-infected with –uninfected patients

2.1 Introduction

South Africa (SA) is presently ranked among the 35 highest TB incidence and prevalence countries worldwide (Global Health, 2006), with 458 persons living with TB per 100 000 population. Of the 9 provinces of SA, KwaZulu-Natal (KZN) boasted the highest incidence of TB and HIV/TB co-infection in 2002, and was among the 3 highest HIV incidence regions in the country (DOH, 2002, DOH, 2004). The leading causes of death in the province remain TB and HIV/TB with mortality due to pulmonary TB surpassing all other opportunistic infections. While comparatively less significant, spinal TB still impacts greatly on morbidity and mortality particularly since spinal deformity and paralysis is a common complication.

Clinical investigations of spinal TB rank the condition as the most important site of extra-pulmonary disease (Sinan et al., 2004). The escalating HIV epidemic exacerbates an already sizeable pulmonary, and more recently expanding extra-pulmonary, epidemic (te Beek et al., 2006). More alarming is the increase in the number and location of extra-pulmonary sites including musculoskeletal, the extent of pathogenesis and incidence of drug resistant disease (Houshian et al., 2000).

Studies of spinal TB infections have largely focused on radiological findings and clinical reports (Govender et al., 2000a, Chang et al., 1989, Dass et al., 2002, Pertuiset et al., 1999, Srivastava and Sanghavi, 2000, Villoria et al., 1992). Few have focused on the Southern African epidemic, where the prevalence and incidence of both HIV and TB is high, and where HIV-1 subtype C is the predominant genetic clade (Govender et al., 2001b, Govender et al., 2001a).

The main objective of this chapter was to contrast selected clinical, haematological and demographic factors of HIV-uninfected with HIV-infected patients diagnosed with TB spondylitis, in order to identify related markers unique to the latter group.

2.2 Methods

2.2.1 Study population

This study was approved by the Biomedical Ethics Research Board of the University of KZN (Post-graduate reference #: H112/02). Patients, from a primary referral hospital (King George V Hospital, KZN), who consented to participate in the study, were recruited at the Spinal Unit. This referral institute receives patients from all peripheral hospitals in KZN and the Eastern Cape. Approximately 150 patients are treated at this institute per year; approximately 66% of this number requires surgical interventions due to pain, progressive deformity despite conservative treatment, and neurological deficit. Of the patients presenting with infective spondylitis, approximately 30-40% are HIV co-infected. Only patients, who fulfilled the selection criteria, following a detailed clinical evaluation, were recruited into the study. The sample size was determined by the rate of intake over a 16-month period (July 2003-November 2004). The selection criteria were as follows:

2.2.1.1 Inclusion Criteria

1. Clinically and radiologically diagnosed TB spondylitis patients
 - a. Clinical diagnosis
 - i. Axial (spine) pain
 - ii. Deformity (Gibbus)
 - iii. Constitutional symptoms (loss of weight, loss of appetite and night sweats)
 - b. Radiological diagnosis (Fig. 2.1a,b)
 - i. Large paraspinal abscess involving multiple vertebral bodies
 - ii. Bone destruction, collapse of the vertebrae, and deformity
 - iii. Compression of the spinal cord
2. Patients who consented to participate in the study
3. Patients already initiated on 5-24 weeks of standard anti-TB chemotherapy
4. Infective spondylitis confined to ≤ 4 contiguous vertebral bodies
5. Patients who consented to HIV testing
6. Patients >18 months of age

2.2.1.2 Exclusion Criteria

1. Patients with active pulmonary tuberculosis or foci outside of the spine
2. Patients who were ARV naïve
3. Patients with concomitant immunosuppressive conditions (Diabetes, chronic steroid use, chemotherapy, congenital disorders)

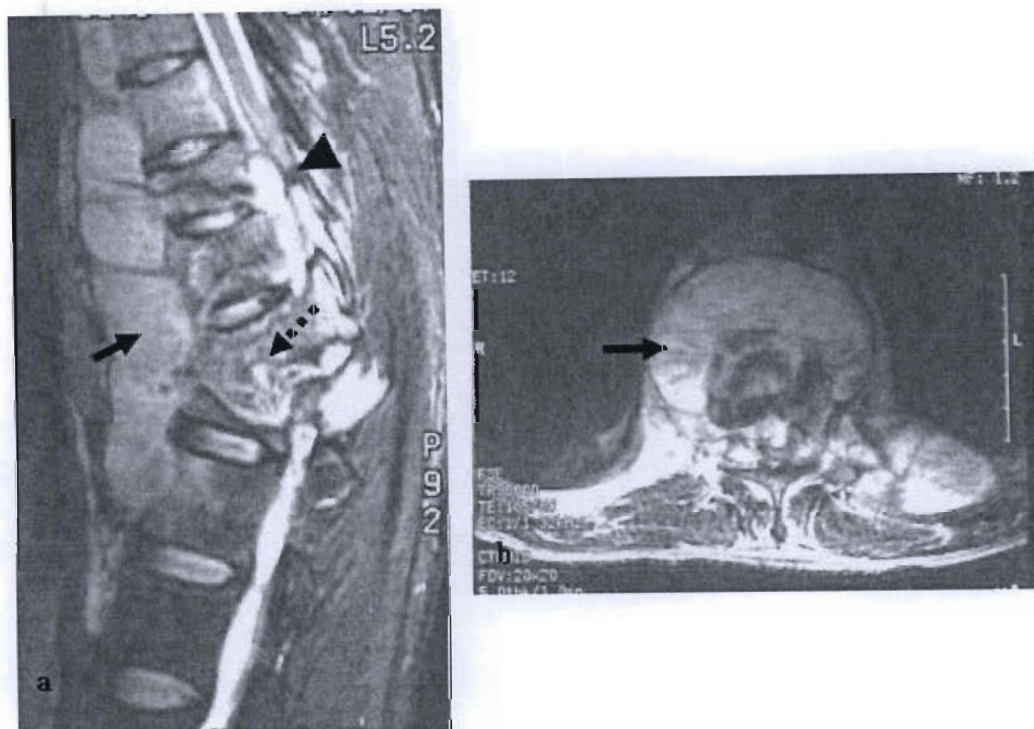


Figure 2.1: MRI micrographs of an HIV-infected patient showing a large extradural granuloma (arrow) involving multiple vertebral bodies in (a) longitudinal and (b) cross section. Bone destruction (dashed arrow), collapse of the spinal column resulting in deformity, and compression of the spinal cord (arrow head) is evident.

2.2.1.3 Treatment interventions

Interventions and treatment strategies for all patients recruited into the study were identical. All patients received pre-operative nutritional support (mean = 2 weeks) range 2-4 weeks) to achieve safe haemoglobin and albumin levels (>100 g/l and >300 g/l, respectively), as well as standard anti-TB therapy (600 mg Rifampicin, 400 mg Isoniazid, 1500 Pyrizinamide 1500 and 1200 mg Ethambutol daily) for a mean period of

8 weeks (range = 5 – 24 weeks). HIV-infected patients were antiretroviral (ARV) naïve, even those conforming to the SA guidelines for treatment initiation.

Surgical interventions involved removal of the diseased vertebral bodies, debridement and insertion of a femoral allograft in order to stabilize the spinal column. In selected cases, the allograft was further reinforced with instrumentation.

2.2.2 Specimen collection and processing

Blood (EDTA anti-coagulant) and patient-matched open tissue biopsies were collected from a total of 60 patients (22 HIV-infected and 38 HIV-uninfected) diagnosed with TB spondylitis. All patients were scheduled to undergo vertebral excision and anterior spinal decompression for the treatment of infectious spondylitis-related neurological deficit. Blood and tissue were collected under sterile conditions during surgery (in theatre). Part of the tissue biopsy was submitted to an accredited (South African National Accreditation System, SANAS) private pathology laboratory in addition to a government managed TB pathology laboratory for routine microscopy (Ziehl-Neelsen, Auramine stain) and culture (BACTEC MGIT system). The remaining excised tissue was processed immediately for HIV viral loads, molecular assays (Africa Centre Virology Laboratory [ACVL], Pfizer Molecular Laboratory, DDMRI*) and microscopy (Optics and Imaging Unit, DDMRI). Routine TB diagnostic testing (microscopy and culture) were conducted at the TB pathology laboratories according to their standard operating procedures.

2.2.3 Laboratory and diagnostic assays

Blood (EDTA anti-coagulant) was submitted to a government managed haematology laboratory for routine full blood counts (FBCs). CD4⁺ and CD8⁺ T-cell quantification (FACSCount method) was conducted at the HIV Pathogenesis Program (HPP, DDMRI). CD4⁺ and CD8⁺ T-cell counts were determined using a standard single-platform Beckman Coulter TetraOne method and a Coulter XL flow cytometer. CD4/CD8 testing was not performed on bloods that were clotted or insufficient. HIV

*The Doris Duke Medical Research Institute (DDMRI) houses the Africa Centre Virology Laboratory, the Pfizer Molecular Laboratory and the Optics and Imaging Unit.

serology (Vironosika UniformII [Biomeriux], Gac [Abbott] ELISA), and HIV RNA quantification were performed (ACVL, DDMRI) on all resultant HIV-infected specimens. The investigator conducted all viral load assays, thereby eliminating inter-technologist variability. RNA was isolated from plasma (100µl) and tissue (10-12mg) samples using the guanidine-silica Boom method as per the manufacturers protocols (Nuclisens isolation kit, Organon Teknika). Tissue specimens were homogenized and incubated overnight in assay lysis buffer (9ml) to ensure complete lysis. The Nuclisens HIV-1 QT assay (Organon-Teknika) quantifies HIV-1 RNA copies/ml plasma (detection limit = 40 to >500 000 copies/ml (cp/ml)). As per the manufacturer's protocols tissue viral loads were standardized per unit input mass (mg) which equated to 1ml input volume of plasma.

2.2.4 Statistical analysis

Statistical analyses were performed using the SPSS Version 11 software package for this and all subsequent chapters. Statistical significance was accepted at a 5% level, unless otherwise stated. The distribution curves for all data were generated to distinguish parametric from non-parametric data sets in order to direct subsequent analyses. A comparison of the means using parametric (Student's t test) or non-parametric (Kruskal Wallis) tests was undertaken to investigate differences between the HIV-infected and –uninfected groups, Correlations between the HIV-infected and –uninfected data sets were drawn using the Pearson's and Spearman Rho correlations for non-parametric data sets.

2.3 Results

2.3.1 Patient Demographics

The study population was predominantly HIV-uninfected (63%) and female (68%) (Table 2.1). All patients presented with an extra-dural granuloma, vertebral collapse and progressive pathology that was unresolved following a mean of 8 weeks of anti-TB therapy (range = 5-24 weeks). At least two contiguous vertebral bodies (thoracic,

lumbar or, more rarely, cervical spine) were involved in all patients. The thoracic spine (T5-T12) was implicated in >80% of the cohort (Patient records, results not shown). Onset of disease was insidious in the majority of cases (65%). Paralysis was rare (5%), with varying levels of neurology and deformity (no neurology to weakness and numbness) (Fig. 2.1a, b). Clinical and nutritional status was monitored against albumin (Alb) (normal range = 3.4-5.4 g/dL) and erythrocyte sedimentation rates (ESR) (normal range = males: 1-13mm/hr; female: 1-20mm/hr). ESR and Alb levels also determined the patient's readiness for surgery.

Table 2.1: Demographics patients clinically diagnosed with TB spondylitis contrasting HIV-infected (HIV⁺) and -uninfected (HIV⁻) patients.

	All patients (n=60)	HIV⁻ (n=38)	HIV⁺ (n=22)
Mean Age in years ± SD* (range)	32 ± 19 (2 – 71)	34 ± 21 (2 – 71)	29 ± 15 (2 – 65)
Male (%)	17 (28)	12 (32)	9 (50)
Female (%)	41 (68)	24 (63)	9 (50)
Missing data (%)	2 (4)	2 (5)	0 (0)
Paediatric (%)	13 (22)	10 (26)	3 (14)
Adult (%)	43 (72)	24 (63)	19 (86)
Missing data (%)	4 (7)	4 (11)	0 (0)

* = Standard Deviation

2.3.2 TB diagnosis

Results from two routine TB pathology laboratories were in agreement and reported 68% of the cohort to be *Mycobacterium* culture negative (Table 2.2). Consequently, these specimens could not be classified as drug sensitive or resistant and there was no change to the patient's treatment regimes. Of the culture positive specimens, drug resistance against Isoniazid (6 HIV-infected and 2 HIV-uninfected) and streptomycin (2 HIV-infected and 1 HIV-uninfected) were noted. Culture identified NTMs occurred in the HIV-uninfected group alone (Table 2.2). A positive culture result was associated

with higher plasma viral loads ($r=0.568$; $p<0.05$) in HIV-infected patients. Routine microscopy was positive in one case, an HIV co-infected paediatric patient (Patient 32). The association between elevated viral loads and an Mtb infection was notable in the 0-30 year age group but not among patients older than 30 years ($p>0.05$).

Table 2.2: Findings of TB culture identification (Bactec MGiT) and drug reactivity

	All patients (n=60)	HIV- (n=38)	HIV+ (n=22)
Culture identification			
No result (%)	41 (68)	27 (71)	14 (64)
Mtb (%)	16 (27)	8 (21)	8 (36)
NTM (%)	3 (5)	3 (8)	0 (0)
Drug reactivity			
Isoniazid			
Sensitive (%)	14 (23)	8 (21)	2 (9)
Resistant (%)	4 (7)	2 (5)	6 (27)
No result (%)	42 (70)	28 (74)	14 (64)
Ethambutol			
Sensitive (%)	16 (27)	8 (21)	8 (36)
Resistant (%)	2 (3)	2 (5)	0 (0)
No result (%)	42 (70)	28 (74)	14 (64)
Rifampin			
Sensitive (%)	17 (28)	9 (24)	8 (36)
Resistant (%)	1 (2)	1 (3)	0 (0)
No result (%)	42 (70)	28 (74)	14 (64)
Streptomycin			
Sensitive (%)	2 (3)	2 (5)	0 (0)
Resistant (%)	3 (5)	1 (3)	2 (9)
No result (%)	55 (92)	35 (92)	20 (91)

NB: the BACTEC MGiT assay was conducted in a pathology laboratory by trained technologists following their laboratory Standard Operating Procedures.

2.3.3 Viral loads and CD4+/CD8+ T-cell counts

A comparison of viral loads and CD4/CD8+ T-cell counts indicated that tissue and plasma viral loads were positively correlated ($r=0.52$; $p=0.01$) but were not significantly ($p>0.01$) different (Table 2.3). However, when tissue viral loads in paediatric and adult patients were compared, they were significantly ($p<0.05$) greater in former group (Table 2.3). A positive correlation between tissue viral loads and platelet counts ($r = 0.600$;

$p=0.02$) over all HIV-infected patients was noted. Interestingly, a negative association was demonstrated when comparing tissue viral loads with the age of the patient ($r=-0.458$; $p=0.03$) and percentage CD3 counts ($r=-0.79$; $p<0.01$) illustrating that an immature, depressed or compromised immune response can be associated with unrestrained viral replication in co-infected tissue.

CD4/CD8+ T-cell counts were available for 65% of the cohort due to unacceptable, insufficient sample or delay in sample delivery to the testing site (Table 2.3). CD4 T-cell counts were in the normal range (> 500 cells/ μ l) in 40% of all patients in the cohort ($n=60$). This trend was observed in the HIV-infected (41%) and -uninfected (40%) groups alike. Only 4 HIV-infected patients displayed CD4+ T-cell counts below 200cells/ μ l. When comparing absolute CD4+ T-cell counts, the HIV-uninfected group displayed significantly ($p<0.05$) greater levels when compared with the HIV-infected patients. CD4/CD8 ratios were largely inversed in the HIV-infected group (mean = 0.50 cells/ μ l; range= 0.10-1.29 cells/ μ l) and significantly lower ($p<0.01$) in comparison to the HIV-uninfected group (mean = 1.16 cells/ μ l; range= 0.34-2.23 cells/ μ l) (Table 2.3) providing an indication of their immune compromised status.

2.3.4 Clinical outcomes

Clinical recovery was noted in 56/60 patients, and ranged from full recovery 46/60 patients, partial recovery in 10/60 patients and no recovery in the remaining 4 patients. Recovery patterns were defined as follows:

1. Full recovery - recovery of neurological deficit to full mobility, resolution of disease pathology and incorporation of allograft
2. Partial recovery – recovery of neurological deficit to limited degree allowing for limited range of motion
3. No recovery – no resolution of neurological deficit

There were no significant differences in the rates and scope of clinical recovery patterns in the HIV-infected, compared with the -uninfected patients.

Table 2.3: Viral loads and CD4+/CD8+ T-cell counts contrasting HIV-infected and uninfected patients

	All patients (n=60)	HIV- (n=38)	HIV+ (n=22)
Log plasma viral load in copies/ml (range) Overall (n=22) Adults (n = 19) Paediatric (n = 3)			4.14±1.59 (<LDL – 6.40)* 4.02±1.63 (<LDL-5.79)* 4.84±0.85 (4.20-6.39)*
Log tissue viral load in copies/ml (range) Overall (n=22) Adult (n = 19) Paediatric (n = 3)			4.20±1.85 (<LDL – 7.18)* 3.94± 2.36 (<LDL-8.15)* 6.63±1.55 (8.08-9.18)*
CD4 distribution (percentage of total population) <500 cells//µl >500 cells//µl Missing data	15 (25%) 24 (40%) 21 (35%)	7 (18%) 15 (40%) 16 (42%)	8 (36%) 9 (41%) 5 (23%)
Median Abs CD4+ counts in cells/µl (range) Adults (n=31) Paediatric (n=8) Missing data (n=17)	619 (100-1370)* 945 (400-2254)*	643 (281-1370)* 1390 (400-2254)*	496 (100-1081)* 885 (474-1005)*
Mean % CD4+ counts in cells/µl (range) Adults (n = 37) Paediatric (n = 10) Missing data (n=9)	34.0 (8.8-72.0)* 27 (12.0-51.0)*	643 (281-1370)* 1390 (400-2254)*	28.0 (8.8-53.0)* 21.37 (12.0-23.0)
Median Abs CD8+ counts in cells//µl (range) Adults (n = 31) Paediatric (n = 8) Missing data (n=17)	661 (194-1990)* 1314 (315-2512)*	511 (194-1712)* 1155 (315-2469)*	904 (314 – 1990)* 1925 (879–2512)*
Median CD4:CD8 ratio (range) Adult (n = 35) Paediatric (n = 10) Missing data (n=11)	0.80 (0.10-2.23)* 0.99 (0.34– 1.75)*	1.04 (0.40-2.23)* 1.23 (0.34–1.75)*	0.51 (0.10-1.29)* 0.46 (0.41– 0.50)*

Key: * = significant difference

2.4 Discussion

The findings of this study, demonstrated a 37% incidence of HIV co-infection among patients diagnosed with TB spondylitis, which was consistent with provincial and global demographic of HIV/TB co-infection (WHO, 2006). This cohort of 60 patients was representative of the patient intake at King George V (KGV) referral hospital, which received patients from the KZN and Eastern Cape regions. Annual spinal TB intake at KGV is 150 patients per year, approximately 100 of which require surgical interventions. The KZN region has the highest HIV and TB incidence rates of the 9 provinces of South Africa (Ige et al., 2005). This cohort was also representative of a significant portion of the nationwide disease burden of infectious spondylitis.

This study demonstrated that clinical and radiological markers of TB spondylitis between HIV-infected and –uninfected patients are distinctive, on presentation, yet outcome measures following surgery are not HIV specific. All patients presented with an extra-dural granuloma, vertebral collapse and reported limited response to conventional anti-TB therapy. Despite pre-surgery anti-TB therapy, progressive pathology and neurological involvement was evident, particularly in the HIV-infected group. However, there was no difference in the clinical outcomes following surgical intervention, enhanced nutritional support and sustained anti-TB drug therapy when comparing HIV-infected with –uninfected or adult with paediatric patients. The primary determinant of outcomes was their clinical status on presentation. In general, patients with severe deformity and neurological deficit fared worse than those with pain and limited pathological manifestations. This was independent of HIV status. The findings of this study were consistent with previous reports for KZN (Govender et al., 2000a, Govender et al., 2001b), (Govender et al., 2001a), which described a more severe disease pathology in HIV/TB co-infected patients.

In the present study, plasma and tissue viral loads were in excess of $4\log_{10}$ copies/ml despite pre-operative anti-TB therapy (mean 8 weeks), improved clinical status, CD4+ T-cell counts $>500\text{cps}/\mu\text{l}$ in $>40\%$ of cases and pauci-bacillary disease. These findings were consistent with a report by Wolday et al (Wolday et al., 2003) who demonstrated high viral loads following anti-TB chemotherapy despite clinical resolution of the pulmonary TB infection. However, unlike the Wolday et al (Wolday et al., 2003)

cohort, the prognosis and clinical outcomes of the HIV-infected patients in this study were favourable and comparable to the HIV-uninfected group. The present findings demonstrated that age was a significant contributor to site-specific disease pathogenesis. HIV replication in paediatric patients proceeded unrestrained as demonstrated by significantly greater tissue viral loads when compared with the adults ($p < 0.05$). The significant inverse correlation between tissue viral loads and age confirmed this finding even in patients with CD4+ T-cell counts of >500 cells/ml. Physiological and developmental differences between adult and paediatric patients, particularly with regards their immune responses, may account for these findings. High viral load at the site of infection, as demonstrated in the present cohort, may indicate site-specific immune or drug mediators, which up-regulate viral replication. As a result more virulent viral variants and escape mutants are likely to emerge.

This investigation substantiated previous findings from KZN (Govender, 2005, Govender and Charles, 1987) and emphasised the efficacy of enhanced nutritional status, surgical removal of the infected lesion and conventional anti-TB therapy to favourable clinical outcomes, even in immune compromised hosts (CD4 < 200 cell/ml). Although four patients displayed diminished immune responses and none of the patients in the cohort ($n=22$) received ARVs (the SA national rollout had not reached this population at the time of sample collection) there were no significant differences in either CD4+ T-cell counts or patient outcomes between HIV-infected and -uninfected patients. Patients were thus reliant, solely, on nutritional support, surgery and anti-TB chemotherapy for disease resolution. This suggests other mediating factors to clinical outcomes. A CD4+ T-cell count less than 200 cells/ μ l has been used as a preoperative indicator of the level of risk of post-operative wound infection and poor clinical outcomes, as it reflects both the vigour of the host immune response and the stage of HIV pathogenesis in co-infected patients (Bono, 2006). Also, diminished immune responses were shown to aggravate the localized pathology resulting in irreversible neurological damage in certain instances (Jutte et al., 2005). Nutritional deficiency impacts on both the cellular and humoral immune responses manifesting in altered chemotactic and phagocytic potentials and the relative abundance of immune cells (Cunningham et al., 2006). In developing nations, utilizing ARVs to elevate the host immune response of co-infected patients may not be accessible, practical or economical. Delaying ARVs also has the additional benefit of prolonging ARV therapy prior to

development of drug resistance. Thus these findings would suggest that the treatment regime applied to patients in this cohort may be adopted as a practical, effective and economical strategy for co-infected patients particularly since neither previous nor concurrent ART is required.

The large proportion of culture-negative (68%) and AFB-negative microscopy (98%) findings may either reflect an emergence of NTM disease in the local setting or the impact of pre-surgical anti-TB therapy. An escalating HIV pandemic coupled with severe immune-suppression as a result of malnutrition has culminated in an increase in atypical and/or opportunistic spinal infections particularly in developing nations (Archibald et al., 1998, Bono, 2006, Castagnola et al., 1997) (Looney, 2005). Atypical opportunistic pathogens have been implicated more often than Mtb (39%) in blood stream infections (Archibald et al., 1998) thereby enabling haematogenous spread to remote anatomical sites such as the spine. An association between CD4+ T-cell counts <50cells/ μ l and atypical Mycobacterial infections primarily among HIV-infected patients, has been shown (MacGregor et al., 1999). Moreover, TB spondylitis is characteristically a pauci-bacillary disease. In contrast to pulmonary TB, musculoskeletal disease is AFB negative in approximately 60% of cases (Walsh et al., 2004). Pre-operative anti-TB therapy initiated, at peripheral referring clinics and/or hospitals (mean = 8 weeks; range = 5-24 weeks), is likely to have contributed to the lack of confirmatory laboratory diagnoses in this cohort (Section 2.3.2). Initiation of anti-TB therapy in endemic developing nations and patients at a high index of suspicion often precedes laboratory confirmation of the disease (Jutte et al., 2005). As demonstrated in the present cohort, this severely complicates diagnosis and must be taken into account in paucibacillary disease. Moreover, immune suppression, while a known risk factor for TB, elicits a greater number of false negative tuberculin reactions and diagnoses (Jutte et al., 2005).

2.5 Summary

The findings of the present cohort confirmed previous findings in the KZN region of positive outcomes in clinically and radiologically diagnosed TB spondylitis patients following nutritional support, surgery and anti-TB therapy. These outcomes were

independent of CD4+ T-cell count, HIV status and viral load. Clinical and radiological markers remain the primary distinguishing features of HIV co-infection as no laboratory markers were distinctive. Pre-surgical anti-TB therapy was shown to be effective at reducing bacterial load but was unable to halt or resolve disease pathology. High viral replication rates were indicated by higher tissue versus plasma viral loads. Favourable clinical outcomes following the treatment strategy outlined in this chapter are achievable even in immune compromised hosts and in the absence of ARTs. Although, spinal TB is AIDs-defining, it can effectively be treated while still delaying ARTs.

Chapter 3 - Microscopy

3.1. Introduction

Granuloma formation is the hallmark of an effective immune response against a pathogenic or irritant challenge (DesJardin et al., 2002a) (Kaufmann, 2002). It acts to contain active disease and prevent dissemination. A typical granuloma is infiltrated with immune cells (Adams, 1983), and is surrounded by an outer cuff of fibrosis (Fig. 1.1) (Roitt et al., 2001).

The primary effector cells against a TB infection are CD4+ T-cells and phagocytes, including macrophages and macrophage-derived cells. As disease progresses, macrophages differentiate into epithelioid cells, which later coalesce to form multinucleated giant cells (Roitt et al., 2001). Phagocytes may be distinguished by their expression of the CD68 endosomal/lysosomal-membrane restricted antibody (Ab). These include macrophages from the germinal centre, tonsils, splenic red pulp, dermal connective tissue, hepatic Kupffer cells; alveolar macrophages and blood monocytes (Roitt et al., 2001). Although histopathological studies of pulmonary and pleural TB are well documented (Beers and Berkow, 1999, Engers et al., 1986, Kaufmann, 2002, Raja, 2004) related non-clinical investigations of the spine are rare (Govender et al., 2000a). Since spinal disease is debilitating, and results in severe pathology and neurological damage (Govender, 2005) understanding the disease pathology is important to future treatment strategies and patient outcomes.

This study hypothesised that HIV co-infection significantly impairs macrophage infiltration of spinal granulomas and induces ultrastructural changes reflecting greater degeneration, necrosis and caseation. It also proposes that histopathological features of co-infected granulomas may be distinguished from HIV-uninfected specimens at similar stages of pathology. Spinal granuloma biopsies were investigated at a light and electron microscope level to address these questions.

3.2. Methods

3.2.1 Study population

As described previously (Chapter 2, Section 2.2.2-2.2.3), epidural granuloma biopsies were collected at surgery, from 60 patients (38 HIV-uninfected and 22 HIV-infected) (Fig. 3.1). A sub-set of this population was processed for histological (n=35) and ultrastructural (n=13) evaluation.

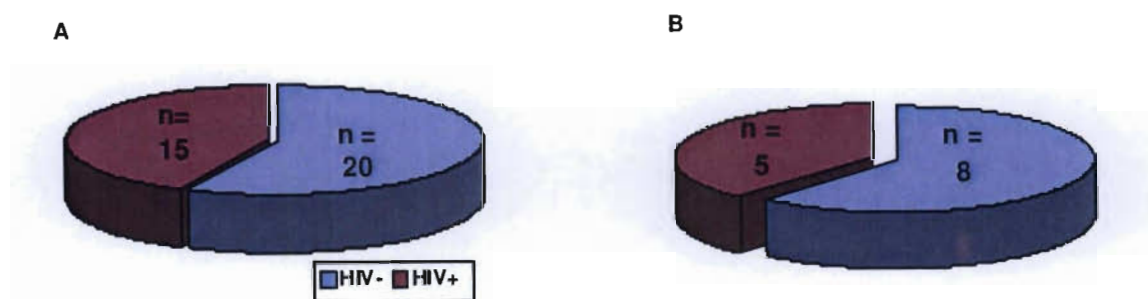


Figure 3.1: Graphical illustration of the patient cohort selected for (A) histopathological (Ziehl Neelsen, Haematoxylin & Eosin, Periodic Acid Schiff staining), CD68 immunolocalisation (Immunohistochemistry) and (B) ultrastructural investigations (Transmission Electron Microscopy).

3.2.2. Tissue processing for Light Microscopy (LM)

3.2.2.1 Tissue collection and pre-processing

Tissue biopsies collected at surgery were fixed immediately to minimize morphological aberrations resulting from post-surgical decay. Samples were cut into $\pm 1\text{cm}^3$ and $\pm 0.5\text{cm}^3$ blocks. The former ($\pm 1\text{cm}^3$) were immersed in 5% buffered formal-saline (41% formaldehyde/0.9% NaCl, 1:8 v/v) (Drury and Wallington, 1980) for routine light microscopy (LM).

3.2.2.2 Processing for light microscopy

Following a 12-24 hour incubation in 5% buffered formal saline (5% formaldehyde/0.9% NaCl, 1:8 v/v), specimens were processed for light microscopy by conventional means (Drury and Wallington, 1980, Bunea and Zarnescu, 2001). Serial sections of 2 and 3µm thickness were cut on a Jung RM2135 microtome (Leica, UK), and prepared for treatment with an appropriate staining technique.

3.2.3. Histopathology

3.2.3.1 Histopathology techniques

Following re-hydration, serial sections (3µm thickness) were stained with Haematoxylin and Eosin (H&E), Ziehl-Neelsen (ZN) and Periodic Acid Schiff (PAS) stains. Detailed descriptions of these techniques appear in Drury and Wallington (Drury and Wallington, 1980). Appropriate positive and negative control specimens were stained concurrently. Stained tissue sections were permanently mounted in distyrene tricresyl phosphate xylene (DPX) (Sigma, St. Louis, MO). The H&E stain was used to investigate the general histology of the specimens while ZN stains localised acid fast bacilli (AFB) such as *Mycobacterium tuberculosis*. Since the PAS stain localizes glycogen, mucin, mucoprotein, and glycoprotein of fungi, as well as human basement membranes, capsules and blood vessels. In this study, the PAS technique was applied to investigate possible fungal infections. Please consult Drury and Wallington (Drury and Wallington, 1980) for detailed descriptions of the H&E, ZN and PAS procedures.

3.2.3.2 Evaluation of histopathology

All analyses were confirmed by an independently pathologist¹ blinded to the HIV status of the specimens to reduce potential observer bias. The presence of granulomas, caseation/ necrosis, lymphocytes, epithelioid cells and plasma cells were scored

¹ Dr Ashwin Bramdev, Specialist Pathologist, Lancet Laboratories, Durban, SA

according to their relative prevalence in the infected tissue. Morphometric quantification of these pathological features and cell types allowed for the specimens to be divided into distinct patterns on the basis of their prevalence in the tissue sections. They were classified as moderate to profuse when their distribution in the field of interest was >45% or sparse to moderate when their prevalence accounted for 0-45% of the region of interest. Specimens were further classified as displaying histopathological features diagnostic or atypical of TB forming the basis of histopathological comparisons between HIV-infected and –uninfected specimens. The presence or absence of AFBs was not taken into consideration during these analyses in order to ensure an unbiased evaluation of the occurrence and severity of histopathological features consistent with a TB infection in both study groups.

3.2.4. Immunohistology

3.2.4.1 CD68, CD4 and CD8 immunolocalisation

Serial sections (2µm thickness), picked up on poly-L-lysine coated glass slides (Sigma, St. Louis, MO) were used to immunolocalize CD68, CD4 and CD8 Ab (Appendix B). The CD68 peptide is an 110kDa-glycosylated trans-membrane glycoprotein located in the lysosomes and endosomes of non-Langerhans histiocytes, monocytes, macrophages and multi-nucleated giant cells (Pulford et al., 1989). The commercially available antibody (Clone KP1, Dako) was used in this study. The CD4 marker (Clone IF6, Novocastra) was used to localise CD4+ T-cells. This antibody is a monoclonal prokaryotic transmembrane glycoprotein specific to the CD4+ T-cell population. The CD8 antibody marker (Clone C8/144B, Dako) was used to localise CD8+ T-cells. The antibody is a synthetic peptide, which consists of 13 c-terminal amino acids from the cytoplasmic domain of the CD8 polypeptide.

The primary antibody (CD68, CD4, CD8) was immunolocalised in re-hydrated tissue sections using a modified indirect immuno-peroxidase assay. Phosphate buffered saline (PBS) was used throughout the procedure, with the exception of the chromogen step, which required Tris-HCl (0.05M). The detailed methodology used is described in Appendix B. Each section was incubated with the appropriately diluted (1:1000 in 10%

milk protein) primary antibody and visualised using the Dako LSAB® kit (DAKO) and the chromogen diaminobenzidine (DAB). Replacement of the primary antibody with non-immune sera of the same IgG class produced no immunoreactivity (Fig. 3.6 b, c). Normal lymph node tissue sectioned as positive (Fig. 3.6a) controls were included.

3.2.4.2 Image analysis

Image analysis was performed using the Soft Imaging System (SIS) software package. This software system allows the user to acquire digital images, store them in a database, process and analyse them by performing various spatial and colorimetric measurements. A defined area or field referred to as the region of interest (ROI) was chosen for analysis in which the Ag-Ab-chromogen immunoprecipitate complex appeared as a collection of particles. These particles are essentially a quantity of connected pixels within a defined range of colour values, which correlate well image structure.

A quantitative analysis of CD68 immunoreactivity and a qualitative analysis of CD4 and CD8 reactivity were performed. Following capture, images were calibrated to a defined magnification. The frame area was defined to limit image processing and analysis to the region of interest. Segmentation was first conducted to selectively define the brown DAB chromogen from the background tissue based on selective thresholds, which were set interactively in a histogram. Phase analysis, a quantitative measure of intensity of the area of the defined phase or colour thresholds, was then performed according to a grey-scale (range = 0–255 dens units) hence not true colour densitometry. All micrographs are represented at their initial magnifications.

3.2.5 Transmission Electron Microscopy (TEM)

3.2.5.1 Tissue processing for TEM

The specimens analysed by TEM were processed in the Reichert Lynx automated EM tissue processor (Leica, UK). Tissue was fixed in 4% glutaraldehyde/5%

paraformaldehyde in 0.2M sodium cacodylate buffer, dehydrated through a graded ethanol series, prior to embedding in Spurr epoxy resin (Spurr, 1969) (Sigma, St. Louis, MO) according for 48 hours at 60°C. Ultra-thin tissue sections (50nm thickness) were cut with glass knives on an RM ultra-microtome (Leica, UK) and collected onto 200-mesh copper grids prior to contrast enhancement with saturated ethanoic uranyl acetate and Reynolds lead citrate (Reynolds, 1963).

3.2.5.2 TEM viewing and analysis

The Jeol JEM-1011 Transmission electron microscope (TEM) was used to visualize and digitise the ultrastructural features of the tissue. The iTEM imaging software enabled image acquisition and morphometric analyses.

3.3. Results

3.3.1 Histopathology

3.3.1.1 Patient demographics

The demographics of the cohort is outlined in Table 3.1. Conventional culture identification was negative in a large proportion of both the HIV-infected (66.7%) and – uninfected (75%) groups (Table 3.1). The mean percentage CD4+, CD8+ T-cell counts and CD4/CD8 ratios were significantly greater in the HIV-uninfected group ($p < 0.01$) while median CD4+, CD8+ cells were in the normal range for both paediatric and adult patients apart from CD4+ T-cell counts in the HIV-infected group (Table 3.1).

Table 3.1: Demographics of patients evaluated histopathologically, following H&E staining of granuloma biopsies

	All patients (N = 35)	HIV⁻ group (N = 20)	HIV⁺ group (N = 15)
Age in years ± SD (range)	34.1 ± 20.3 (2 – 71)	36.1 ± 23.7 (2 – 71)	31.9 ± 16.5 (2 – 65)
Gender			
Male (%)	12 (34.3)	9 (45)	3 (20)
Female (%)	21 (60.0)	9 (45)	12 (80)
Missing	2 (5.7)	2 (10)	0 (0)
Culture identification			
No growth (%)	25 (71.4)	15 (75)	10 (66.7)
Mtb (%)	8 (22.9)	3 (15)	5 (33.3)
MOTT (%)	2 (5.7)	2 (10)	0 (0)
Median CD4+ T-cell count (cells/μl)			
Adult	571	643	379
Paediatric	680	1390	474
Median CD8+ T-cell count (cells/μl)			
Adult	898	874	920
Paediatric	1177	1474	879
Median CD4/CD8 ratio			
Adult	0.52	0.86	0.44
Paediatric	0.92	1.14	0.46
Log plasma viral loads ± SD (copies/ml)	N/A	N/A	4.19 ± 1.30 (3.61- 5.74)*
Log tissue viral load ± SD (copies/ml)	N/A	N/A	6.32 ± 1.37 (3.00 - 8.15)*

SD = standard deviation; Mtb = *Mycobacterium tuberculosis*; MOTT = *Mycobacterium* other than *tuberculosis*; *=range

3.3.1.2 Ziehl-Neelsen stain

The Ziehl-Neelsen (ZN) stain was employed to identify AFBs in TB-infected tissue. The stained bacilli in the control section appeared bright red against a background of blue-stained cytoplasm (Fig. 3.2a). All specimens (both HIV-infected and –uninfected) were devoid of AFBs (Fig. 3.2b).

3.3.1.3 PAS stain

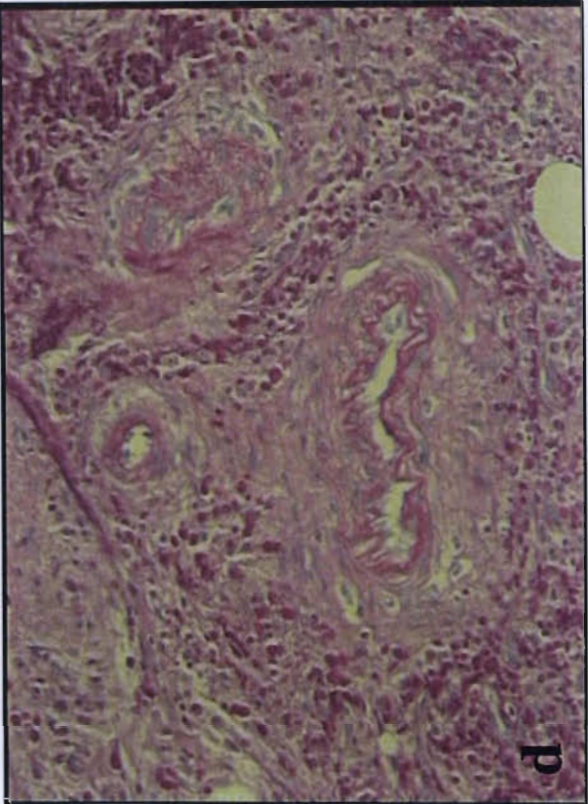
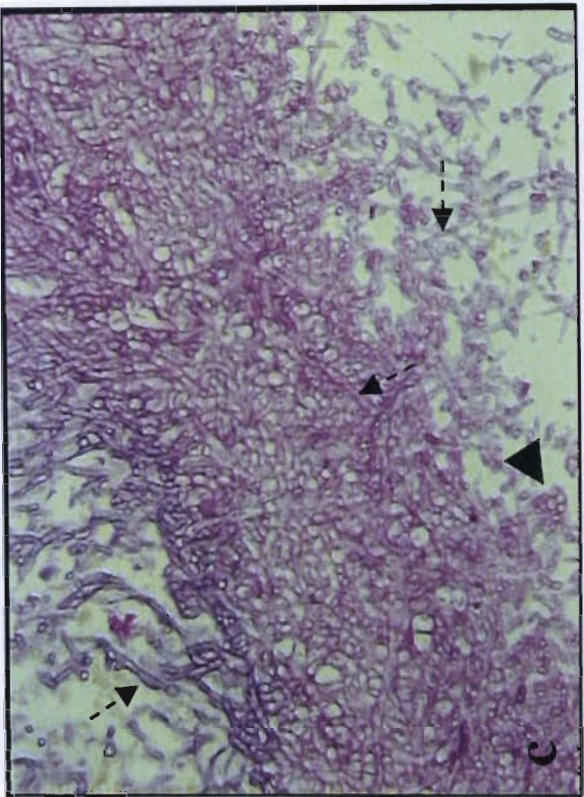
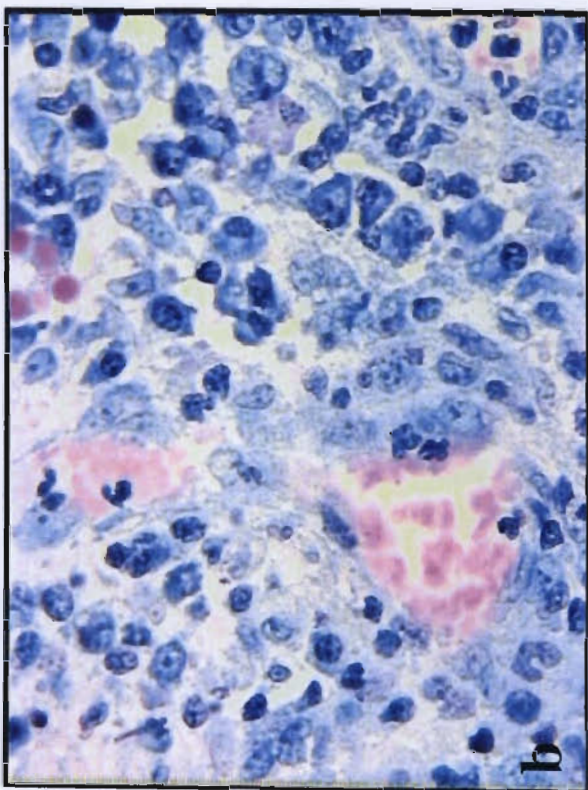
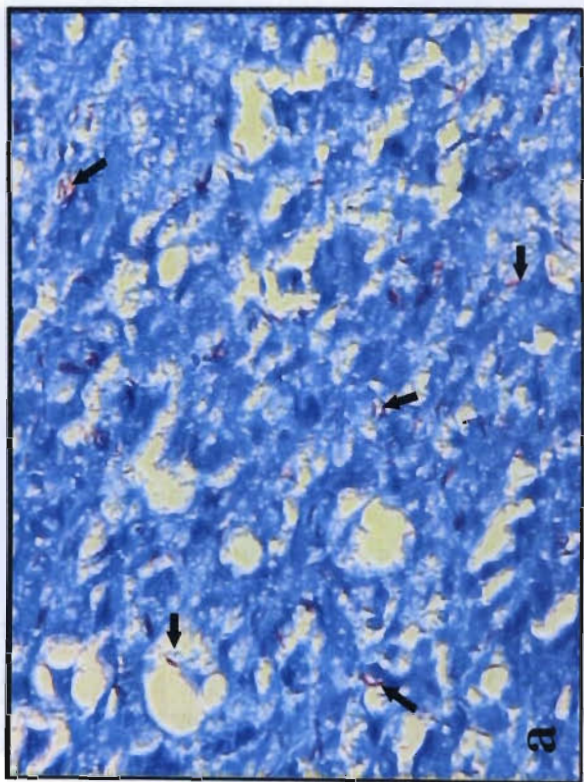
The absence of AFBs following ZN staining alluded to a possible atypical opportunistic pathogen. To investigate this possibility, sections were stained with PAS to detect / exclude mycoses. Although hyphae and fruiting bodies were clearly visible in the positive control (Fig. 3.2c) there was no evidence of fungal infection among any of the HIV-infected or HIV- negative patients in this cohort (Fig. 3.2d).

3.3.1.4 Semi-quantitative analysis of H &E stained specimens

Despite the negative AFB findings, histopathology findings revealed that 91.4% of specimens (HIV-infected and -uninfected) displayed features typical of a TB infection. Granuloma formation, caseation and lymphocyte infiltration were detectable at varying degrees (Table 3.2). Discrete compartments were discernable and the four zones, characteristic of granulomatous tissue, were identified. These zones comprised of:

1. An inner acellular core of caseation composed of highly necrotic tissue
2. A cellular zone consisting of macrophages, giant cells (Fig. 3.3), and lymphocytes (Fig. 3.3).
3. A sheath-like layer composed of epithelioid and plasma cells and
4. An outer fibrotic zone that formed a thickened physical barrier to the spread of infection

An overview of the histopathological findings is presented in Table 3.2. The absent/sparse distribution pattern of granuloma formation predominated in the HIV-infected patients (Table 3.2) while the moderate/profuse pattern was common to uninfected patients (73.3% versus 55.0%; $p < 0.05$). CD4+ T-cell counts were associated with greater granuloma formation in the HIV-uninfected group ($r = 0.635$; $p=0.027$). Caseation was noted in the HIV-infected group however, there was no statistically significant difference in the median degree of caseation when compared with the HIV-uninfected group ($p>0.05$).



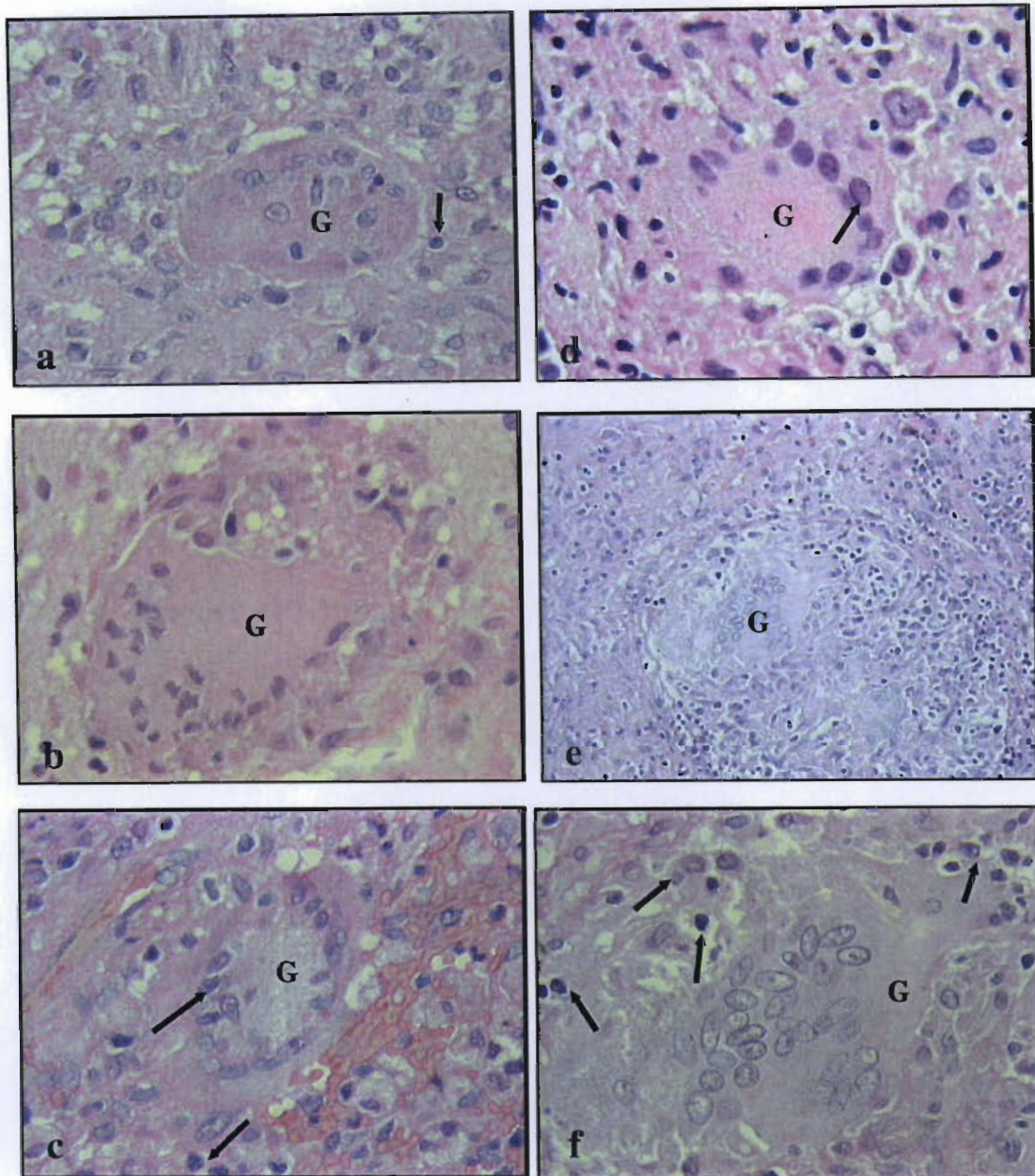
HIV⁻ GroupHIV⁺ Group

Figure 3.3: Light micrographs of H&E stained sections depicting variable morphologies of multi-nucleated giant cells (G) in HIV-uninfected (a, b, c) and HIV-infected specimens. Nuclei distribution was peripheral in b, c and d, and diffuse in a, e and f. Note the polymorphonuclear lymphocytes (arrows). [Initial Mag. a-f: x100]

Table 3.2: Pattern and cellular composition of epidural granuloma biopsies following hisopathological evaluation of H&E stained specimens.

Tissue Morphology	Absent to Sparse No of patients (%)	Moderate to Profuse No of patients (%)
All patients (N=35)		
No. of Granulomas	20 (57.1)	15 (42.9)
Degree of Caseation	20 (57.1)	15 (42.9)
Lymphocytes	16 (45.7)	19 (54.3)
Epithelioid cells	25 (71.4)	10 (28.6)
Plasma cells	33 (94.3)	2 (5.7)
HIV⁻ group (N=20)		
No. of Granulomas	9 (45.0)	11 (55.0)
Degree of Caseation	11 (55.0)	9 (45.0)
Lymphocytes	11 (55.0)	9 (45.0)
Epithelioid cells	17 (85.0)*	3 (15.0)*
Plasma cells	19 (95.0)	1 (5.0)
HIV⁺ group (N=15)		
No. of Granulomas	11 (73.3)	4 (26.7)
Degree of Caseation	9 (60.0)	6 (40.0)
Lymphocytes	5 (33.3)	10 (66.7)
Epithelioid cells	8 (53.3)*	7 (46.7)*
Plasma cells	14 (93.3)	1 (6.7)

*Statistically significant difference between HIV-infected and -uninfected group; $p < 0.01$

3.3.2 Immunohistology

3.3.2.1 CD68 immuno-localisation

CD68 immuno-reactivity was variable (sparsely (Fig. 3.5a, b), moderately (Fig. 3.5c, d) or densely (Fig. 3.5e, f) distributed) within CD68⁺ cells. Positive reactivity was observed in specimens infiltrated with mononuclear cells and well-formed granulomas (Fig. 3.5a, c, d) distinctive of the HIV-uninfected group. Reactivity was also noted in highly necrotic and caseous specimens particularly among the HIV co-infected group (Fig. 3.5b, d, f). Multi-nucleated giant cells were observed in both HIV-infected (Fig. 3.6a) and -uninfected specimens (Fig. 3.6b). Negative controls displayed no immune-reactivity (Fig. 3.4).

Image analysis quantified the distribution of the chromogen as a percentage of the area of interest (AOI) and the intensity of immunoreactivity, as a measure of the percentage CD68 reactivity and antibody aggregation respectively (Table 3.3). Although not statistically significant ($p > 0.05$), HIV-1 co-infected specimens displayed greater percentage CD68 reactivity compared with the HIV-uninfected group (Table 3.3). Despite this association, higher plasma viral loads were negatively correlated with maximum area of immuno-reactivity ($r = -0.580$; $p < 0.05$). The data also demonstrated a weak association between the mean intensity of CD68 reactivity and higher percentage CD4⁺ T-cell counts ($r = 0.400$; $p = 0.048$).

Table 3.3: Quantitative evaluation of CD68 immuno-reactivity in HIV-infected and -uninfected spinal TB-infected granuloma biopsies

	All patients (N = 35)	HIV- group (N = 20)	HIV+ group (N = 15)
Percentage of CD68 immuno-precipitation (%/ field)	6.19 ± 3.81 (1.05 – 15.69)	5.30 ± 3.68 (1.05 – 12.42)	7.39 ± 3.76 (2.83 - 15.69)
Mean intensity of CD68 immuno-precipitation per field¹ (dens units)	178.14 ± 24.48 (143.62 – 221.66)	181.00 ± 25.24 (143.83 – 221.66)	174.32 ± 23.74 (143.62 - 214.74)

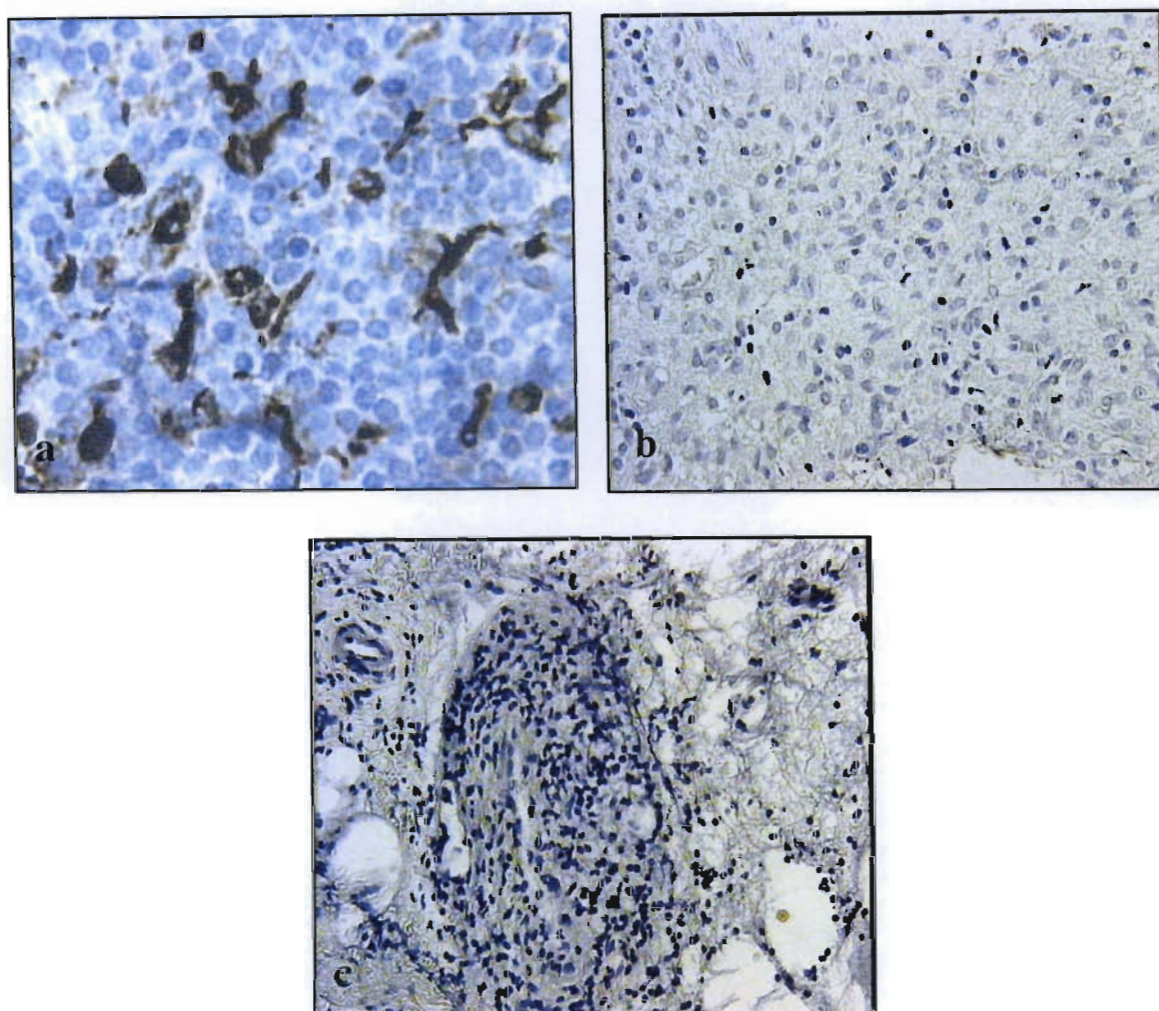


Figure 3.4: Light micrographs of (a) normal lymph node showing CD68 immunoreactivity (positive control). Figures b and c show an absence of CD68 immunoreactivity and represent (b) the buffer control and (c) the replacement of the primary antibody with non-immune sera of the same IgG class [Initial Mag. a-c: x40].

HIV-infected

HIV-uninfected

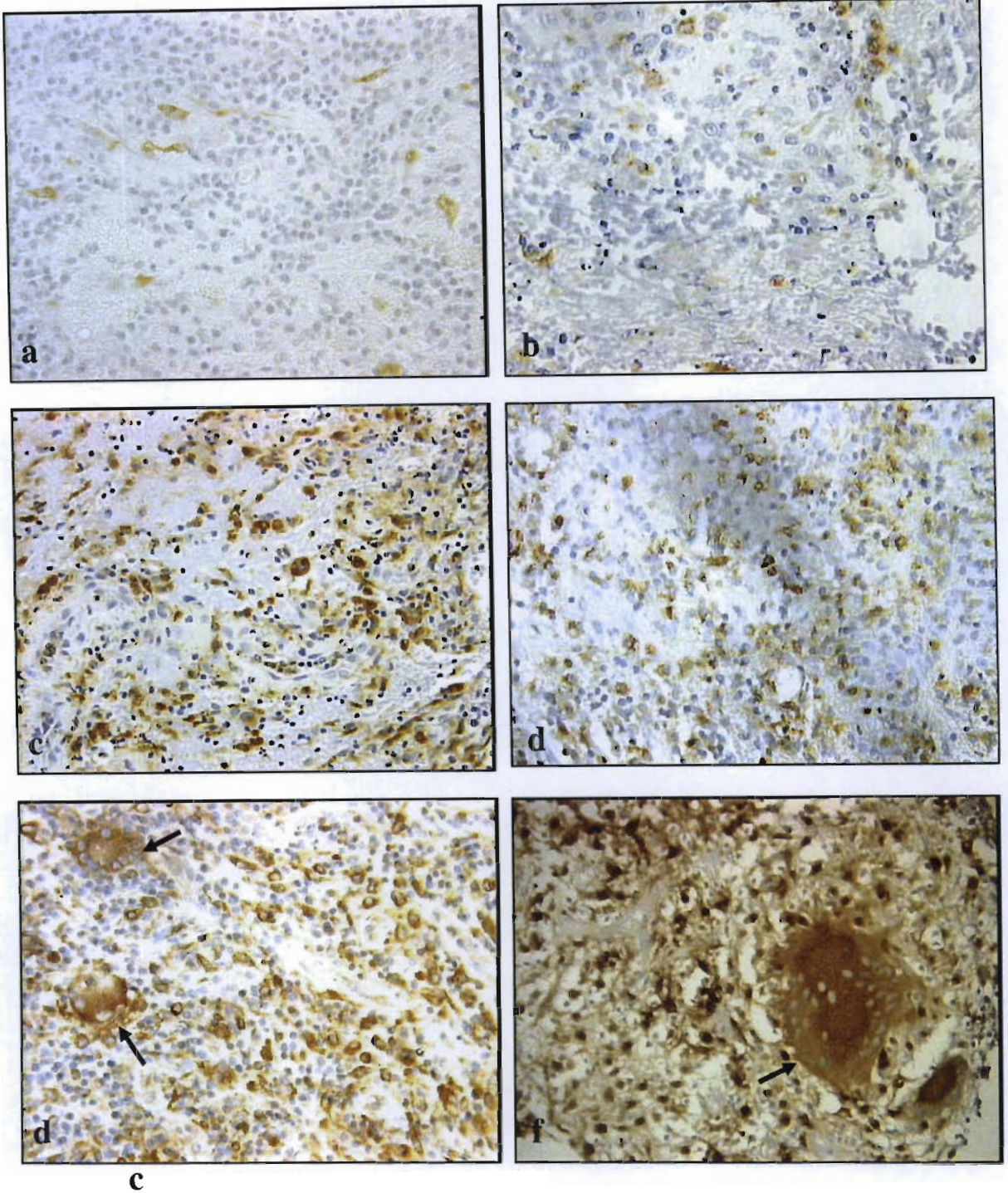


Figure 3.5: Light micrographs illustrating (a,b) sparse, (c,d) moderate and (e,f) dense CD68 immunoreactivity in (b, d, f) HIV-infected and (a, c, e) HIV-uninfected specimens. Note (e, f) giant cells (arrows) observed in both groups. [Initial Mags. a-f: x20].

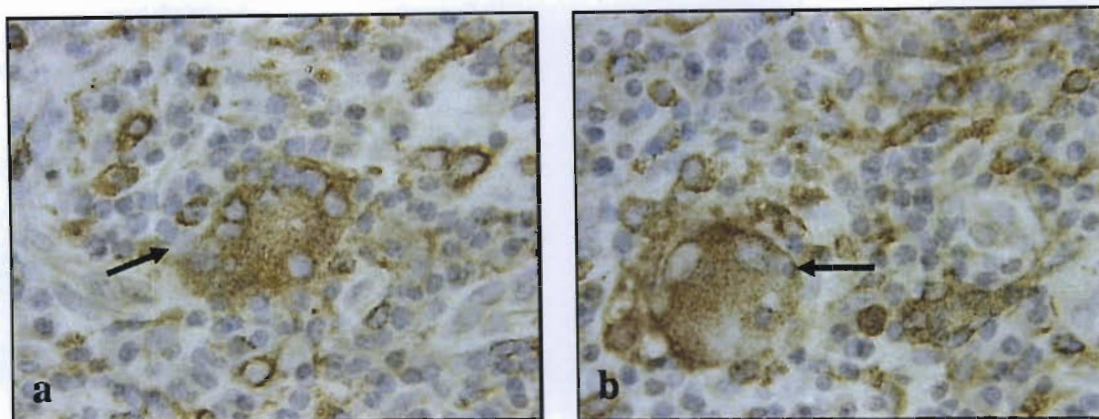


Figure 3.6: Light micrographs indicating CD68 immunoreactive cells in (a) HIV-infected and (b) –uninfected specimens. Note the immunoprecipitation of CD68 in multi-nucleated giant cells (arrows) [Initial Mag. a-c: x100].

3.3.2.2 CD4 immunolocalisation

Both HIV-uninfected (Fig 3.8a-c) and –infected (Fig. 3.8 d-f) specimens displayed sparse or an absence of immune-reactivity to the CD4 antibody. Lymph node tissue served as the positive control while substitution of the primary antibody with non-immune sera of the same IgG produced an absence of immune-reactivity (Fig. 3.7a).

3.3.2.3 CD8 immunolocalisation

Both HIV- uninfected (Fig 3.9a-c) and – infected (Fig 3.9d-f) specimens were reactive to CD8 antibody. Greater immune reactivity was observed in the HIV-infected (Fig 3.9d) compared with the -uninfected group (Fig 3.9a). CD8 immune reactive cells were noted scattered (Fig 3.9a), surrounding granulomas (Fig 3.9b), centres of caseation (Fig 3.9e, f) and adjacent to a giant cell (Fig 3.9c).

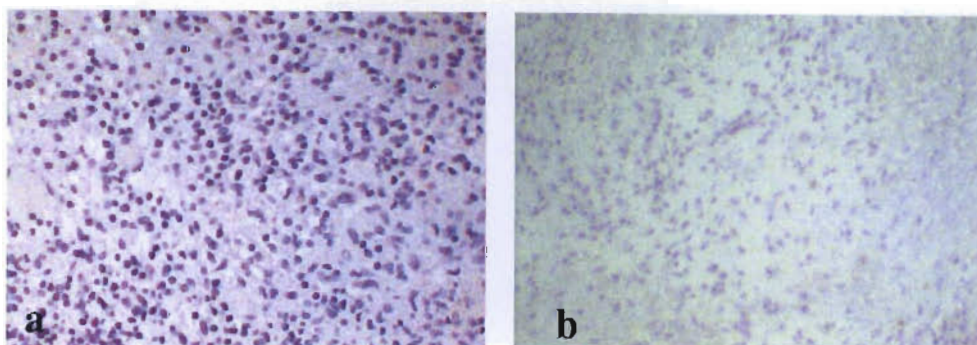


Figure 3.7 : Micrographs showing an absence of CD4 (a) and CD8 (b) immunoreactivity and represent the replacement of the primary antibody with non-immune sera of the same IgG class [Initial Mag. a,b: x20].

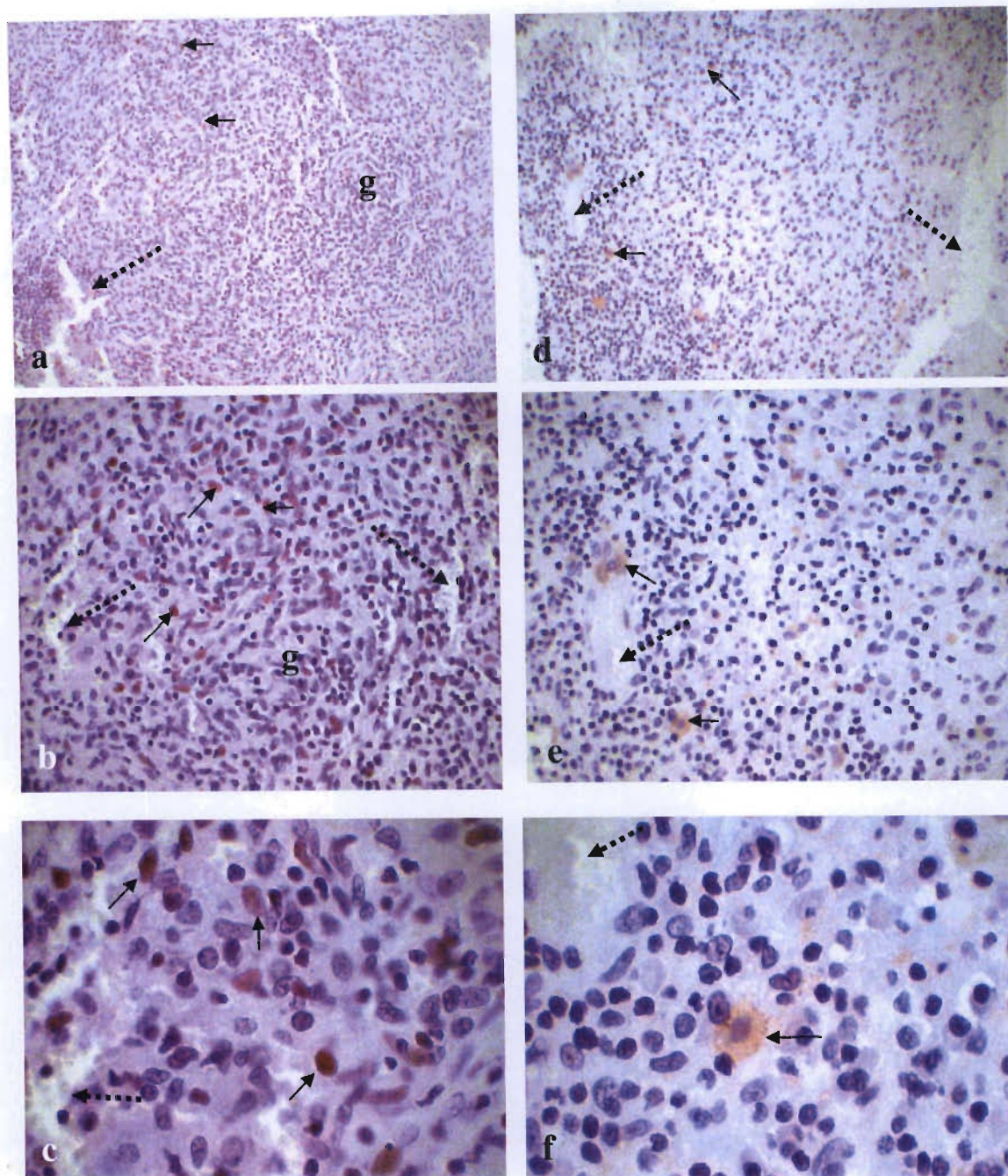


Figure 3.8: Micrographs of illustrating CD4 immunoreactivity (arrows) in (a-c) HIV-uninfected and (d-f) HIV-infected specimens. Note the (a, b) presence of granulomas, and (a-c) greater immune reactivity in the HIV-uninfected specimens compared with (d) sparse distribution of reactive cells, (arrow heads), extensive caseation and (d, e) an absence of granuloma formation in the HIV-infected group. [Initial Mag. a, d: x 20; b, e: x40; c, f: x100]

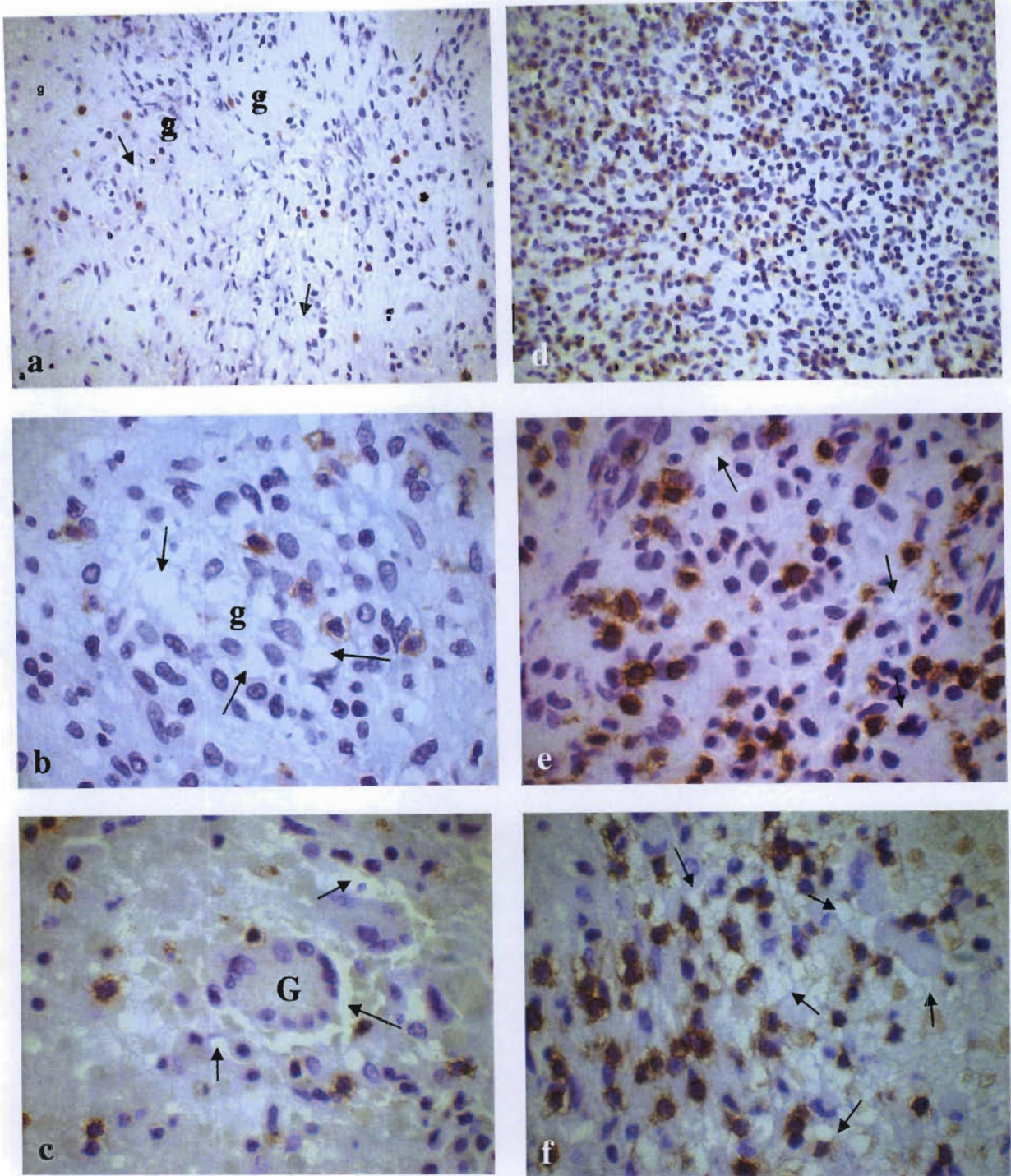


Figure 3.9: Micrographs illustrating CD8 immune reactivity in HIV-uninfected (a, b, c) and HIV-infected (d, e, f) specimens. Sparse immune reactivity was noted in HIV-uninfected specimens (a-c) but greater reactivity was observed in the HIV-infected (d-f) group. Note granuloma formation (g) and a giant cell (G) in the HIV-uninfected specimens; (a-f) and caseation (arrows) in both groups. [Initial Mag: a, d: x20; b, e: x40; c, f: x100]

3.3.3 Transmission electron microscopy (TEM)

3.3.3.1 Ultrastructural features in HIV uninfected specimens

Semi-thin toluidine blue-stained sections of an HIV-uninfected (Fig 3.10a-c) and an –infected (Fig 3.10d-f) specimen revealed granuloma formation (Fig 3.10b,e), immune cell infiltration (Fig 3.10b,c,e,f), giant cell (Fig 3.10c) and extensive caseation (Fig 3.10a-f). In contrast to the HIV-infected group, macrophages and immune cells (Fig. 3.11) were pervasive in 3 HIV-uninfected specimens (Patients 2, 13, 21). Various polymorphonuclear leucocytes and immune cells (Fig. 3.11) were present including granulocytes (Fig. 3.11a), neutrophils (Fig. 3.11b) and plasma cells (Fig. 3.11c). Lymphocytes were noted (Fig. 3.11d) with characteristically large nuclei and a thin rim of cytoplasm. Multinucleated giant cells were detected in 5 HIV-uninfected samples (Patients 1, 9, 17, 19, 23). Evidence of apoptosis, nuclear necrosis and pyknosis of the nucleus was also observed (Fig. 3.12a-f) in both HIV-infected and –uninfected groups.

3.3.3.2 Ultrastructural features in HIV-infected specimens

Four HIV-infected specimens (Patients 12, 16, 20, 22) were largely caseous consisting of amorphous material mixed with cells at varying stages of necrosis (Fig. 3.10d-f; 3.12). A qualitative ultrastructural evaluation confirmed the histological findings of the HIV-infected versus the –uninfected group consistent with a pathogenic insult. Despite the occurrence of features similar to the HIV-uninfected group, the severity in the HIV-infected group was more extensive and degenerative. A multinucleated cell was detected, (Fig. 3.13a) localised together with plasma and epithelioid cells in an HIV-infected specimen (Fig 3.13b,c) (Patient 14). Nuclear changes included peripheral heterochromatin aggregation (Fig. 3.12e), micro-segregation of heterochromatin (Fig. 3.12f), and nucleolemma swelling, (Fig. 3.12f) occurring, predominantly, in the HIV-infected group. A predominance of degenerative features such as dilated cisternal whorls of rER (Fig. 3.14b,c) and profuse cytosolic ribosomes (Fig. 3.15fa) was evident in the majority of HIV-infected specimens. There was evidence of an amorphous intracisternal content indicating that oedema was not the cause of ER swelling. Other

specimens showed cisternal inclusions of Russell bodies (Fig. 3.15c-e). Lysosomes were widely distributed in both HIV-uninfected and -infected specimens (Fig. 3.16a-d) and contained lysed material or debris (Fig. 3.16a, b), intact bacilli (Fig. 3.16c, d, 17c), and/or viral-like particles (Fig. 3.17a-c). Virus-like particles measuring 100-207 μ m in diameter (Fig. 3.17a-c) were characterised by their size and morphology. Lysosomal inclusions of 500-1500nm in diameter and resembling bacilli with thickened cell walls were also observed within the same lysosomal compartment as the virus-like particles (Fig. 3.17c).

3.3.3.3 Ultrastructural features in both HIV-uninfected and infected specimens

Plasma cells (Fig. 3.11c) and giant epithelioid cells (Fig. 3.13b,c) were rich in ER. The distribution of cisternae in the latter ranged from closely packed parallel stacks (Fig. 3.14a). Mitochondria (Fig. 3.15a-f) varied in shape and size, were double membrane-bound and contained cristae. In addition, mitochondria were observed in close proximity to ER and within dilated cisternal whorls of ER (Fig. 3.15). Degenerative mitochondria in the form of myelin bodies (Fig. 3.18a, b), were noted intracellularly or as part of the degenerative cellular debris. Intra- (Fig. 3.19a) in addition to a wide distribution of collagen fibrils (Fig. 3.19b) occurred in both HIV-uninfected and – positive specimens. Collagen deposits displayed characteristic cross-striations (Fig. 3.19b).

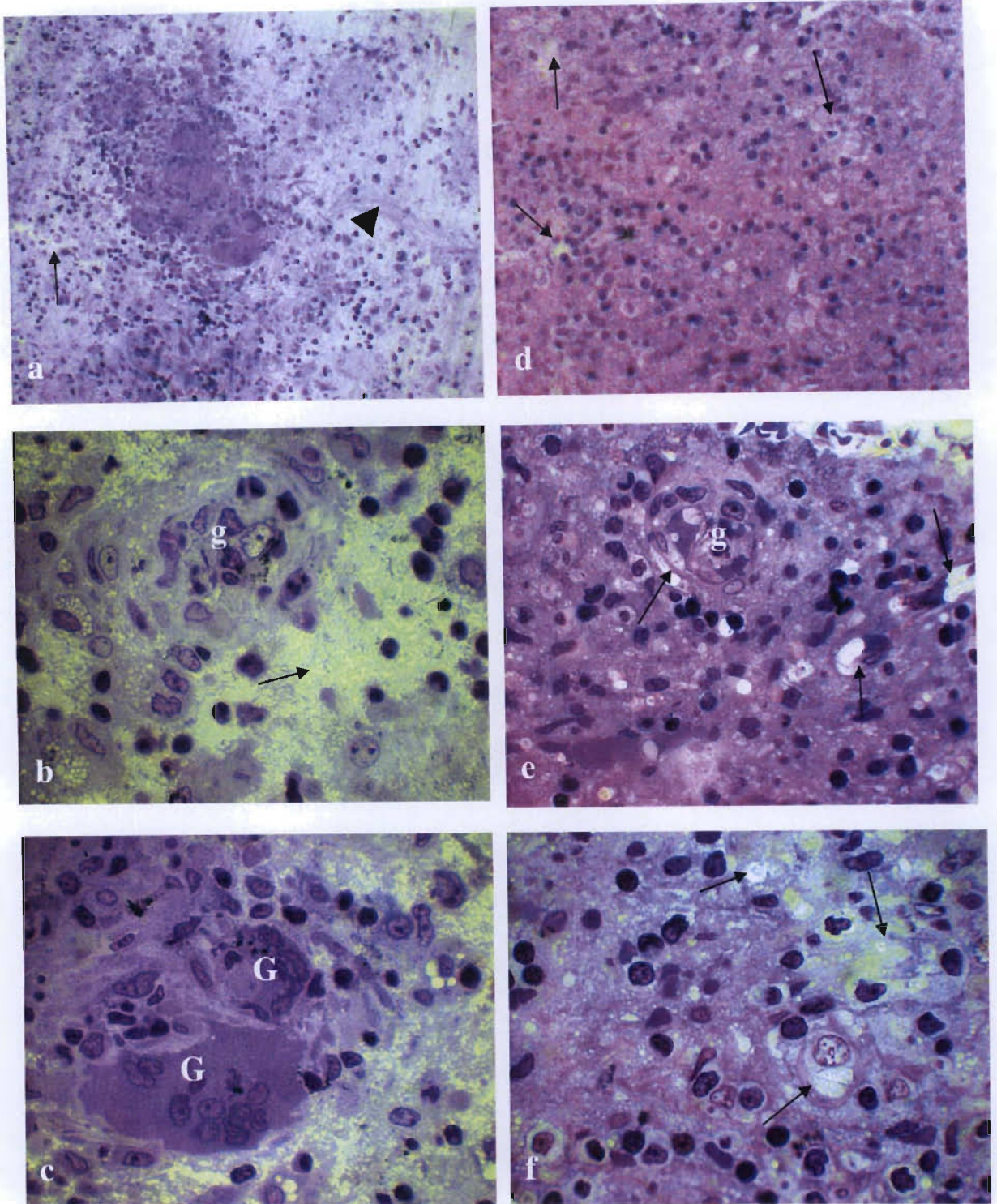


Figure 3.10: Representative micrographs of toluidine blue stained semi-thin sections of (a-c) HIV-uninfected and (d-f) HIV-infected specimens. Note (b,e) granuloma formation, (arrows) caseation and an infiltration of immune cells (b,c,e,f) in both HIV-uninfected and -infected sections. Giant cells (G) and fibrosis (arrow head) were observed in the HIV-uninfected section. [Initial Mag. a,d: x20, b,e: x40; c,f: x100].

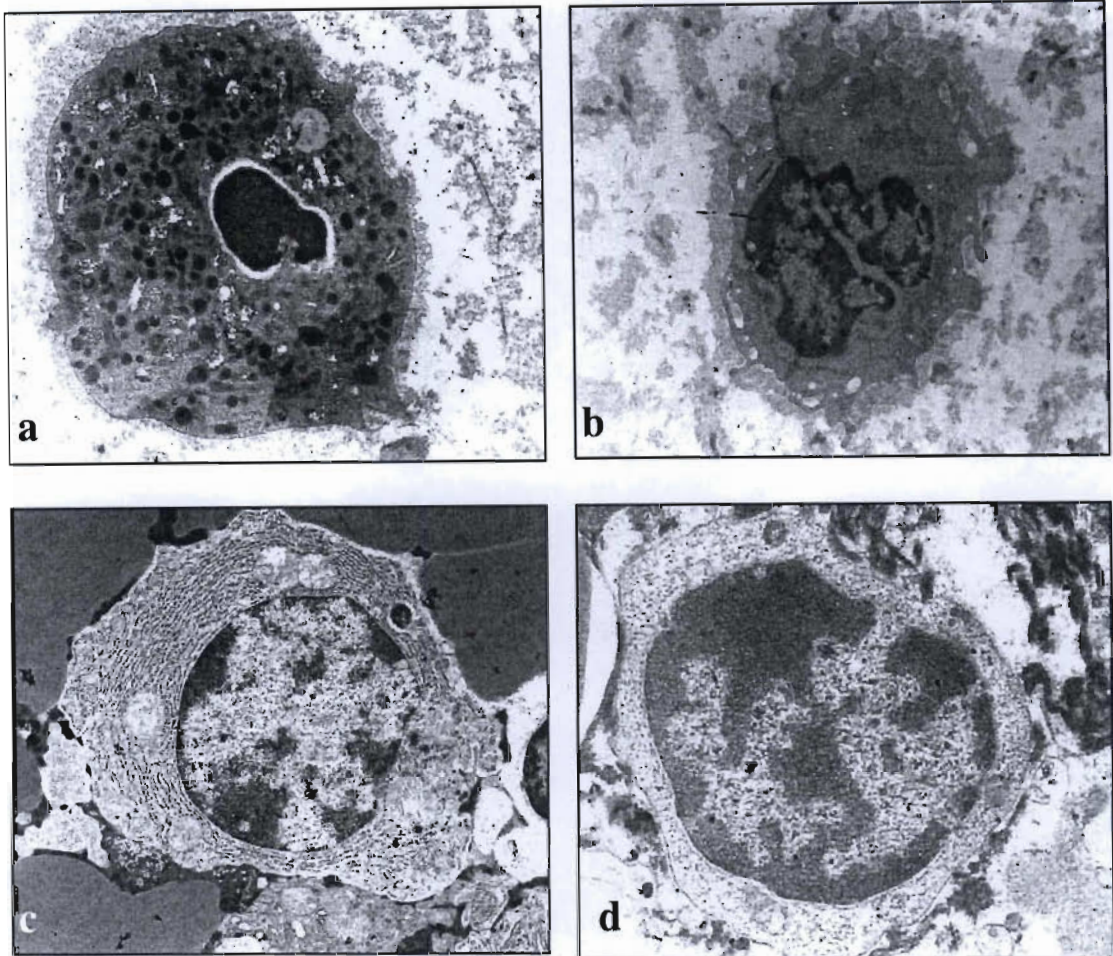


Figure 3.11: Electron micrographs depicting (a) a granulocyte (basophil or eosinophil) with granular cytoplasmic inclusion, (b) a neutrophil with characteristic multi-lobed nucleus, (c) a plasma cell displaying characteristic profusion of ER and (d) a lymphocyte which is a-granular, has a large nucleus with a thin rim of cytoplasm [Initial Mag. a: x15 000, b: x12 000, c: x10 000, d: x12 000].

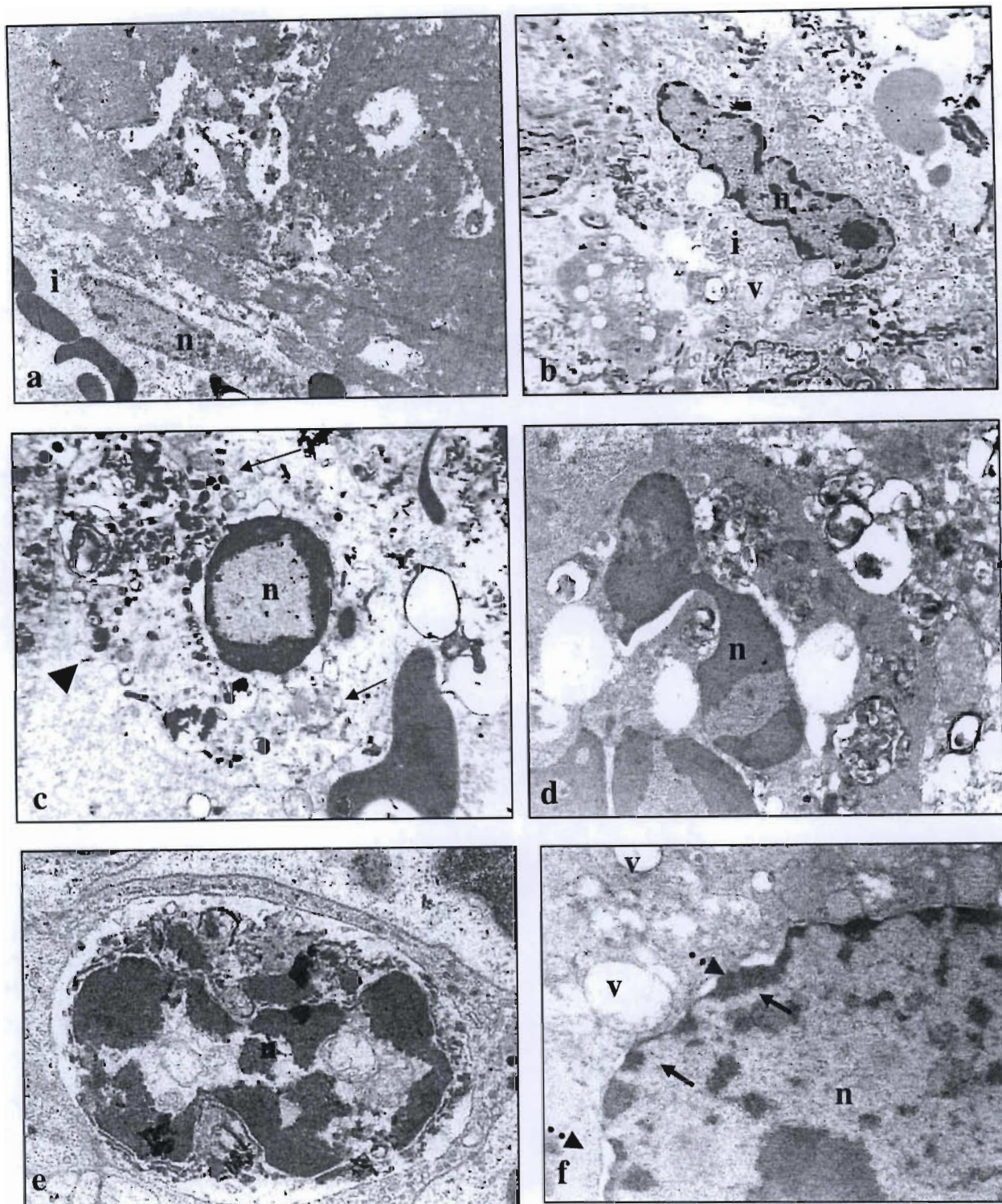


Figure 3.12: Electron micrographs illustrating the predominance of typically necrotic features such as degenerative nuclei (n), vacuolation (v), extensive intra- (i) and extracellular (e) fibrin deposits observed in (a,b) HIV-infected specimen. Nuclear features such as (c) apoptotic nucleus, (d) heterochromatin degranulation (e) a pyknotic nucleus and (f) oedma of nucleolemma (dashed arrows), vacuolation (v), and cytoplasmic lysis were also observed. Note micro-segregation of the heterochromatin (arrows). [Initial Mag. a: x3 000, b,c: x2 500; d, e: x8 000, f: x25 000].

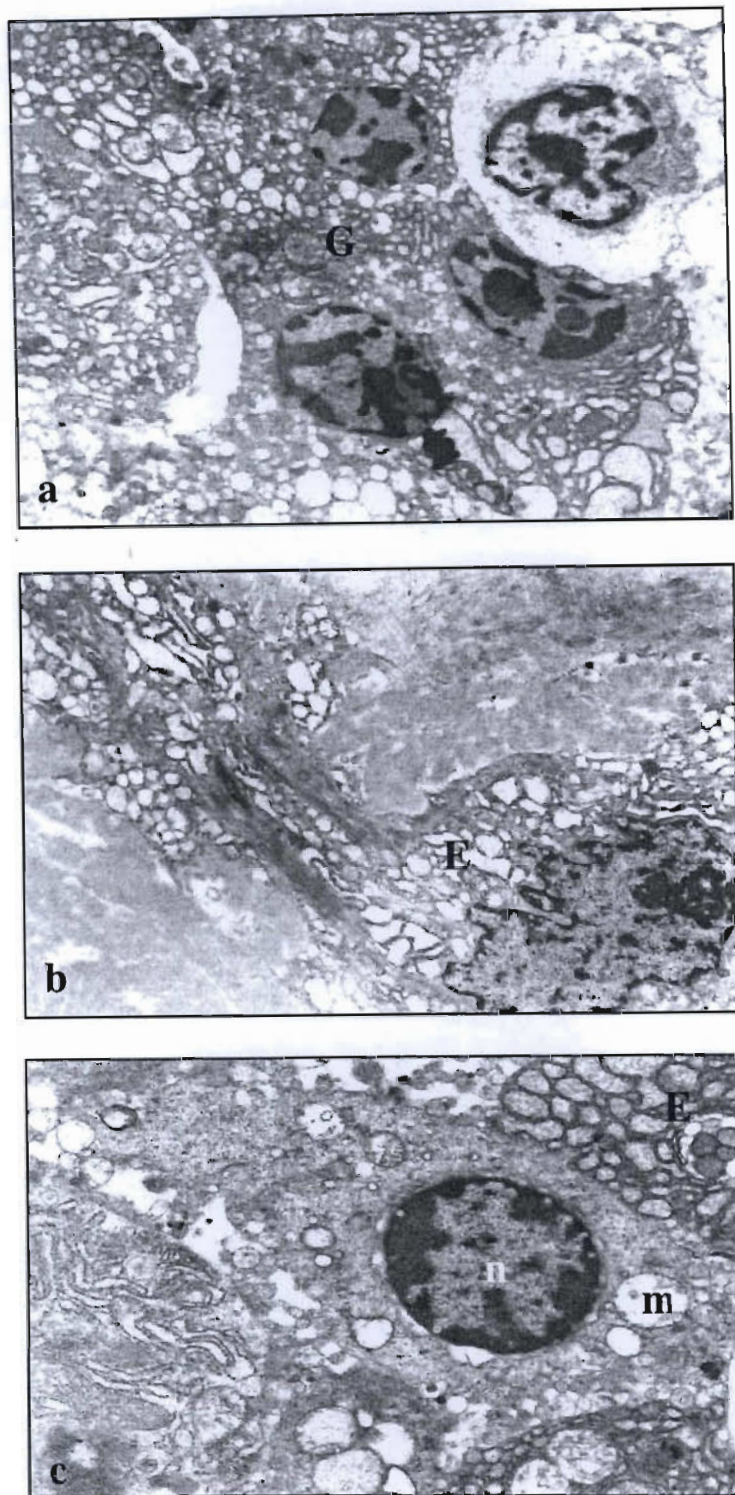


Figure 3.13: Electron micrograph showing (a) a mult-nucleated giant cell (G) observed in an HIV-infected specimen, (b,c) epithelioid cells (E), (c) an apoptotic nucleus (n) and degenerative mitochondria (m)[Initial Mag. a: x 8 000; b, c: x 4 000]

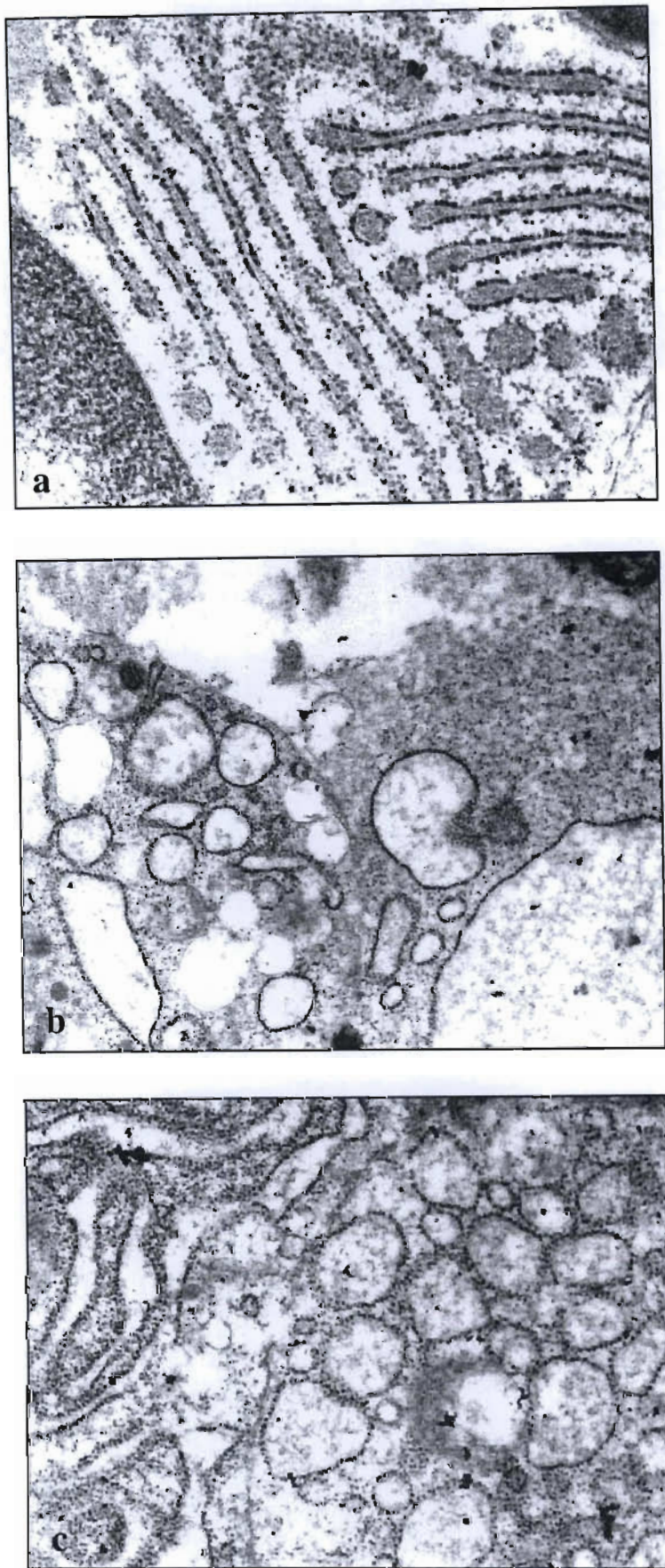


Figure 3.14: Electron micrographs illustrating ER distribution in (a) in an HIV-uninfected and (b, c) HIV-infected specimens. Figures b and c illustrate a predominance of dilated cisternal pools of rER with an amorphous content. [Initial Mag. a: x30 000; b, c: x25 000]

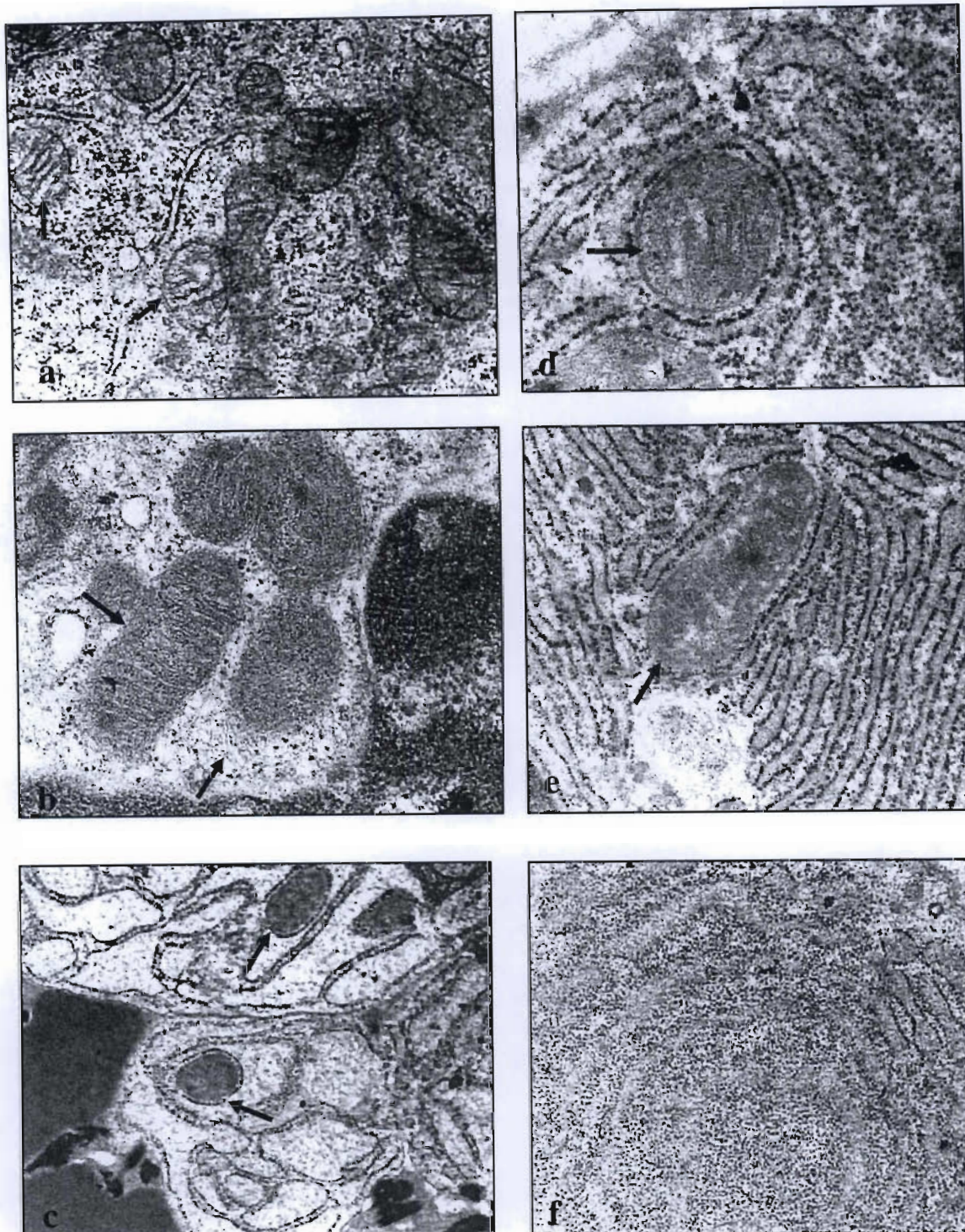


Figure 3.15: Electron micrographs illustrating cisternal inclusion of Russell bodies (arrows) in (b,c) HIV-uninfected and (c-e) HIV-infected specimens. Note the close proximity of ER to mitochondria and dilated cisternae and (f) profuse accumulation of free cytosolic ribosomes in an HIV-infected specimen. [Initial Mag. a: x25 000, b: x60 000; c: x15 000; d: x60 000, e: x40 000f: x30 000]

HIV-negative

HIV-positive

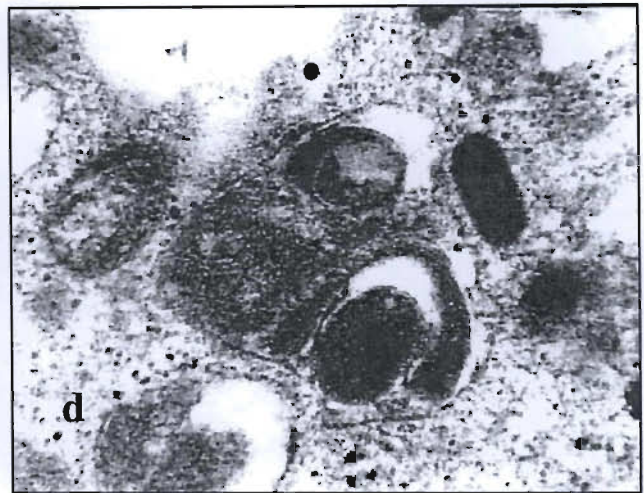
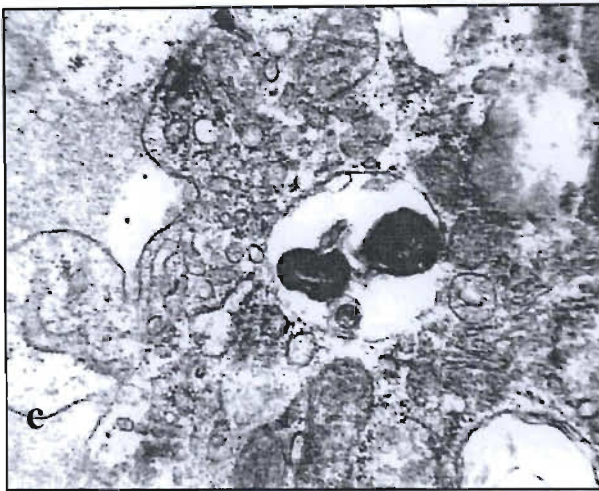
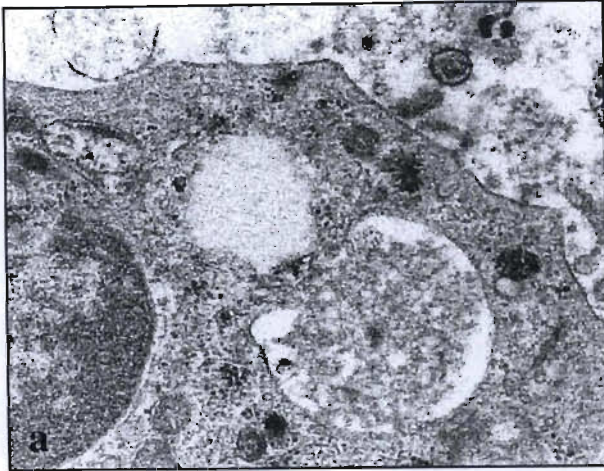


Figure 3.16: Electron micrographs illustrating lysosomal activity in (a,c) HIV-uninfected and (b,d) HIV-infected specimens. Note degenerative material and debris in a and b, whilst c and d contain phagocytosed degenerative bacilli (arrows) [Initial Mag. a: x50 000, b: x15 000, c: x20 000, d: x50 000].

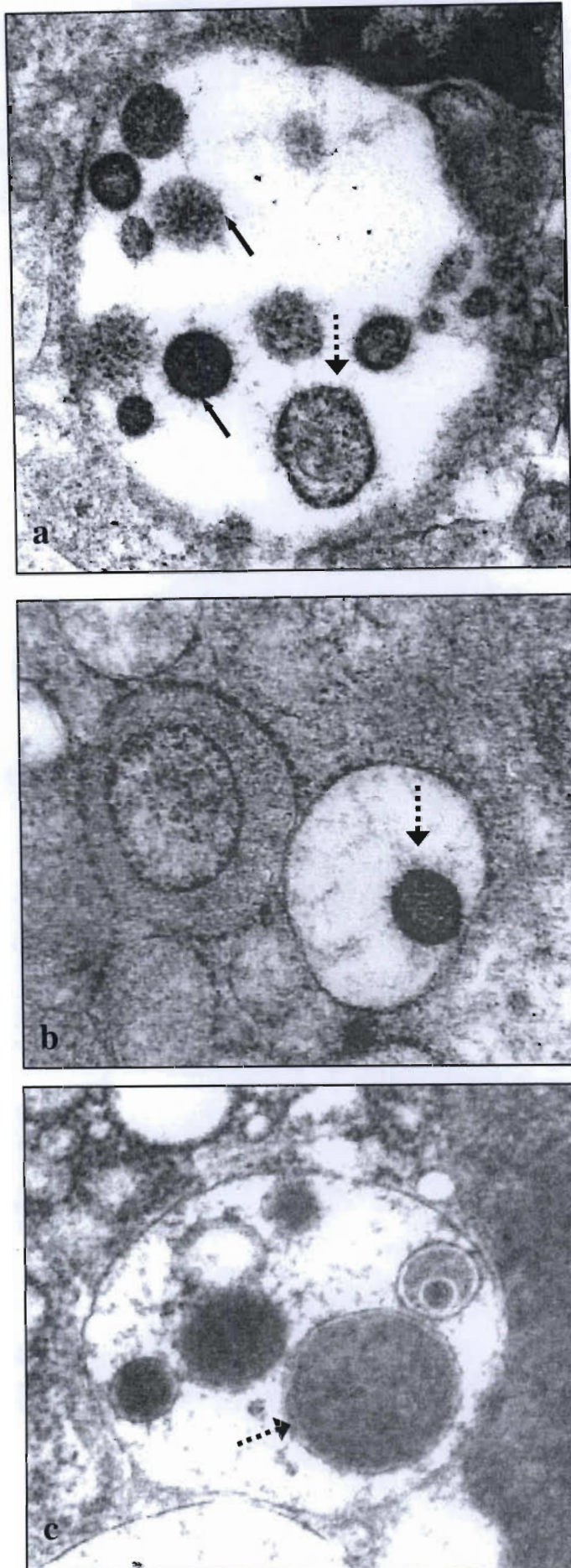


Figure 3.17: Electron micrographs showing (a,b) intra-lysosomal viral-like particles (arrows). Note the (c) thickened cell walls of the bacilli-like inclusions (dashed arrows). [Initial Mag. a: x 50 000, b: x 40 000, c: x6 000]

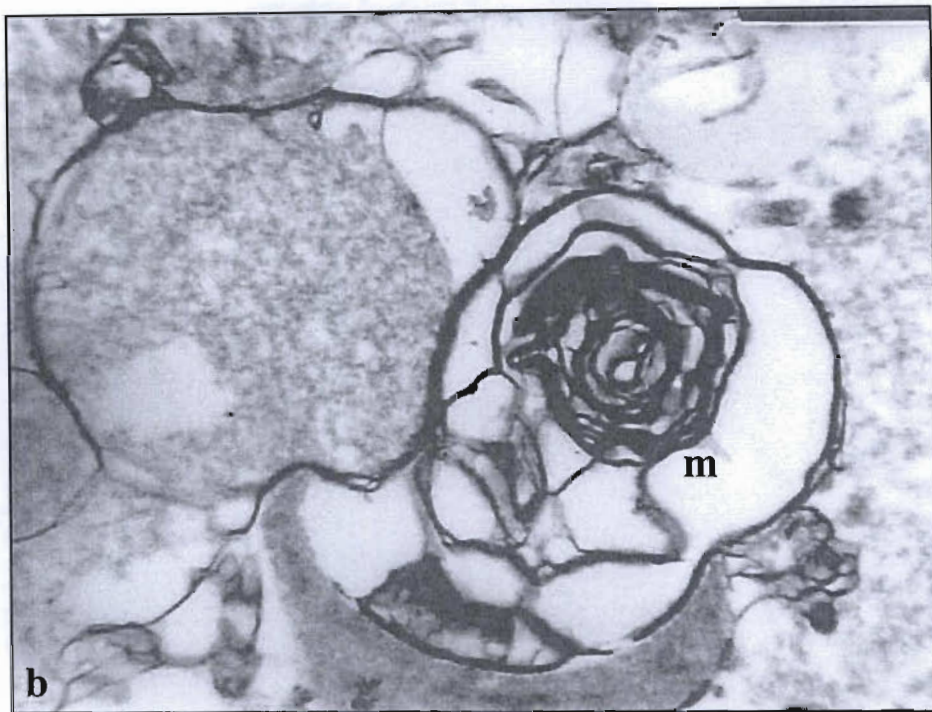
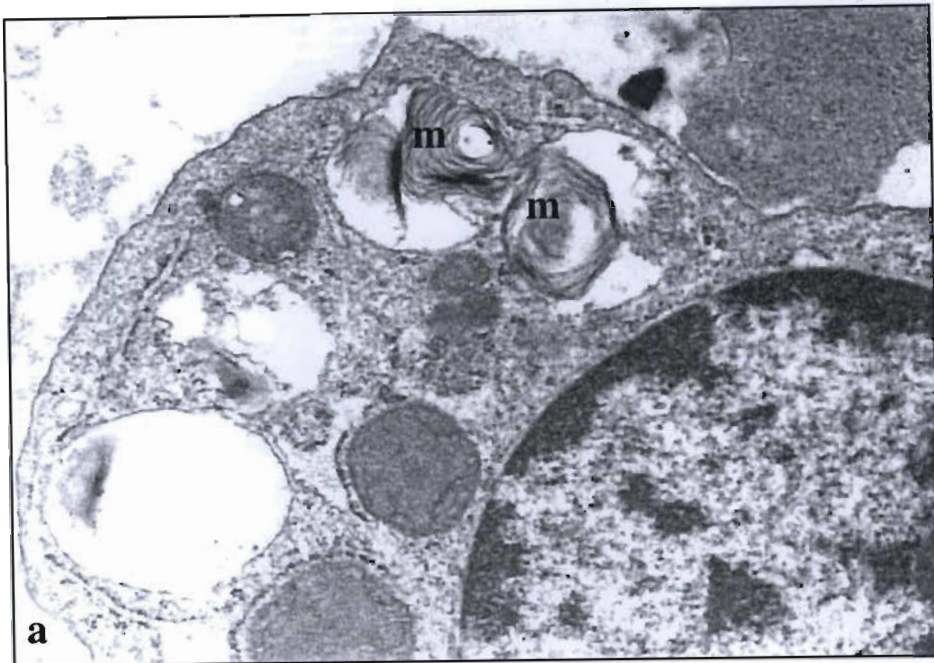


Figure 3.18: Electron micrographs of (a) myelin body (m) occurring intracytoplasmically in an HIV-uninfected specimen and (b) within degenerative debris of an HIV-infected specimen. [Initial Mag. a: x25 000, b: x20 000]

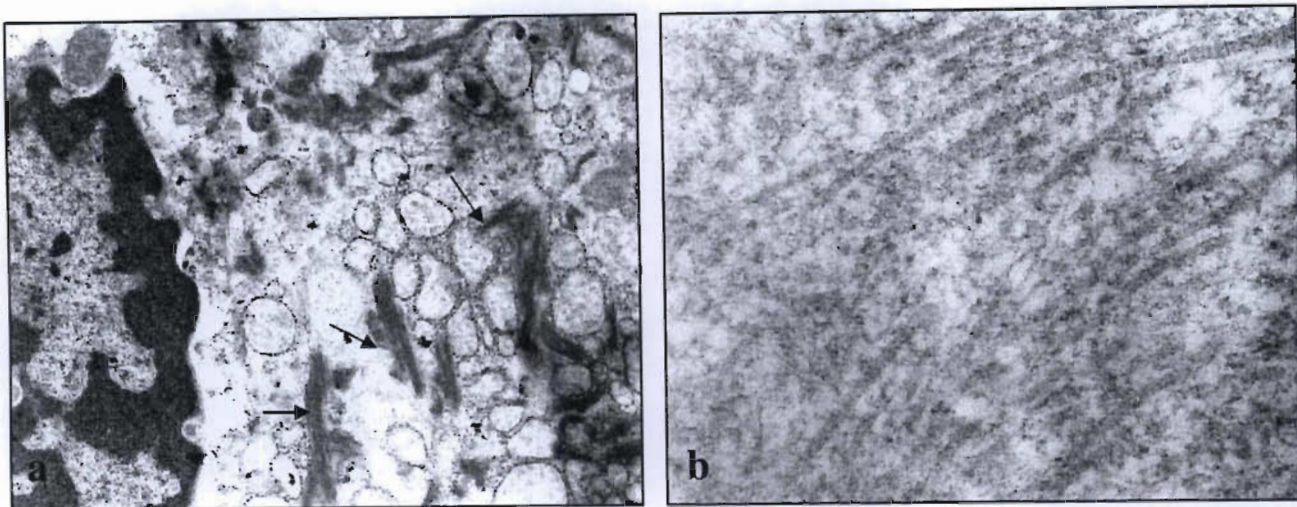


Figure 3.19: Electron micrographs displaying (a) intracellular fibrin bundles (arrows) and (b) collagen showing the regular periodicity of the cross-striations typical of the collagen fibrils. [Initial Mag. a: x40 000, b: x30 000].

3.4 Discussion

3.4.1 Histopathological features

No statistical difference in granuloma formation between HIV-infected and uninfected patients could be demonstrated in the present cohort. It has been proposed that TB infections specifically, lepromatous lesions, do not differ pathologically between HIV-infected and -uninfected patients with comparable levels of cellular infiltration such as epithelioid and giant cells (Faye et al., 1996, Lawn et al., 2002). At a radiological level they present similarly despite a more rapid disease progression in the co-infected patient (Kurisaki, 2000, Villoria et al., 1992, Villoria et al., 1995). The findings of the present study were consistent with these findings.

Histopathological evaluations of HIV-infected and -uninfected specimens in the present study, were consistent with previous descriptions of granuloma presentation as comprising of solid epithelioid cells, one or more giant cells and fibroblastic proliferation with central or no necrosis; or minute, peculiar foci of necrosis with little cellular material in co-infected patients (Roitt et al., 2001). This is despite differences in anatomical sites of infection. Atypical features of TB-infection were noted in the

present study such as a predominance of plasma cells. While the anti-TB immune response is cell-mediated other bacterial species elicit a humoral or Ab-mediated response in which plasma cells play a prominent role (Roitt et al., 2001). Their presence may therefore be indicative of an atypical mycobacterial infection.

A further atypical feature of a TB infection was the predominance of epithelioid cells in selected specimens where significant association between greater tissue granulation and reduced epithelioid cell infiltration was demonstrated. The importance of epithelioid cells to an effective anti-TB immune response has been demonstrated in murine models of pulmonary TB (Dormans et al., 2004). In addition, epithelioid cells predominate in non-caseating granulomas (Fujita et al., 2000), illustrating their protective role. However, others have reported HIV localised to macrophages and epithelioid cells of TB co-infected patients (Kurisaki, 2000) demonstrating that this protective role may be inevitably altered or disrupted in HIV/TB infected patients.

Multi-nucleated giant cells, the hallmark of an effective anti-TB immune response (Jordaan et al., 1994), were identified at both the light and ultrastructural level primarily in the HIV-uninfected group. However, in HIV pathology, CD4⁺ T-cells also aggregate to form a giant cell referred to as a syncytium (Huff, 2003). The induction of syncytia formation is characteristic of CXCR4-tropic HIV variants (Korber et al., 1994) and heralds late-stage disease. While syncytia formation is usually associated with T-lymphocytes, it has also been reported in Langerhans cells, which may also occur in TB granulomas (Jordaan et al., 1994, Stingl et al., 1990). CD⁺ T-cell depletion progresses rapidly in HIV-infected patients as a result of apoptosis, giant cell formation and degeneration of these aggregations of multiple CD4⁺ cells (Roumier et al., 2003). Interestingly, Roumier et al, (Roumier et al., 2003) were able to map HIV-initiated syncytia apoptosis to the mitochondrial pathway.

3.4.2 ZN staining

Spinal TB is a pauci-bacillary disease (Walsh et al., 2004), which contributed to the negative AFB (ZN technique) findings in this study. It is also plausible that the paraspinal tissue evaluated in the present study were biopsied from pauci-bacillary regions within the spinal lesion. Others have described micro-anatomical niches within

granulomas where foci of bacilli occur at the surface of a TB cavity or in lesions connected to an airway, rather than in the cellular or necrotic regions (Kaplan et al., 2003). Moreover, the oxygen concentration within the tissue can not equate to that of pulmonary tissue and related airway structures. Bacterial populations in the spine are expected to therefore be impacted accordingly and this was demonstrated, in the present study, by the absence of AFBs in specimens.

Secondly, patients were maintained on conventional anti-TB therapy and enhanced nutritional support prior to surgery and sample collection. Consequently, site-specific bacterial loads would have been radically reduced. Since the epidural tissue is an oxygen-deficient environment relative to pulmonary tissue, bacterial clearance may be augmented a low oxygen content greatly enhances the sterilizing activity of drugs such as pyrazinamide (PZA) (Wade and Zhang, 2004). On the other hand, persistence of approximately 8% viable bacilli has been noted in pulmonary TB granulomas even with anti-TB chemotherapy (Fenhalls et al., 2002a).

Finally, in paediatric patients and those with HIV co-infection, demonstration and isolation of AFBs is rare (Anane and Grangaud, 1992) and challenging. The use of molecular technology to diagnose (Kivihya-Ndugga et al., 2004) and localise (Fenhalls et al., 2002a) Mtb has shown notable success over conventional microscopy. Negative AFB staining, but positive immunolocalisation of Mtb antibody (Humphrey and Weiner, 1987) and DNA (Fenhalls et al., 2002a) suggested the persistence of cell wall deficient (Hulten et al., 2000) or remnants of lysed bacilli (Kaplan et al., 2003) in infected tissue. It may therefore be argued that similar bacterial remnants were present in this study resulting in negative ZN findings.

3.4.3 Immunolocalisation of CD68 cells

The importance of monocyte/macrophage phagocytic cells to the anti-TB immune response focused IHC investigations in the present study on the CD68⁺ cells representing phagocytic/endocytic cells. While, other cell types, such as the CD4⁺ and CD8⁺ T-cells play a significant role particularly at the initial stages of infection (Mehta et al., 1996), the primary cells targeted and exploited by the bacilli are macrophages. It

was hypothesised that macrophage infiltration in spinal TB granulomas would parallel that of pulmonary disease and this was demonstrated both histopathologically (H&E) and by immunolocalisation of CD68+ cells. It was also anticipated that disparate CD68+ immunoreactivity patterns would be noted in HIV-infected and -uninfected specimens. However, this hypothesis was refuted, following a quantitative analysis of CD68 immunoreactivity.

It would be misleading to assume that the prevalence of CD68 immuno reactive cells in both study groups represented an active and robust immune response since it must be remembered that infected macrophages, despite their presence, may simply be incapable of a killing-response and consequently ineffectual against the pathogen (Barker et al., 1996). Since the CD68 peptide is a glycosylated lysosomal membrane protein involved in endocytosis and lysosomal trafficking (Barois et al., 2002, Kobayashi et al., 2002) localised within immature dendritic cells (Barois et al., 2002) and epithelial cells (Povysil et al., 2001) its localisation provided a relative indicator of lysosomal activity. While not significant, greater distribution and reactivity of CD68 was noted in the HIV-infected compared with the –uninfected group. Thus lysosomal activity, while greater, did not result in efficient resolution of disease, rather, greater pathology, necrosis and degeneration. It may be speculated that lysosomal function was altered or redirected by HIV co-infection.

The present study demonstrated CD68 immunoreactivity in regions rich in mononuclear cells, granulomas and in highly necrotic specimens. These findings were consistent with those of Kaplan et al, (Kaplan et al., 2003) who described abundant CD68⁺ cells in regions of the granuloma with greater phagocytic activity such as the surface of the granuloma cavity and fibrotic zone. In addition, the authors (Kaplan et al., 2003) reported remnants of degenerative or necrosed macrophages in the necrotic zone. Similarly, the present study demonstrated that caseous, necrotic regions devoid of cells but rich in cellular debris were CD68 antibody reactive. Moreover, ultrastructural investigations in the present study demonstrated bacilli within intra-cellular membrane-bound compartments rather than in the extra-cellular matrix. Others have localised bacilli and bacterial DNA within CD68+ cells of macrophage morphology (Kaplan et al., 2003) and giant cells (Fenhalls et al., 2002a, Fenhalls et al., 2002).

In addition, tissue macrophages represent a source of productive HIV infection. It has been reported that replication efficiency and the accumulation of mutations is elevated in patients with OIs commonly associated with AIDS such as *M.avium* and *P.carinii* (Orenstein et al., 1997). The paradoxical increase of viral load at late-stage disease, despite severely depressed CD4+ T-cell counts, has been attributed to viral reservoirs in monocyte/ macrophage-derived cells (Cassol et al., 2005, Orenstein et al., 1997). The greater infiltration of CD68+ cells in the spinal granulomas may represent a significant potential for viral sequestration within these cells.

3.4.4 Immunolocalisation of CD4 and CD8 cells

In direct contrast with CD8 immune reactivity, CD4 reactivity was sparse or absent in the present study. Both cell types are essential to an effective immune response against acute infection although the specific role of CD8+ cells remains controversial (Chan and Flynn, 2004).

A reduced or depleted CD4+ T-cell population is usually associated with HIV infection therefore was not an unexpected finding in the HIV co-infected group. However, in the HIV-uninfected group, depletion of CD4+ T-cells and a sparse distribution of CD8+ T-cells were atypical. However, when one considers its insidious onset spanning, it is clear that the paradigm of acute infection cannot be applied to spinal TB – rather the disease represents a chronic infection. As demonstrated in a murine model, there is an influx of CD4+ and CD8+ during the first weeks of infection however as the infection progresses, the role of CD8+ cells become more prominent (Gonzalez-Juarrero et al., 2001). Similarly, immunolocalisation of CD4 and CD8 cells in the present study demonstrated sparse CD4 reactive cells but greater CD8 reactive cells. Although controversial, the contention of Gonzalez-Juarrero et al, (2001) that CD8 cells perpetuate chronic disease may, therefore, be applicable to the present study. Earlier studies have established similar kinetics where CD4+ T-cells predominate in the acute phase and CD8+ cells in the chronic phase (Hernandez Pando et al., 1996).

The HIV-uninfected group displayed distinct granuloma formation and less caseation. The findings of CD4 and CD8+ T-cell immunolocalisation were therefore consistent

with the observed histopathology in the present study since these cells contribute significantly to IFN γ production (Villarreal-Ramos et al., 2003), which ensures a robust immune response and efficient clearance of disease. In pulmonary-TB patients displaying cavitation, the percentage CD8⁺ T-cell is significantly greater ($p < 0.05$) than in their non-cavitary counterparts in which CD4⁺ T-cells are also elevated (Mazzarella et al., 2003). Not only do T-cells mediate the immune response against infection, but they also aid in the killing of infected macrophages (Oddo et al., 1998). Consequently, the pathogenicity of the bacilli is reduced. In this way, depletion of T-cells contributes to the greater pathogenesis and infectivity of TB in HIV co-infected individuals (Kaplan et al., 2003). These findings may also account for the more severe pathologies in co-infected spinal TB patients in the present study.

Lymphocytes and cells of macrophage appearance were observed in close proximity in the present study. Interestingly, it has been shown that the proximity of HIV-infected macrophages and CD4⁺ T-cells in HIV/TB co-infection, activates HIV-1 LTR (Nakata et al., 2002) thereby initiating viral replication.

3.4.5 Ultrastructural features

3.4.5.1 Ultrastructural features of TB

Recently, Dahl (Dahl, 2005) described the ultrastructural features of *Mtb* from nutrient cultures. However, caution must be applied when viewing clinical specimens since the fine structural details of the bacilli can be well characterised in culture but may be more difficult to discern them in clinical samples particularly those receiving treatment. Particles resembling intact bacteria were noted in the present study. Since the cell walls of phagocytosed bacilli undergo lysis within phagolysosomes, morphological and functional changes result (Gerasimov et al., 2003), thus observation of a double membrane cell envelope for instance would be exceptional in treated clinical samples. The findings of the present study may thus represent morphologically altered bacilli.

3.4.5.2 Ultra-structural features of the granulomas

A prominent feature of both groups, particularly the co-infected specimens, was the presence of distended ER within epithelioid, plasma and immune cells. Dilation of ER is a result of either ingress of water into the cell or storage of secretory proteins within the ER (Ghadially, 1982). In the former instance, the contents appear more electron-lucent, while in the latter, the ER appears electron-dense. It is hypothesised that the latter results from a rate of protein synthesis which exceeds the capacity of the cell's transport mechanism; a mechanical or enzymatic defect; or production of an atypical peptide (Ghadially, 1982).

It can be speculated that enhanced production of immune complexes or up-regulated viral replication may be responsible for the distended ER in the present study. Thus the inclusion peptides may be comprised of up-regulated secretory products, or atypical peptides such as viral proteins. In the context of an active TB infection, particularly in association with HIV, elevated immunoglobulin production is highly likely. Moreover, Facke et al, (Facke et al., 1993) reported immature virus-like particles resembling the intracisternal A-type particles (murine endogenous retrovirus elements) within the ER of infected cells. These viral particles were non-infectious while those observed extracellularly had the typical morphology of mature viral particles. Others have demonstrated similar findings where a single amino acid substitution in the matrix gene redirects virion assembly and budding to cytoplasmic vacuoles identified as Golgi elements (Facke et al., 1993, Freed and Martin, 1994).

An additional account for the distended ER may lie in changes associated with cell injury. Atypical appearance of ER has been correlated with early cytological changes due to cell injury; while dilation and vesiculation of ER is a known feature of apoptosis (Ghadially, 1982). Whatever the reason, hypertrophy of the ER is a manifestation of altered cellular activity. In the present study, increased prevalence of rER in epithelioid and plasma cells exemplified increased cellular activity and upregulation of protein synthesis. The present investigation therefore, reflected possible HIV exploitation of the cellular protein synthesis machinery and the host's attempt to mount an effective immune response.

The mitochondrial changes and disruption observed in this study conforms to similar reports in HIV-infected cells (Raidel et al., 2002). In their study (Raidel et al., 2002), the ultrastructural and consequent degenerative changes to the cellular mitochondria were attributed to HIV-1 *tat* protein. Both TB and HIV, contribute to mitochondrial changes induced by oxidative stress. The multi-centric structures bound by a single membrane were identified in this study as myelin bodies and indicated mitochondrial degeneration and increased turnover. Duan et al, (Duan et al., 2002) associated mitochondrial membrane disruption with necrosis, increased bacterial replication and a failed antibacterial response. Hernandez-Pandor et al, (Hernandez-Pando et al., 2001) also presented evidence of the role of mitochondria in disease progression.

The present cohort of HIV-infected patients did not receive ARVs therefore the observed mitochondrial changes cannot be attributed to their toxic effects. It is, however, known that nucleoside reverse transcriptase inhibitors (NRTIs) disrupt mtDNA and resultant enzyme malfunction (Lewis et al., 2003, Nolan et al., 2003). Commonly, abnormalities of the mitochondria in treated patients included accumulation of glycogen, reduction or loss of cristae and matrix density (Verucchi et al., 2004). Morphologically, the mitochondria appear swollen, the cristae and internal structures are disrupted, and there is acute thinning of the mitochondrial membrane (Tolomeo et al., 2003). Recently, it was demonstrated that an anti-TB drug, Isoniazid (INH), also induces mitochondrial dysfunction in hepatic cell lines (Bhadauria et al., 2007). Thus, it is more likely that the mitochondrial pathologies observed in the present study result not from ARV toxicity but reflect the impact of either, anti-TB chemotherapy or the immune pressures.

The occurrence of Golgi complexes, which function to condense, modify and package peptides transported from the ER into secretory granules, illustrated that cells were functional and actively involved in protein synthesis. High concentrations of Golgi complexes furthermore, reiterate the increased cellular activity alluded to by the morphology of the ER. This may be due to either immune activation or active HIV replication and the exploitation of the cell's replication mechanisms by the virus.

Both intra- and extra-cellular fibrin was noted in both HIV-infected and –uninfected specimens. Fibrosis is typical of late stage granuloma formation (Mornex et al., 1994), is important in preventing disease dispersion (Adams, 1983) and is initiated by leukocyte infiltration (Kelley, 1990, Kovacs, 1991). However, fibrosis increases the risk of permanent tissue and organ damage of adjacent structures (Kovacs, 1991, Mornex et al., 1994) and is arguably the main cause of post-treatment morbidity (Jayasankar et al., 2002). While not all granulomas develop a fibrotic cuff, fibrosis was clearly demonstrated in both HIV-infected and uninfected specimens representing a discrete anatomical compartment of co-infection where both the viral and bacterial latency may be sequestered.

3.4.5.3 Apoptosis, necrosis and related nuclear changes

Characteristic nuclear changes noted in the present cohort were all indicative of necrosis and apoptosis while pyknosis and karyolysis, in particular, are characteristic of a viral infection (Ghadially, 1982). In an HIV/ herpes virus co-infected patient, ultrastructural features included marginated and condensed chromatin (Guccion and Redman, 1999). Since the authors failed to characterise HIV-induced ultrastructural changes, these features cannot solely be attributed to the herpes virus. Moreover, comparable findings demonstrated in the present study suggested that HIV co-infection also contributed to the morphological changes described by Guccion and Redman (Guccion and Redman, 1999).

Mycobacterial infection induces both apoptosis and necrosis of infected monocytes and macrophages (Gil et al., 2004). Monocyte-derived macrophages for example, undergo apoptosis in response to an Mtb infection (Ciaramella et al., 2004), cells from reactive TB-exposed individuals undergo apoptosis, while cells from actively-infected patients undergo both apoptosis and necrosis (Gil et al., 2004). However, more virulent strains of bacilli disrupt the process of apoptosis (Fratuzzi et al., 1999) or induce necrosis instead, resulting in uncontrolled TB replication (Duan et al., 2002). Macrophage apoptosis is advantageous since it not only retards bacterial replication but may also reduce tissue damage (Fratuzzi et al., 1999, Kornfeld et al., 1999). In addition, halting the release of intracellular debris and promoting the sequestration of bacilli in apoptotic

bodies prevents the spread of disease (Chen et al., 2006, Fratazzi et al., 1999). In a TB infection alone, TNF α mediated macrophage apoptosis is protective, but in HIV co-infected patients high TNF α concentrations up-regulate HIV replication.

While features consistent with apoptosis were observed in selected specimens, necrosis predominated in the present study. A primarily necrotic rather than apoptotic response would substantiate the gross pathology and tissue damage in the absence of high bacterial numbers that were observed in the present cohort. It was therefore demonstrated that cell death in TB spondylitis regardless of HIV status,, reflects previous reports in pulmonary TB (Chen et al., 2006).

Enlarged nucleoli or nucleolar hypertrophy are strongly associated with an up-regulation of protein synthesis particularly in relation to malignancy (Ghadially, 1982). Like features were evident among HIV-infected specimens in this study. Whether these changes were reflective of active processes or events that had drawn to a close yet still manifest morphologically, can be speculated upon. The induction of increased protein synthesis may have occurred via an activated immune response in an attempt to control progressive disease or may reflect HIV exploitation of the cellular machinery for its own replication cycle.

3.4.5.4 Viral and bacterial sequestration

Evidence of sequestration of bacilli in membrane-bound intra-cellular compartments was noted in the present study, suggesting a possible bacterial reservoir. They were observed containing degenerative material of varying electron density in addition to bacilli- and virus-like particles in the present study.

Lysosomes are single membrane bound organelles involved in the intracellular digestion of various endogenous and exogenous materials including pathogens (Ghadially, 1982). Consequently, functional disruption, by preventing the fusion of phagosome and hydrolase-containing lysosomes (Ghadially, 1982), is the primary mechanism by which the bacilli is able to evade destruction and induce latency (Nguyen and Pieters, 2005). Others describe lysosomes in rCSF-treated monocytic cells where a number of electron-

dense lysosomal compartments were observed (Gendelman et al., 1988). Similar observations were made in the present study.

Viral-like particles were identified, at the ultrastructural level, in the present study, but varying in size and morphology. This finding represents the first report of intracytoplasmic viral particles in spinal TB/HIV co-infected granulomas suggestive of a sequestered compartment of latent infection. This was not an unexpected finding since viral particles exist in varying stages of maturation (Pudney and Song, 1994), may vary in size from 100nm (Raposo et al., 2002) to 207nm (Briggs et al., 2003) and may occur as single- and double-cored particles ranging in size from 134nm to 159nm (Briggs et al., 2003). Consequently, viral morphology, *in vivo*, is diverse and it is rare that viral particles are observed depicting the typical core structure.

The virus-like particules observed in the present study may represent phagocytosed/endocytosed virions or particles misdirected to bud into the vacuole by polymorphisms in the viral genome. It has been shown that alveolar macrophages (AM) from HIV/pulmonary TB infected patients endocytosed/phagocytosed both bacilli and viral particles but the phagolysosome formation and acidification of these vacuoles was halted (Mwandumba et al., 2004). Viral particles have been observed within phagocytic and non-phagocytic endosomal vacuoles of epithelial cells (Bourinbaier and Phillips, 1991, Deretic et al., 2004) plasma cells (Weiss, 2002) and follicular dendritic cells as well as in their cytoplasm (Tacchetti et al., 1997). In an earlier study Pudney and Song (Pudney and Song, 1994) localised virions specifically to endocytic compartments. The receptor mediating endocytosis is independent of CD4 but is a Langerhans-associated surface molecule (Dezutter-Dambuyant et al., 1991).

A further account for these intra-lysosomal virus-like particles may lie with its assembly and budding properties within non-T-cells. Studies have shown virions to bud into the lumen of late endosomes in macrophages (Lindwasser and Resh, 2004, Meltzer et al., 1990a, Meltzer et al., 1990b, Meltzer et al., 1990c, Ono and Freed, 2004, Pelchen-Matthews et al., 2003, Raposo et al., 2002). Gendelman et al, (Gendelman et al., 1988) also noted several immature virions in a transition period preceding viral assembly in these vacuoles. The mechanisms of virion budding and assembly therefore, mimic that of internal vesicle formation in macrophages (Raposo et al., 2002). Fusion of these

multi-vesicular compartments with the plasma membrane actually facilitates the release of huge numbers of virions (Raposo et al., 2002). These viruses acquire MHC class II proteins and CD63 when budding into these compartments, thus acquiring a mechanism for immune evasion and forming an important reservoir of escape mutants. Recruitment and fusion of macrophages and lymphocytes to form multi-nucleated giant cells, furthermore, aids in the spread of virions

It was important to note that prevention of phagosome/lysosome fusion commonly associated with TB latency and survival may also enhances HIV infectivity and virulence. In these instances, HIV infection is facilitated via the endocytic pathway (Wei et al., 2005) enhancing viral infectivity by up to 400-fold and is independent of viral isolate and co-receptor usage. Both HIV and TB disrupt the steps during or after the synthesis of P13P, a membrane-bound signalling molecule that controls endosomal and phagosomal sorting (Deretic et al., 2004). Not only is phagosome maturation halted, but HIV replication and assembly in macrophages is enhanced.

3.4.6 Diagnostic relevance of microscopy

The utility of microscopy over more advanced immuno-localisation methods such as flow cytometry has been demonstrated. Microscopy is highly functional demonstrating specific cellular morphologic features otherwise indistinguishable by any other means (Ghadially, 1982), (Giulian et al., 1995). The value of antibody localisation using immuno-histochemistry (IHC), in the clinical, as well as, the research setting has proven highly sensitive and specific (Gutierrez et al., 1999) for a range of immune cells and pathogens. The related technology is, moreover, constantly evolving and advancing (Bunea and Zarnescu, 2001) allowing an ever-increasing range of applications. Unfortunately one of the primary restrictions to microscopy is the lack of appropriate and specific markers for various cells and their products. The findings of this study demonstrated the efficacy of IHC and TEM as adjunct diagnostic and research tools, particularly in pauci-bacillary disease and diagnostically problematic specimens (Walsh et al., 2004).

3.4.7 Limitations of the study

This study was conducted in a small subset of the cohort which, if expanded, may have yielded greater statistical power to the subsequent analyses. It may be argued that the efficiency of immune localisation in wax embedded sections may not be as informative as cryosections. This is because processing and embedding tissue biopsies may result in disruption or blocking of antigen sites while cryoblocks are unprocessed, flash-frozen specimens. The former requires preparation and treatment to expose antigen sites while the latter can be treated with the antibody of interest, directly and is often more sensitive and specific (Barrett et al., 1993). However, the utility of IHC on wax embedded sections has been demonstrated (Pudney and Anderson, 1995). Authors argue that while cryosections may afford many advantages, the loss of structural features, and the biohazard risk of unfixed specimens make this an unattractive alternative. It is for these reasons that paraffin-embedded wax blocks were chosen for this investigation. Since any histological and ultrastructural investigation represents a snap-shot of the disease condition, these investigations allowed a study of TB spondylitis at late stage disease when progressive pathology had resulted in extensive tissue damage. Monitoring disease progression in HIV-infected and -uninfected patients would be an important contribution to the understanding of both HIV and TB pathogenesis, but was beyond the scope of this investigation.

3.4.8 Summary

This report constitutes the first histopathological and ultrastructural description of clinically and radiologically diagnosed infectious spondylitis contrasting, the HIV-infected and -uninfected specimens. Patients biopsied were at similar clinical stages of disease despite greater immune competence ($>CD4+/CD8+$ T-cell counts) in the HIV-uninfected group. All specimens were AFB negative, illustrating the diagnostic limitation of the ZN stain when applied to pauci-bacillar diseases such as infectious spondylitis. These findings confirm that negative TB microscopy and culture findings reported in previously (Chapter 2). There was no association between negative AFB microscopy and HIV co-infection. The PAS stain, used to demonstrate the presence of fungal pathogens, was negative in all instances confirming a bacterial mediator of

disease. Whether the organisms were all *Mtb* or included non-tuberculous *Mycobacterium* (NTMs) was not established at this juncture.

Histopathological features were diagnostic of spinal TB in the majority of cases (91.4%) and were associated with HIV status. The atypical features noted in the remaining patients may be a result of non-tuberculous pathogens. Pathologies ranged from distinctive granuloma formation and simple necrosis to caseation and suppuration similar to features typical of pulmonary TB. Also consistent with previous findings of pulmonary TB was that the HIV-infected group displayed greater caseation relative to their HIV-uninfected counterparts. However in direct contrast to pulmonary TB, distinctive differences between the HIV-uninfected and –infected groups were demonstrated in the current study. These included limited granuloma formation, and an inverse correlation between granuloma formation and epithelioid, as well as lymphocyte infiltration. Immunolocalisation of CD68+ cells, however, illustrated that the phagocytic/endocytic potential within the lesions was not related to HIV status. However, as expected, CD4 and CD8 immune reactivity was HIV related. Moreover, the distribution and reactivity of CD4 and CD8 localisation was strongly suggestive of chronic rather than an acute TB infection, which is consistent with the insidious nature of spinal TB.

Ultrastructural evaluation was indicative of either, necrosis and degeneration or a strong cellular immune response. The most distinctive feature associated with HIV co-infection was grossly dilated ER. Virus-like particles and bacilli were identified based on their size and morphology at the ultrastructural level representing a novel finding in association with TB spondylitis. Demonstration of virus- and bacterial-like particles within membrane-bound intracellular compartments was suggestive of sequestration of the pathogens and a possible reservoir. Extensive fibrosis and collagen deposits were noted, affirming the similarity in histological and ultrastructural features between infectious spondylitis and pulmonary TB. Thus the hypothesis, that macrophage infiltration is impaired by HIV co-infection, was rejected. However, ultrastructural differences comparing HIV-infected versus the –uninfected specimens, confirmed the hypothesis that HIV co-infection manifests as greater degeneration and caseation, at an ultrastructural level.

Chapter 4 - Bacterial Genotyping

4.1 Introduction

Timely interventions in TB patients even in extra-pulmonary disease, significantly improves patient morbidity and mortality regardless of HIV status (Alvarez et al., 2004, Day et al., 2004b) illustrating the importance of accurate and rapid diagnosis. Despite technological advances, traditional TB diagnostic methods (Garg et al., 2003), still prevail, which may not be robust enough to detect paucibacillary diseases, such as spinal TB. In addition, symptoms of an Mtb infection may intersect with those of other pyogenic diseases (Swaminathan, 2004) further obscuring diagnosis.

Infectious spondylitis results from a range of pathogenic and opportunistic organisms such as Mtb, *M. avium* and *M. intracellulare* (Collins, 1989, Beggs et al., 2000, Sakatani, 2005, Koh et al., 2005, Lima et al., 2005, Morita et al., 2005). In pulmonary disease, Mtb and HIV are closely linked (Ferreira et al., 2002) however, atypical or non-tuberculous Mycobacterial infections are increasingly being associated with HIV-uninfected, immune compromised individuals (Corbett et al., 1999a, Henry et al., 2004). Whether HIV co-infection impacts the occurrence of non-tuberculous or atypical opportunistic infections in the context of spinal TB has yet to be established.

The recent sequencing of the full genomes of several bacteria has assisted in developing molecular techniques including genotyping and restriction fragment length polymorphisms (RFLP) (Hazbon, 2004, Brown et al., 2005, Zink et al., 2003, Zink and Nerlich, 2004, Zink et al., 2005) for diagnostic purposes.

Previous clinical observations have reported the occurrence of pyogenic (Charles et al., 1989) and cryptococcal (Govender and Charles, 1987, Govender et al., 1999a, Govender et al., 1999b) spinal infections in KZN, SA. This chapter examines the hypothesis that atypical bacterial spondylitis is associated with HIV-infection using a broad-range bacterial genotyping assay.

4.2 Methods

4.2.1 Specimen collection

As described earlier (Chapter 2, Section 2.2.2-2.2.3), open biopsies of granulomatous tissue were collected from 60 patients (38 HIV-negative and 22 HIV-positive) during spinal decompression and submitted to two independent accredited pathology laboratories for conventional AFB microscopy and culture identification (BACTEC MGIT Bacterial Identification assay; Beckton Dickenson,).

4.2.2 DNA isolation

4.2.2.1 DNA isolation from clinical samples

DNA was isolated directly from 10-20mg of fresh-frozen granulomatous tissue (Fig. 4.2) using the Qiagen DNA Mini Kit. Tissue was macerated in Lysis Buffer, further homogenized using a QiaShredder (Qiagen, Hilden, Germany) and the resultant homogenate was applied to the Qiagen DNA isolation spin columns (Qiagen, Hilden, Germany). The isolation procedure complied with the manufacturer's protocol, and DNA was eluted in 25µl sterile, filtered deionised water. DNA samples were quantified on a 1% agarose gel.

In cases where the isolation procedure was unsuccessful, other isolation methods were attempted including the PrepMan Ultra reagent (Applied Biosystems, Foster City, Calif., USA); 5% Chelex (Sigma, St. Louis, MO); a TE_{0.1} (10mM Tris-HCl and 10⁻⁴M EDTA) buffer/Chelex (5%w/v)/ProteinaseK protocol; and an alkaline-lysis buffer method. In order to purify and concentrate the DNA, a precipitation using glycogen (1µl); sodium acetate (3M) and absolute ethanol was performed. Thereafter, samples were centrifuged (maximum speed; 20 minutes), washed in cold 70% ethanol and re-suspended in 50-100µl sterile / filtered water.

4.2.2.2 DNA isolation from pure culture

The human MDR1 gene was amplified from extracted DNA to determine the integrity of the extracted product (results not shown). For samples not amplifiable directly from clinical samples, DNA was isolated from pure bacterial cultures (BACTEC MGIT culture). All positive cultures were heat inactivated at 95⁰C for 10 minutes at the TB pathology laboratory prior to transportation (at -20⁰C) to the molecular facility where subsequent DNA extraction and genotyping was conducted.

Bacterial DNA was isolated using the Qiagen DNA Mini Kit (Qiagen, Hilden, Germany) from heat-inactivated pure cultures according to the manufacturer's protocol for bacterial cultures. The PrepMan Ultra Sample Preparation Reagent (Applied Biosystems Inc., Foster City, Calif., USA), was applied to those specimens that failed to provide an ample DNA yield. In instances where the yields were low, DNA was precipitated as described in Section 4.2.2.1.

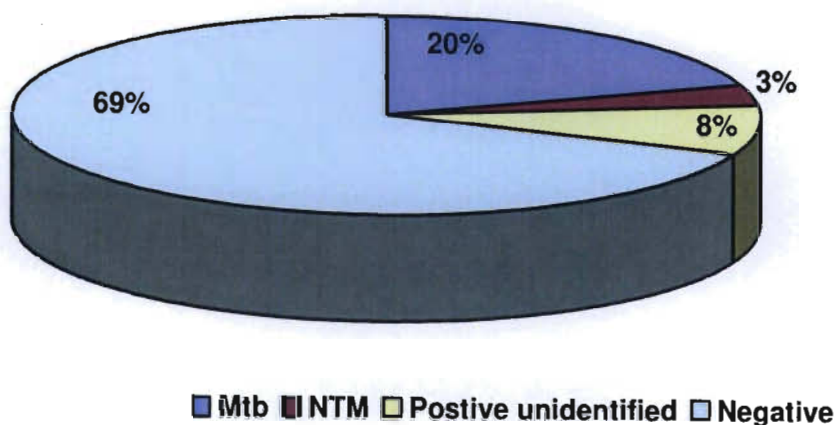


Figure 4.1: Graphical representation of diagnostic culture findings (BACTEC MGIT system), illustrating the large proportion of negative results and a predominance of *Mycobacterium tuberculosis* (Mtb) isolates. Non-tuberculous Mycobacteria (NTM) were also represented.

4.2.3 16S rDNA PCR amplification

4.2.3.1 In-house PCR

An in-house PCR adapted from Edwards et al (Edwards et al., 1989) was used to amplify the extreme 5' and 3' regions (primers A and H) of the 16s gene. The reaction mix is described in Table 4.1.

The thermal cycling conditions included a 12 minute denaturation at 94⁰C, 36 cycles of 95⁰C for 30 seconds, 55⁰C for 45 seconds and 72⁰C for 1minute with a final extension of 72⁰C for 7 minutes.

Table 4.1: A description of the components of the in-house PCR reaction amplifying a region of the 16S rDNA gene.

Reagent	Final Concentration	Volume (μ l)
Water		10.25
10x PCR buffer		2.5
MgCl ₂ (2.5mM)	1.5mM	1.5
dNTP (200 μ M)	4 μ M	0.5
Primer A (25 pmol/ μ l)	2.5pmol/ μ l	2.5
Primer H (25 pmol/ μ l)	2.5pmol/ μ l	2.5
AmpliTaq Gold (5U/ μ l)	0.05U	0.25
Sample (~1ug/ μ l)		5.0
Total volume		25

4.2.3.2 MicroSeq 500bp 16S rDNA Bacterial Identification

The MicroSeq 500bp 16S rDNA Bacterial Identification Kit (Foster City, Ca., USA) was used as per the manufacturer's protocol to produce a 460-560bp product at the 5' end of the gene (Fig. 4.2). Briefly, equal volumes (15 μ l) of the PCR module reaction mix and the isolated DNA were subjected to the thermal cycling conditions as per the manufacturer's protocol. The precise primer sequences are trademark protected as part of the Bacterial Identification Kit and, as such, are not available to customers. A 500bp segment at the 5' end of the 16S rDNA gene was targeted. Negative (water) and positive (1ng/ μ l *E.coli*) controls were included in each PCR reaction. The presence of the amplified product (460-560bp) from each PCR reaction was confirmed on an agarose gel thereafter purified using the Qiagen PCR Clean-up Kit (Qiagen, Hilden, Germany).

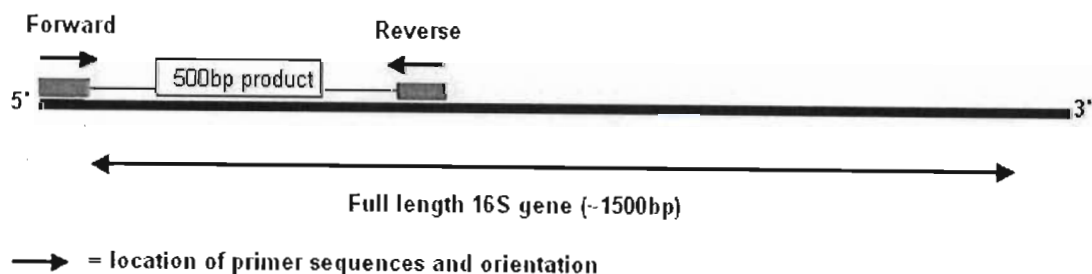


Figure 4.2: An illustration of the 500bp region targeted by the MicroSeq 500bp 16S rDNA Bacterial Identification Kit and sequenced from pure bacterial cultures.

4.2.4 Cycle sequencing

4.2.4.1 Sequencing reaction

Following quantification on an agarose gel, the purified PCR products were subjected to the sequencing reaction. One reaction for each strand (Forward and Reverse) was prepared by adding 7 μ l purified PCR product to 13 μ l of the sequencing module reaction mix (ABI, Foster City, Calif. USA). The thermal-cycling conditions were as per the manufacturer's protocol.

4.2.4.2 Clean-up of sequencing product

Following the cycle sequencing reaction in a 96-well plate, excess dye terminators and primers were removed. Fifty-five micro litres of freshly prepared solution of 3M-sodium acetate in absolute Ethanol (4% v/v) was added to each sample, the wells were sealed, vortex-mixed and centrifuged (3000g; 20 minutes). Plates were inverted onto absorbent paper to remove supernatant and centrifuged inverted at 150g for 5 minutes. Fresh preparations of 70% ethanol were added to each well (150µl/well); vortex-mixed and centrifuged (3000g; 5minutes). Inverting the plates onto absorbent paper and centrifuging (150g; 1-1.5minutes) removed excess ethanol. Plates were dried at room temperature for 1-5 minutes in the dark; thereafter sealed and stored at -20°C .

The purified extension products were re-suspended in 10µl Hi-DiTM Formamide (Applied Biosystems Inc., Foster City, Calif., USA), denatured (5 minutes at 95°C) and sequenced on the ABI 3100 genetic analyzer (Applied Biosystems Inc., Foster City, Calif., USA).²

4.2.4.3 Sequence analysis

The resultant 16S rDNA sequences were codon aligned (Clustal W, GDE). Basic Local Alignment Search Tools (BLAST) at the National Centre of Biotechnology Information (NCBI), Ribosomal Differentiation of Medical Micro organisms (RIDOMM) and DNA Data Bank of Japan (DDBJ) sequence repositories compared these 16S sequences with published reference strains. An agreement between the query and reference sequences (NCBI, RIDOMM and DDBJ) of greater than 90% was accepted as a positive match.

Confirmatory phylogenetic reconstruction was performed using an appropriate model selected in MODELTEST 3.0 and PAUP4b10 bioinformatics software tools. Trees were bootstrapped using 100 replicates and rooted against an *E.coli* bacterial strain. The resultant trees were viewed in Treetool and Treeview. The reference sequences from the NCBI repository that were used in the construction of the tree included

² For more information regarding the Big Dye terminator sequencing reactions, refer to the ABI PRISM Automated DNA Sequencing Chemistry Guide (PN 4305080).

M.tuberculosis (AJ536031, NC000962, X58890, S45301); *M.africanum* (AF480605); *M.kansasii* (AY43075); *M.intracellulare* (AJ536036, M61682, X52927); *M.avium* (AJ536037); *M.chelatum* (Z46664); *M.xenopi* (AJ536033); *M.fortuitum* (AY513243, AY457066); *M.flavescens* (X52932); *Nocardia* spp. (X80611, X80610); *B.sanguinis* (AJ628351); *B.casei* (AY468375); *Stenotrophomonas maltophilia* (AF533952); *Pseudomonas* spp. (AJ551156); *Vibrio parahaemolyticus* (X56580, AH000275, S83383, S83386, S83391); *E.coli* (NC000913); *Klebsiella pneumoniae* (DQ185604); *Serratia marscescens* (AY498856); Uncultured environmental bacilli (AB193886); *Propionibacterium acnes* (NC006085); *Salmonella typhimurium* (DQ153191); *Streptococcus pneumoniae* (AM157443) and *E.coli* (J01859).

4.3 Results

4.3.1 Culture identification

Nineteen (31.1%) of the granuloma specimens were positive for a mycobacterium by BACTEC MGIT culture: 12 (19.7%) for *Mycobacterium tuberculosis*, and 7 (11.4%) for non-tuberculosis Mycobacteria (NTMs).

4.3.2 Patient demographics of genotyped specimens

Of the nineteen samples that were culture positive, nine (47.4%) yielded non-analyzable 16S rDNA sequences with multiple stop codons. The remaining cultures (52.6%) provided high-quality sequence data and the demographic, immunological and microbial data for these patients are listed in Table 4.2. These were predominantly adult patients (80%) who were largely HIV-uninfected (70%). The mean age in this subset (n= 10) was 43 years (range, 2-71 years) while 2 patients were <3 years old. All patients, irrespective of their HIV-1 status, had inversed (or substantially reduced) CD4/CD8 T-cell ratios. Two HIV-1-negative patients (1 Mtb-infected (Patient 36) and one with an unclassified pathogen (Patient 23)) displayed CD4+ lymphopenia.

4.3.3 16S rDNA genotyping

There was agreement between BACTEC MGIT culture identification and genotyping in 4 of 10 culture positive specimens (3 Mtb and 1 NTM). As detailed in Table 4.2, three isolates identified as *Mycobacterium tuberculosis* (Mtb) (Patients 26, 32 and 36) by the BACTECT MGIT system were subsequently confirmed by 16S rDNA genotyping. The 16S rDNA sequence from patient TB21 was identified as Mtb by BACTEC MGIT culture but was homologous with *Mycobacterium intracellulare* following BLAST analyses. Isolates that did not segregate with any published sequences in any of the 3 repositories accessed was given the designation “unclassified”.

4.3.4 Phylogenetic analysis

Bootstrap values were significant and strongly supported the tree (Fig. 4.3). Phylogenetic reconstruction confirmed the genotyping (BLAST) findings in all 10 cases. Three isolates (Patients 26, 32 and 36) clearly clustered with Mtb, 1 (Patient 21) with *M.intracellulare* and another with (Patient 24) *M.fortuitum*.

Table 4.2: Patient demographics corresponding to the 10 isolates that yielded analyzable 16S rDNA 500bp sequences.

Patient Number	Age (years)	Gender	HIV status	CD4 (cells/ μ l)	CD8 (cells/ μ l)	Viral Load (copies/ml)	BACTEC MGIT Culture Identification	16S rDNA Genotyping
TB5	65	M	P	135.4	920.6	13 000	NR	NH
TB18	2	M	N	2254.2	2469.0	-	NTM	<i>S.maltophilia</i>
TB21	47	M	N	NR	NR	-	MTB	<i>M.intracellulare</i>
TB23	23	M	N	497.8	614.8	-	MTB	NH
TB24	71	M	N	954.5	1711.6	-	NTM	<i>M.fortuitum</i>
TB26	39	M	N	NR	NR	-	MTB	MTB
TB32	2	M	P	1004.8	2512.0	2 500 000	MTB	MTB
TB33	50	F	P	376.4	443.7	210 000	MTB	<i>S.marcescens</i>
TB36	28	F	N	487.9	488	-	MTB	MTB
TBS4	20	F	N	685.0	1043.0	-	NR	<i>B.sanguinis</i>
Mean \pm SD (range)	34.7 \pm 23.98 (2-71)			799.5 \pm 655.05 (135.4-2254.2)	1275.34 \pm 851.23 (4443.7-2512)			

NR = no result available; M = male; F= female; P = positive; N = negative; NTM = Non-tuberculous Mycobacteria; NH = no significant homology to published sequences

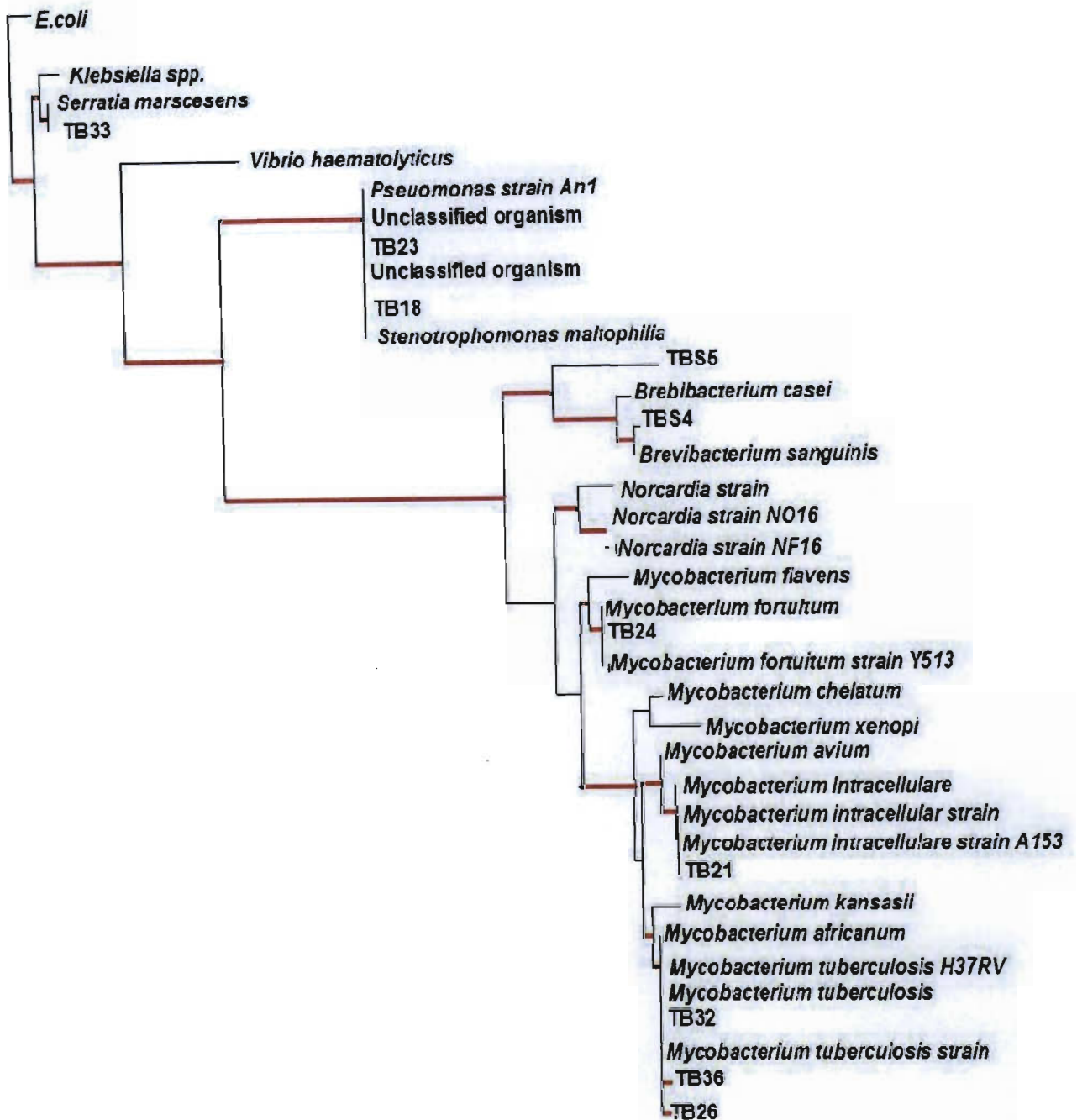


Figure 4.3: Representative Maximum Likelihood tree showing clustering of 3 isolates with *M.tuberculosis* 1 each with *M.intracellulare*, *M.fortuitum*, *B.sanguinis*, *S.maltophilia* and *S.marscesens*. Two previously uncharacterised isolates clustered with environmental bacilli. Branches indicated in Red correspond to a bootstrap value of $\geq 75\%$. The tree was rooted against *E.coli* (J01859)

4.4 Discussion

4.4.1 Main findings

This study confirmed equal proportions of infections that were attributed to Mycobacteria as there were atypical environmental pathogens. There was no association between atypical opportunistic spondylitis and HIV co-infection. These findings were consistent with those of Corbett et al (Corbett et al., 1999a), (Corbett et al., 1999b) in their study of South African mine workers. Others (Govender and Charles, 1987, Mahaisavariya et al., 2005) have also reported NTM spondylitis in which patients were HIV-negative yet severely immune-suppressed as a result of malnutrition and socio-economic factors. In addition, results of the present study were consistent with recent studies in India (Jesudason and Gladstone, 2005, Karak et al., 1996) (Mousa, 2003) (Van der Spoel van Dijk et al., 2000) where a higher prevalence of non-Mtb pulmonary and extra-pulmonary infections were reported in immune suppressed, HIV-1 negative patients from low socio-economic settings. In contrast similar escalations in developed nations were attributed primarily to HIV co-infection and improved diagnostic assays (Jesudason and Gladstone, 2005). The impact of nutrition, demonstrated in Chapter 2 of this dissertation, was emphasised by the findings of bacterial genotyping and a lack of association with HIV immune-suppression.

Although Mtb and HIV co-infections have been significantly linked (WHO, 2006), a similar association was not established in the present cohort. However, in agreement with other reports of spinal Mtb infections (Arnold et al., 1997), (Uppal et al., 2004), patients in the current cohort, reported a previous or current pulmonary component particularly among the adult patients. While patient CD4 and CD8 counts were indicative of a relatively robust immune response their CD4/CD8 ratios were inversed or reduced. CD4+ lymphopenia was noted in two HIV-uninfected patients further implicating poor nutrition in immune suppression in this cohort. Lower than expected CD4+ counts that normalise following anti-TB therapy have previously been reported in HIV/pulmonary TB co-uninfected patients (Uppal et al., 2004). This would suggest factors, other than HIV-1, may be independent mediators of CD4+ depletion and immune suppression.

Failure to isolate bacteria from 68.9% of specimens in the present cohort highlighted the limitations of culture diagnosis when applied to spinal TB and treated specimens (Mousa, 2003) (Perronne et al., 1994). These findings were consistent with previous reports demonstrating a low incidence of culture positive findings in spinal TB (Migliori et al., 1998). Traditionally, negative diagnostic assays associated with spondylitis have been attributed to its paucibacillary state. In addition, all patients in the present cohort received pre-surgery anti-TB therapy and enhanced nutritional support which is likely to have significantly reduced the bacillary counts.

This findings of this study indicated that a multi-disciplinary approach to diagnosis of TB spondylitis would be advantageous. The utility of a multi-disciplinary approach, including the use of 16S genotyping, was demonstrated by Lu et al, (Lu et al., 2000) in the diagnosis of bacterial CSF infections. The previous chapter (Chapter 3) demonstrated that, despite being AFB negative, histopathological (H&E staining) features distinguished typical from atypical Mycobacterial infections (Chapter 3). Histopathological findings for Patients 23, 24 and 33 provided evidence for an atypical or opportunistic infection and genotyping confirmed these findings. However, in the remaining patients, including the NTMs, histopathological features were diagnostic of TB. Failure to accurately identify the organism delays and/or prevents initiation of individualized treatment strategies and exerting a significant impact on patient outcomes (Duttaroy et al., 2004). There is little clinical difference, between infections resulting from *Mtb* and atypical bacterial infections of the spine (Duttaroy et al., 2004) necessitating robust diagnostic assays to distinguish them. Thus alternative or supplementary diagnostic methods may be extremely important to accurate, timely diagnosis and implementation of appropriate treatment strategies.

4.4.2 Mycobacteria

4.4.2.1 Mycobacterium tuberculosis (Mtb)

M.tuberculosis is an established human pathogen (Jesudason and Gladstone, 2005) and has a long history in the context of spondylitis (Zink et al., 2005). In the present era of HIV and elevated incidence of immune suppression, disseminated mycobacterium

infections are prevalent in both developed and developing countries (Reller et al., 2002). *M.avium* and *M.intracellulare* commonly cause disease in developed countries while Mtb is a widespread infective agent in less developed nations (Archibald et al., 1998), (Archibald et al., 1999),(McDonald et al., 1999). Patients presenting with Mtb mediated spondylitis usually report a previous active pulmonary infection (42%; $p<0.001$) and display a high rate of neurological complications (32%; $p<0.001$) (Perronne et al., 1994).

The present study reported Mtb infection in two HIV-infected patients; both patients displaying severe, progressive pathology and insidious onset suggesting exploitation of a diminishing immune response. Interestingly, Mtb seems to have targeted the very young (2-year-old) and the elderly (50 year old) immune-compromised host in the present study. The immunological status of dually infected spinal TB patients is variable (Belzunegui et al., 2004) with no significant difference in immunological markers between HIV-infected and uninfected patients (Govender et al., 2000a). Clinical outcomes, even in HIV-infected patients remain positive following surgery, nutritional support and anti-TB therapy (Belzunegui et al., 2004) (Govender et al., 2000a). In direct contrast, others have demonstrated severe disease pathology in co-infected patients (Archibald et al., 1998, Archibald et al., 1999) (Govender et al., 2000a) (McDonald et al., 1999) (Perronne et al., 1994).

4.4.3 Atypical organisms

The present study illustrated an insidious emergence of atypical opportunistic infections of the spine in the local setting. Clinical accounts of spinal NTM infections (*M.fortuitum*) have been reported in severely malnourished HIV-negative patients (Duttaroy et al., 2004) substantiating these findings. Isolation of atypical bacterial infectants has previously been attributed to contamination, transient colonizers (Duttaroy et al., 2004) or minor variants. A likely source of infection may be the domestic, informal or rural environment where a high index of exposure to large numbers of opportunistic pathogens may be encountered. Thus, non-tuberculous Mycobacterial infections remain a significant problem to both HIV-uninfected and infected individuals, alike (Henry et al., 2004).

4.4.3.1 Non-tuberculous Mycobacteria (NTM)

The findings of the present study reiterate those of Corbett et al (Corbett et al., 1999a) which reported a high incidence of non-tuberculous Mycobacteria (NTMs) with no correlation between HIV infection, and NTMs. In addition, there was no significant difference between the average CD4 counts between HIV-positive and -negative patients nor were clinical outcomes following surgery and anti-TB therapy dissimilar between the groups. Others have shown that patients with an *M.kansasii* infection display high CD4+ T-cell counts and positive outcomes in the short term (Corbett et al., 1999a). The impact of the HIV epidemic on related incidence rates cannot be discounted (Corbett et al., 1999b), (Ferreira et al., 2002) and must be included among the primary risk factors of related pathologies.

NTMs comprise more than 95 species of naturally occurring saprophytic organisms causing 4 types of human diseases: TB-like pulmonary infections; extra-pulmonary infections of the lymph nodes, skin and soft tissue; multifocal disease and infections in immune suppressed hosts (Karak et al., 1996). The incidence of related pathology has escalated in recent years due, in part, to the HIV epidemic but also to improved diagnostic assays (Jesudason and Gladstone, 2005), malnutrition and poverty. While developed countries have noted a decline in the incidence of NTMs, current incidence rates in developing countries remain ambiguous (Ferreira et al., 2002). The primary reason for the lack of clarity is, often, failure to identify the infective organism to species level (Ferreira et al., 2002). A further concern is that NTMs are resistant to a number of conventional anti-TB therapies culminating in increased treatment failure, disease morbidity and mortality.

It may be argued that the NTM and environmental bacilli isolated in this cohort may represent infection with multiple opportunistic pathogens. Dual Mycobacterial and pyogenic infections of the spine are rare (Govender and Charles, 1987), (Mousa, 2003). A Mycobacterial and concurrent *Nocardia asteroides* and *Moraxella catarrhalis* spinal infection has been reported (Mousa, 2003) while a similar pathology involving Mtb and *Cryptococcus neoformans* was described in the local setting (Govender and Charles, 1987). TB infections involving 2 to 4 Mycobacterial organisms, in the same patient, have been reported in India (Raja, 2004). In the era of the HIV pandemic, super-

infections may not be as unusual as previously assumed. Indeed, immune suppression, psoas muscle calcification, low-virulence infectants, treatment non-responders and recurrent infections feature among the risk factors for multiple bacillary infections.

4.4.3.1.1 *M.intracellulare*

The present study identified an *M.intracellulare* isolate in an HIV-negative patient confirming the predilection *M.intracellulare* for HIV-uninfected hosts, even in an HIV endemic region. *Mycobacterium intracellulare*, together with *M.avium* constitutes the *M.avium* complex (MAC) and accounts for an excess of 85% of human pathogenesis in HIV-infected and –uninfected patients (Beggs et al., 2000). They have been implicated in an increasing number and severity of infections at pulmonary, extra-pulmonary and osteoarticular sites (Beggs et al., 2000), (Collins, 1989) (Koh et al., 2005) (Raszka et al., 1994a) (Raszka et al., 1995) (Raszka et al., 1994b) (Scoular et al., 1991). Associated infections are independent of geographical and anatomical sites (Sakatani, 2005) and have been linked with HIV-uninfected individuals (Collins, 1989) (Guthertz et al., 1989) (Kyriakopoulos et al., 1997) (Raszka et al., 1994b, Yakrus and Good, 1990) and HIV-infected individuals (Singh et al., 2007). Crowle et al (Crowle et al., 1992) attributed the greater incidence of *M.intracellulare* infection among HIV-negative patients to differences in relative infectivity of macrophages.

4.4.3.1.2 *M.fortuitum*

Genotyping identified a *Mycobacterium fortuitum* infectant in one patient of the present cohort. These organisms are obligate pathogenic, atypical, rapidly growing mycobacterium and are characteristically resistant to the majority of anti-TB chemotherapeutic measures (Matos et al., 2004), (Vincurova et al., 1984). These organisms pose significant therapeutic challenges often requiring aggressive surgical interventions and appropriate anti-bacterial therapies (Duttaroy et al., 2004), (Muthusami et al., 2004), (Vincurova et al., 1984). Similar interventions were necessary in the *M.fortuitum*-infected patient in the present study.

Related pulmonary disease has been noted (Katoch, 2004), while infections at various anatomical sites occur with greater regularity than other NTMs (Chakrabarti et al., 1990) (Muthusami et al., 2004). However, infection of the spine is rare (Duttaroy et al., 2004), (Smith, 1976) (Vincurova et al., 1984) with no associated predisposing factors.

4.4.4 Environmental bacteria

Pyogenic infections of the spine involving non-Mycobacterial organisms have been documented (Arnold et al., 1997), (Perronne et al., 1994) even in the local setting (Charles et al., 1989). Indeed, infections of the spine have implicated pyogenic organisms (61%) more often than Mtb (39%) (Perronne et al., 1994). Diabetes is the primary predisposing factor with immune suppression and HIV infection exerting an equal influence on incidence rates (Arnold et al., 1997).

4.4.4.1 *Serratia marcescens*

One patient in the present cohort was infected with *Serratia marcescens*, an opportunistic environmental pathogen also associated with hospital-acquired and nosocomial infections (Iwaya et al., 2005). A primary risk factor of infection is immune-suppression and was, accordingly, displayed in the present study (CD4+ count < 500 cells/ μ l). Related infections hold a high probability of fatality (Hadjipavlou et al., 2002). Infection of the spine has been reported but relates primarily to surgical contamination (Carmichael, 2003), (Hadjipavlou et al., 2002) (Liu et al., 1994) or trauma (Lowe et al., 1989). Clinical presentation is comparable to TB spondylitis. These similarities are likely due to both organisms sharing selected biochemical features that are important to virulence (Hejazi and Falkiner, 1997), (Hejazi et al., 1997). Using 16S rDNA genotyping, Lu et al, (Lu et al., 2000) identified *S. marcescens* in CSF emphasizing the utility of genotyping at distinguishing atypical pathogens. The present study further confirmed the utility of 16S genotyping.

4.4.4.2 *Brevibacterium sanguinis*

Brevibacterium sanguinis is an emerging human pathogen rarely isolated from clinical samples (Gruner et al., 1993), (Gruner et al., 1994) (Janda et al., 2003) (McCaughey and Damani, 1991, Wauters et al., 2004), and was identified in a patient of the present cohort. In addition, these organisms are extremely difficult to isolate, therefore their incidence may be severely underestimated. It was thus a novel and significant finding in the context of infectious spondylitis and the local setting.

These gram-positive organisms are characterized by an irregular, rod-shaped morphology; are non-acid-fast obligatory aerobic organisms (Janda et al., 2003) and propagate preferentially at 25-37⁰C (Wauters et al., 2004). They are generally undetectable on conventional AFB diagnostic tools. It is therefore not surprising that the *B.sanguinis*-infected patient in the present study was AFB and culture negative. Infections from *Brevibacterium* species such as *B.caseii* have been reported but are believed to be underestimated due to diagnostic constraints (Castagnola et al., 1997).

Representatives of the genus are commonly associated with milk products or human skin (Gruner et al., 1994). They had no relevance to human pathogenesis prior to 1991 when the first case of a catheter infection was reported (McCaughey and Damani, 1991). Subsequent isolation has been primarily from blood (Janda et al., 2003) suggesting that haematogenous spread to a remote site such as the spine is not improbable. In general, *Brevibacteria* infections are associated with the immune compromised host particularly those with cancer or HIV infection (Castagnola et al., 1997), (Janda et al., 2003) (Wauters et al., 2004). Wauters et al, (Wauters et al., 2004) isolated *B.sanguinis* from 4 immuno-compromised hosts including patients with HIV infection, a Hickman catheter, an acute myeloid leukaemia and a febrile child. These patients had no invasive procedure and no injury therefore a nosocomial source of infection was discounted. Similarly no prior nosocomial source was identified in the present study.

4.4.4.3 *Stenotrophomonas maltophilia*

An unusual finding in the present study was a *Stenotrophomonas maltophilia* infection. This is an environmental pathogen, which displays notable morbidity, limited pathogenicity and may be fatal (Looney, 2005). These organisms are associated with nosocomial infections, the aquatic and agricultural environments (Looney, 2005) (Skorska et al., 2005). The organism is an airborne contaminant of valerian root farming and sewage treatment plants (Prazmo et al., 2003), (Skorska et al., 2005) suggesting an agricultural or aquatic source of exposure in the *S.maltophilia*-infected patient in this cohort.

Individuals with impaired immunity are at highest risk (Looney, 2005). Sites of human infection include soft tissue (Bello et al., 2005) the respiratory tract (Schewe et al., 2005) bloodstream, skin, urinary tract, endocardium (Dignani et al., 2003) and small bowel (Hellmig et al., 2005). Since these organisms rarely infect pulmonary tissue the absence of active pulmonary involvement was not unexpected in the present study. Infections with *S.maltophilia* also pose therapeutic challenges since they readily acquire and transfer antibiotic resistance. This may provide an explanation for the progressive and gross pathology noted in the *S.maltophilia*-infected patient and their failure to respond to pre-surgery anti-TB treatment.

4.4.5 Previously uncharacterised bacteria

Two of the organisms isolated in the present study did not display homology to published sequences in all 3 repositories accessed. In addition, sequences that were presented as possible matches were rejected since phylogenetic analysis failed to yield clustering patterns supported by acceptable bootstrap values. The failure to genotype these organisms may be a consequence of a mixed bacterial population. Alternatively, these organisms could represent previously uncharacterised variants. It must be remembered that the universality of current sequence repositories may be questionable as they may not be representative of a wide range of genera. Sequences generated in this study were submitted to 3 independent sequence repositories. Of the 3, RIDOM provides a comprehensive and high quality database of bacterial isolates (Mellmann et

al., 2003). Mellmann et al, (Mellmann et al., 2003) demonstrated a 71% specificity of RIDOM compared with the ABI Microseq databases (26%) and Genbank (59%). The greatest criticism of sequence repositories is that they often contain erroneous entries, as there is no quality control. Submitting query sequences to multiple sites, as performed in the present study, is thus an important verification exercise. In addition, the organisms isolated in this study may be variants of existing environmental pathogens and obligate infectants that have, as yet, not been characterized fully.

4.4.6 The utility of 16S rDNA genotyping

Various novel tools have, to date, been applied to TB diagnosis in an attempt to enhance the efficiency of conventional assays. 16S genotyping was attractive for several reasons. Firstly, the almost universal occurrence of this gene, which is common to all Eubacteria, enables the identification of aerobic and anaerobic bacilli, Mycobacteria and even fungi. Secondly, it is a more accurate and objective means of identification as it does not selectively isolate a specific organism or category of organism. Finally, rare, recombinant or quasi-species may be identified through genotyping.

Unfortunately, the cost of genotyping and the need for specialised personnel makes this application inaccessible to many health care systems particularly in marginalized developing nations. An additional disadvantage of 16S genotyping is that this gene is homogenous in *M.bovis*, *M.microti*, *M.tb tuberculosis* and *M.africanum*. There is thus a need for multiple database searches and the inclusion of different genotypes in the analysis of a wild type. This limitation was accounted for in the present study by multiple sequence repository searches (RIDOMM, NCBI, DDBJ).

4.4.7 Limitations of the study

A primary limitation is that the extraction and amplification of the 16S rDNA gene was unsuccessful directly from clinical samples. It is assumed that amplification of a specific region has been optimized to detect template down to a single copy, however, not all reactions proceed optimally and in most instances, only reach 60-70% efficiency.

Detection of low copy number templates may therefore fail. In treated patients the copy number of bacterial DNA will be severely diminished. Drug therapy may also result in DNA fragmentation and mitochondrial dysfunction (Bhadauria et al., 2007) further complicating PCR amplification and genotyping.

While the quality of DNA in a subset of samples was screened using the MDR1 gene, the entire sample population was not. This was a limitation as it is unknown whether the remaining clinically-derived DNA samples were negative due to poor template quality or simply an absence of bacterial DNA. In addition, other extraction methods such as TrizolPlus (Invitrogen, Corp., San Diego, CA) or conventional phenol-chloroform extraction may yield better results and must be considered in future studies of this kind. These extraction methods were not available for use at the time of this study.

It may be argued that the environmental bacilli isolated in this study were contaminants. Every precaution was taken to eliminate this possibility. A strict chain of sterility was maintained from the point of tissue excision, to the routine laboratory where cultures were inoculated and maintained. In addition, this study was the only investigation genotyping bacterial species in the molecular laboratory where the work was conducted ensuring that no amplicon contamination was possible.

Finally, the small sample size may not reflect the larger demographic. However, it must be remembered that infectious spondylitis is not as prevalent as pulmonary TB. The sample population of this cohort represents a substantial sample size compared with other settings (Pertuiset et al., 1999).

4.5 Summary

This study extends the findings from other socio-economically constrained nations, by demonstrating a higher incidence of NTMs among HIV-uninfected patients in the context of infectious spondylitis. Consequently, it may be speculated that host susceptibility and immune suppression relates more to high indices of exposure to opportunistic pathogens, which is exacerbated by poor nutrition rather than HIV

infection. In addition, these findings demonstrated that the range of organisms implicated in infective spondylitis is vast, ranging from *Mtb* to previously uncharacterised environmental bacilli. Broad-range genotyping provides an important and useful adjunct to conventional diagnostic tools particularly in cases of treatment failure, progressive pathology and diagnostically challenging cases.

Chapter 5 - HIV-1 Heterogeneity

5.1 Introduction

A more rapid HIV disease progression has been strongly associated with high viral loads and greater heterogeneity, low CD4+ counts and higher mortality rates (Collins et al., 2000) in pulmonary TB. Similar associations have, as yet, not been established for extra-pulmonary TB.

At transmission, HIV isolates are homogenous but transform to a progressively more heterogeneous population (Learn et al., 2002). Co-infections, particularly TB, greatly influences HIV evolution since immune activation at sites of TB co-infection is frequently associated with a marked increase in HIV-1 replication and viral dissemination (Collins et al., 2002b), (Toossi et al., 2001a) (Toossi et al., 2001b). This increased activity, is mediated by pro-inflammatory mechanisms, such as TNF α -stimulated transactivation of the viral LTR (Hoshino et al., 2002). Studies have provided evidence to suggest that infection with *M.tuberculosis* results in increased heterogeneity and compartmentalization of HIV-1 in pulmonary tissue. This is followed by dissemination of lung-derived variants into the systemic circulation, leading to more rapid disease progression and increased mortality (Collins et al., 2002a). However, findings in TB-infected pulmonary tissue were not conclusive of viral compartmentalisation.

This chapter tested the hypothesis that TB co-infection induced HIV-1 viral compartmentalization and divergent evolution in the anatomically remote spinal granulomas of the co-infected patients in the present cohort. It was hypothesised that the fibrotic encapsulation of the spinal granuloma provides a unique permissive compartment for divergent viral evolution and latency. This investigation represented the first investigation of the role of the epidural granuloma as an anatomical site of virus evolution.

5.2 Materials and Methods

5.2.1 Patient subset

All 22 HIV-infected patients were included in these investigations. Matched plasma and tissue specimens were processed and PCR amplified, concurrently. Only those specimens which yielded paired plasma and tissue sequences were analysed further (n=7).

5.2.2 Sample preparation

HIV-1 RNA was isolated from plasma (100µl) and tissue (10-12mg) samples using the guanidine-silica Boom method (Nuclisens isolation kit, Organon Teknika) as per the manufacturer's protocol.

5.2.3 Reverse transcription

HIV-1 RNA templates, extracted as described above (5.2.2) were reverse transcribed using 200 U of Superscript II reverse transcriptase (Invitrogen, Paisley, UK) as per the manufacturer's protocol. A 20-µl reaction with 1x reaction buffer, 10 mM dithiothreitol (DTT), and 0.5 mM of each deoxynucleoside triphosphate (dNTP) was prepared (Gordon et al., 2003).

5.2.4 PCR amplification

The C2V5 *env* region was amplified from cDNA in a nested-PCR using MK605 (5'-AATGTCAGCACAGTACAATGTACAC-3'; positions 6945 to 6969) and CD4R2 (5'-TATAATTCACCTTGTCCTCAATTGTCC-3'; positions 7652 to 7675) as outer primers and M13F-ES7 (5'-tgtaaacgacggccagtCTGTAAATGGCAGTCTAGC-3'; positions 7002 to 7021) and M13R-ES8 (5'-caggaaacagctatgaccCACTTCTCCAATTGTCCCTCA-3'; positions 7648 to 7668) as inner primers. Each reaction contained 1x PCR buffer, 2.0

mM MgCl₂, 0.2 mM each deoxynucleoside triphosphate, 2.5 pmol of each primer, and 1.25 U of Amplitaq Gold (Gordon et al., 2003). Thermocycling conditions were as outlined in Table 5.1.

Table 5.1: Thermocycling conditions for the amplification of an approximately 621bp region of the HIV-1 *env* gene

Temperature (°C)	Time		Cycles
95	13 min	Denaturation	1
95	30 sec	Denaturation	
65	45 sec	Annealing	
72	60 sec	Extension	
95	30 sec	Denaturation	
60	45 sec	Annealing	
72	60 sec	Extension	
72	10 min	Final extension	

Amplified DNA was verified by agarose gel electrophoresis, purified on either a Microcon (Amicon) or Qiagen PCR purification kit (Qiagen, Hilden, Germany) spin column using the manufacturer's protocols and quantified by agarose gel electrophoresis (Appendix D1 and 2).

5.2.5 Cloning and sequencing the C2V5 region

The purified ~621bp PCR product (50ng/μl) was cloned using the TOPO TA Cloning® kit (Invitrogen, San Diego, Ca., USA) which utilizes the pCR®2.1-TOPO plasmid vector in a 5-minute, one-step cloning reaction (Evans et al., 2004). Chemically competent *E.coli* cells were transformed and plated to distinguish single colonies. Positive cultures were PCR screened using the ES7 and ES8 primers. The plasmids of positive clones were extracted using an alkaline lysis method (Appendix C) and

subsequently sequenced on an automated ABI 3100 genetic analyzer (Applied Biosystems Inc., Foster City, Calif.) with M13 (Forward and Reverse) sequencing primers and the Big-Dye terminator cycle sequencing reaction (Applied Biosystems Inc., Foster City, Calif.) (Gordon et al., 2003). The cycle sequencing reaction was as per manufacturer's instructions. A minimum of 5 sequences per compartment were selected for further analysis. Generating clones in two instances failed as the vector did not incorporate the insert despite repeated cloning reactions. As a result, 7 pairs of tissue- and plasma-derived sequences were generated.

5.2.6 Sequence analysis

Consensus sequences were obtained, from the electropherograms, in Phred and Phrap (Linux) and compared with *env* sequences concurrently, or previously amplified in our laboratory (Y137011-AY137073, AY196497), in order to eliminate any possibility of contamination. In order to further exclude any potential contamination, newly derived sequences were compared to subtype reference strains in the Los Alamos database (http://hiv-web.lanl.gov/content/hiv-db/SUBTYPE_REF/align.html) and to HIV-1 subtype C sequences previously amplified in our laboratory.

Phylogenetic trees were constructed using all sequences generated (plasma- and tissue-derived) and selected reference sequences, following degapping (Degap option) in PAUP* Version 4.0b10. Trees were generated (PAUP* Version 4.0b10) using the F84 substitution model and the neighbour-joining method. To examine intra-subtype relationships, appropriate evolutionary models selected with the Akaike identification system executed in MODELTEST 3.0, were used to construct neighbour-joining and maximum likelihood (ML) trees. One of three (TVM, GTR or K84) basic models was used; each modified to include/exclude invariable and variable site distributions (γ) and a α -shape. Trees were rooted against the -AF110967 (C-96BW05.02 isolate) strain of HIV-1 subtype C and viewed in Treetool and Treeview. Bootscanning (1,000 replicates), the Recombination Identification Program (RIP) and Simplot were used to assess reproducibility and test for inter- subtype recombination.

Mean distances among nucleic acid sequences were determined using a Kimura two-

parameter model calculated with the DNADIST program of PHYLIP. Amino acid diversity was measured using a Poisson distribution method implemented in the same MEGA package. P-values for diversity measurements were calculated by applying the *t* test to the distance matrix of each data set. Three independent software programs were used to calculate the ratio of synonymous to non-synonymous amino acid substitution as a measure of natural selection pressure at the protein level. These programs included SNAP and MEGA, which calculate a synonymous-to-non-synonymous (ds/dn_n) substitution ratio, and Codeml, which calculates a w (dn_n/ds_s) value. In Codeml, a tool included in the PAML software package, four different codon-based substitution models; M0 (one ratio), M1 (neutral), M2 (selection) and M3 (discrete); were used to assess positive selection, as previously described (Gordon et al., 2003). High rates of synonymous mutations ($\omega < 1$) were indicative of conservation and a strict requirement for biological function, while high rates of non-synonymous substitution ($\omega > 1$) were attributed to adaptive change in response to selection pressure. To identify signature patterns that were characteristic of granuloma tissue, nucleotide sequences were translated aligned and the consensus sequences analyzed using the Viral Epidemiology Signature Pattern Analysis (VESPA) program. Granuloma and blood sequences were also screened for the presence of important functional domains using Prosite, a database of protein families (De Oliveira et al., 2003, Gordon et al., 2003).

Variations of the Swofford-Maddison and Slatkin-Maddison (Swafford, 2002) tests were used to determine whether the HIV-1 *env* sequences derived from spinal granuloma tissue differed significantly from those found in the plasma of the same patient. The null hypothesis for these analyses was that the distribution of sequences was due to chance rather than tissue compartmentalization. The rate of migration between the two compartments was determined with the Likelihood Analysis with Metropolis Algorithm using Random Coalescence (LAMARC) software package. The Ancestry of the sequences was determined in Migrate, a program available in the LAMARC package. LAMARC software is based on models that use Maximum Likelihood (ML) estimates of population dynamics over all likely lineages and ancestries and extends coalescence theory to encompass migration events (Beerli and Felsenstein, 2001), (Beerli and Kuhner, 2000).

5.2.7 Optimisation of experimental assays

A corresponding tissue and plasma derived amplified PCR product could not be generated for 4 plasma- and 4 tissue-derived products. In 9, neither a PCR product nor an analysable sequence could be generated. Several strategies to generate analysable products were attempted, including:

1. The HIV RNA extraction was repeated using the Nuclisense NASBA extraction protocol and varying input volumes of plasma or masses of tissue (Section 5.2.2).
2. RNA was eluted in the elution buffer provided in the kit or in depC treated water (Section 5.2.2)
3. Multiple cDNA synthesis assays (Section 5.2.3) were undertaken and varying input volumes of template RNA was used
4. Template concentration was titrated (Section 5.2.4) in order to dilute out inhibitors
5. Increased input volume of template was used in the PCR amplification assay (Section 5.2.4)
6. Running a known positive confirmed the presence of inhibitors (Section 5.2.4)
7. Cloning reactions were repeated using varying input concentrations of amplified product (30ng/ μ l, 45ng/ μ l, 50ng/ μ l) (Section 5.2.5)
8. Cloning was repeated using failed templates and a new cloning kit, which contained a new set of reagents and competent *E.coli* cells.
9. Plasmids were re-extracted using an alkaline lysis methods (Appendix C)

5.3 Results

5.3.1 Clinical data

All HIV-infected samples were selected for analysis of viral diversity. However, PCR amplification failed to yield a positive amplified product in 11 unmatched plasma and tissue-derived samples. The remaining 11 specimens yielded an acceptable PCR product but failure to obtain positive inserts (2 samples) and/or analysable sequences (2 pairs), resulted in a final samples size of 7 plasma and tissue-derived sequence pairs.

Appendix D1 is an illustration of an agarose gel of the PCR amplified C2-V5 env region.

Table 5.2 shows the demographic characteristics and laboratory results of this subset of patients. Four patients were *M. tuberculosis* positive; and three had an unclassified *Mycobacterium* infection (Table 5.2). Clinical data was only available for 4 (3 paediatric; 1 adult) of the 7 patients. Of the paediatric group, Patient12, a 2-year-old female presented with no neurological deficit (Frankel E) and clear chest sounds indicating no pulmonary involvement. Patient25 was a 3-year-old female who experienced spastic paraplegia and was immobile for 3 months prior to presentation. Patient32, a 2-year-old male, whose TB contact was parental, was observably malnourished with oral thrush and nodes clearly indicative of advanced AIDS. This patient was diagnosed with milliary TB. Both Patients25 and 32 displayed symptoms of a possible pulmonary component. One adult patient; Patient16, (28-year-old male), was symptomatic for 3 months prior to admission. On presentation he was incontinent, immobile (Frankel A), febrile and experiencing sharp chest pain.

Table 5.2: Demographics and basic laboratory parameters of study population subjected to HIV-1 heterogeneity

Patient number	Age (years)	CD4 (cells/ μ l)	CD8 (cells/ μ l)	CD4:CD8 ratio	Plasma viral load (\log_{10} copies/ml)	Tissue viral load (\log_{10} copies / ml)	Culture Identification
Tb12 ^F	2	474	879	0.50	4.2	8.08	No isolate obtained
Tb16 ^M	28	No result available	No result available	No result available	4.48	6.52	No isolate obtained
Tb20 ^F	25	100	661	0.15	5.74	5.41	Mtb
Tb22 ^F	32	379	1848	0.20	4.36	4.98	Mtb
Tb25 ^F	3	855	1925	0.46	4.26	N	No isolate obtained
Tb32 ^M	2	1004	2512	0.41	6.40	9.18	Mtb
Tb38 ^F	43	No result available	No result available	No result available	4.95	8.15	Mtb
Mean (Adults)	32.0 \pm 7.87 (25 – 43)	240 \pm 197.6 (100 - 379)	1255 \pm 839.1 (879 - 2512)	0.18 \pm 0.04 (0.15 \pm 0.20)	4.88 \pm 0.63 (4.36 – 5.74)	6.26 \pm 1.41 (4.98 – 8.15)	
Mean (Paediatric)	2.3 \pm 0.58 (2 – 3)	788 \pm 278.5 (474 - 1004)	1772 \pm 827.1 (879 - 2512)	0.46 \pm 0.05 (0.41 \pm 0.50)	4.95 \pm 1.25 (4.20 – 6.40)	7.42 \pm 2.17 (5.00 – 9.18)	
Mean (Overall)	19.3 \pm 16.8 (2 – 43)	569 \pm 372.5 (100-1004)	1565 \pm 773.5 (661-2512)	0.34 \pm 0.16 (0.15 – 0.50)	4.91 \pm 0.84 (4.20 - 6.40)	6.76 \pm 1.71 (4.98 – 9.18)	

Key: F = female; M = Male; Mtb = *Mycobacterium tuberculosis*

5.3.2 Sequence analysis

A total of 108 sequences (58 plasma and 50 tissue-derived sequences) were analysed. The number of plasma- and tissue-derived sequences from each compartment was mismatched but it was ensured that a minimum of 5 sequences from each compartment were represented in the analysis. A representative agarose gel of a PCR reaction from the plasmids of Patient12 generated from plasma and tissue-derived sequences appears in Appendix D2.

5.3.2.1 Inter- and intra-patient Diversity

Measures of the percentage diversity between plasma and tissue derived sequences for each patient (intra-patient diversity, Table 5.3) revealed greater diversity among adult (range = 4.1% to 20.7%; median = 11.0%) compared with the paediatric study group (range = 0.8% -5.5%; median =4.0%) due primarily, to extensive variation in the tissue sequences of 2 patients (Patient 20 and 22). These findings supported a clear separation of plasma and spinal sequences into distinct mono-phyletic clusters in 66.6% (2/3) paediatric and 75% (3/4) adult patients (Table 5.3). The percentage diversity comparing all plasma versus tissue-derived sequences (distance between the compartments) was greater in the former (15.3 ± 1.1 % versus 12.5 ± 1.0 %).

5.3.2.2 Phylogenetic Reconstruction

To demonstrate the inter-relatedness and the subtype of HIV variants, phylogenetic trees were constructed using published HIV-1 reference sequences (Los Alamos database) representing subtypes A, B, C, D, F1, F2, H, J and K (Fig. 5.1). This confirmed that all sequences were HIV-1 subtype C. As expected plasma and tissue-derived sequences from the same patient were more closely related to each other (Fig. 5.2) than to those derived from unrelated HIV-1 C-infected patients.

Table 5.3: Mean diversity measures of tissue- and plasma-derived sequences from paediatric and adult patients

	% diversity in plasma		% diversity in tissue		% diversity of tissue-vs-plasma	
	Mean	Median	Mean	Median	Mean	Median
<u>Intra-patient diversity</u>						
Paediatrics	3.0 ± 0.0	2 (1-6)	3.0 ± 0.0	3 (1-4)	4.0 ± 1.0	4 (0.8-5.5)
Adult	4.0 ± 1.0	5 (0-7)	1.0 ± 0.0	1 (0-1)	12.0 ± 1.0	11 (4.1-20.7)
Overall	4.0 ± 1.0	4 (0-7)	1.0 ± 0.0	1 (0-4)	8.1 ± 1.0	5.5 (8.0-20.7)
<u>Inter-patient diversity</u>						
Overall	15.3 ± 1.1		12.5 ± 1.0			

Phylogenetic reconstruction of individual patient sequences (Fig. 5.3a-g) revealed three clustering patterns. In 3/7 datasets (Fig. 5.3a; 5.3c; 5.3f), tissue-derived sequences were more tightly clustered relative to plasma sequences while, in a further 2 datasets, plasma sequences were widely dispersed relative to the tissue (Fig 5.3e; 5.3g). Plasma and tissue-derived sequences fell into two distinct phylogenetically separated lineages suggesting tissue compartmentalization and independent viral evolution. The greater branch lengths separating the two compartments in the adult group may reflect a longer period of co-infection and divergent evolution in adults. In the remaining 2 datasets (Fig. 5.3b and 5.3d), plasma and tissue-derived sequences was intermingled illustrating migration or trafficking of HIV-1 variants between compartments. These findings were supported by bootstrap values of >75%, for both ML and neighbour-joining (NJ) trees.

In addition, phylogeny confirmed the findings of the diversity analysis showing unrestricted bi-directional migration between plasma and tissue in 2 patients (Fig. 5.3b; 5.3d) and viral sequestration in the rest of the patients. Distinct monophyletic clustering and separation of variants, according to anatomical site, was observed, predominantly among adult datasets with one exception, Patient38 (Fig 5.3d); displaying limited migration between plasma and tissue.

Correlating the clinical status of patients with their respective migratory and clustering patterns indicated anatomical sequestration was more common to patients at a more acute clinical state such as Patient16 (Fig. 5.3f) and Patient25 (Fig. 5.3a). Ultrastructural investigations (Chapter 3; Section 3.3.3) of related tissue specimens of these patients revealed features characteristic of necrosis and apoptosis; including nuclear changes, extensive myelin bodies and degenerative mitochondria. Furthermore, extensive fibrin deposits were observed in Patient 16 (Fig 5.3f) and 22 (Fig. 5.3g), both displaying distinct sequestration of viral variants according to anatomical site. On the other hand, sequences from patients who were clinically stable, such as Patient12 (Fig. 5.3b), were intermingled, indicative of migration between compartments. While all patients displayed indications of dilated rough endoplasmic reticulum (rER), grossly dilated rER was noted, particularly, in Patient 12 (Fig. 5.3b). In addition, ultrastructural (Chapter 3; Section 3.3.3) evaluation revealed moderate to profuse polyribosome infiltration in three of the four adult (Patient 16 [Fig. 5.3f], 20 [Fig. 5.3e], 22 [Fig. 5.3g]) specimens compared with none of the paediatric specimens. Patient 20 (Fig. 5.3e) displayed distinctive features of degeneration with moderate/profuse polyribosomes and myelin bodies. Characteristic features of apoptosis and necrosis were observed in this patient, who, in addition, displayed discrete monophyletic clustering of plasma and tissue-derived sequences, and greater diversity in this compartment (Table 5.3).

Despite a negative AFB microscopy findings, in all cases, specimens displayed features (H&E, Section 3.3.1) diagnostic of TB, including granuloma formation, caseation and lymphocyte infiltration. All patients, apart from Patients 22 (Fig. 5.3g) and 32 (Fig. 5.3c), displayed elevated epithelioid cell infiltration. This was indicative of advanced pathogenesis and an attempt at containing the spread of disease.

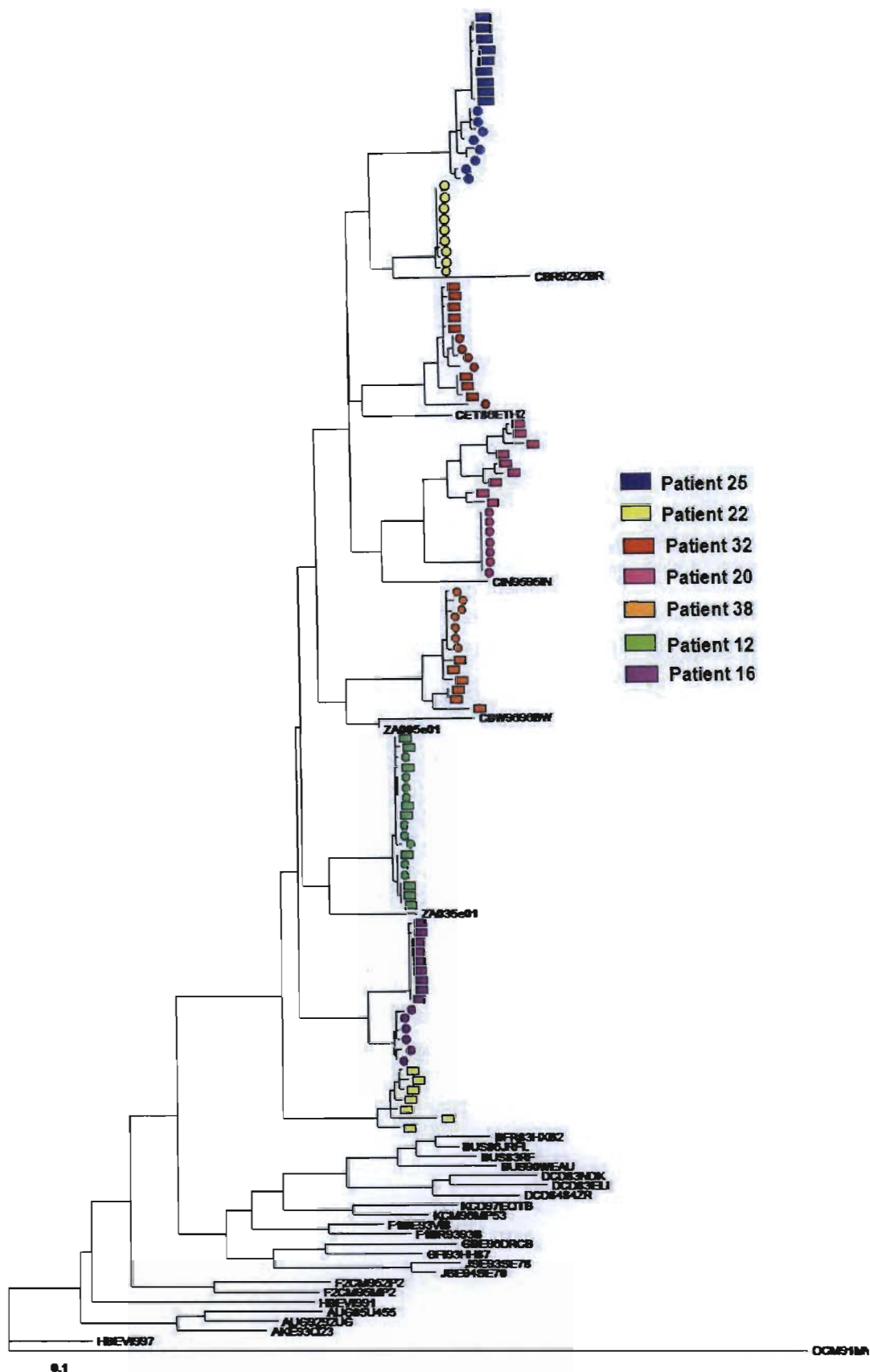


Figure 5.1: Phylogenetic tree of plasma- and tissue-derived sequences **against** HIV1 reference sequences representing HIV-1 subtypes A, B, C, D, F1, F2, H, J and K. Sequences generated in this study clustered with HIV-1 subtype C variants from South Africa (ZA) and Bothswana (BW).

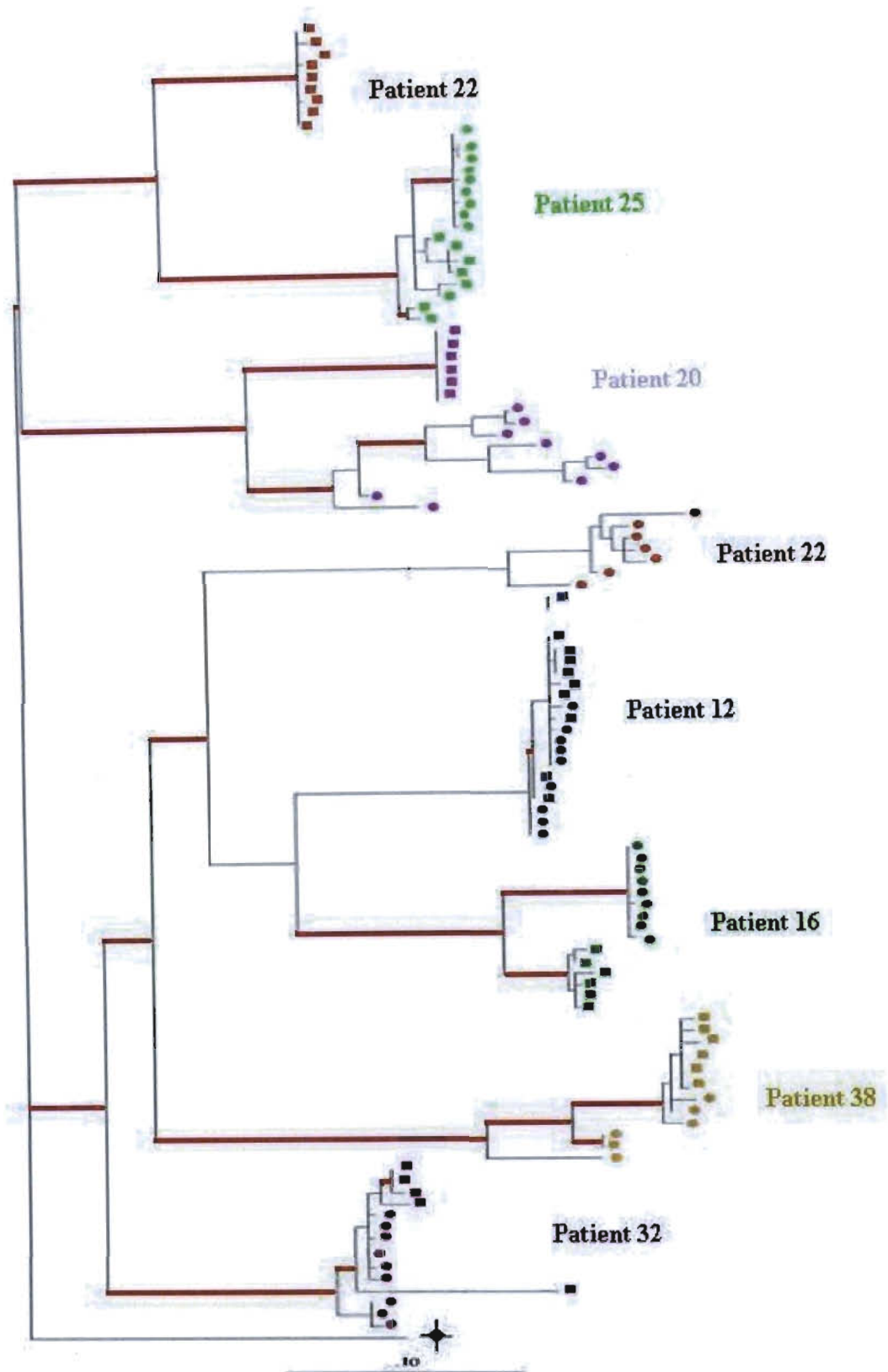


Figure 5.2: Phylogenetic tree illustrating monophyletic clustering of sequences from each patient. Plasma (dots) and tissue (squares) sequences formed discrete clusters or intermingled with each other. Trees were rooted against the -AF110967 (◆, C-96BW05.02) isolate. Branches in Red represent bootstrap values >80%

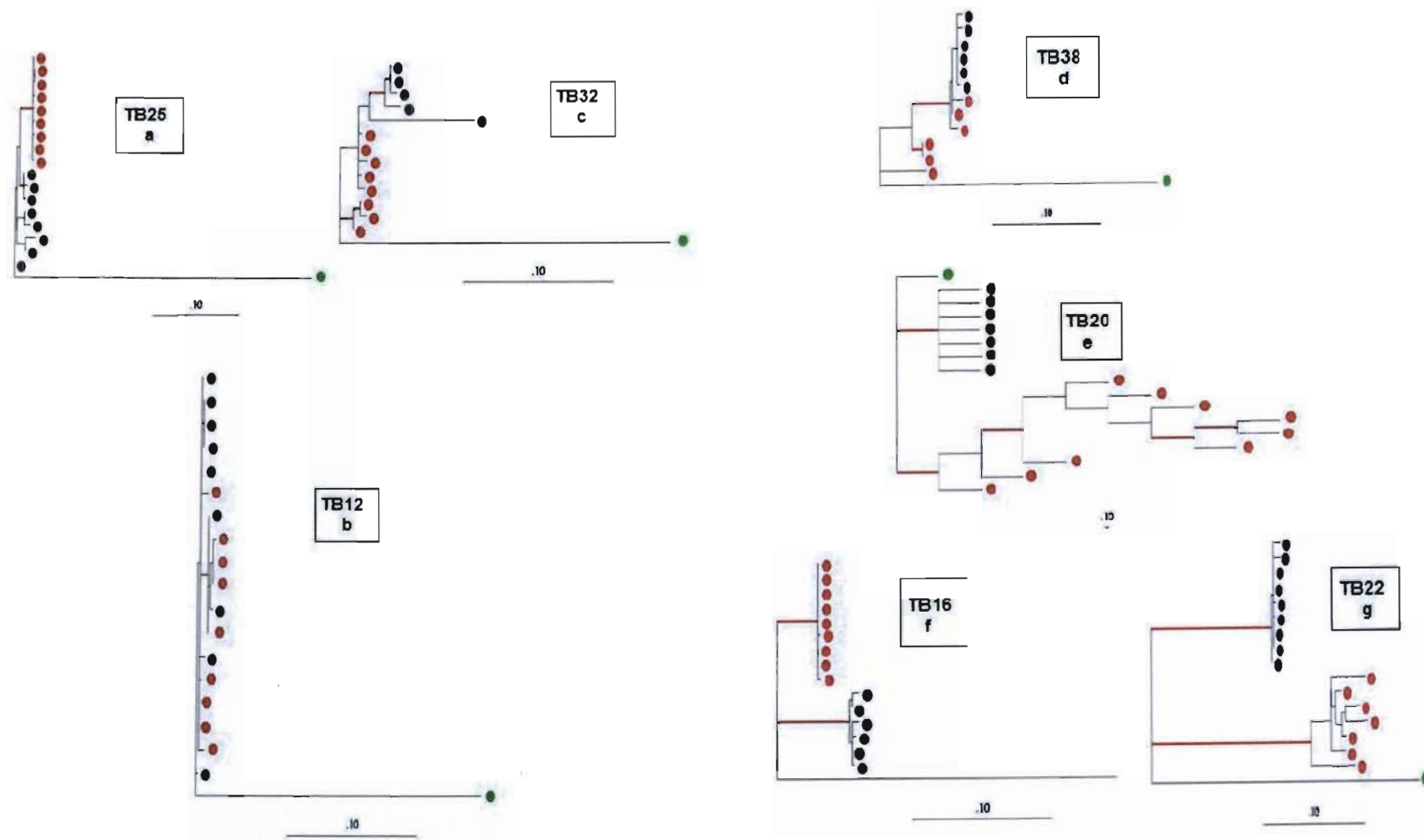


Figure 5.3: Phylogenetic trees of plasma- (dots) and tissue- (squares) derived sequences of individual patients illustrating monophyletic clustering of sequences from each compartment. Plasma- and tissue-derived sequences formed (a, e, f, g) discrete clusters or (b, c, d) intermingled with each other. Trees were rooted against the -AF110967 (†, C-96BW05.02) isolate. Branches in Red represent bootstrap values >80%

5.3.2.3 Analysis of migration (M) and coalescence (Θ)

In patients Patient12, 16 and 25, analysis of migration (Table 5.4) suggested movement of viral isolates from tissue (1893.49; 248.81; 209.79 migrations per generation respectively) to plasma (377.27; 25.20; 150.12 respectively) while in patients Patient20 and 22 the direction of migration was reversed (Table 5.4). Coalescence data verified these findings. Greater coalescence forces (Θ) were computed for tissue sequences in Patients 12 ($\Theta = 0.04$), 16 ($\Theta = 0.222$) and 25 ($\Theta = 0.20$) but for plasma in Patient20 ($\Theta = 0.224$) and 22 ($\Theta = 0.514$). Greater than 10-fold differences were noted between the migration rates from plasma-to-tissue compared with tissue-to-plasma (Table 5.4). No migration or coalescence data was generated for 2 patients (Patient32 and 38) and relates to the level of variation of the datasets.

Table 5.4: Migration rates and measures of coalescence forces comparing plasma- and tissue-derived sequences

	Migration rate per generation (M)		Coalescence Force (Θ)	
	From plasma to tissue	From tissue to plasma	Plasma (γ)	Tissue (γ)
Patient 12	377.27	1893.49	0.002 (3.40)	0.04 (3.40)
Patient 16	25.20	248.81	0.002 (0.568)	0.222 (0.568)
Patient 20	2334.91	33.03	0.224 (2.172)	2.43E-06 (2.172)
Patient 22	152.62	15.95	0.514 (0.123)	0.005 (0.123)
Patient 25	150.12	209.79	0.03 (6.77)	0.20 (6.77)
Patient 32	No result generated			
Patient 38	No result generated			

$\gamma = 4N_e m$ where N_e = effective population size and m = migration rate

5.3.2.4 Analysis of putative functional sites

In comparison to other putative functional sites evaluated, N-glycosylation sites occurred most frequently in both plasma and tissue-derived sequences (Table 5.5). There were no statistical differences in the number of sites, between the plasma- and tissue-derived sequences, although a greater number of N-glycosylation, protein kinase C phosphorylation (PKC), amidation, sulphation and the gram positive anchoring nuclear RGD binding sites (RDG) occurred in the plasma, compared with tissue sequences of adults. However, in the paediatric group, N-glycosylation, casein kinase II phosphorylation (CK2) and PKC sites were more abundant in the tissue compared with plasma sequences (Table 5.5). Sequences from the spinal granuloma and plasma derived virions showed equal conservation of N-glycosylation sites, with the exception of Patient 32, which lacked this site in both plasma and tissue.

Table 5.5: The mean percentage putative functional sites in tissue- and plasma-derived sequences

	N-glycosylation		Myristoylation		PKC		CK2		Amidation		Sulphation		RGD binding	
	Pla	Grt	Pla	Grt	Pla	Grt	Pla	Grt	Pla	Grt	Pla	Grt	Pla	Grt
Paediatric	14.3	14.7	4.7	4.3	4.3	5.0	2.0	2.7	0.3	0.3	0.7	0.3	0.0	0.0
% diff	3*		3		7*		7*		0		3		0	
Adults	14.5	12.8	4.5	4.5	5.8	3.5	3.8	3.5	0.3	0.0	0.5	0.0	0.3	0.0
% diff	18		0		23		3		3		5		3	

* = number of sites in tissue exceeds number in plasma derived sequences

There was no statistical difference in the mean of the overall charge of the V3 loop ($p > 0.05$) when comparing plasma and tissue-derived sequences, nor were there

differences in the overall charge between paediatric and adult groups (Table 5.6). Despite amino acid substitutions, biochemical properties at the specific sites remained unchanged (Fig. 5.4; Table 5.6). No changes were tolerated in residue 13(R), which remained highly conserved in both compartments.

Table 5.6: The mean charge over the V3 loop of the *env* gene in plasma- and tissue-derived sequences

	Average charge	
	Plasma	Tissue
Patient 12	5.0	4.6
Patient 16	3.9	4.0
Patient 20	4.2	5.0
Patient 22	4.1	4.0
Patient 25	3.9	4.0
Patient 32	4.0	4.2
Patient 38	4.4	3.9
Average Paediatric	4.3 ± 0.61	4.3 ± 0.31
Average Adults	4.2 ± 0.21	4.2 ± 0.52

Apart from 2 plasma sequences (Patient 20) where a 2 amino acid insertion resulted in a shift in sequence from CCR5-like to CXCR4-like variant, isolates were all CCR5-tropic (Fig. 5.4). These insertions resulted in disruption of the GPGQTF signature pattern (subtype C) of the tip of the V3 loop. All other plasma and tissue derived sequences retained the characteristic KZN-subtype C signature pattern, with the exception of Patient 20 (GIGGIPGWTF) (Fig. 5.3) and 38 (GPGQAF). The first (G), third (G) and last (F) residues were highly conserved, regardless of the anatomical site and relative number of polymorphisms, alluding to their conformational importance. The cystein residues (C) were conserved in all plasma and tissue sequences (Fig. 5.4; Table 5.7).



Figure 5.4: A representative image of the plasma (p) and tissue (g) derived sequences of the V3 loop from Patient 20 illustrating the two amino acid insertion (*) in two plasma sequences and the resultant disruption of the GPGQ signature of the hairpin loop of the V3 region. Disruption of this region resulted in the downstream disruption of the third amino acid residue (^) which determines viral tropism.

Table 5.7: Details of polymorphisms of the residues determining tropism and overall charge of the V3 loop indicating properties of the residues and their respective charges

	11S residue		13R residue		25E residue	
	Plasma	Tissue	Plasma	Tissue	Plasma	Tissue
Patient 12*	S ⁿ	S ⁿ	R ^b	R ^b	L ⁿ	L ⁿ
Patient 16 [#]	S ⁿ	S ⁿ	R ^b	R ^b	L ⁿ	L ⁿ
Patient 20 [#]	S ⁿ	G ⁿ	R ^b	R ^b	K ^{b,1}	Sⁿ
Patient 22 [#]	S ⁿ	S ⁿ	R ^b	R ^b	D ^a	D ^a
Patient 25*	S ⁿ	S ⁿ	R ^b	R ^b	E ^a	E ^a
Patient 32*	S ⁿ	S ⁿ	R ^b	R ^b	A ⁿ /D ^{a,2}	D ^a
Patient 38 [#]	S ⁿ	S ⁿ	R ^b	R ^b	A ⁿ /D ^{a,3}	D ^a
Overall - Paediatric	N	N	B	B	A	A
Overall - Adult	N	N	B	B	A	A

N = neutral; A = acidic; B = basic; 1 = 30% sequences = K; 2 = 40% sequences = A; 3 = 50% sequences = A; # = adult; * = paediatric, n = neutral residue, b = basic residue, a = acidic residue

5.3.2.5 Positive selection

Of the 7 patients studied, sequences from all paediatric and 3/4 adult patients displayed neutral selection. Evidence of positive selection was demonstrated in only one of the four adult patients. These findings may prove more significant should longitudinal comparisons be made. Unfortunately follow-up samples were not available for this study therefore such comparisons were not possible.

5.4 Discussion

5.4.1 Main findings

This investigation was undertaken to explore the impact of HIV and TB co-infection at a remote, isolated compartment on viral evolution. Although the prognostic value of greater viral diversity has been questioned (Poss et al., 1998) it is unquestionable that greater heterogeneity increases the incidence of CTL escape mutants; and drug resistant, fitter or more virulent strains of HIV. The hypothesis that HIV/TB co-infection induces the generation of such variants was tested. The findings of this study demonstrated separation of HIV variants into 2 discrete monophyletic clusters in 5/7 patients. This was highly suggestive of compartmentalisation and restricted trafficking into and out of the spinal TB granuloma in the majority of the patients. These findings confirmed the hypothesis that epidural HIV/TB co-infection leads to divergent viral evolution within the granuloma. Furthermore, the present investigation represents novel findings as this phenomenon has not been previously demonstrated in the context of infectious spondylitis.

In addition, tissue derived variants appear to have evolved independently following a single introduction, the source of which is likely haematogenous. Phylogenetic analysis of sequences from paediatric and adult patients demonstrated greater divergence between plasma and tissue derived sequences in the latter. This is a likely consequence of a mature, more robust immune response in adults compared with children. The findings of this study, further suggested that differences between the plasma- and tissue-derived strains do not translate to putative conformational and functional differences.

Since patients in this cohort did not receive ARVs, there was no impact of drug selection pressure.

5.4.2 Anatomical compartmentalisation

This report represents the first account of viral sequestration within epidural infectious spondylitis. Anatomical compartmentalisation has been conclusively demonstrated in the brain (Korber et al., 1994), semen (Choudhury et al., 2002) and lymph nodes and strongly suggested in the lungs (Collins et al., 2000), genital tract, cerebrospinal fluid (CSF) (Cinque et al., 2001) (Stingele et al., 2001) and blood (Zhu et al., 1996). For instance, despite establishing that cerebrospinal fluid (CSF) harbours diverse viral variants compared with plasma in early disease and in the presence of opportunistic infections, conclusive evidence for compartmentalisation in the CSF was lacking (Cinque et al., 2001). There are however indications that trafficking of various cell populations as well as the physical barriers of the central nervous system (CNS) and CSF mediate divergent evolution at these sites.

Clinical and histopathological findings of related patients revealed an association between clinical status and viral sequestration where anatomical compartmentalisation was common to patients at advanced disease pathology. These patients additionally displayed ultrastructural features consistent with a pathogenic insult such as extensive necrosis, apoptosis, and degenerative cellular organelles including mitochondria and rER. Grossly dilated rER, suggestive of up-regulated protein synthesis, synthesis of proteins atypical to the cell or storage of atypical peptides within these cellular organelles was noted in acutely infected patients, particularly Patient12, who displayed viral sequestration. This implies that the virus may be actively exploiting the replication machinery of the host. How this impacts on viral evolution remains to be established. Interestingly, patients who displayed distinct monophyletic clustering of viral isolates also displayed features of fibrosis which heralds late stage granuloma formation and an attempt by the immune response to physically isolate the infection. An alternative account for elevated protein synthesis may therefore be a reflection of elevated synthesis of immune modulators. It would therefore appear that this physical barrier creates a permissive environment for divergent viral evolution. Moreover, a lack of

association between viral diversity and CD4+ T-cell counts, but correlation with CD68+ infiltration ($p < 0.05$) indicated the, possible, importance of macrophages and macrophage-derived cells, in promoting viral sequestration. The impact of patient clinical status was illustrated by Patient 38, who presented with miliary TB, and displayed three ancestral lineages (Fig. 5.3) of HIV isolates, representing the circulating haematological variants and variants from remote anatomical sites of infection, including the spinal granuloma.

The findings of the present study are similar to that of Collins et al, (Collins et al., 2002a) who investigated viral heterogeneity in pleural TB/HIV co-infected patients. In both instances, physical separation (pleural space and spinal granuloma) was consistent with divergent evolution in blood and TB infected tissue. An additional commonality is the high improbability that a localised HIV infection prior to TB infection occurred in the pleura and spinal tissue. The influx of leukocytes, followed by activated lymphocytes and macrophages, including an HIV-infected cellular sub-population, is the likely mechanism of initial entry of HIV. Latent HIV may then be activated by cytokines such as tumour necrosis factor alpha (TNF- α) and monocyte chemoattractant protein (MCP) as a result of constant immune stimulation by the TB infection (Collins et al., 2002b). Moreover, TB increases the susceptibility of HIV cellular targets particularly lymphocytes, to infection and the transmission of macrophage-tropic variants in the lower mucosa (Santucci et al., 2004).

Korber et al (Korber et al., 1994) suggest the range of variants within compartments is sensitive to immune pressure, changes in tropism and replication efficiency. When disease is established, the tropism of virions is restricted to the particular cell population that carried the virus into the compartment, therefore a distinct “clade”, with corresponding tropism, emerges (Korber et al., 1994). Macrophage-tropic isolates (CCR5 exploiting) predominate in TB-infected individuals (Santucci et al., 2004) but conservation of R5- to X4-utilizing variants was demonstrated even with progressive disease (Collins et al., 2002a). Similarly, in the present cohort, variants were all R5-utilizing variants with the exception of two sequences (Patient 20) which demonstrated amino acid residues and overall charge of the V3 loop characteristic of an X4-utilising variant (Fouchier et al., 1992). The impact of the bacterial infectant was illustrated, *in vitro*, by a *Mycobacterium avium*-induced increase in HIV-1 replication and CCR5

expression rates (Wahl et al., 1998). In contrast, CXCR4 expression was up-regulated in Mtb/HIV infected alveolar macrophages (Hoshino et al., 2004). Patient 32 in the present cohort was infected with Mtb as identified by 16S rDNA genotyping (Chapter 4) and demonstrated divergent viral evolution with two distinct clusters of tissue-derived sequences (Fig. 5.2c). Whether these clusters are indicative of an emerging population expressing a switch of co-receptor usage may be established by an appropriate bioassay, but this was beyond the scope of this investigation. Differences between these findings may be a result of disparate assay platforms (*in vitro* versus *ex vivo*) and infective organism (*M.avium* versus Mtb). It may additionally reflect an expansion of the initial infecting variant rather than a switch from one co-receptor to another as suggested by reports of the expansion of R5 tropic variants (Morris et al., 2001), (Santucci et al., 2004). Morris et al, (Morris et al., 2001) established that despite advanced disease in a South African cohort of HIV subtype C/ TB infected individuals, there was no change in co-receptor usage suggesting up regulation of macrophage-tropic R5-utilising variants. In contrast, expansion of macrophages and microglial cell populations was associated with distinct sequestration of brain and blood viral variants while, lymphocyte infiltration correlated with unrestricted migration between the compartments (Korber et al., 1994). Others reported distinct sequestration of macrophage-tropic variants in the nervous system and testicles demonstrated in autopsied individuals (Sanjuan et al., 2004). The present findings include a predominance of R5-tropic variants but a dual-tropic (R5/X4) variant (Patient 20) was also observed. Since dual tropism suggests a transitional variant between the non-syncytium inducing macrophage tropic (R5) to the syncytium inducing T-cell tropic (X4) isolates (Doranz et al., 1997) their presence in this cohort may suggest a similar phenomenon.

Collins et al (Collins et al., 2002a) were unable to conclusively demonstrate compartmentalisation of HIV in pleural tissue, a site that is infiltrated by activated leukocytes in response to TB infection. Spinal tissue on the other hand is infiltrated by macrophages that mature to giant cells and epithelioid cells. It is thus most likely that the predominant cell type infiltrating the infected tissue is one of the primary determinants of tissue-localised divergent HIV evolution. Increased viral heterogeneity in circulation has been attributed to migration of pleura-derived isolates as a result of increased viral replication at the site of TB infection and chronic immune stimulation

(Collins et al., 2002a). Most notable in this study is that infiltrating cells were activated leukocytes and that these cells, a large proportion of which would be HIV infected, were not present in the pleura prior to TB infection. Furthermore, the close contact between HIV-infected lymphocytes and TB-infected macrophages at the site of infection invariably leads to interaction between them and ultimately to HIV activation and up-regulation (Toossi et al., 2004a).

5.4.3 Putative functional sites

In the present cohort, functionally important residues were highly conserved despite the variability in flanking regions. For instance, cysteine residues known to be essential to the formation of functional V3 and V4 loop conformations (Leonard et al., 1990) were highly conserved in both plasma and tissue derived sequences. Similarly, residues that determine tropism and CD4 binding were also highly conserved. The role of the V3 loop in viral tropism, cytopathicity, replication efficiency and fusion is known (Korber et al., 1994). Polymorphisms at various sites throughout the *env* gene can potentially compel conformational changes to the *env* protein (Korber et al., 1994). Ultimately these polymorphisms may be inactivating. Therefore despite highly variable domains within this region certain residues remain conserved alluding to their functional importance.

5.4.4 Inter- and intra-patient diversity

This study has shown a greater inter-patient diversity in plasma- (mean = 15.3% \pm 1.1) compared with tissue- (mean = 12.5% \pm 1.0) derived sequences. In addition, intra-patient diversity of plasma- versus tissue-derived sequences was greater in adult (mean = 12.1% \pm 1.0) compared with paediatric patients (mean = 4.0% \pm 1.0). Others have calculated mean intra-patient diversity at 2.6% (range = 0-13.6%) in subtype C viruses (Shankarappa et al., 2001) and compares poorly with the figures calculated in the present study.

The present study reported greater plasma-tissue diversity compared with tissue-tissue and plasma-plasma measures (Table 5.3). Similarly, when evaluating diversity of

sequences derived from brain and blood, Korber et al (Korber et al., 1994) reported greater blood-versus-blood (4.9%) compared with brain-versus-brain (3.5%) intra-patient diversity, while blood-versus-brain (7.7%) diversity was greater than both these comparisons. Inter-patient diversity comparing blood-vs-blood (15.2%) was greater than brain-vs-brain diversity (11.8%) (Korber et al., 1994) similar to the findings of the present study which compared plasma (15.3%) and tissue (12.5%) derived sequences. Both inter- and intra- patient diversity measures in the present study closely approximated those reported by Korber et al (Korber et al., 1994). Collins et al (Collins et al., 2002a) determined greater diversity in pleura compared with blood-derived sequences but not to significance. Unlike Collins et al (Collins et al., 2002b), there was no correlation of the degree of diversity with CD4 and viral loads in the present cohort.

The ancestry of the lineages of sequences derived from plasma and tissue was determined using coalescence. Coalescence describes the phenomenon of converging lineages to a common ancestor with temporal regression until all lineages merge to a point representing the most recent common ancestor of all the sequences (Rodrigo et al., 1999). The time for populations to coalesce depends primarily on the rate of migration between compartments and secondarily on population dynamics (Rodrigo et al., 1999). A lack of divergence with preservation of essential residues; termed convergence of variants, was demonstrated in the tissue compartment and among paediatric patients of the present cohort. However, in two patients (Patient 32 and 38) migration and coalescence data could not be generated, the reason for which may relate to the variation in the dataset. Datasets with little or no variation biases the dataset making almost any migration parameter possible (Beerli and Felsenstein, 1999). Adding either more sequences or base pairs may overcome this bias. Similarly, a high theta value that estimates coalescence will generate a high number of migration events, which expand to levels beyond the capacity of the data analysis resources (Beerli and Felsenstein, 1999) culminating in the collapse and/or failure of the analyses.

5.4.5 Phylogenetic reconstruction

Phylogenetic analysis revealed two clustering patterns namely, monophyletic tight clustering in 5/7 and mixing or migration in 2/7 patients (Fig. 1a-g). These findings

were consistent with those of Korber et al (Korber et al., 1994). In both instances, the phylogenetic trees generated were evidence of divergent evolution in the respective anatomical compartments. In contrast to the findings of Collins et al, (Collins et al., 2002a), the evidence for monophyletic clustering of viral variants was unambiguous in the present study. In their study (Collins et al., 2002a) frequent migration events were unidirectional from pleura to blood representing a direct mechanism for systemic HIV heterogeneity. Comparable migration events also occur in ARV treated patients as a result of poor drug penetration to the tissue and the emergence of drug resistant isolates (Wang et al., 2000). In these instances heterogeneity is more pronounced in tissue compared with the plasma (Devereux et al., 2002). In contrast, we show limited migration events (2/7 patients, 29%) indicative of limited contribution to systemic viral heterogeneity, but tissue heterogeneity was more pronounced as demonstrated by Devereux et al, (Devereux et al., 2002).

The findings of this study illustrated greater branch lengths between the monophyletic clusters of plasma and tissue-derived sequences in adult compared with the paediatric patients. This was indicative of greater evolutionary distance between the monophyletic clusters and their corresponding viral quasispecies. The association between clinical status and clustering patterns was most noticeable in Patient 32 (Fig. 5.3) who presented with millitary TB. The corresponding viral variants manifested as three distinct sequence clusters representing three separate lineages. A pulmonary component was suspected but had not been confirmed at the time of surgery. Ultrastructural morphology was indicative of acute infection and degeneration including polyribosome infiltration, necrosis and apoptosis.

5.4.6 Positive selection

There was no evidence for positive selection in the majority of the patients in the present cohort (6/7 patients). These findings confirm those of Collins et al, (Collins et al., 2002a) and Sanjuan et al, (Sanjuan et al., 2004) where the findings were suggestive but not conclusive of positive selection in HIV/TB co-infection. In all three reports, including the present study, an *ex vivo* sample was collected from the respective organs, either during surgery (Collins et al., 2002a) or at autopsy (Sanjuan et al., 2004). It is

likely that positive selection was not a recent event thus; polymorphisms evident in present viral populations were likely fixed at some point in the distant past relative to viral evolution. Sampling at a single point along the timeline of the emerging population will not reflect this evolutionary process. Genetic drift is a possible model to account for the divergent evolution between the tissue and plasma variants, however this hypothesis also requires longitudinal sampling which was not possible once the granuloma was surgically removed in the present study.

It has been established that pulmonary TB exerts selection pressure on HIV evolution (Collins et al., 2000). This emphasises that a more established HIV infection may result in greater selection pressure as opposed to a recent introduction or an acute TB infection. HIV evolves continuously in the lung a process that, in most instances, precedes the TB infection. An HIV infection which precedes the TB infection represents a pre-existing viral load prior to TB and is currently recognised as a significant contributor to outcomes (Day et al., 2004a). The authors, in the South African setting, demonstrated that TB infection was correlated with a minor increase in HIV viral load, and suggest that this would not impact on outcomes in the individual. Instead they propose it may exert an important influence on HIV transmission and disease progression at a population level. It is therefore important to investigate, longitudinally, polymorphisms that arise in dually infected individuals. The most compelling rationalization of a lack of positive selection is that of Leitner and Albert (Leitner and Albert, 1999), who propose that positive and purifying selection may result in less than expected heterogeneity. Since certain substitutions are not tolerated, they are eliminated, thereby reducing the genetic variation in the population. For example, changes in receptor fit of the *env* gene is a potential source of selection pressure since it impacts on the evasion of the immune response (Williamson, 2003), but these variants soon become fixed and expand to a homogenous population. The lack of evidence for either positive or purifying selection in this study would therefore imply a more variable genetic pool – a circumstance that would be highly beneficial to viral evolution and fitness.

Finally, it is likely that the occurrence of positive selection could not be demonstrated because the analysis of the C2-V5 region rather than portions thereof would not have detected variations in individual regions. Different regions of the *env* gene have been

shown to experience varying rates of positive selection (Mani et al., 2002) (Williamson, 2003). The V1-V2 region has the highest adaptive rate (1 event/2.5 months) followed by the C2-V3 (1 event/5.9 months.) and V4-V5 regions (1 event/12.2 months.) (Williamson, 2003). Accordingly, calculating the rate of positive selection over the entire *env* gene or a substantial portion thereof may not fully illustrate selection pressures exerted at individual sites. There are strong associations between the stage of disease and the frequency and strength of positive selection (Ross and Rodrigo, 2002). The phenomenon of variable positive selection and evolutionary rates is reiterated at protein coding regions over the entire HIV genome (De Oliveira et al., 2003). In addition, analysis of HIV subtype B and C sequences revealed that *tat* and *rev*, rather than the *env* gene are under positive selection (De Oliveira et al., 2003). It is thus, conceivable that the consequence of selection pressure would be evident in these early multiply spliced regulatory proteins in the present study.

5.4.7 Limitations of the study

Since the diseased bone and tissue are removed at surgery, longitudinal tissue samples were not available. Moreover, a single sampling event excluded the impact of sampling bias (Mani et al., 2002) such that measures subsequently conducted on blood and tissue were accurate point determinations of the *in vivo* dynamics.

A further criticism of this study may be that viral heterogeneity was demonstrated following a single PCR amplification and cloning event therefore may have selectively amplified the dominant population. Minor variants, which may become dominant in time and in response to drug or immune selection pressure, would have been excluded from the analysis. However, it must be remembered that while a selection bias may be represented by the data, viral compartmentalisation and divergent viral evolution was demonstrated by this single PCR and cloning event. Multiple PCR reactions would act simply, as a confirmation of these findings. Cloning multiple PCR products would serve to further clarify ambiguous patterns of divergence therefore, the findings of this study may actually be an underestimate of the true occurrence of viral sequestration in this population.

5.5 Summary

This investigation demonstrated restricted migration between TB infected spinal granuloma and blood in 5 of 7 spinal TB and HIV co-infected patients indicative of divergent HIV-1 evolution in spinal granulomas. These findings are novel in the context of HIV/TB co-infection and in relation to infectious spondylitis.

Chapter 6 - General Discussion and Conclusion

6.1 Patient cohort and related clinical findings

In the first of four experimental chapters, the incidence, clinical features and demographics of a cohort of HIV-uninfected and –infected TB spondylitis patients was presented in order to investigate the differences between HIV-infected and –uninfected groups. The number of cases of TB spondylitis treated per annum (150pa) at King George V Hospital far exceeds previous related reports from other regions (Table 6.1). Approximately 70% of these patients undergo spinal decompression, insertion of an allograft and instrumentation, if required (Patient files, data not shown). These surgical interventions are implemented in addition to conservative anti-TB therapy. Approximately 30-40% is HIV co-infected. Despite a high rate of co-infection, severe neurological involvement and disability patients recover clinically following conventional anti-TB therapy and surgery regardless of HIV status.

In light of the abovementioned as well as international statistics (Table 6.1), a patient cohort of 60 patients was an adequate coverage of the general population. In addition, the rate of HIV co-infection noted in this study, corresponded with previous reports in the same demographic (Govender et al., 2001a, Govender et al., 2001b) as well as, the incidence of HIV, TB and HIV/TB co-infection in the local region (81, 118, 119). In the present study, co-infection accounted for 37% of the study population consistent with global and national figures (Ige et al., 2005, WHO, 2006), in addition to the demographics of the referral hospital in question (30-40%). The impact of co-infection is also evident in developed nations with TB defined as the leading cause of HIV related deaths and the most common AIDS defining condition of 2002 in UK (Health_Protection_Agency_Tuberculosis, 2004).

The findings of this study illustrated the devastating effect of infectious spondylitis on the primary economically viable demographic (mean age = 32 years) in KZN. This cohort was largely comprised of young adults who presented with spinal deformity and

neurological deficit particularly in co-infected individuals. It has previously been reported that the highest incidence rates of co-infection occur in younger individuals between the ages of 25-44 years (de O Liverato et al., 2004). In KZN, incidence rates following a population-based HIV survey in a rural community reported highest incidence rates among men aged 25-30years (8.0 per 100 person years) and peaked at 25 years for women (Barnighausen, 2007). Again, this group represents an important demographic of the country and suggests a significant social, economic and political consequences. The findings of the present study highlighted a similar circumstance in relation to infectious spondylitis and HIV co-infection.

In addition, the present study demonstrated the efficacy of enhanced nutritional support and surgical interventions at resolving an AIDS defining disease with advanced pathology even in patients with CD4 T-cell counts <200 cells/ μ l. The scientific outcomes of this study had no clinical bearing on the patient cohort since no change to the therapeutic interventions was implemented subsequent to the findings of this investigation. The same surgical, nutritional support and therapeutic regimes were implemented in all patients and sample collection was conducted at the same time point i.e. at surgery, in all instances. These findings were consistent with previous investigations of spinal TB in KZN which reported similar recovery rates and clinical outcomes in HIV-infected and uninfected patients (Govender, 2005). This is perhaps due to the similarity in the patient demographic, and therapeutic interventions implemented. Despite being ARV naïve, patients recovered full or partial neurology (Chapter 2); and incorporated bone grafts. There was no difference in the scope of or time to clinical recovery between HIV-infected and uninfected patients nor between adult and paediatric patients. These findings do not detract from the benefits of ARVs but illustrate that they can be delayed in TB spondylitis/HIV co-infected patients. The impact of ARVs on patient mortality rates have yet to be determined but it was shown recently that mortality rates decline significantly following the implementation of an ARV programme in a rural KZN community (Herbst, 2007). Moreover, the findings of present study was comparable to that of Arora et al, (Arora et al., 2006) who reported positive clinical outcomes in anti-TB treatment non-responders (on 3 months of conventional anti-TB therapy) and those with reactivation disease following conventional therapy. Others also reported conventional anti-TB therapy to be effective at varying degrees ranging from 15%-73% (Butler et al., 2006, Tasova et al., 2006).

This study reiterates the clinical benefits of anti-TB chemotherapy without ARV treatment since patients in this cohort recovered clinically with graft incorporation and, neurological improvement despite the absence of an ARV intervention. Treatment with anti-TB chemotherapy in the absence of ARVs, in co-infected patients has an acute impact on disease progression (Kirschner, 1999) with rebound of T-cell and macrophage populations and concurrent declines in viral loads in the context of pulmonary TB (Martin et al., 1995). This treatment option will be especially attractive to developing nations particularly in Africa and Asia as TB chemotherapies are more economical than ARVs. In addition, treatment is not encumbered by adverse drug interactions between selected ARVs and anti-TB chemotherapies. An equally imperative consideration to successful treatment is suppression of bacterial growth rather than enhancing the bacterial death/killing rate (Kirschner, 1999) since increased killing of macrophage-associated bacilli severely depletes immune cell populations. Consequently, mounting an effective “killing response” against the bacterial pathogen is likely to pose a greater risk to positive clinical outcomes.

The present study confirmed that surgical interventions can be effective in both HIV-infected and –uninfected patients over a range of CD4 T-cell counts and viral loads (Chapter 2). Of the approximately 150 cases treated annually at KGV, conservative treatment resolves disease in 30% of patients (Chapter 2). Surgery is necessary in the remaining patients. Spinal TB is AIDS defining and represents late stage disease. For this reason, it is widely accepted that HIV-TB co-infected patients are poor candidates for surgery, as they present with depleted CD4+ T-cell populations, a severely depressed immune response and advanced disease. However previous studies (Govender et al., 2000a, Govender et al., 2001b, Govender et al., 2001a) have demonstrated that sero-positive status and stage of HIV disease need not be a disincentive to surgical interventions even at advanced disease when CD4+ T-cell counts are <200 cells/ μ l. The present study confirmed these findings.

Table 6.1: Summary of recently published reports of infectious spondylitis

Reference	N	Time frame	Infective organisms	Cohort	Setting
The present study	61	2001-2003	Mycobacteria, environmental bacilli (Chapter 4)	Adult and paediatric	KZN, SA
Tasova et al, 2007 (Tasova et al., 2006)	40	1997-2003	TB	Adult	
Butler et al, 2006 (Butler et al., 2006)	48	1992-2004	Pyogenic bacteria	Adult	Ireland
Arora et al, 2006 (Arora et al., 2006)	51		TB	Adult	India
Cormican, et al, 2006 (Cormican et al., 2006)	21		TB	Adult	UK

Key: N = number of patients in cohort, TB = tuberculosis, KZN,SA = KwaZulu-Natal, South Africa, UK = United Kingdom

An additional novelty of the present cohort was the ability to incorporate bone grafts even in immune-suppressed patients with CD4 T-cell counts <200cells/ μ l (Chapter 2). Unlike conventional allografts, those used in this cohort were devoid of all immunogenic material since all marrow and other material are removed leaving only dead bone. In addition, fragmented autologous rib material was inserted into the femoral allograft before insertion, which prevented a rejection response. Not only does this strategy preclude the possibility of host rejection but the grafts may serve an immunogenic role. Ironically, patients lacking mature T-cells do not reject tissue transplants (Roitt et al., 2001) therefore HIV depletion of the CD4+ T-cell subpopulation may in fact, aid in graft incorporation. The staging of surgery was determined by the nutritional status of patients as reflected by their albumin and ESR markers (Chapter 2) - not their CD4+ T-cell counts. Yet, clinical outcomes were positive regardless of CD4+ counts implying that ESR and albumin levels may serve as useful surrogate markers of clinical outcomes in addition to directing therapeutic interventions.

6.2 Histopathology and ultrastructural morphology of spinal TB and HIV

The second experimental chapter aimed to distinguish histopathological and ultrastructural features of HIV-uninfected from HIV-infected extra-dural granulomas. An additional objective was to assess the efficacy of conventional microscopy diagnosis in paucibacillary and treated spinal TB disease. The immunohistological and ultrastructural findings of this chapter represented novel work in the context of spinal infections since related evaluations of extra-dural granulomas has, to our knowledge, not been previously undertaken.

The Ziehl-Neelsen (ZN) technique, for the detection of acid-fast bacilli, failed to demonstrate AFBs in this cohort (Chapter 3). The complete absence of staining was contrary previous reports, however, low detection rates using this technique have been published. Previous reports have indicated specificity in untreated pulmonary (Kivihya-Ndugga et al., 2004) (Zajac and Melcher, 1991) and spinal disease (Tasova et al., 2006) of 50-80% and 47% respectively. Assessment of clinical samples by direct staining and microscopy is widely used and purported to be one of the more economical and rapid techniques presently available to disadvantaged nations (Kivihya-Ndugga et al., 2004). However, the present findings indicated that adjunct diagnostic assays must be applied to spinal TB for an accurate, conclusive and timely diagnosis largely due to its paucibacillary characteristic. As a result of failed laboratory diagnosis patients in this study were initiated on therapy based on clinical and radiological findings rather than histopathology or TB culture. This was consistent with previous reports where presumptive signs and progressive insufficiency directed diagnosis (Anane and Grangaud, 1992). On the other hand, the disadvantage of using presumptive diagnostic signs is that most NTMs produce granulomas identical to those induced by *Mtb* while those of other infectants such as fungi, are very similar.

It may be speculated that the gross tissue and bone pathologies observed at surgery emerged prior to treatment and were not resolved with anti-TB chemotherapy. This is despite the impact of treatment on bacillary counts. A more severe pathology correlates strongly with high bacterial numbers in infected murine pulmonary tissue but not to the delayed type hypersensitivity (DTH) response (Dormans et al., 2004). But DTH is the proverbial “double-edged sword” since it aids in bacterial eradication while advancing

tissue damage (Kobayashi et al., 2002). In addition, TNF α promotes a robust immune response if it is primarily Th1 mediated but induces tissue damage in a mixed Th1/Th2 response (Hernandez-Pando and Rook, 1994). The latter is characteristic of an immune response against TB and of late-stage disease when the immune response is failing.

In keeping with previous findings the present study demonstrated granuloma formation and features diagnostic of TB in HIV-infected and -uninfected specimens (Hakawi and Alrajhi, 2006) (Tasova et al., 2006) However, features that were not diagnostic of a TB infection were more common to the HIV-uninfected group. This represented novel findings in the context of spinal TB and an atypical finding in the context of HIV pathogenesis since it is known that HIV-infected individuals are more susceptible to opportunistic infections as a direct result of a compromised immune response (Cassol et al., 2005). In developing resource limited settings, poverty and malnutrition result in severely depressed immune responses and susceptibility to opportunistic pathogens. This coupled with the high index of exposure to a range of environmental and nosocomial bacteria may account for greater NTMs and OIs among HIV-uninfected patients. Unlike pulmonary TB (Ulrichs and Kaufmann, 2002) greater caseation was not associated with greater immune suppression and HIV co-infection in the present study. This may reflect the pauci-bacillary nature of TB spondylitis, pre-surgery TB treatment, enhanced nutritional support or the impact of a remote anatomical site of infection on disease pathology.

Epithelioid cell infiltration occurred commonly and represented an additional atypical finding of a TB granuloma. Epithelioid cells associated with giant cells and fibrin, have been reported in cases of CNS (Kurisaki, 2000), pulmonary (Fujita et al., 2002) and gastric (Jain et al., 2000) TB. This may allude to chronic or latent infection as it has been stated that in granulomas rich in epithelioid cells, there exists an enduring but unpredictable equilibrium between the host and bacilli (Fayyazi et al., 2000). The spinal granuloma also contained macrophages and lymphocytes in close proximity. It has been shown that the spatial association between HIV-1-containing macrophages and CD4⁺ lymphocytes promotes HIV-1 LTR activation and disease progression (Nakata et al., 2002). CD4 and CD8 immunolocalisation further suggested the chronic nature spinal TB. This is highly plausible when one takes into account the insidious onset of disease in the present cohort. Others have reported similar temporal variations in

CD4⁺ and CD8⁺ cellular infiltration to the site of disease in chronically infected murine subjects (Gonzalez-Juarrero et al., 2001, Hernandez Pando et al., 1996). The predominance of CD8 reactive cells in both HIV-infected and uninfected specimens also represented a possible source of IFN γ and a greater cytotoxic potential (Gonzalez-Juarrero et al., 2001, Hernandez Pando et al., 1996) that persisted even in CD4 diminished or depleted specimens. In addition, the relative distribution of CD4⁺ and CD8⁺ cells was associated with the HIV-status of the patient.

A novel finding of the present study was virus- and bacilli-like particles within membrane bound intracellular compartments. It is known that *Mtb* becomes established in occupant-friendly lysosomes within macrophages (Deretic et al., 2004). Latent infection may re-emerge on suppression of the immune response and in co-infected patients represents a significant hazard. Removal of the granuloma removes latently infected cells ensuring complete eradication of the disease since anti-TB therapy does not ensure complete killing of bacteria. The demonstration of HIV within intracellular compartments was an additional novel finding and may be accounted for by the predator-prey analogy of Pope and Haase, (Pope and Haase, 2003). By decimating the very cellular sub-population on which it depends the virus reverts to latency until the immune system is able to rebuild its CD4⁺ T-cell population. Since co-infection accelerates disease pathogenesis, survival dynamics necessitate that either or both HIV and TB revert to survival behaviour rather than disease transmission and progression. The findings of this study support the occurrence of a similar phenomenon in this cohort.

Both apoptosis and necrosis were noted in 4 of 5 HIV-infected specimens at the ultrastructural level. Apoptosis and necrosis are regarded as extremes of the anti-TB immune response where apoptosis is associated with disease containment while necrosis leads to disease dissemination (Chen et al., 2006). In the context of TB, apoptosis is protective against disseminated disease (Chen et al., 2006, O'Sullivan et al., 2007), however in the HIV context, apoptosis leads to immune cell depletion and progressive pathology (Rodrigues et al., 2003). In both instances, mitochondria and lysosomes play an integral role in the apoptotic pathway (Roumier et al., 2003). The present study demonstrated significant mitochondrial changes and lysosomal activity in the HIV co-

infected TB spondylitis cohort. This association between pathology and apoptotic features in TB spondylitis was a novel finding.

6.3 Bacterial genotyping and identification

Bacterial genotyping, to our knowledge, has not been attempted directly from clinical samples in the context of infectious spondylitis. This study represented the first such attempt and aimed to classify the infective organisms in this cohort in order to test the hypothesis that atypical or NTM infections are more common to HIV-infected compared with –uninfected individuals.

Since this was a broad screen of the infective organisms in a region with a high index of exposure, the 16S rDNA gene was chosen as the target gene. Genotyping using the 16S gene was successful directly from pulmonary specimens (Harris and Hartley, 2003) however proved unsuccessful in the context of spinal disease. The paucibacillary nature of spinal disease coupled with pre-surgery anti-TB treatment is expected to have significantly reduced bacterial numbers and chemotherapy-induced DNA fragmentation and nicking provided poor quality template for PCR amplification.

Genotyping from pure cultures proved more successful and yielded unexpected results. A large proportion of atypical and NTM infections was detected alluding to a disturbing emerging trend. Of note was that the greater majority of atypical or NTM infections were common to the HIV-uninfected patient disproving our initial hypothesis. However, a recent study has similarly shown that genetic diversity of Mtb strains was not associated with HIV-infection and related immune suppression (Godreuil et al., 2007). While OIs and NTMs represent a concern to the HIV-uninfected, they represent a devastating impact to the HIV-infected population. The pathogenesis of HIV/NTM co-infection results in severe gross and ultrastructural pathologies as demonstrated in the present study and may be due to increased monocyte recruitment to the site of infection by MAC organisms which create a permissive environment for the progression of both diseases (Hale-Donze et al., 2002).

HIV and TB exacerbate each other in a vicious cycle. Similarly, HIV may advance the

transmissibility of zoonotic infections in human, with the result that co-infected patients become super-spreaders of disease (Weiss, 2003). The ability of HIV to promote transmissibility has already been observed in the TB epidemic and it is feared that other opportunistic infections will manifest a similar dynamic. Demonstrating the occurrence of environmental and NTM infections in the present study emphasised the reality of this possibility in KZN. Their association with HIV-uninfected hosts further illustrates the enormous impact of depressed nutritional status and associated immune-suppression on disease susceptibility.

6.4 HIV heterogeneity and divergent viral evolution

In the final experimental chapter of this study, the impact of co-infection at a remote anatomical site on HIV evolution was investigated. It was hypothesised that co-infection results in immune selection pressures which induce divergent viral evolution and that the fibrotically encased granuloma forms an isolated viral compartment. Thus the Red Queen Hypothesis which states that a species must continue to evolve into multiple quasispecies to retain or gain fitness advantage. (Collins et al., 2002b) was tested.

Analysis of *env* sequences from plasma and granulomas indicated up to 20% intra-patient diversity in this cohort. A 20% variation in the *env* gene within a clade is not unusual and is continually expanding (Gaschen et al., 2002). However, as we have shown, variability within an individual patient illustrates the fundamental constraint to the development of effective vaccines and therapeutic interventions (Morgado et al., 2002) which is the reason for the current concerted and worldwide effort to clarify the enormous global variability of the HIV genome (Gaschen et al., 2002).

The direct impact of anti-TB drugs on HIV evolution was not explored in this investigation despite the paucity of related investigation in current literature. However, it may be speculated that the immune reconstitution and increased TNF- α production, induced by anti-TB therapy, would impact HIV replication and evolution. As a result, HIV diversity at the site of infection is expected to be high. Progressive granuloma formation leads to fibrosis in an attempt to contain disease progression creating a

sequestered site of immune stimulation. As the primary site of infection, drug levels will be elevated locally. Therefore, while anti-TB drugs may not impact HIV evolution directly, the immune response stimulated invariably exerts immune selection pressure on the HIV genome (De Oliveira et al., 2003, Price et al., 1997).

A novel finding of the present study was the demonstration of virus-like particles within intra-cellular membrane-bound compartments. This coupled with recent reports of the persistence of TB in macrophages and their ability to activate the HIV-LTR region (Toossi et al., 2004a) suggests a site of latent infection and possibly low-level viral replication within macrophages. Greater HIV diversity in dually infected pulmonary TB patients as opposed to patients infected with HIV alone (Collins et al., 2000) further implies this association. The findings of this study confirmed a similar phenomenon in the context of spinal TB. Viral compartments represent an important source of escape mutants, drug resistant or more virulent variants and the spinal granuloma may epitomize such a compartment.

It is important to note that this study was conducted in a region (KZN, SA) that is predominantly HIV-1 subtype C infected. Findings of this study will invariably differ from similar studies of HIV heterogeneity in TB co-infected patients from other regions since they were predominantly conducted subtype B infected specimens. Our rationale in exploring spinal TB and its impact on HIV stems from the fact that despite the intensive search for HIV specific immune factors, no appropriate vaccine and/or immunotherapy has thus far been developed (Fauci, 2003). A further novelty of this study is that it is the first investigation to demonstrate divergent viral evolution in a HIV/TB co-infected tissue and in extra-dural granulation tissue.

6.5 Future studies and Recommendations

6.5.1 Future studies

The role of neutralising antibody (Ab) in TB and HIV pathogenesis have, until recently, been highly contentious with the prevailing outlook being that Ab responses play a

minimal to no role in the immune responses. The complexity then lies in distinguishing the TB, HIV and TB/HIV Ab responses and how they impact on each other. Therefore, elucidating the related response at the remote anatomical site of infection and the subsequent response induced by allografts insertion, bone regeneration and the impact on HIV would be of great interest.

Recent investigations in South Africa associated specific HLA variants and VitD receptors with resistance to pulmonary TB infection (Lombard et al., 2006). Similar studies in spinal TB may provide an account for the high incidence of spinal TB in KZN relative to global rates and the possible role of HLA molecules in susceptibility to infection, disease progression and recovery rates. Moreover, the region has recently seen an explosion of xMDR-TB (Sidley, 2006) which has yet to impact spinal disease. Clarifying this would greatly expand present knowledge of the disease.

6.5.2 Recommendations

The HIV/TB-infected granuloma was surgically removed in patients in this cohort while conservative treatment is the norm in co-infected pulmonary and extra-pulmonary disease. The present study demonstrated that co-infected spinal granulomas and macrophages infiltrating these granulomas serve as reservoirs of latent HIV and TB infection. Despite a depressed immune response and severe pathology, patients recovered clinically, incorporated bone grafts and deformity was corrected. Removal of these viral and bacterial reservoirs may prevent disease resurgence and continued immune stimulation. In addition, this may account for the positive clinical outcomes observed in this cohort. Therefore, surgical interventions and the use of albumin levels and ESR rates as predictors of clinical outcomes, rather than CD4+ T-cell counts and HIV-1 viral loads, may prove beneficial for all forms of TB. This study illustrated that surgical interventions cannot be excluded even in seemingly immune-compromised co-infected patients. Addressing HIV and TB co-infection is of critical importance and is unquestionably an enormous challenge in countries such as South Africa and India. Novel approaches to manage this disease in underprivileged, susceptible populations, must be implemented (Narain and Lo, 2004). The approaches described in the present study proved clinically beneficial regardless of HIV-status and should be considered.

Both applied and basic science approaches, such as the present study, towards improved diagnosis, a better understanding of their disease pathogenesis and recognizing correlates of immunity, will aid in developing vaccines and treatment interventions (Leke, 2001).

6.6 Limitations of the study

The study was conducted with patients on anti-TB medication, which may have impacted on the immune response, disease pathology and HIV evolution. Diagnosis of the infective organism was also impacted.

This study demonstrated divergent viral evolution in spinal granulomas with distinct separation of quasispecies into anatomically defined lineages. A criticism is that these findings may reflect a selection bias since they were deduced from a single PCR amplification. However, several confirmatory bioinformatics analyses were conducted to demonstrate divergent evolution including phylogeny and measures of diversity, coalescence and migration (Chapter 5). Multiple PCR amplification and corresponding cloning events would simply serve as confirmation of these findings. Previous studies confirming viral heterogeneity in brain, lymph nodes, blood and semen also reported viral heterogeneity and compartmentalisation following a single PCR amplification (Korber et al., 1994, Collins et al., 2002a).

6.7 Conclusion

This study has highlighted that infectious spondylitis is a significant public health concern in KZN and affected both HIV-infected and –uninfected individuals alike (Chapter 2). Ultra-structural evaluation, which demonstrated virus-like particles within intra-cellular compartments of macrophage-infiltrated granulomas, indicated that the virus was able to exploit the survival strategies of the bacilli to advance its own latent state and replication requirements (Chapter 3). Immunolocalisation of selected immune cells confirmed the spinal TB to be a chronic infection. The need for adjunct diagnostic assays to identify atypical bacterial infectants was highlighted and the utility of a broad-

range genotyping tool was illustrated (Chapter 4). Opportunistic infections and NTMs were more common to HIV-uninfected patients, highlighting that immunosuppressive factors such as poor nutrition and poverty may play an equally important predisposing role as HIV-co-infection in KZN. Finally, divergent viral evolution was demonstrated following a comparison of plasma- with tissue-derived HIV sequences (Chapter 5). Greater viral diversity was demonstrated in tissue as compared to the blood and was associated with greater CD68+ cellular infiltration indicating a possible role for macrophages and monocytes in divergent viral evolution. The implications of this phenomenon are important to viral latency, therapeutic failure and clinical decline.

Two primary conclusions can be drawn from this study. Firstly, fibrous encapsulation of the TB –infected granuloma creates a unique microenvironment infiltrated with immune cells, virus and bacilli. Secondly, and in support of our initial hypothesis, this environment is permissive for divergent viral evolution. This has not previously been demonstrated in TB spondylitis. Encapsulated extra-dural granulomas may thus represent a new viral compartment. Studies to further define the phenotypic and genotypic characteristics of viruses that replicate in this isolated anatomic compartment in addition to defining the related immune responses are required.

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Appendix A

Details of the functions and localisation of HIV structural, regulatory and accessory proteins (adapted from Kuiken et al, (Kuiken et al., 2002)

HIV/SIV PROTEINS			
NAME	SIZE	FUNCTION	LOCALIZATION
Gag MA	p17	membrane anchoring; env interaction; nuclear transport of viral core. (myristylated protein)	virion
CA	p24	core capsid	virion
NC	p7	nucleocapsid, binds RNA	virion
	p6	binds Vpr	virion
Protease (PR)	p15	gag/pol cleavage and maturation	virion
Reverse transcriptase (RT), RNase H	p66 p51	reverse transcription, RNase H activity	virion
Integrase (IN)		DNA provirus integration	virion
Env	gp120 gp41	external viral glycoproteins bind to CD4 and secondary receptors	plasma membrane, virion envelope
Tat	p16/p14	viral transcriptional transactivator	primarily in nucleolus/nucleus
Rev	p19	RNA transport, stability and utilization factor (phosphoprotein)	primarily in nucleolus/nucleus shuttling between nucleolus and cytoplasm
Vif	p23	promotes virion maturation and infectivity	cytoplasm (cytosol, membranes) virion
Vpr	p10-15	promotes nuclear localization of preintegration complex, inhibits cell division, arrests infected cells at G2/M	virion, nucleus (nuclear membrane?)
Vpu	p16	promotes extracellular release of viral particles; degrades CD4 in the ER; (phosphoprotein only in HIV-1 and SIVcpz)	integral membrane protein
Nef	p27-p25	CD4 and class I downregulation (myristylated protein)	plasma membrane, cytoplasm (virion?)
Vpx	p12-16	vpr homolog (not in HIV-1, only in HIV-2 and SIV)	virion (nucleus?)

Appendix B

Modified peroxidase method for Immuno-localisation of CD68 antibody

1. Pre-warm PBS and bake slides for 45-60min on a hot plate and place immediately in xylene and take through the rehydration protocol outlined in Section 3.2.2.3; Table 3.2
2. Place in warmed sodium citrate buffer (10mM, pH6.0) and microwave at 100%, 85⁰C for 10 minutes, then stand at room temperature (RmT) for 25 minutes.
3. Rinse in deionised water. Blot dry around section and circle area of interest with DAKO PAP pen
4. Place in peroxidase blocker (H₂O₂: methanol, 20:80 v/v) for 5min at RmT
5. Wash in PBS, place in PBS bath and blot dry around section
6. Incubate in 10% casein (in PBS) for 30 minutes at RmT
7. Wash in PBS, place in PBS bath and blot dry around section
8. Add dilute primary antibody (+/- 200µl/ section) to the section and incubate in a moisture chamber at 4⁰C, overnight
9. Wash in PBS, place in PBS bath and blot dry around section
10. Add dilute secondary antibody provided in the LSAB kit (DAKO) and incubate for 30 minutes at RmT in a moisture chamber
11. Wash in PBS, place in PBS bath and blot dry around section
12. Add prepared horse radish peroxidase (HRP) provided in the LSAB kit to each section, and incubate for 30min at room temperature in a moisture chamber
13. Wash in PBS, place in PBS bath and blot dry around section
14. Add approximately 200µl DAB chromogen to each section and incubate at room temperature for 10 minutes, or until a brown colour is observed macroscopically and microscopically (max. 20min)
15. Wash in PBS, place in PBS bath and blot dry around section
16. Add 200µl haematoxylin to each section and incubate at room temperature for 90sec.
17. Place slides into changes of hot (not boiling) water (stand in second change for 90-120 sec); thereafter place under running tap water for 3-5min, before dehydrating (Section 3.2.2.3) and placing a cover-slip over section using DPX.

Appendix C

Alkaline lysis method of plasmid isolation

Reagents

Solution 1 (Glucose / Tris / EDTA):

	Mw	mass
50mM Glucose	180.16	0.901g
25mM Tris.Cl	157.64	0.394g
10mM EDTA	372.2	0.372g

Make up to 100ml. Autoclave before use.

Solution 2

Make up stock solutions of NaOH and SDS:

	Mw	mass	water
2M NaOH	40	8.0g	100ml
10% SDS		10g	100ml

Add 10ml of 2M NaOH to 10ml 10%SDS and make up to a final volume of 100ml. Prepare immediately before use.

Solution 3 (KAc)

Prepare a stock solution of potassium acetate. Store at 4-8⁰C

	Mw	mass	water
5M potassium acetate	98.15	24.55	50ml

Make up the following:

5M potassium acetate	60.0ml	30.00ml
Glacial acetic acid	11.5ml	5.75ml
Water	28.5ml	14.25ml
Total volume	100.0ml	50.00ml

Method

- Pour 1.5ml of the LB culture into an Eppendorf tube (store the rest at 4-8⁰C)
- Centrifuge at full speed for 1min
- Aspirate the SU – as dry as possible
- Resuspend the pellet in 100µl of Solution 1
- Stand at room temperature (RT) for 5min

- Add 200 μ l fresh Solution 2
- Mix by inverting the tube 2 –3 times (DO NOT vortex)
- Stand on ice for 5min
- Add 150 μ l cold Solution 3
- Vortex with the tube inverted for 10sec
- Stand on ice for 5min
- Centrifuge at full speed for 5min
- Transfer the SU to a fresh tube
- Add an equal volume of phenol/chloroform to the SU
- Vortex
- Centrifuge at full speed for 2min
- Transfer the supernatant to a fresh tube a
- Add 2x the volume ethanol at RT
- Vortex
- Stand at RT for 2min
- Centrifuge at full speed for 5min
- Aspirate the SU and stand inverted on a paper towel
- Add 1ml 70% ethanol
- Vortex
- Centrifuge at full speed for 2min
- Aspirate the SU
- Allow to air-dry
- Re-constitute the pellet with 25 μ l sterile water containing 1 μ g/ml RNase A
- Vortex and quick spin
- Store at -20°C

Appendix D

Agarose Gels of C2-V5 env amplification products

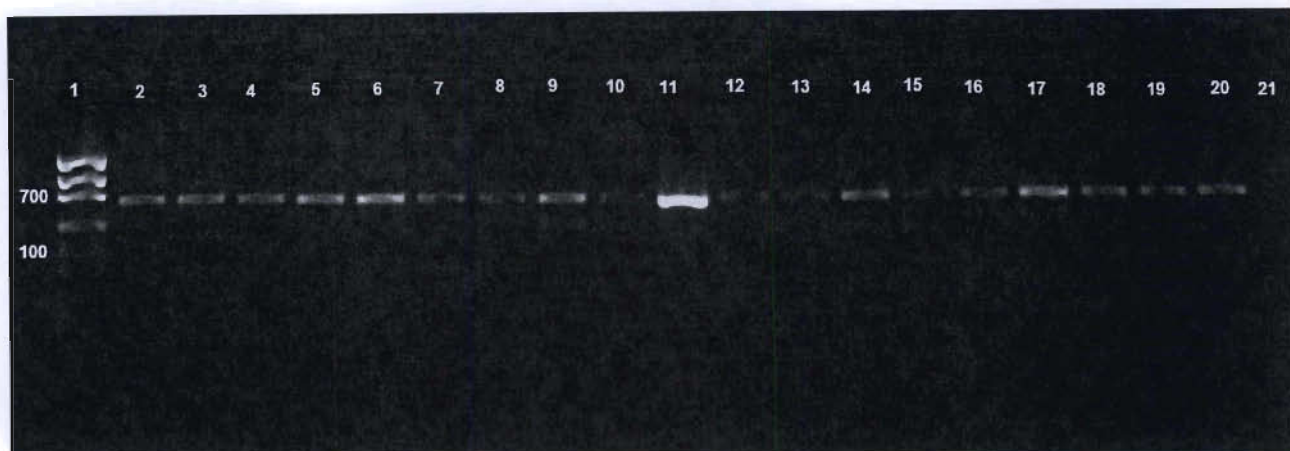


Figure D1: An illustration of an agarose gel of the approximately 621-700bp PCR amplified fragment of the C2-V5 HIV-1 env region against Molecular Weight Marker XIV (100bp ladder, Lane 1). The fragments illustrate the un-purified PCR product from plasma samples of Patient 12 (Lanes 2-4), Patient 16 (Lanes 5-7), Patient 20 (Lanes 8-10), Patient 22 (Lanes 11-13), Patient 25 (Lanes 14-16), Patient 32 (Lanes 17-19), Patient 38 (Lane 20) and a no-template control.

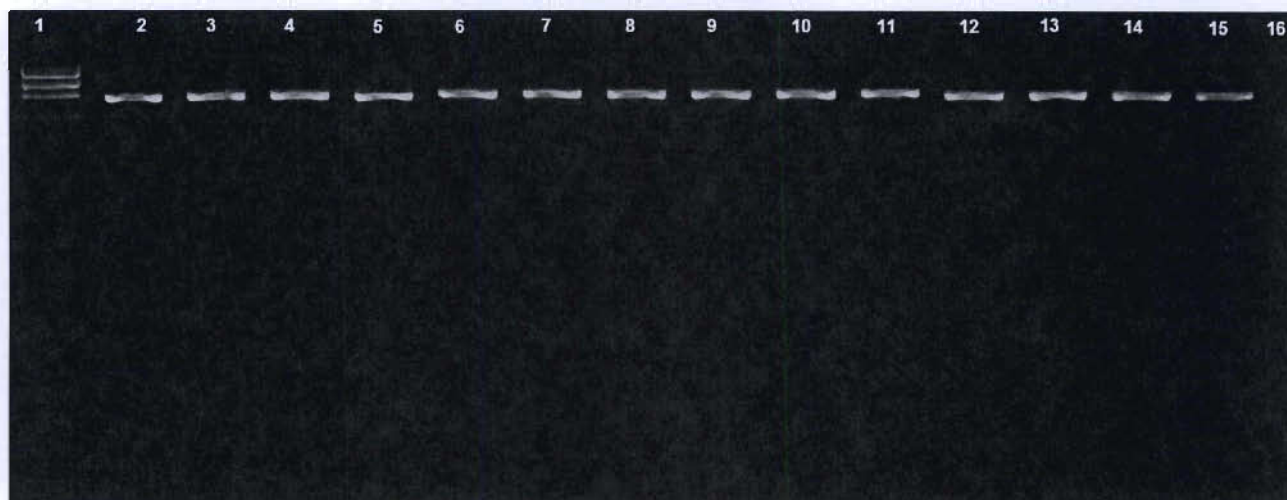


Figure D2: An illustration of an agarose gel depicting a screening PCR reaction amplifying the C2-V5 region of the HIV-1 env gene from plasmid DNA of clones which was subsequently purified in preparation for sequencing. Lane 1 represents Molecular Weight Marker XIV (100bp ladder) and the clones representing plasma- (Lanes 2-11) and tissue-derived (Lanes 12-15) sequences of Patient 12. Lane 16 is the no-template control.