Theses Digitisation:
https://www.gla.ac.uk/myglastron/research/enlighten/theses/digitisation/
This is a digitised version of the original print thesis.

Copyright and moral rights for this work are retained by the author
A copy can be downloaded for personal non-commercial research or study, without prior permission or charge
This work cannot be reproduced or quoted extensively from without first obtaining permission in writing from the author
The content must not be changed in any way or sold commercially in any format or medium without the formal permission of the author
When referring to this work, full bibliographic details including the author, title, awarding institution and date of the thesis must be given
IMMUNODEFICIENCY AND HIV-1 INFECTION IN HAEMOPHILIA

RAJAN MADHOK MBChB MRCP

Thesis Submitted for the degree of MD

University of Glasgow

MARCH 1989

University Dept of Medicine

Glasgow Royal Inf.
The following papers based on work included in this thesis have been published.

1 Froebel K.S., Madhok R., Forbes C.D., Lennie S.E., Lowe G.D.O., Sturrock R.D.
   Immunological Abnormalities in haemophilia: are they caused by American factor VIII concentrate.

   Extranodal lymphoma in a haemophiliac negative for antibody to HIV.

   Impaired cell mediated immunity in haemophilia in the absence of infection with human immunodeficiency virus.

   HTLV III antibody in sequential plasma samples - from haemophiliacs 1974 - 84.
   Lancet 1985;i:524 - 525.

   HTLV - III seropositivity in European hemophiliacs exposed to factor VIII concentrate imported from the USA.

   Immunosuppression following alloantigen exposure: A role for Fc - Y receptor blocking antibodies?

7 Froebel K., Madhok R., Forbes C.D.
   Immunodeficiency in Scottish haemophiliacs.

8 Gracie J.A, Froebel K., Madhok R., Lowe G.D.O., Forbes C.D
   AIDS: An Overview.

9 Froebel K., Lowe G.D.O., Madhok R., Forbes C.D.
   AIDS, hepatitis B virus and haemophilia.
   Lancet 1984;i:632.

    Lack of HIV - 1 transmission by casual contact.
The following papers included in this thesis have been submitted for publication or are in preparation:

   B cell abnormalities in haemophilia in the absence and presence of HIV - 1 infection.
   (Submitted for publication)

   Serum IgG levels in haemophilia - a useful marker of chronic liver disease?
   (submitted for publication).

   Impaired capacity to produce interleukin 2 in haemophilia.
   (submitted for publication)

   Longterm outcome of infections transmitted by clotting factor concentrates in a well defined cohort of haemophiliacs.
   (submitted for publication)

   Lymphopenia in haemophilia in the absence of HIV - 1 infection.
   (submitted for publication)

6. Madhok R.
   In vivo and in vitro effects of factor concentrates on the immune system.
   In: Mannucci P., (ed) Replacement therapy in Haemophilia - Problems and Solutions (in press).

   Histometric studies on the cellular infiltrate at the site of tuberculin skin tests on haemophilia patients seropositive and seronegative for HIV.
   J.Clin Path (in press)

As a direct result of my work in the areas studied I was invited to edit and write the following:

   Blood, Blood Products and AIDS.
2 Madhok R and Forbes C.D.
The treatment of haemophilia: a double edged sword.
In: Costello (ed). Progress In Haematology Balliere Tindall London (UK)
(in press)

3 Madhok R and Forbes C.D.
Clinical aspects of the hemophilias. In: Ratnoff O.D and Forbes C.D. (eds) Grune and Stratton (USA)
(in press)
TABLE OF CONTENTS

List of Tables

List of Illustrations

Acknowledgments (General)

Acknowledgments of collaborative work

Chapter 1  Introduction

Aims of Thesis

Chapter 2  Patients and analysis.
  2.1  The West Of Scotland Adult Haemophilia Centre
  2.2  Treatment Policies and Clinic Visits
  2.3  Study Population
  2.4  Demographic Characteristics of Recruited Cohort.
  2.5  Number of Patients with inhibitors.
  2.6  Use of blood products
  2.7  Liver Disease
  2.8  Hepatitis B Infection
  2.9  Materials and Methods
  2.10 Statistical Methods

Chapter 3

3.1  Do clotting factor concentrates have a direct "chemical" effect on lymphocyte function in vitro.

Summary

3.1.1  Introduction

3.1.2  Methods

3.1.3  Results

3.1.4  Discussion

3.2  Immunosupression following replacement therapy in haemophilia - a role for Fc Y-receptor blocking antibodies.
Chapter 4

4.1 The epidemiology of HIV-1 infection in West West of Scotland.

Summary

4.1.1 Introduction
4.1.2 Methods
4.1.3 Results
4.1.4 Discussion

4.2 Risk factors for HIV-1 infection haemophilia

Summary

4.2.1 Introduction
4.2.2 Methods
4.2.3 Results
4.2.4 Discussion

4.3 The ability to isolate HIV-1. Is it governed by the anti-viral immune response?

Summary

4.3.1 Introduction
4.3.2 Methods
4.3.3 Results
4.3.4 Discussion

4.4 Human T-cell lymphotropic virus - 1 (HTLV-1) is not implicated in the T cell subset aberration in haemophilia.

Summary

4.4.1 Introduction
Methods

Results

Discussion

Chapter 5

5.1 Serum HIV - 1 antigenaemia - Clinical and serological correlations.

Summary

5.1.1 Introduction

5.1.2 Patients and Methods

5.1.3 Results

5.1.4 Discussion

5.2 The Antibody response to HIV - 1 includes a protective antibody dependent cell mediated cytotoxicity (ADCC) response to the envelope proteins.

5.2.1 Introduction

5.2.2 Patients and Methods

5.2.3 Results

5.2.4 Discussion

Chapter 6

6.1 Haemophilic Mortality - The Impact of HIV - 1 infection.

Summary

6.1.1 Introduction

6.1.2 Methods

6.1.3 Results

6.1.4 Discussion

6.2 Does treatment with clotting factor concentrates predispose to clinical immunodeficiency in the absence of HIV - 1 infection?

Summary

6.2.1 Introduction
6.2.2 Patients and Methods
6.2.3 Results
6.2.4 Discussion

6.3. HIV - 1 related disease in haemophiliacs at the West of Scotland haemophilia Centre.
Summary
6.3.1 Introduction
6.3.2 Patients and Methods
6.3.3 Results
6.3.4 Discussion

Chapter 7
7.1 Impaired cell mediated immunity in HIV - 1 antibody negative haemophiliacs.
Summary
7.1.1 Introduction
7.1.2 Methods
7.1.3 Results
7.1.4 Discussion

7.2 Impaired cell mediated immunity in HIV - 1 antibody negative haemophiliacs - is it due to liver disease ?
Summary
7.2.1 Introduction
7.2.2 Methods
7.2.3 Results
7.2.4 Discussion

7.3 Impaired in vivo cell mediated immunity in HIV - 1 antibody negative haemophiliacs - Fact or Fiction.
Summary
7.3.1 Introduction
7.3.2 Materials and Methods
7.3.3 Results
7.3.4 Discussion

Chapter 8

8.1

Summary

8.1.1 Introduction
8.1.2 Patients and Methods
8.1.3 Results
8.1.4 Discussion

8.2 The immunoregulatory ratio in haemophilia

Summary

8.2.1 Introduction
8.2.2 Methods
8.2.3 Results
8.2.4 Discussion

8.3 Cofactors in the CD 4+ve T cell decline in HIV-1 infected haemophiliacs.

Summary

8.3.1 Introduction
8.3.2 Methods
8.3.3 Results
8.3.4 Discussion

Chapter 9

9.1 Lymphocyte activation and proliferation in haemophilia - the effect of HIV-1 infection, liver disease and treatment.

Summary

9.1.1 Introduction
9.1.2 Methods
9.1.3 Results
9.1.4 Discussion
9.2 The effects of non-infectious HIV-1 lysate on lymphocyte function.

Summary

9.2.1 Introduction
9.2.2 Methods
9.2.3 Results
9.2.4 Discussion

9.3 Concanavalin A induced lymphocyte proliferation in haemophiliacs.

Summary

9.3.1 Introduction
9.3.2 Methods
9.3.3 Results
9.3.4 Discussion

Chapter 10 Interleukin 2 levels in the absence and presence of HIV-1 antibody in haemophilia.

Summary

10.1.1 Introduction
10.1.2 Methods
10.1.3 Results
10.1.4 Discussion

Chapter 11 Serum immunoglobulin levels in haemophilia.

11.1

Summary

11.1.1 Introduction
11.1.2 Methods
11.1.3 Results
11.1.4 Discussion

11.2 B cell function in haemophilia.

Summary
11.2.1 Introduction
11.2.2 Methods
11.2.3 Results
11.2.4 Discussion

References.
ACKNOWLEDGMENTS - General

I should like to thank the following colleagues without whose support and help this thesis could not have been completed:

Mrs. E. Ballantyne
Ms. B. Burns
Dr. H. A. Capell
Sister D Campbell
Prof. C. D. Forbes
Dr. E. Follett
Dr. J. A. Gracie
Mr. A. Jenkins
Prof. A. C. Kennedy
Dr. G. D. Lowe
Sister I McDougal
Dr. M. Melbye
Mr. F. Spiers
Mr. J. Smith
Dr. R. D. Sturrock
Dr. L. Soo

Glasgow University
Ctr. Rheumatic Dis.
Haemophilia Unit
Dundee Medical School
Hepatitis Ref. Laboratory
Glasgow University
Glasgow University
Glasgow University
Glasgow University
Haemophilia Unit
NCI (Bethesda USA)
Glasgow University
Glasgow University
Glasgow University
Haemophilia Unit

The present and former staff of the Centre for Rheumatic Diseases.

I am indebted to Miss. A. Tierany for her patience and expert help in typing the manuscript.

Without the support of the patients attending the West of Scotland haemophilia unit this work would not have been possible and I am grateful for their help.

The work presented in this thesis would not have been possible without the financial support of the Scottish Hospital Endowments Research Trust.
Acknowledgments for provision of materials:

Mr. Christie  Armour Pharmaceuticals (UK).
Dr. R. C. Gallo  National Cancer Institute (USA).
Dr. R. Jarrett  Glasgow University.
Miss R. Spooner  Oxford Haemophilia Centre.

Acknowledgment Of Collaborative Work:

<table>
<thead>
<tr>
<th>Chapter</th>
<th>Authors</th>
<th>Institutions</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.2</td>
<td>Dr. M. Forwell, Dr. G. Sandilands</td>
<td>Western Inf., Glasgow</td>
</tr>
<tr>
<td>4</td>
<td>Dr. E. Follett, Miss L. Wallace, Mr. G. Scotland, Dr. H. Laird, Dr. M. Melbye</td>
<td>Hepatitis Lab., &quot;</td>
</tr>
<tr>
<td>7.3</td>
<td>Dr. G. Lowe, Prof. S. Beck</td>
<td>Dundee Uni., &quot;</td>
</tr>
<tr>
<td>8</td>
<td>Dr. A. Burnett</td>
<td>Royal Inf., Glasgow</td>
</tr>
</tbody>
</table>
LIST OF ILLUSTRATIONS

FIGURE 1.2.1 The Coagulation Pathway
1.3.1 Activation Of Factor X By Factor Xa
1.3.2 Activation Of Factor IX
2.1.1a Treatment Use - Factor VIII
2.1.1b Treatment Use - Prothrombin Complex Concentrates.
3.1.1 The Effect Of IPFC(VIII) On Lymphocyte Transformation.
3.1.2 Preincubation Of PBMC With IPFC(VIII).
3.1.3 Time Course Of Lymphocyte Proliferation.
3.1.4 Citrate Ions Do Not Affect Lymphocyte Proliferation.
3.1.5 A Comparison Of Purified And Highly Purified Concentrates.
3.1.6 IPFC (IX) - Affect on Lymphocyte Proliferation.
3.1.7 Response Of Jurkat Cells Cultured In The Presence (IPFC (VIII) or Monoclate.
3.1.8 Effects Of IPFC (VIII) And Monoclate On IL 2 Production.
3.2.1 IgG Anti Lymphocyte Antibodies By EA Rosette Inhibition Assay.
3.2.2 IgG Anti Lymphocyte Antibodies In Haemophiliacs With And Without HIV - 1 Antibody.
3.2.3 Inhibition Of PHA Blastogenesis By Haemophiliac IgG.
4.1.1 Principles Of Available Serological Tests For HIV - 1.
4.1.2 A Typical Positive Western Blot Result.
4.1.3 Cumulative Annual Incidence of HIV 1 Antibody.
4.1.4 Retrospective Serum Results.

4.3.1 T Cell Subsets At Times Of HIV - 1 Isolation.

5.1.1 Western Blot Showing Declining p24 Antibody.

5.1.3 Western Blot Showing Persistent p24 And p15 Reactivity.

5.1.4 Western Blot Showing Absent p15 But Persistent p24 Antibody.

5.1.5 Western Blot Showing Resurgence Of p24 Antibody In 1988 & Persistence To p15.

5.2.1 ADCC Of Transfected Cells In HIV - 1 Antibody Positive And Negative Patients.

6.1.1 The Annual Death Rate Per 1000 Haemophiliacs For Each Year Studied.

6.1.2 Age Specific Mortality In Haemophilia.

6.1.3 Age Distribution Of Registered Patients.

6.1.4 Annual Death Rate Per 1000 Due To Infection Including HIV - 1.

6.1.5 Annual Mortality For The Two Most Common Causes Of Death In Haemophilia.

6.3.1 Platelet Count In Thrombocytopenic Patients.

7.1.1 The DNCB Score In Haemophiliacs

7.1.2 Mean Annual Dose Of Clotting Factor Concentrate Used And The DNCB Score.

7.2.1 The Compound Skin Score In HIV - 1 Antibody Positive And Negative Patients.

7.2.2 The Compound Skin Score In Relation To The Mean Annual Dose Of
Treatment Used.

7.2.3 The Compound Skin Score In Relation To The Severity Of Liver Disease.

8.1.1 The Initial Lymphocyte Count In HIV - 1 Antibody Positive And Negative Patients.

8.1.2 The Lymphocyte Count At The End Of The Study.

8.2.1 The Total T - Cell (CD 3+ve) Count.

8.2.2 The T Helper Cell (CD 4+ve) Count In HIV - 1 Antibody Positive And Negative Patients.

8.2.3 The T Suppressor/Cytotoxic (CD8+ve) In HIV - 1 Antibody Positive And Negative Patients.

8.2.4 The CD 8+ve Count In HIV - 1 Antibody Positive With Reduced And Normal CD 4+ve Cell Count.

8.3.1 Sequential CD 4+ve Cell Count In HIV - 1 Antibody Positive Patients.

8.3.2 Sequential CD 8+ve Cell Count In HIV - 1 Antibody Positive Patients.

9.1.1 PHA Induced PBMC Activation And Proliferation In Haemophiliacs.

9.1.2 The Proliferative Response To PPD In Haemophiliacs.

9.2.1 PHA Induced PBMC Proliferation In The Presence Of HIV - 1 Lysate.

9.2.2 PPD Induced PBMC Proliferation In The Presence Of HIV - 1 Lysate.

9.2.3 IL 2 Production In The Presence Of HIV - 1 Lysate.

9.3.1 The Concanavalin A Proliferative Response In Haemophiliacs.

10.1.1 Interleukin 2 Production In Treated Haemophiliacs.

11.1.1 Serum Immunoglobulin Levels At The Start of The Study.
11.1.2 Serum Immunoglobulin Levels At The End Of The Study.

11.1.3 Grade Of Liver Disease And Serum IgG Levels In HIV - 1 Negative Haemophiliacs.

11.2.1 Basal Immunoglobulin Production In Haemophiliacs And Controls.

11.2.2 PWM Stimulated Immunoglobulin Production In Haemophiliacs And Controls.
### LIST OF TABLES

<table>
<thead>
<tr>
<th>TABLE</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.4.1</td>
<td>Factor Deficiency And Clinical Severity in Haemophilia.</td>
</tr>
<tr>
<td>1.5.1</td>
<td>Factor VIII Containing Blood Products: Potency And Purity.</td>
</tr>
<tr>
<td>1.5.2</td>
<td>Protein Composition Of Factor VIII Concentrates.</td>
</tr>
<tr>
<td>1.6.1</td>
<td>Life Expectancy In Haemophilia.</td>
</tr>
<tr>
<td>1.7.1</td>
<td>Infections Transmitted By Blood Transfusion.</td>
</tr>
<tr>
<td>1.7.2</td>
<td>Studies Reporting Serum ALT Levels In Haemophilia.</td>
</tr>
<tr>
<td>1.7.3</td>
<td>Liver Biopsies In Haemophilia.</td>
</tr>
<tr>
<td>1.7.4</td>
<td>Serial Liver Biopsies In Haemophilia.</td>
</tr>
<tr>
<td>1.7.5</td>
<td>Immune Function Abnormalities In AIDS.</td>
</tr>
<tr>
<td>1.7.6</td>
<td>Mechanisms Of Immune Suppression By HIV - 1.</td>
</tr>
<tr>
<td>2.3.1</td>
<td>The Reasons For Not Recruiting Registered Patients.</td>
</tr>
<tr>
<td>2.4.1</td>
<td>Characteristics Of Study Population.</td>
</tr>
<tr>
<td>2.4.2</td>
<td>The Severity Of Factor Deficiency In Patients Studied.</td>
</tr>
<tr>
<td>2.7.1</td>
<td>Clinical Features Of Patients With Grade 1 Liver Disease.</td>
</tr>
<tr>
<td>2.7.2</td>
<td>Characteristics Of Patients In Liver Disease Groups.</td>
</tr>
<tr>
<td>2.8.1</td>
<td>Numbers Of Patients With Serum Markers Of Hepatitis B Infection.</td>
</tr>
<tr>
<td>3.1.1</td>
<td>The Effect Of IPFC (VIII) On Lymphocyte Proliferation.</td>
</tr>
<tr>
<td>3.1.2</td>
<td>Kinetics Of IPFC (VIII) Inhibition Lymphocyte Proliferation.</td>
</tr>
</tbody>
</table>
The Effect Of IPFC (VIII) On Cell Viability.

A Comparison Of Different Factor VIII Concentrates.

The Results of ELISA Serological Tests To Detect Anti IgG HIV - 1 Antibody.

Results Of Western Blots And Immunofluoresence Test In Samples Positive In Any ELISA.

Comparison Of The Characteristics Of HIV - 1 Antibody Positive And Negative Patients.

Comparison Of Factor VIII And Factor IX Deficient Patients.

Comparison Of HIV - 1 Antibody Positive And Negative Patients.

The Association Of Concentrate Source And HIV - 1 Status.

The Number of Infusions Of Each Implicated Batch Used By Treated Patients.

Comparison Of Number Of Deaths Obtained From Death Certificates And Oxford Haemophilia Centre.

The Number Of Deaths In Haemophilia A and B Patients.

Numbers Of Registered Patients Over 50 Years With Factor VIIIC Deficiency.

Causes Of Death.

Causes Of Death In Patients Prospectively Studied.
A and B Patients.

6.1.3 Numbers Of Registered Patients Over 50 Years With Factor VIIIC Deficiency.

6.1.4 Causes Of Death.

6.2.1 Causes Of Death In Patients Prospectively Studied.

6.3.1 Infections Associated With HIV - 1 Infection.

6.3.2 CDC Classification Of Patients Studied.

6.3.3 Walter Reed Classification Of Patients Studied.

7.2.1 Results Of Skin Testing In HIV - 1 Antibody Positive And Negative Patients.

8.1.1 Differences Between Patients With And Without Lymphopenia At The Start Of The Study.

8.1.2 Liver Disease, HIV - 1 Status And The Total Lymphocyte Count.

8.2.1 A Comparision Of Haemophiliacs In Whom T cell Subsets Were Counted & Not Counted.

8.2.2 T Cell Subsets In Haemophiliacs And Controls.

8.3.1 Sequential T Cell Subsets In HIV - 1 Antibody Positive Haemophiliacs.

8.3.2 Sequential T cell Subsets In HIV - 1 antibody Negative Haemophiliacs.

8.3.3 A Comparision Of Patients With A Low And Normal CD-4 +ve Cell Count.

8.3.4 CD - 8 + ve Cell Count In Haemophiliacs (HIV - 1 Ab.+ve) With Low And Normal CD 4 +ve Cell Count In 1986 And 1988.

9.1.1 A Comparision Of Haemophiliacs In Whom In Vitro Lymphocyte Function Was Studied And Not Studied.
9.1.2 A Comparision Of Haemophiliacs Studied According To HIV-1 Status.

9.1.3 Differences Between HIV - 1 Antibody Positive Patients With Low Normal And Normal Response To PHA.

9.1.4 A Comparision Of Characteristics Of HIV - 1 Antibody Negative Haemophiliacs With Low Normal And Normal Response To PHA.

9.3.1 The Characteristics Of Patients In Whom The Concanavalin A Response Was Studied.

9.3.2 A Comparision Of HIV - 1 Antibody Positive Patients With Low Normal And Normal Concanavalin A Response.

9.3.3 A Comparision Of HIV - 1 Antibody Negative Patients With Low Normal And Normal Concanavalin A Response.

10.1.1 Characteristics Of Patients Studied

11.1.1 Serum Immunoglobulin Levels.

11.2.1 A Comparision Of Patients Studied And Not Studied.

11.2.2 A Comparision Of HIV - 1 Antibody Positive Patients Studies And Not Studied.

11.2.3 Basal And Pekeweed Mitogen Stimulated Immunoglobulin Levels.

11.2.4 In Vitro B Cell Function In HIV - 1 Negative Patients According To Severity Of Liver Disease.

11.2.5 Basal And SACA Stimulated Values.
Table 1.4.1. Clinical Presentation.

<table>
<thead>
<tr>
<th>Factor Level (u/ml)</th>
<th>Bleeding</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.01</td>
<td>Recurrent spontaneous</td>
</tr>
<tr>
<td>0.01-0.05</td>
<td>Occasional spontaneous mild trauma surgery</td>
</tr>
<tr>
<td>0.05-0.5</td>
<td>Trauma surgery</td>
</tr>
</tbody>
</table>

The severity of the bleeding diathesis usually parallels the severity of the disorder. Occasional patients are found in whom the clinical grade of defect does not match assayed levels.
1.1 INTRODUCTION

1.1.1 The use of blood products in haemophilia - A double edged sword.

Haemophilia is an inherited disease predisposing the individual to prolonged and excessive bleeding. The only satisfactory treatment in severe haemophilia is replacement of the missing factor with blood products. Frequent use of blood products however is associated with a substantial risk of acquiring life threatening blood borne viral infections. For example, infection with the human immunodeficiency virus type 1 (HIV-1) carries a 30-75 percent chance of progression to AIDS in five years and non - A,non - B (NANB) infection results in chronic active hepatitis or cirrhosis in approximately 25 percent of infected patients (1, 2).

On the other hand without treatment severe haemophiliacs would have a life expectancy that is half that of the general population and most would develop severe crippling musculoskeletal deformities (3).

To fully appreciate the risks and benefits of available blood products administered to haemophiliacs requires first, an understanding of why haemophiliacs bleed; second, a balanced evaluation of benefits as well as risks of blood products presently used and third, an appraisal of alternatives that may favourably influence outcome.
1.2 Why haemophiliacs bleed

Haemophilia is the commonest of the congenital haemorrhagic diseases. The term describes two disorders: factor VIIIC deficiency (Haemophilia A, Classical Haemophilia) and factor IX deficiency (Haemophilia B or Christmas disease). The estimated incidence of factor VIIIC deficiency is approximately 1 in 10,000 live births, factor IX deficiency is ten times less common (3).

Both are due to single gene defects on the X chromosome resulting in absence of the specified protein or a molecule with reduced activity. The mode of inheritance is sex linked.

To understand why haemophiliacs bleed requires a basic knowledge of how factor VIIIC and factor IX participate in coagulation and the nature of the deficiency. Both proteins are part of the intrinsic pathway of coagulation which consists of several inactive zymogens, and cofactors that are serially activated in response to exposure to collagen or other negatively charged components of subendothelial connective tissue. Specifically factor VIIIC and factor IX are involved in the mid-phase of the coagulation cascade in the conversion of factor X to Xa (Figure 1.2.1).
THE COAGULATION PATHWAYS

- Kallikrein → Prekallikrein
- HMW Kininogen → XII → XIIa
- XIIa → HMW Kininogen → XI → XIa
- XIa → IX → IXa
- IXa → VIII
- VIII → Xa
- Xa → X
- X → VII
- VII
- Tissue Thromboplastin + VII
- Phospholipid Ca++ → Prothrombin
- Prothrombin → Thrombin
- Thrombin → Fibrinogen
- Fibrinogen → Fibrin
- Fibrin
- Cross-linked Fibrin

Feedback loops

Figure 1.2.1
1.2.1 Factor VIIIC
Factor VIIIC is specified by a gene on the X chromosome. In vivo, factor VIIIC circulates non-covalently bound to von Willebrand factor, (factor VIII vWF) a large multimeric glycoprotein essential for platelet adhesion to subendothelium (4). The exact binding site of factor VIIIC to factor VIIIvWF is not known but has been localized to somewhere within the first 272 amino acids of factor VIIIvWF (5). Factor VIII vWF functions exclusively as a carrier molecule of factor VIIIC and plays no part in factor VIIIC cofactor activity.

1.2.2 Structure of factor VIIIC
The cloning of the complementary DNA (cDNA) and gene for human factor VIIIC indicates that factor VIIIC is synthesized as a single polypeptide chain of 2332 amino acids plus a leader sequence of 19 amino acids (6-9). There are 25 potential carbohydrate binding sites to asparagine: if all sites are occupied the molecular weight for the glycoprotein would be approximately 330,000 (± 20,000). This is similar to previous biochemical estimates of the molecular weight of factor VIIIC (10, 11).

The cDNA sequence of factor VIIIC shows that the mature peptide has two different homologous domains (Fig 1.2.1), A and C. The A domains have approximately 30 percent homology and C domains are 40 percent homologous.
Interestingly, the A domain also shows 30 percent homology with caeruloplasmin, whether this domain complexes with copper or other heavy metal ions is not known (6,7). Furthermore, the cDNA sequence of factor V shows homology to caeruloplasmin (12,13). This structural and functional similarity of factor Va and VIIIa as cofactors suggests that caeruloplasmin, factor V and factor VIIIC may have arisen from a common ancestral gene.

1.2.3. Biosynthesis of factor VIIIC
Organ transplant studies in canine haemophilia and the serendipitous observation of normalization of factor VIIIC after orthotopic liver transplantation in a severe haemophiliac with chronic hepatitis suggest that factor VIIIC is synthesized in the liver (14-21). This has been confirmed by detection of factor VIIIC messenger RNA in liver but not other tissues (22). In-situ hybridization studies and immuno-cytochemical studies show that hepatocytes and not Kuppfer or sinusoidal endothelial cells synthesize factor VIIIC (23-26).

1.2.4 Biological Function of Factor VIIIC
Factor VIIIC is a component of the intrinsic pathway of coagulation (Figure 1.3.1) activation of factor VIIIC is a prerequisite in activation of factor X (27-29). The reaction requires calcium ions and phospholipids, in-vivo phospholipid surface is provided by platelets and other
cell membranes (30). No other physiological functions have been attributed to factor VIIIC (31).

1.2.5 Factor VIIIC Activation

Activated factor VIIIC has no intrinsic catalytic activity, therefore the mechanism by which factor VIIIC accelerates proteolysis by factor IXa of factor X is as a binding protein for factor IXa and X in a conformation favouring catalysis (31)). To participate in clotting reactions variable amounts of the B domain have to be cleaved (32). Thrombin is the most potent protease known to activate factor VIIIC (27-29,33-34). Thrombin activates factor VIIIC bound to factor VIIIvWF, upon activation or binding to a phospholipid surface factor VIIIC dissociates from factor VIIIvWF. Activated factor VIIIC increases Vmax of catalysis of factor X almost 20,000 fold (29, 35).

Factor VIIIC is initially cleaved by thrombin after Arg-740 to yield heavy (M.Wt. 90,000) and light chain fragments (M.Wt.80,000) (32). Heavy and light chain fragments originate from amino and carboxy terminals of the precursor molecule respectively and are linked by calcium and/or other cations. Further thrombin digestion cleaves after Arg-372 of the heavy chain and, after light chain Arg 1689 (32). Which of these cleavages actually results in factor VIIIC activation is still unclear (32-36). Factor Xa acts at sites cleaved by thrombin and two additional sites have also been proposed (32).
In vitro factor IXa activates factor VIIIC. The physiological relevance of this activation is unclear as the reaction requires high concentrations (37).

1.2.6 Inactivation of activated factor VIIIC

Factor VIIIC is probably inactivated in vivo by proteolytic degradation (28, 33). Several proteases degrade activated factor VIIIC: the most important is activated protein C (320). Protein C cleaves factor VIIICa heavy chain at a single site but does not degrade the light chain (32). Protein C activation of (by thrombin) is regulated by thrombo-modulin at the endothelial cell surface (38).

Non-proteolytic degradation of activated factor VIIIC has been described in vitro, but its physiological role is not known.

1.2.7 The molecular defect in haemophilia A

A single gene disorder such as haemophilia A may arise from gene deletions or a point mutation resulting in synthesis of a inactive molecule. Defective molecules (factor VIIIC antigen, cross reacting material [CRM]) can be detected by immunoassays using human anti-VIIIC antibodies (39-50). Attempts to identify inactive factor VIIIC or factor VIIIC antigen (cross reacting material or CRM,factor VIIICAg) in haemophiliacs have provided conflicting results. Initial studies found CRM in a
small minority of moderate haemophiliacs (39-42), whereas subsequent studies have found CRM in all patients tested (43, 44).

More recent studies using sensitive immunoradiometric and electroimmunoassays show that the majority of severe factor VIIIC deficient haemophiliacs are CRM negative, whereas almost all patients with moderate to mild haemophilia A have CRM present in amounts that are comparable to or exceeding circulating factor VIIIC of levels (45 - 50).

The implications of these findings are that the functional defect in mild to moderately affected patients is due to presence of a defective molecule. Both missense and non-sense point mutations are described in mild-moderately affected patients (51-52). In CRM negative patients and therefore the vast majority of severe patients, the molecule is absent. Antonarakis et al have screened 240 unrelated males for abnormalities in the factor VIII gene detectable by restriction analysis. Significant deletions were found in 14 patients and point mutations in 12 (53). More recently two unrelated severe patients have been described who had insertions of Li elements in exon 14 of the factor VIII gene (54).

1.3.1 Factor IX

Factor IX is a glycoprotein with a molecular weight of 55,000 of which 20 percent is carbohydrate (55). It is a
single polypeptide chain of 415 amino acids (56 - 61). The complete amino acid sequence of factor IX has been deduced from a cDNA clone (59). It shows striking amino acid homology to both vitamin K dependent clotting factors and other serine proteases - suggesting that this larger family of proteins were probably derived from a single ancestral gene (56).

Based on these homologies factor IX can be considered to have 4 distinct domains. Starting at the amino terminal these include:

1. The Gla Domain: In this domain 12 of the glutamine acid residues are carboxylated to gamma carboxyglutamic acid (61). These residues are important for calcium dependent phospholipid binding which is critical for complex formation with activated factor VIIIC and for subsequent conversion of factor X to factor Xa (62). This however, is not the only calcium binding site, high affinity calcium binding sites are also found in the first growth factor domain (63).

2. The Growth Factor Domain: This region consists of two homologous domains arranged in tandem (61). Both show limited homology to epidermal growth factor (61). Similar domains are found in other serine proteases. The function of these growth factor domains is not known.
The first growth factor domain of factor IX contains another unusual residue B-hydroxyaspartic acid (Bha) at position 64 (63). About 25 percent of human factor IX contains Bha, the remainder contains aspartic acid (63). It has been suggested that Bha participates in calcium binding but direct evidence for this is lacking (63).

3. The Activation Peptide Domain: This domain contains 35 amino acids and shows considerable polymorphism particularly at the third amino acid (64). Factor XIa cleaves factor IX at two sites in this region (61).

4. The Catalytic Domain: This region is common to all serine proteases and all share a common mechanism of proteolytic activation. Cleavage of the Arg-Val bond at Arg-35 allows the amino terminal Val residue to interact with Asp-104 (65). This interaction allows a conformational change in the active site increasing its catalytic activity (65).

1.3.4 Biosynthesis of Factor IX

Factor IX is synthesized by hepatocytes (57). The initial factor IX translational product contains an amino terminal extension or signal peptide which is cleaved in the rough endoplasmic reticulum (57). Glycosylation of factor IX occurs co-translationally and during transit of the protein via the Golgi apparatus (57). Further post-translational modifications includes gamma carboxylation
of glutamic acid residues and hydroxylation of aspartic acid at residue 64 (66).

1.3.5 Biological function of factor IX

Factor IX can be activated by either factor XIa and calcium or by factor VIIa tissue factor complex (67-75). Activation of factor IX is schematically shown in figure 1.3.2.

Factor IXa can activate factor X in the absence of cofactors, however, in the presence of cofactors (calcium, phospholipid, and activated factor VIIIC) the rate of conversion is accelerated almost 200,000 fold (30).

The interaction with calcium probably occurs at two sites, the Gla domain (contains 16 calcium binding sites) and a Gla independent site - this second site is thought to be Bha reactive at position 64 (76). The mechanism of interaction between activated factor VIIIC and factor IX is not known, but it has been shown that the activated factor IX beta form is more accessible for binding than activated factor IX alpha form (77).

In vitro studies have shown that factor IXa can activate factor VIIIC, the physiological significance of this is not clear (78). Other factor IX and activated factor IX interactions include: binding to antithrombin III and
FIGURE 1.3.1. The activation of factor IX by factor XIa and Ca$^{2+}$ or factor VIIa results in cleavage of an Arg$^{145}$ - Ala$^{146}$ bond and formation of IXK. Cleavage of Arg$^{180}$ - Val$^{181}$, results in formation of IXa beta and release of activation peptide. RVV = Russell's Viper Venom giving factor IXa beta. L = Light chain. H = Heavy chain.
endothelial cells (79). The biological significance of these interactions are not known but may be elucidated by studying factor IX variants.

1.3.6 The molecular defect in haemophilia B

By using immunological rather than functional assays for factor IX, Farth et al in 1986 and subsequently others showed that plasma from some factor IX deficient patients contained antigen or CRM reactive factor IX antibody (80-83). Patients therefore can be further phenotyped into categories using a clinical, functional and immunological measures of the amount of factor IX present. Most of the recent advances in identifying defects in haemophilia B have been made in patients who have circulating CRM. Some examples of the known variants are given below.

Factor IX Chapel Hill

Factor IX Chapel Hill was first described in 1978 (84). The molecular defect is substitution of histidine for arginine at position 145 (85). As shown in Figure 1.3.2. this is the first cleavage site in normal factor IX activation. Thus, factor IX Chapel Hill is not activated normally, only the Arg^{180} - Val^{181} bond is cleaved giving rise to a activated factor IX alpha molecule in which the activation peptide remains attached to the light chain (86,87). This variant has only 20 percent of the clotting activity of normal activated factor IX beta (86,87).
Factor IX Alabama
As in factor IX Chapel Hill in this variant there is a point mutation, glycine is substituted for aspartate at position 47 (88). It is not known how this affects function but interaction with phospholipid is normal.

Haemophilia B Leyden
This variant is clinically functionally and immunologically distinct from others. Clinically, affected individuals are severely affected till puberty and have undetectable levels of factor IX antigen (90). However, after puberty both factor IX antigen and coagulant levels increase to greater than 50 per cent. The genetic defect is not known but presumably occurs in the regulatory portion of the gene.

Unlike the variants described above, which are CRM +ve, and are due to point mutations, some CRM -ve variants have been shown to be due to gene deletions and associated with inhibitors. However the association between gene deletions and inhibitors has only been found in 9 of the 23 patients characterized (92 97).

1.4 Treatment of bleeding episodes:

The impaired ability in haemophilia A and B to generate intrinsic factor X - activating complex predisposes the individual to bleeding. The frequency and severity of
bleeding episodes parallels the deficiency in circulating factor levels, Table 1.4.1.

1.4.1 Principles of treatment

The aim in treating bleeding episodes is to raise the circulating level of factor VIIIC/IX into a haemostatic range. This can be achieved by using blood products or synthetic drugs. The type of treatment chosen will depend on 1. the degree of deficiency in the patient's plasma, 2. the severity of injury or extent of surgery, 3. the bleeding site, 4. the presence of inhibitors, 5. the plasma volume of the patient, 6. the potency of the material to be used, 7. the biological half life of the blood product.

1.4.2 Blood products

The recognition by Addis in 1911 that the defect in haemophilia resides in plasma and not platelets was a landmark in the rational management of haemophilia (98). Interestingly, blood transfusion had been suggested in the treatment of congenital bleeding disorders by Schonlein in 1832 and the first report of successful use of blood in the treatment of such a disorder was reported in 1840 (99,100). This form of therapy did not however become widely practiced and by the beginning of this century serum was the only blood component in use. In retrospect it seems likely that the success claimed for
serum therapy was probably due to its use in haemophilia B patients (101).

Plasma was subsequently shown to be superior to whole blood (102, 103). However, both blood and plasma had the obvious limitations of availability and because of short lived benefit, volume overload was a common problem. In addition their use had frustrating practical difficulties.

The further development of treatment paralleled improvements in blood banking and evolution of plasma fractionation on a scale ample to match clinical needs. Development was accelerated by the pressing needs of a plasma volume expander during World War II. A byproduct of albumin fractionation, Cohn Fraction 1 was found to be rich in fibrinogen and antihaemophilic factors, contains 50iu factor VIIIC/g of protein (104). Although this fraction was a significant improvement over plasma, its use was limited by variability of the method and expense. The use of Cohn fraction 1 was therefore restricted to treatment of life threatening bleeding episodes and for surgery.

Cohn fraction 1 was rapidly superseded by Blombeck fraction 1-0, which contain 100iu factor VIIIC/g protein (105). These and animal plasma products formed the mainstay of haemophilia therapy for the next thirty years.
The most important single achievement in the care of haemophiliacs was the observation by Pool and Robinson that the cold insoluble precipitate after controlled freeze-thawing of plasma was rich in fibrinogen and factor VIIIC (102). The importance of this observation was the potential of freeze-thawing providing a cheap, reproducible method for large scale production of factor VIII concentrates. Modifications of the method to improve purity, increase recovery, and prevent complications have been natural developments.

1.5 Factor VIII and IX containing blood products

A large number of different factor VIII containing blood products are now available. Despite initial apparent diversity, they can be classified into three main categories based on (i) factor VIIIC potency, (ii) protein content and (iii) specific activity (the ratio of potency/protein content). The three subtypes are equivalent in their clinical effect, Table 1.5.1.

Cryoprecipitate

In the preparation of cryoprecipitate, human plasma is frozen at -40°C to -70°C within six hours of blood collection and allowed to thaw at 2-4°C. The precipitate is collected by centrifugation and is either refrozen, alternatively it is redissolved in citrate buffer or a small amount of supernatant plasma and frozen. The
cryoprecipitate may be prepared from single donations or pooled (four to ten units). Pooled preparations can be either used directly or sterile filtered and freeze dried. The potency, protein content and specific activity of cryoprecipitate compared to other factor VIII concentrates are shown in Tables 1.5.1 and 1.5.2.

Some commercial manufacturers have scaled up the above method by pooling several hundred litres of plasma and freeze drying the precipitate. By using such methods the specific activity can be increased to 0.3iu factor VIIIC/mg.

Advantages of small donor pool cryoprecipitate are: its simplicity in preparation, the relatively low cost and by selection of donors hepatitis risk can be reduced. It is the treatment of choice in mild haemophiliacs who cannot receive desamino desarginine vasopressin (DDAVP). In both the UK and the USA cost of cryoprecipitate preparation is similar to that of intermediate purity factor concentrates (107,108). The main disadvantages of cryoprecipitate are the variability in potency between batches, the higher storage costs and the inability to heat sterilize.

Intermediate purity factor VIII concentrates

Cryoprecipitate is the starting material in preparation of all factor VIII concentrates. The cryoprecipitate
is washed with a glycine-ethanol-citrate buffer to extract protein contaminants and then subjected to cold precipitation associated with aluminum hydroxide adsorption to remove the prothrombin complex of proteins (110,11). Intermediate purity concentrates are rich in fibrinogen making reconstitution a problem and limiting concentrations achieved in solution to 15iu factor VIIIC/ml. The characteristics of such preparations are shown in Tables 1.5.1 and 1.5.2 (112).

Intermediate purity concentrates are the most commonly used blood products in the treatment of haemophilia A. Their main advantages are: ease of clinical use and a high yield for economic use of plasma. Disadvantages include a high risk of transmitting viral infections in the unheated state and the additional potential risk of recurrent alloantigen exposure.

High purity factor VIIIC concentrates

High purity factor VIIIC concentrates have a specific activity greater than 1iu factor VIIIC/mg. The first generation were prepared by further precipitation of intermediate concentrates with polyethylene glycol (113,114). Such high purity concentrates contain no albumin and reduced amounts of alpha and beta globulins. The main beta globulin that remains is fibronectin Table 1.5.2. The main disadvantage of such clotting factor concentrates is the lower factor VIIIC
### Table 1.5. Potency, purity and yield of factor VIII preparations.

<table>
<thead>
<tr>
<th>Preparation</th>
<th>VIII:C potency (u/ml)</th>
<th>Specific activity (u/ml)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh frozen plasma</td>
<td>1</td>
<td>0.017</td>
<td>100</td>
</tr>
<tr>
<td>Cryoprecipitate</td>
<td>3-6</td>
<td>0.1-0.3</td>
<td>35-50</td>
</tr>
<tr>
<td>Intermediate-purity</td>
<td>15-40</td>
<td>0.5-0.9</td>
<td>20-35</td>
</tr>
<tr>
<td>concentrate</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High-purity</td>
<td>20-40</td>
<td>&gt;1</td>
<td>8-23</td>
</tr>
<tr>
<td>concentrate</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 1.5.2. Protein composition of factor VIII preparations.

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Fibrinogen</th>
<th>Fibronectin</th>
<th>IgG</th>
<th>IgM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cryoprecipitate</td>
<td>60-70</td>
<td>20-25</td>
<td>5-8</td>
<td>1-2</td>
</tr>
<tr>
<td>Intermediate-purity</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>concentrate</td>
<td>42-50</td>
<td>15-35</td>
<td>10-12</td>
<td>2-5</td>
</tr>
<tr>
<td>High-purity concentrate:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>First generation</td>
<td>0-60</td>
<td>17-46</td>
<td>6-30</td>
<td>1.5-4</td>
</tr>
<tr>
<td>Second generation</td>
<td>&lt;1-80</td>
<td>2-30</td>
<td>&lt;3-30</td>
<td>5-15</td>
</tr>
</tbody>
</table>
yield because of the purification process which increases financial cost of production.

Affinity column purified factor VIII concentrates

Highly purified factor VIII concentrates have been recently manufactured using murine monoclonal antibodies and affinity chromatography. The manufacture of the product available in the UK takes advantage of the fact that, in normal plasma the procoagulant part of the factor VIII molecule is physically bound to von Willebrand factor. A monoclonal antibody specific to an epitope on the vWF protein is bound to insoluble Sepharose beads and poured into a gel column for chromatography. When cryoprecipitate is run through the column, the antibody binds to the vWF molecule. Factor VIIIC is separated from vWF by calcium and eluted from the column. The purified factor VIIIC is concentrated by ultrafiltration and further purified by additional affinity chromatography. The final product is stabilized with human albumin. The specific activity of the final product is 15.2 iu/mg protein and is due entirely to the albumin added (115).

Porcine factor VIII

The use of porcine and bovine factor VIII concentrates has been largely abandoned due to the high incidence of side effects (116). However a relatively new, highly
purified polyelectrolyte porcine preparation has proven to be quite effective and has a much lower incidence of side effects (116, 117). Its main use is in patients with factor VIIIC inhibitors.

1.5.4 Factor IX concentrates (Non activated prothrombin complex concentrates)

The early concentrates used in the treatment of haemophilia B were prepared from ethylenediamine tetraacetate (EDTA) anticoagulated plasma adsorbed with tricalcium phosphate, eluted with citrate and dried with ethanol (118). The starting material most commonly used now is cryoprecipitate supernate, the vitamin K dependent proteins are adsorbed onto diethylaminoethyl (DEAE) - cellulose and eluted with phosphate or citrate buffers. The final product is lyophilized. It contains relatively little factor VIIIC but has approximately equal amounts of factors II, IX and X (121).

In order to limit possible side-effects related to presence of thrombin and other activated factors some manufacturers add heparin.

Activated Prothrombin Complex Concentrates (APCC)

The manufacture of APCC was stimulated by anecdotal reports of the success of prothrombin complex concentrates in controlling bleeding episodes in a factor
VIIIC deficient patients with an inhibitor (122). The concentrate used represented specific lots of prothrombin complex concentrate which had been withdrawn because of potential thrombogenic complications. Further use of these batches in patients with inhibitors provided similar results and stimulated the production of prothrombin complex concentrates which were deliberately activated in a controlled fashion to produce concentrates with so called factor VIIIC bypassing activity (123). Controlled studies have shown that prothrombin complex concentrates can control joint bleeds in up to 50 percent of episodes in patients with factor VIIIC inhibitors (124 -127)

Two activated prothrombin complex concentrates are available commercially - FEIBA (Immuno, Austria) and Autoplex (Hyland Laboratories). Both significantly reduce haemophilic and normal plasma clotting time (non-activated PTT), while non-activated complexes produce only a modest decrease. Both products have been shown to be effective in clinical trials (126.127)

1.6 Synthetic drugs

1.6.1 Des deamino desarginine vasopressin

To date the only satisfactory alternative to blood products in the treatment of factor VIIIC deficiency is the pharmacologic analogue of vasopressin deamino 8 D
desarginine vasopressin (DDAVP) (130-135). Theoretically factor VIIIC deficient patients who have the capacity to produce factor VIIIC (ie CRM positive patients) should show increases in factor VIIIC, DDAVP is only satisfactory in securing haemostasis in patients with a mild deficiency (131-135). The response is dose dependent and reaches a maximum at 0.3 ug/kg increasing levels by four fold. The response lasts several hours and is more prolonged for factor VIIIvWF than factor VIIIC or factor VIIIAg (133). The mechanism of action is not known. It is often used in conjunction with tranexamic acid to inhibit induction of fibrinolysis (131,133).

1.6.2 Anabolic steroids

Initial studies with the attenuated anabolic steroid, danazol showed that administration of danazol for 2 weeks increased factor VIIIC and factor IX levels in patients with haemophilia A and Christmas disease respectively (136). All five treated patients showed rises in deficient factors ranging from two - six times basal levels. This has not been confirmed. A similar attenuated anabolic steroid, stanozolol showed only minor increases. Its use was further limited by an increased incidence of soft tissue haematomas presumably due to increased fibrinolytic activity (137).

1.6.3 Fibrinolytic inhibitors
Decreasing fibrinolytic activity has the theoretical advantage of off setting the imbalance created by factor VIIIC and IX deficiency. Two such drugs have been used in haemophiliacs, epsilon amino caproic acid (EACA) and tranexamic acid. Both inhibit binding of plasmin to fibrin.

The results in preventing spontaneous bleeds with both drugs have provided contradictory results long term use at present cannot be justified (138 - 142). Studies of chronically inflamed haemophilic synovium show increased plasminogen activator activity and it has been suggested that EACA may reduce bleeding during the chronic synovitic stage (143). The principal use of these agents however has been restricted to reducing dental socket bleeding after surgery (144 -149).

1.7 A balanced assessment of the risks and benefits of treatment:

1.7.1 Benefits
There is little doubt that appropriately used the blood products discussed in section 1.5 correct the haemostatic defect in haemophilia. The immediate benefits in controlling haemophiliac bleeding are well established. However, benefit in the long term also requires to be measured. The influence of clotting factor concentrate use on life expectancy and joint disease are therefore reviewed.
Factor deficiency only, moderately affected patients had a life expectancy of 71.5 years. The lower median life expectancy in Sweden was for patients with a severe inhibitor. The data shown is for haemophiliacs of all severities and includes patients with

<table>
<thead>
<tr>
<th>Study Country Year</th>
<th>No. of Patients</th>
<th>Median Life Expectancy</th>
<th>Normal Life Expectancy</th>
<th>Expressed</th>
<th>Rate/1000</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bradstock USA 1980</td>
<td>-</td>
<td>62.9</td>
<td>56.8</td>
<td>75.6</td>
<td>1985</td>
</tr>
<tr>
<td>Marsee UK 1983</td>
<td>-</td>
<td>62.9</td>
<td>56.8</td>
<td>75.6</td>
<td>1985</td>
</tr>
<tr>
<td>Ixkender Portugal 1982</td>
<td>-</td>
<td>4.8</td>
<td>43</td>
<td>72.8</td>
<td>1985</td>
</tr>
<tr>
<td>Camps Chile 1980</td>
<td>-</td>
<td>64.9</td>
<td>43</td>
<td>73.4</td>
<td>1980</td>
</tr>
</tbody>
</table>

Table 1.7.1. Life expectancy studies in haemophilia.
1.7.2 Life Expectancy

Studies that have reviewed life expectancy in haemophilia are shown in Table 1.7.1 (150 - 154). In two studies the additional information necessary to compare results was not available (150,151). The data from the remaining three studies is comparable and therefore only the findings drawn by Rizza and Spooner on 5098 haemophiliacs UK are presented (154):

1. The median life expectancy for a severe factor VIIIC deficient haemophiliac was 69.2 years and 79.2 years for a moderately affected patient. Median life expectancy of an English male was 72.8yrs. Therefore by 1976 - 80 haemophiliacs in the UK were expected to have a near normal life expectancy. Similar results were found in Sweden and Finland (152,153).

2. The overall annual death rate for severe haemophilia A (VIIIC < 2%) was 6.3 per thousand for 1976-80. No cumulative UK data was available prior to 1969 but can be implied from data published by Ikkala on haemophilic deaths in Finland, where annual death rate decreased from 8.6 in 1930-39 to 4.8 per thousand in 1970-79 for children under ten years of age. Similarly in Sweden it was found that death rates in severe haemophiliacs was no different from Swedish males.
Therefore, the use of blood products particularly cryoprecipitate and clotting factor concentrates was associated with a reduced mortality in haemophilia.

1.7.3 Joint Disease
Excessive bleeding may occur from any site in haemophiliacs, however, spontaneous haemorrhages into joints, muscles and the renal tract are the most frequent (3). Clinical experience and several studies have shown that joint disease is the single most important cause of morbidity from bleeding in haemophilia. The incidence parallels the severity of the bleeding tendency and up to 90 percent of patients with a severe deficiency have had this complication (153).

The benefits of early use of clotting factor concentrates in relieving symptoms of an acute haemarthrosis are well established. However, the impact of the early use and the amount of clotting factor concentrate used on the progression of the arthritis and frequency of haemarthrosis is not known but is being evaluated prospectively (156).

An interim analysis at two years of this prospective study shows that:

(i) The dose and type of replacement material does not affect frequency of bleeds.
(ii) Multiple joints are involved and there is a progressive deterioration with age.
(iii) The clinical findings do not parallel the severity of radiological damage.
(iv) The degree of radiological damage is independent of dose used to treat the patient.

The only firm conclusion therefore that can be drawn at present is that clotting factor concentrates are effective in treating acute haemarthrosis but do not appear to significantly alter the natural history of joint disease but may delay its onset (156).

1.8. Complications of treatment with blood products

Complications that may result from the use of blood products include:

1. Infective: The major infective complications include hepatitis and human immunodeficiency virus 1 infections.
2. Non-Infective: These can be further subdivided into those with an immunological basis and those independent of an immune mechanism.

1.8.1 Infectious Complications of treatment with blood products:

After the widespread use of blood transfusion therapy in the 20th Century, data soon accumulated that implicated blood as a vehicle for the transmission of infectious
organisms. In 1943 Beeson reported jaundice occurring in patients 1-4 months after transfusion with blood or plasma and suggested an infectious aetiology (157). The list of infectious organisms has gradually expanded, Table 1.8.1.

The biology of the infectious agent determines whether they are transmitted via cellular or cell free blood products. Haemophiliacs have a lifelong dependence on cell free blood products and are therefore particularly at risk of acquiring hepatitis, (due to hepatitis B and the non A, non B [NANB] viruses) and acquired immunodeficiency syndrome (AIDS) due to human immunodeficiency virus - 1 (HIV-1).

Hepatitis
Post-transfusion hepatitis is a well recognised and important complication of blood transfusion. It is estimated that in the USA alone between 200,000 - 300,000 cases occur each year. To fully appreciate the current understanding of hepatitis in haemophiliacs several major observations that have shaped and altered the course of post-transfusion hepatitis merit recounting.

First, and perhaps most important was recognition of the importance of donor source and greater hepatitis risk of commercial blood donations. The seminal observations in this area were by Allen and Kunin (158.159).
### Table 1.8.1. Infectious agents transmitted by transfusion.

**Viral**
- Hepatitis A
- Hepatitis B
- Hepatitis non-A, non-B
- Cytomegalovirus
- Epstein-Barr virus
- Serum parvovirus
- HTLV-I/HIV

**Parasitic**
- Malaria
- Filariasis
- Trypanosomiasis
- Toxoplasmosis
- Babesiosis

**Trepomnemal**
- Syphilis

**Bacterial**
- Multiple organisms
Second, the chain of events initiated by the discovery of hepatitis B surface antigen (HBs Ag, Australia antigen) by Blumberg and his associates in 1965; the association of this antigen with viral hepatitis (Blumberg 1967); the subsequent linking of this agent with type B hepatitis; the findings by Gocke 1970, and Okochi 1970 that transfusion of HBs Ag - positive blood was associated with hepatitis; the demonstration that exclusion of HBs Ag - positive blood and commercial donors reduced post-transfusion hepatitis by 85 percent and finally development of sensitive radio immunoassays for HBsAg detection in selecting blood donors (160 - 166).

The third major development was a test for the hepatitis A virus (HAV) in 1973 and subsequent recognition that non-B hepatitis cases were not due to HAV but due to a newly recognised, hepatotropic virus or groups of viruses that were designated non-A, non-B (NANB) (167 - 169). NANB now accounts for 85-98 percent of episodes of post-transfusion hepatitis.

Hepatitis B
The frequent exposure of haemophilic patients to hepatitis B virus (HBV) is evidenced by the high incidence of positive serological tests for HBV (170). The current situation regarding HBV infection can be summarized as follows:
First, risk of HBV infection is strongly correlated with use of large pool concentrates and is greater with plasma obtained from paid donors (171).

Second, application of sensitive tests for detection of HBsAg to screen units of donated blood and plasma has resulted in a notable reduction in HBV infection, after first transfusion of concentrate. Prior to 1975 the attack rate was greater than 80 percent; more recent studies show a 75 percent reduction (172). Thus occurrence of HBV infection has not yet been entirely eliminated.

Third, most severe haemophiliacs are exposed to HBV before ten years of age. Acute infection is often asymptomatic or occasionally associated with mild non-specific illness (172). On the other hand mild haemophiliacs who acquire infection usually do so later on in life and in such cases infection is more often symptomatic (172). The reason for this difference may not be a function of age but could be related to the acquisition of partial immunity conferred by passive transfusion of HBV antibody from previous transfusions of clotting factor concentrate.

Fourth, the finding that the proportion of HBsAg carriers in treated haemophiliacs is no higher than in non haemophiliacs with a similar exposure to HBV is reassuring (172). However, it must be noted that carriers
are at a greater risk from chronic liver disease, not only from HBV but also in their greater susceptibility in acquiring delta virus infection (173). Such patients may benefit from treatment with recombinant alpha interferon - its efficacy however needs to be more fully established especially in the context of HIV-1 infection (172 - 174).

Finally, the efficacy and safety of HBV vaccine is well established in conferring immunity for at least five years. It is therefore potentially possible to eliminate further HBV infections.

NANB hepatitis
One widely accepted epidemiological definition of NANB hepatitis is a rise in serum alanine aminotransferase (ALT) above twice the upper limit of normal on at least two occasions at least two weeks apart in the absence of any known cause (176). Using such a definition the attack rate after transfusion of the first batch of unheated concentrate approaches 90 percent (177).

The risk of acquiring NANB hepatitis is significantly greater with clotting factor concentrates than cryoprecipitate and there is a greater risk with clotting factor concentrates fractionated from paid plasma donations rather than voluntary donations (178).
Complications of NANB hepatitis:

The importance of NANB infection lies not in acute manifestations, but in its chronic sequelae. In non-haemophilic patients who acquire NANB, persistent or fluctuating elevations in ALT remain for at least three years in up to 70 percent of patients (179). Characteristically rises in ALT are fluctuating in nature with the periodicity in fluctuations decreasing with progression to chronicity. Percutaneous liver biopsy studies in such patients invariably show features of chronic hepatitis and in up to 20 percent of biopsied patients progression to cirrhosis occurs (180). The difficulty in distinguishing severe chronic persistent hepatitis from chronic active hepatitis limits the significance of firm conclusions being attached to a finding of chronic hepatitis.

Similar natural history observations are more difficult to obtain in haemophiliacs: first, the very nature of the disease necessitates frequent transfusions and therefore the date of first infection often is not known; second at least two agents have been implicated in haemophilic NANB hepatitis and third clotting factor concentrates in the absence of infection may result in a chemical/allergic hepatitis (181,182).

Studies of serum ALT have been reported by several groups, Table 1.8.2 (183 - 192). The wide variations in percent prevalence of abnormal serum ALT level reported
Table 1.8.2. Studies with >50 patients evaluating frequency and persistence of abnormal transaminase levels in haemophilia.

<table>
<thead>
<tr>
<th>Author (Ref)</th>
<th>No. of Patients</th>
<th>Cross sectional % with abnormal liver enzymes</th>
<th>Longitudinal % with abnormal liver enzymes &gt;6mths</th>
<th>Percent with Hepatitis B surface Antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cederbaum (183)</td>
<td>1332</td>
<td>72</td>
<td>23.6</td>
<td>78</td>
</tr>
<tr>
<td>Webster (184)</td>
<td>261</td>
<td>57</td>
<td>28</td>
<td></td>
</tr>
<tr>
<td>Rickard (185)</td>
<td>243</td>
<td>34</td>
<td>8</td>
<td>63</td>
</tr>
<tr>
<td>Kim (188)</td>
<td>103</td>
<td>79</td>
<td>51</td>
<td>77</td>
</tr>
<tr>
<td>Levine (186)</td>
<td>98</td>
<td>69.4</td>
<td>69.4</td>
<td>33.6</td>
</tr>
<tr>
<td>Mannucci (187)</td>
<td>91</td>
<td>45</td>
<td></td>
<td>66</td>
</tr>
<tr>
<td>Yannitsitos (189)</td>
<td>101</td>
<td>47</td>
<td></td>
<td>61.3</td>
</tr>
<tr>
<td>Hillgartner (190)</td>
<td>54</td>
<td>42.5</td>
<td></td>
<td>90</td>
</tr>
</tbody>
</table>
may be due to differences in patient selection or the natural history of the fluctuations in serum ALT. More meaningful information has been provided by longitudinal studies. There is agreement in statistically comparing longitudinal studies Table 1.8.2 that between 20-30 percent of patients have persistent serum ALT elevations. In the vast majority clinical signs of chronic liver disease were absent.

Studies of liver biopsy material show that the magnitude of rise in serum ALT does not reflect the nature of hepatic lesions, for example Carr et al found that the level of AST was the same in cirrhosis and chronic persistent hepatitis (192). However, additional information can be obtained by reviewing the pattern of rise; persistently raised levels of above 150 percent of normal levels for >6 months indicate chronic hepatic disease - disappointingly, this does not distinguish between chronic persistent and chronic active. Clinical examination in such individuals for stigmata of chronic liver disease may help but these findings are infrequently present.

Fluctuating levels tend to indicate less severe liver disease; in a biopsy study of 15 patients with such a pattern, White et al found that despite presence of inflammatory portal tract lesions in all patients, only one patient had chronic active hepatitis, 11 chronic persistent hepatitis and the remainder no additional
changes were present (193). The latter group after two years had normal ALT levels this did not help in distinguishing lesions, although as a group mean ALT levels were lower.

The interpretation of serum ALT levels in haemophiliacs is further complicated by the ability of clotting factor concentrates to induce a non-infectious hepatitis (182).

Liver biopsies in haemophiliacs have provided worrying but controversial data. The divided opinions on the interpretation of findings are best illustrated in the choice of titles of published series.

"Liver disease in haemophilia: An overstated problem." (194)
"Progressive liver disease in haemophilia: An understated problem." (195)

What then are the sources of controversy and are there any agreements. Liver biopsy or autopsy data have been reported in 278 patients, sequential biopsy data were available in 38 patients. The studies reviewed are shown in Table 1.8.2.

Clinically important information can only be obtained from one study (2). In this collaborative, worldwide study biopsy (or autopsy) obtained material from both treated and untreated patients was independently
<table>
<thead>
<tr>
<th>Key</th>
<th>N/S=not stated, B=biopsy, A=autopsy, Co=cooperative, I=independent, numbers in brackets are percentages</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>S/N</td>
<td>S/N</td>
</tr>
<tr>
<td></td>
<td>6.6</td>
</tr>
<tr>
<td></td>
<td>2 (1)</td>
</tr>
<tr>
<td></td>
<td>32</td>
</tr>
<tr>
<td></td>
<td>5 (8)</td>
</tr>
<tr>
<td></td>
<td>5 (8)</td>
</tr>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>2 (2)</td>
<td>S/N</td>
</tr>
<tr>
<td>2 (2)</td>
<td>YES</td>
</tr>
<tr>
<td></td>
<td>N/S</td>
</tr>
<tr>
<td>2 (2)</td>
<td>1 (15)</td>
</tr>
<tr>
<td></td>
<td>S/N</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
</tbody>
</table>
interpreted by four histopathologists, using predefined and accepted criteria for histological grading. Despite these preconditions it was found that agreement in final diagnosis between all four pathologists was reached in only 55 percent of cases. The major area of disagreement was in classification of chronic persistent and chronic active hepatitis. These lesions accounted for between 7-72 percent of diagnosis in other studies. Furthermore in up to 10 percent of cases reported by Aledort et al the cause of liver disease was not directly attributable to NANB or hepatitis B infection - a finding in keeping with some other reports, Table 1.8.2.

Despite this there are areas of agreement and several conclusions can be drawn. First, cirrhosis occurs in about 15 percent of patients, the nine percent figure from the cumulative Sheffield studies is not statistically different than that reported by Aledort (2). Mannucci et al also report a similar incidence (203). The incidence of cirrhosis in haemophiliacs is therefore not significantly different from that reported in the outcome of individuals who acquire NANB after elective cardiac surgery (204).

Second chronic hepatitis (without distinguishing between chronic active hepatitis and chronic persistent hepatitis) occurred in 71 - 91 percent of patients. The prevalence of chronic hepatitis between the Aledort study and others showed considerable differences, in particular
Table 1.8.4. Serial liver biopsy studies.

<table>
<thead>
<tr>
<th>Author (Ref)</th>
<th>1st biopsy</th>
<th>2nd biopsy</th>
<th>Age at 1st Bx</th>
<th>Interval mths</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aledort (2)</td>
<td>CPH (5)</td>
<td>CPH (6)</td>
<td>}</td>
<td>N/K</td>
</tr>
<tr>
<td></td>
<td>CAH (6)</td>
<td>CAH (2)</td>
<td>}</td>
<td>N/K</td>
</tr>
<tr>
<td></td>
<td>CIRR (1)</td>
<td>CIRR (2)</td>
<td>}</td>
<td>N/K</td>
</tr>
<tr>
<td>Schimpf (201)</td>
<td>CPH (3)</td>
<td>CPH (4)</td>
<td>24</td>
<td>48</td>
</tr>
<tr>
<td></td>
<td>CAH (2)</td>
<td>CAH (2)</td>
<td>(18-25)</td>
<td>(31-58)</td>
</tr>
<tr>
<td>Hay (195)</td>
<td>CPH (6)</td>
<td>CPH (3)</td>
<td>32</td>
<td>47</td>
</tr>
<tr>
<td></td>
<td>CAH (4)</td>
<td>CAH (2)</td>
<td>(26-28)</td>
<td>(31-58)</td>
</tr>
<tr>
<td></td>
<td>CIRR (0)</td>
<td>CIRR (5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mannucci (197)</td>
<td>CPH (8)</td>
<td>CPH (8)</td>
<td>12</td>
<td>36</td>
</tr>
<tr>
<td></td>
<td>CAH (2)</td>
<td>CAH (2)</td>
<td>(6-15)</td>
<td>(36-36)</td>
</tr>
<tr>
<td></td>
<td>CIRR (1)</td>
<td>CIRR (1)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The median and interquartile ranges are shown for age and interval.

N/K = not known
CPH = chronic persistent hepatitis
CAH = chronic active hepatitis
CIRR = cirrhosis
Chronic lobular and aggressive hepatitis was included under CAH.

22 1st biopsies = CPH - 2nd biopsy: CPH=17, CAH=3, CIRR=2.
12 1st biopsies = CAH - 2nd biopsy: CPH=5, CAH=4, CIRR=5.
2 1st biopsies showed CIRR - 1 had died, he had concommitant delta in one the outcome is not known.
there were major differences in lesions classified as chronic persistent hepatitis and chronic active hepatitis. On comparing the cumulative Sheffield results with those of Aledort et al, it was found that considerably greater numbers of cases had been classified by the Sheffield group as chronic active hepatitis than in the Aledort data.

An additional potential source of error in the cumulative Sheffield data was the inclusion two patients who had granulomatous lesions on biopsy (a feature that is not recognised in the spectrum of histological abnormalities reported in NANB hepatitis).

The best estimate of severe liver disease defined as the presence of cirrhosis and chronic active hepatitis in haemophilia therefore is 22 percent (cirrhosis = 15%; chronic active hepatitis = 7%). A more fundamental question in planning for the health care of haemophiliacs is the prognosis of chronic hepatitis which accounts for up to two thirds of patients that are biopsied.

Four studies had reported on 38 serial biopsies, the cumulative results from these studies are shown in Table 1.8.3 (2,195,201,203). Accepting the difficulties in histological differentiation of chronic active hepatitis and chronic persistent hepatitis if individuals with these lesions were considered as a group (n=34), six (18%) showed clinical or histological progression to
cirrhosis, in 11 (32%) the histological diagnosis did not change, three (9%) showed a histological deterioration. Chronic hepatitis therefore progresses to cirrhosis in 32 percent of cases.

**Human immunodeficiency virus - 1**

The acquired immunodeficiency syndrome (AIDS) is a clinical diagnosis, and is but one manifestation of a spectrum of clinical and subclinical disorders caused by the human immunodeficiency virus - 1 (HIV-1). Haemophiliacs acquire HIV-1 infection from contaminated blood products. Aspects of HIV-1 infection in haemophiliacs, in particular the immune response to HIV-1, and the effects of HIV-1 on the immune system are discussed in subsequent chapters of this thesis. There are however, several aspects worthy of further review.

**Human Immunodeficiency Virus - 1**

HIV-1 is an RNA retrovirus and shares morphological, biological and molecular similarities to the visna virus of sheep, equine infectious anaemia virus and the recently described feline and bovine immunodeficiency viruses. It is also related to recently isolated primate retroviruses such as simian T lymphotrophic virus-III. It is distinct from HIV-2 in several respects, HIV-2 has a more restricted distribution, it is most prevalent in West and Central Africa. Other differences include significant variations in envelope proteins and to a lesser extent core proteins. HIV-2 has similar
routes of transmission and risk groups, it also appears to be more adapted to the host and is less virulent. HIV-2 is more closely related to primate retroviruses than HIV-1. Cross-reactivity between the two viruses occurs in serological tests for HIV-1 but specific serological tests are now commercially available (reviewed 208,209).

Viral Tropism

The accepted sine qua non of HIV-1 infection is the progressive depletion of T-helper cells. The CD4 antigen expressed on human T-helper cells is a major cellular receptor for HIV-1 envelope protein gp120 (207). However, it is now apparent that HIV-1 can infect cells that lack CD4 and is found in endothelial and epithelial cells of seropositive individuals (208-211). Alternative mechanisms may therefore govern infection. Fusion of the target cell membrane with the transmembrane envelope protein gp41 may be one such mechanism. The finding that HIV-1 infection of cells is pH independent supports a fusion process (212). Furthermore, part of the sequence of gp41 is similar to the fusinogenic proteins of paramyxoviruses, and antibodies to gp41 protein neutralize HIV-1 (213,214). The finding that HIV-1 antibodies enhance HIV-1 infection of cells in vitro suggests virus-antibody complexes may permit infection of cells that express Fc or complement receptors (215). Cellular factors may also determine establishment of HIV-1 infection, a single HIV-1 isolate showed
differential levels of productive infection in peripheral blood mononuclear cells from various individuals (216).

The immunopathology of HIV-1 infection
A central dogma in HIV-1 infection has been that immunosuppression and immune function abnormalities in AIDS (Table 1.8.5) are due to progressive depletion of T-helper cells. Several observations have challenged this. First, the number of actively infected blood cells are small, although a larger number may be latently infected. This does not account for the profound T helper cell depletion in AIDS (217). Second, although syncitia formation accounts for the cytopathic effects of HIV-1 in vitro, syncitia have not been demonstrated in vivo (218). Third, many HIV-1 isolates from immuno-compromised patients are not highly cytopathic in vitro (219). Last, T-helper cells from infected individuals but not actively infected show aberrations comparable to infected cells (220). Other mechanisms therefore require to be postulated and these are summarized in Table 1.8.6.

Determinants of disease progression
Considering the biology of HIV-1 it is not surprising that up to 70 percent of infected individuals progress to develop symptomatic immunodeficiency within six years of infection (1). A more interesting aspect is why up to a third of infected individuals remain symptom free. Variables that may potentially affect the course of infection include virulence of the virus strain, the
Table 1.2.5. Immune function abnormalities in AIDS

**T lymphocytes**

(1) Decreased proliferative responses to mitogens, soluble antigens, and allogeneic cells.

(2) Decreased lymphokine production (IL-2, gamma interferon) in response to antigen.

(3) Decreased cytotoxic T lymphocyte activity against virus-infected cells.

**B lymphocytes**

(1) Polyclonal activation with hypergammaglobulinaemia and spontaneous plaque forming cells.

(2) Decreased humoral response to immunization.

(3) Production of autoantibodies.

**Monocytes**

(1) Decreased chemotaxis.

(2) Decreased IL-1 production (or production of an inhibitor of IL-1).

(3) Decreased microbiocidal activity.

**NK cells**

(1) Decreased cytotoxic activity.
Table 1.8.6.  Mechanisms of immune suppression by HIV infection.

Direct mechanisms

(1) HIV cytocidal effect on CD4+ lymphocytes.

(2) Functional defects in infected CD4+ cells:
   (a) decreased expression of cell surface proteins (eg, IL-2 receptor, CD4);
   (b) impaired production of lymphokines such as IL-2 or gamma interferon.

(3) Impaired antigen presentation and/or monokine production by infected macrophages; cell death.

Indirect mechanisms

(1) Generation of suppressor T cells and/or factors.

(2) Toxic or inhibitory effects of viral protein.

(3) Immune complex formation.

(4) Induction of autoimmune phenomena:
   (a) autoantibodies resulting from polyclonal B cell activation or antigen mimicry;
   (b) virus mediated, enhanced immunogeniticy of normal cellular proteins.

(5) Cytotoxic cell activity against viral or self proteins.
antiviral immune response and the inherent sensitivity of the host cell to virus replication.

Several recent findings suggest that the immune response to HIV-1 may be a determinant of outcome. First, exposure to the virus is not invariably associated with infection. This has been shown in haemophiliacs and homosexual men (221,222). Second, in a cohort of homosexuals known to be HIV-1 antibody positive six individuals have become seronegative for antibody and remain asymptomatic. Using the polymerase chain reaction it was shown that all previously had integrated provirus (223). Third, the frequency of virus isolation from peripheral blood is increased in asymptomatic individuals if the cell preparation is depleted of T suppressor/cytotoxic cells (224). Further studies showed that soluble cell secreted factors mediated the response. Taken together these findings suggest that the immune response to HIV-1 may be an important determinant in the outcome of HIV-1 infection.

1.8.2 Non infective complications of replacement therapy in haemophilia.

The non infective complications of replacement therapy can be considered under two broad categories:

1. Immune mediated.
Immune mediated
Complications included in this group are listed in Table 1.8.5. Immune reactions may mediate tissue injury by four types of reactions.

Anaphylactic (Intermediate Hypersensitivity) - Type I Reactions.

Anaphylactic reactions are caused by antigen - antibody reactions occurring on the surface of mast cells leading to release of histamine and other vasoactive amines. The incidence of such reactions has been reported to be as high as 19 percent (224). The majority are mild, manifest as fever and urticaria but in 0.7 percent systemic anaphylactoid reactions occur which may be life threatening (3). The mechanism is not clear but presumed to be to the contaminating immunoglobulins contained within clotting factor concentrates.

Non allergic reactions may also occur and consist of variable subjective complaints of dizziness, headache, and shortness of breath. Such reactions may be due to infusion of vasoactive substances contained within clotting factor concentrates.

Cytotoxic (Humoral Antibody) - Type II reactions.

Autoimmune haemolytic anaemia is the clearest example of a type II reaction. Haemolytic anaemia may occur in
haemophilic patients of blood group A, B or AB after a period of intensive therapy with concentrates (230-231). The haemolytic anaemia is due to the passive infusion of IgM and IgG anti-A and anti-B alloagglutinins present in factor VIII concentrates. Haemolysis usually starts after two or three days of intensive replacement therapy. A positive direct Coombs test is often found (3). More recently, it has been suggested that the high frequency of low levels of haptoglobin in multitransfused haemophiliacs might be related to a certain degree of chronic, clinically unapparent haemolysis (232).

The presence of factor VIII/IX inhibitors (antibodies) can also be included under this category. Factor VIIIC inhibitors develop in approximately 10-15 percent of treated haemophilia A patients (233). In the majority of cases inhibitors are IgG antibodies with kappa light chains (234-237). A few however have lambda light chains or were mixtures of IgG kappa and lambda chains (234, 238, 239). Heavy chain subtyping has shown that IgG4 predominates (238). They are therefore non-precipitating antibodies and do not fix complement. This may explain why patients with an inhibitor do not develop renal damage when infused with factor VIII concentrates (238).

Two types of factor VIIIC inhibitors can be recognized in vitro.
Type I - appear to react with epitopes near the factor VIIIC procoagulant site. The inactivation kinetics of factor VIIIC shows a steep slope and complete factor VIIIC inactivation occurs. Such inhibitors are saturated when sufficient factor VIIIC is added and there is a linear relationship between residual factor VIIIC activity and inhibitor concentration (234, 235, 238, 240).

Type II inhibitors by contrast have complex inactivation patterns. These occur in a minority of haemophilic patients and the majority of non-haemophilic patients with inhibitors (234, 235, 238, 241, 242). It has been suggested that type II inhibitors react with interaction sites of factor VIIIC and factor VIII von Willebrand factor (238, 243).

The majority of factor VIIIC deficient patients have a severe factor deficiency, although inhibitors are reported in patients with moderate - mild haemophilia. The development of inhibitor invariably requires exposure to factor VIIIC, although the amount is quite variable. The majority of patients develop an inhibitor before the age of 10 years (244).

Most patients are "high responders" exhibiting a marked rise in inhibitor concentration following exposure to factor VIIIC. A few are "low responders" never having much of a amanestic response. Inhibitors in high responders
usually persist whereas in low responders several clinical patterns are apparent. The inhibitor may spontaneously disappear or it may persist, the patient gradually becoming a high responder. In some it may remain unchanged and bleeding episodes can thus be continued to be treated with human factor VIII concentrates (3).

There is some evidence to suggest a genetic predisposition to inhibitor development. Inhibitors tend to occur more frequently in brother pairs and in black haemophiliacs. The occurrence of inhibitors before the age of 20 years in up to two thirds of patients also suggests a genetic predisposition (244).

The sensitization to IgG subtypes and variants of complement C4 can also be considered as type II reactions (245, 246).

**Immune Complex (Arthus) Type III Reactions**

Type III reactions are induced by deposition of antigen-antibody complexes in tissues causing an inflammatory reaction via mediators such as complement. The occurrence of serious side-effects with bovine and porcine factor concentrates may have mediated by such reactions. Immune complexes have not been found in human clotting factor concentrates, however, treated haemophiliacs have higher levels than normals of immune
complexes detected by a variety of different methods (247-251). In a few hepatitis B antigen has been identified (247).

The clinical significance of these findings is not clear, haemophiliacs with immune complexes have evidence of complement consumption but true immune complex mediated disease has been only reported infrequently. In one study a correlation between immune complexes and chronic synovitis, liver disease as well as episodes of haematuria was found (248).

Cell-mediated (Delayed Hypersensitivity) - Type IV Reactions

Type IV reactions are mediated by T lymphocytes and in which free antibody plays no part. Delayed hypersensitivity can be tested in-vivo by challenging the immune system with dermal or intradermal antigens. Impaired cell mediated immunity using such tests has been reported in haemophiliacs independent of the HIV-1 antibody status (see Chapter 7).

1.8.3 Non Immune, non infectious complications of clotting factor concentrates

Thrombotic complications with factor IX complex.
As a result of preparative procedures, some zymogens in factor IX concentrates became activated, with raised levels of factor IXa and Xa. Thrombosis both venous and arterial as well as disseminated intravascular coagulation are reported in patients treated with large doses of prothrombin complex concentrates. It has been suggested that patients with chronic liver disease may be predisposed to this complication because of impaired clearance of activated factors (3).

1.9 Safer clotting factor concentrates

The use of clotting factor concentrates in the treatment of haemophiliac bleeding has proven to be a double edged sword. The benefits of treatment are firmly established (section 1.7), on the other hand treatment carries a high risk of transmitting blood borne viral diseases (section 1.8). Moreover, intermediate purity preparations contain substantial amounts of plasma proteins other than factor VIII (Tables 1.5.1 and 1.5.2). The clinical consequences of the repeated administration of this large alloantigen load is not clear. Recurrent alloantigen stimulation is implicated in changes in the immuno-regulatory ratio which may have predisposed some haemophiliacs to acquiring HIV-1 infection (221).

The development of safer clotting factor concentrates has therefore been arrived at by first reducing risk of
acquired blood borne viral infections, and second by reducing alloantigen load.

Measures employed to reduce risk of acquiring viral infections include donor screening for viral markers such as hepatitis B surface antigen or anti-viral antibodies, for example anti-HIV-1 IgG antibody. Such strategies minimize, but do not eliminate the risk of viral infections. Various additional methods have therefore been sought.

Three main approaches have been used:

1. Heat
2. Chemical inactivation
3. Physical exclusion

1.9.1 Heat

Heat treatment is the most common additional virucidal method used. All methods based on heating use temperatures between 60°C and 80°C for 30-72 hours. Dry heat is the term applied when clotting factor concentrates are heated once lyophilized, this is the commoner method applied. Dry heat has also been applied to lyophilized concentrates resuspended in the organic solvent n heptane. The alternative is pasteurization (or wet heating) of concentrate in the aqueous phase with direct heat or a hot vapour. Wet heat has the advantage that less heat needs to be applied to achieve a given virucidal effect. The disadvantage is a lower yield.
This has been partially overcome by addition of stabilizers such as glycine, sucrose or potassium citrate. Their use however reduces heating efficacy by a magnitude of $2 \log_{10}$ (252). The effect of temperature on factor VIII yield is dependent more on temperatures applied rather than duration (252). For instance increasing temperatures from 60°-70° or 80°C causes a 90 percent or greater loss in factor VIII activity. Heating for 72 hours instead of 10 hours at 60°C does not result in additional loss (252).

Hepatitis
Despite donor screening for hepatitis B surface antigen, the use of clotting factor concentrates still carries a risk of contracting hepatitis B virus (HBV) infection. The impact of donor screening therefore has been to delay the onset of contracting HBV infection in patients receiving regular treatment with clotting factor concentrates by one to four years from first receipt of these products (253).

Although pasteurization of albumin (60° for 10 hours) is not associated with HBV infection, the same does not apply to clotting factor concentrates or plasma. Pasteurization of whole plasma at 60°C for 10 hours results in incomplete HBV inactivation (254). Dry heat applied to factor VIII concentrate at 60°C for 20 hours does inactivate HBV. Studies in chimpanzees inoculated with a dry heat inactivated factor VIII concentrate have
confirmed that HBV is inactivated after such treatment (253).

The primary aim in heat treating concentrates is now prevention of NANB hepatitis, consequently a substantial number of patients recruited into virgin patient studies have received prior HBV immunisation. For instance 16 of 26 patients entered into a study evaluating pasteurized factor VIII had been immunized; therefore no firm conclusions can be drawn on effects of newer wet heat treated preparations in preventing HBV infection (256). Dry heat at temperatures less than 60°C is ineffective in preventing infection (257).

The chronic sequelae of NANB hepatitis are a more serious problem in haemophiliacs (section 1.8). In the absence of a serological test the risk of infection can be reduced by screening plasma donations for raised serum ALT level and additional heat treatment. In the light of recent observations of a dry heat method which was shown to be effective in preventing infection in chimpanzees but resulted in hepatitis in animals and haemophiliacs, in vitro observations of efficacy can no longer be regarded to be critical enough and therefore are not reviewed (258).

Haemophiliacs treated with a dry heat treated factor VIII concentrate at 60°C for 30 hours and 72 hours have a higher incidence of NANB hepatitis (257). Treatment with
chloroform carries no additional benefit (257). Heat treatment in the presence of solvent (n heptane) using plasma screened for raised ALT activity appears to be safe but is not effective if plasma with raised ALT levels is used (259).

Preliminary studies with dry heated concentrate at 80°C for 72 hours appear to be safe but the numbers of patients treated meeting the International Committee on Thrombosis and Haemostasis criteria is small (n=13) (257). Heat treatment in the presence of steam appears to prevent NANB hepatitis but not hepatitis B (260). Wet heat treatment for 10 hours at 60°C also appears to be effective in preventing acute NANB hepatitis (257).

HIV-1 inactivation

HIV-1 is a heat sensitive virus. It has been recommended that the duration and amount of treatment applied should aim at a reduction of viral infectious dose by 5 log\(_{10}\) infectious HIV-1 doses/ml (261). Heat inactivation studies show that when a one in ten dilution of cell culture medium of HIV-1 propagated in H-9 cells with a infectivity titre of > 10\(^5\) is added to liquid factor VIII concentrate and heated to 60°C one log\(_{10}\) is inactivated in about 30 seconds (262,263). The rate of thermal decay of wet heat is consistent with first order kinetics (263). In the liquid state, thermal decay is not affected by the liquid matrix or the presence of
stabilizers (263). Dry heat required 32 mins. Heating at 60°C to reduce infectious dose by ten fold (1 log) (263). Longer intervals however have been reported by others. Prince reported that heating lyophilized factor VIII concentrate at 60°C contaminated with a 1 in 10 volume of HIV-1 propagated in a H-9 cell line resulted in 0-1 log₁₀ inactivation at 10 hours and between 2-4 log₁₀ at 72 hours (262). Levy et al also showed a 2-5 log₁₀ inactivation of HIV-1 after 24 hours heating at 68°C (264). Lyophilisation alone had a minimal virucidal effect reducing infectious dose by 0.5-1 log₁₀ (264).

These findings are in keeping with results of prospective surveillance studies of HIV-1 seroconversions with the use of heat treated factor concentrates (257). Dry heat treatment at 60°C for 30 hours (in the absence of donor screening) is inadequate in conferring protection against HIV-1 infection, whereas heat treatment at 68°C for 72 hours (in the absence of donor screening) has not resulted in HIV-1 seroconversions (257). HIV-1 seroconversions have not been reported with pasteurized concentrates (257).

1.9.2 Physical Exclusion

In vitro studies of a purified factor VIIIC concentrate (purity=5iu/mg) prepared by immunoaffinity chromatography using a mouse monoclonal antibody to von Willebrand factor have shown that this method in addition to
removing other plasma proteins is effective in reducing HIV-1 log_{10} titre of added infectious virus by at least 4 log_{10}. A similar reduction occurs for the model viruses Sindbis, Pseudorabies and Vesicular stomatitis virus. Additional dry heat treatment at 60°C for 30 hours inactivates a further 3 log_{10} of HIV-1 (115).

Although availability of such a pure concentrate would appear to be ideal treatment in haemophilia the clinical value of such a preparation is unclear. Preliminary uncontrolled observations suggest that in HIV-1 antibody positive patients the rate of CD4+ve T-cell depletion may be reduced. This, however, requires to be substantiated (269). Initial clinical studies also indicate that the risk of acquiring hepatitis after a first infusion is less than products heated in the lyophilized state at less than 80°C (257). None of the 25 previously untreated patients receiving this product have acquired hepatitis (265).

1.9.3 Chemical Inactivation

Lipid enveloped viruses can be inactivated by a combination of solvent and detergent (Tween 80/ether) without additional heat treatment (266). Use of this combination is however limited as the detergent is difficult to remove after sterilization and ether is difficult to handle on a large scale. An alternative and more acceptable combination is tri(n-butyl) phosphate (TNBP) and sodium cholate, this mixture readily
inactivated HIV-1, hepatitis B and the Hutchison strain of NANB hepatitis virus, in vitro (267). However, one putative strain of the NANB virus is resistant to chloroform treatment and is unlikely therefore to be susceptible to inactivation (268).

After sterilization both TNBP and sodium cholate can be easily removed from treated concentrate. The yield of factor VIII is high and the process is not associated with protein denaturation, however, electrophoretic mobility of some lipoproteins is altered (269). Initial primate studies confirm the in vitro observations and factor VIII concentrates treated with this mixture have been licensed by the USA Federal Drug and Food Administration (267). Preliminary human studies show that concentrate treated with TNBP/sodium cholate is not associated with acute NANB hepatitis (270).

Beta propionlactone with ultraviolet radiation inactivates HIV-1, hepatitis B and the NANB viruses (271-272). This process of sterilization has been used for several years in Germany. It is limited by the potential oncogenic effects of beta propionolactone which preferentially reacts with DNA or RNA by alkylation but does not affect the biological activity of coagulation proteins (272). No HIV-1 seroconversions have been reported with concentrate sterilized with this method (272).
1.10 Bio-free recombinant factor concentrates

Investigators working independently at two separate companies, Genentech and Genetics Institute, have succeeded in isolating and cloning the gene for factor VIII (6-9). The gene has been found to be 186 000 kilobases long and the coding information for circulating factor VIII is spread among 26 exons. The extreme length of the gene and necessity for extensive post translational modification discouraged the use of recombinant bacteria, such as E.coli for manufacture of this protein and thus initial attempts to produce the protein in culture systems have been with mammalian cells. Recombinant factor VIII has been successfully produced in Chinese hamster ovary cells transfected with the coding sequence human factor VIIIC. Factor VIII is then purified to homogeneity by affinity chromatography with a mouse monoclonal antibody to human factor VIII and ion exchange chromatography. The recombinant protein has been shown to be fully functional in that it corrects the defect caused by the missing protein in haemophiliac plasma and, when injected into haemophiliac dogs, has been found to correct the clotting defect and to circulate with a half-life similar to that of native factor VIII. Thus, the evidence accumulating suggested that bio-engineering factor VIII is equivalent to the blood derived protein.
Progress is also being made on bioengineered factor IX. The gene for factor IX has been cloned and several biotechnology companies are trying to develop the bioengineered factor IX product (59). Bioengineered factor IX has an additional complication in that it requires gamma carboxylation of a glutamyl side chain. Because factor VIII deficiency is nine times more prevalent in the population than factor IX deficiency, bioengineering factor VIII is more viable commercially and is thus predicted to be available at an earlier date. There are no definite predictions as to when such products will be widely available; most investigators indicate that a several years' delay is probable.
AIMS OF THESIS:

Background:
Clinical and laboratory evidence of impaired cell mediated immunity has been frequently reported in haemophiliacs treated with clotting factor concentrates. Potential causes include:

1. The viruses transmitted via clotting factor concentrates.
2. A direct chemical "effect" of clotting factor concentrates on the immune system.
3. Alternatively, the frequent extraneous protein load of alloantigens infused may be responsible.

See Figure Aims of Thesis.

Impaired cell mediated immunity may arise due to:

1. Impaired T-helper/inducer cell activity, this may due to quantitative or qualitative changes.
2. Excessive T-suppressor/cytotoxic cell activity, either quantitative or qualitative.
3. Impaired function of monocyte/macrophages.

AIMS

The aims of this thesis were:

1. To study in vivo and in vitro cell mediated immunity in haemophiliacs
2. To determine influences of each of the potential causative factors.
3. To study the immune response and effects of the human immunodeficiency virus - 1 on cell mediated immunity in haemophiliacs.
These aims were achieved by studying prospectively a well defined cohort of haemophiliacs treated with clotting factor concentrates and:

1. Assessing the in vitro effects of clotting factor concentrates on peripheral blood mononuclear cells from immunocompetent individuals (Chapter 3).

2. Establishing the extent of exposure to HIV-1 and determining risk factors for infection in this cohort (Chapter 4).

3. Measuring the presence of HIV-1 antigen and correlating this to the antibody response. In addition, antibody dependent cell mediated cytotoxicity was measured to the envelope protein, gp120 of HIV-1 (Chapter 5).

4. In chapter 6 the impact of HIV-1 infection in haemophilia is discussed and evidence for clinical immunodeficiency was sought in HIV-1 antibody negative patients.

5. In vivo cell mediated immunity was measured in Chapter 7.

6. Quantitative changes in the immunoregulatory ratio were measured (Chapter 8).

7. Qualitative measurements of lymphocyte function were made and the effects of HIV-1 proteins on the immune system in vitro were established (Chapter 9).

8. The capacity to produce the lymphokine interleukin 2 was also measured (Chapter 10).
B cell function is partly controlled by T cells and was therefore assessed (Chapter 11)
CHAPTER 2 Patients and analysis

2.1 The West of Scotland Adult Haemophilia Centre
The patient population studied in this thesis was recruited from the West of Scotland Adult Haemophilia Reference Centre. The Centre serves a population of three million and is responsible for confirming the diagnosis of haemophilia and management of registered patients.

2.2 Treatment Policies and Clinic Visits
2.2.1 The treatment policies of the Centre for acute bleeding episodes are summarized in Table 2.1.1

2.2.2 Clinic Visits:
Prior to 1981 registered patients were seen as bleeding episodes occurred or when haemophilia associated medical problems arose. Patients attended the clinic without prior notice and were seen by the doctor attached to the Unit.

In 1981 all registered patients were seen by me to assess the severity of joint disease. The results and indicated the need for a review clinic. A separate clinic was established, attendance at this clinic was in addition to visits for acute bleeding episodes. The aims of this clinic were:

1. To identify medical problems.
Table 2.2.1. Treatment policy of the Unit.

<table>
<thead>
<tr>
<th>Deficiency</th>
<th>Severity</th>
<th>Type of Product</th>
</tr>
</thead>
<tbody>
<tr>
<td>Factor VIII</td>
<td>Severe/moderate</td>
<td>Factor VIII concentrate</td>
</tr>
<tr>
<td>Factor VIII</td>
<td>Mild</td>
<td>Cryoprecipitate or DDAVP</td>
</tr>
<tr>
<td>Factor VIII with inhibitor</td>
<td>Low titre of inhibitor</td>
<td>High dose factor VIII concentrate</td>
</tr>
<tr>
<td>Factor VIIIC with inhibitor</td>
<td>High titre of inhibitor</td>
<td>FEIBA or factor IX concentrate or porcine factor VIII</td>
</tr>
<tr>
<td>Factor IX</td>
<td>Severe/moderate</td>
<td>Factor IX concentrate</td>
</tr>
<tr>
<td>Factor IX</td>
<td>Mild</td>
<td>Fresh frozen plasma</td>
</tr>
</tbody>
</table>

FEIBA - factor VIII bypassing activity (Immuno Austria)
Porcine factor VIII - used was the highly purified polyelectrolyte porcine preparation (Hyate:C, Speywood Laboratories, UK).
DDAVP - des arginine aminovasopressin (dose 0.3µg per kg body weight).

The dose of blood product used was determined by:
(i) patient weight
(ii) nature and size of bleed
(iii) previous response.
2. To treat chronic musculoskeletal problems identified in the cross-sectional study. It had been previously found that such problems were difficult to manage during an acute bleeding episode.

3. To educate patients on preventive aspects of bleeding episodes.

4. To provide a genetic counselling service for patients and relatives.

5. To make available and advise on the availability of social services the patients were eligible for.

6. To encourage regular attendance at the dental clinic for preventive treatment.

7. In 1982 following reports of the Acquired Immunodeficiency Syndrome, to monitor the clinical and immunological outcome of selected patients (273)

All patients with chronic problems were asked to attend every six months and were seen by me and Dr G D O Lowe.

At each clinic visit bleeding problems that had arisen were discussed, in addition to other medical problems. All patients were examined, and blood withdrawn for the following:

1. Full blood count and differential (Coulter S).

2. Liver function tests which included serum bilirubin, alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase, albumin and total globulins (Technicon Autoanlyser)
3. Hepatitis B serology - hepatitis B surface antigen was measured at each clinic visit and the presence of hepatitis B surface antibody measured in 1982 and 1987/8 (Abbott Diagnostics, Illinois, USA).

4. If indicated, factor VIIIC/IX levels were checked (one stage assay, based on the activated thromboplastin time) and plasma screened for an inhibitor (274, 275).

2.3 Study Population

All patients reported in this thesis had therefore been seen regularly by me since 1981. From this population was drawn a cohort who were prospectively studied from 1982 and retrospectively from 1977-1982. Criteria for recruitment into prospective studies included:

I Inclusion Criteria:

1. Patients with factor VIIIC or IX deficiency who had been treated with a blood product from this Centre during or after 1980. Untreated patients were also included for comparison.

II Exclusion Criteria:

1. Patients who were only temporarily registered.
2. Patients who were registered at our centre but who had transferred to another centre prior to 1982.
3. Patients permanently registered at this centre since 1984.
4. Patients with factor VIIIvWF deficiency.
To confirm that retrieval of clinic records was accurate, the names of registered patients were checked against the annual patient returns for 1980 to 1982 (inclusive) provided by the Oxford Haemophilia Centre.

Of 169 permanently registered patients (42 factor IX deficient; 127 factor VIII:C deficient) 133 were recruited (100 factor VIII:C deficient and 33 factor IX deficient). The reasons for not recruiting the remaining 36 patients are summarized in Table 2.3.1

2.4 Demographic Characteristics of Recruited Cohort
The median age of the cohort was 30 years (range 16-76 years), Table 2.4.1 shows the ages of factor VIII:C and IX deficient patients.

100 were factor VIII:C deficient and 33 were factor IX deficient. The median factor deficiency was 2 percent (Interquartile range [IQR] 0-40 percent), Table 2.4.2. Of these patients 49 were on home treatment.

2.5 Number of patients with inhibitors:
Eight patients had a factor VIII:C inhibitor, seven had a severe deficiency and one was moderately deficient in factor VIII:C. Five were high responders and had required regular treatment with activated prothrombin complex concentrates or porcine factor VIII. Two had required activated prothrombin complex concentrates
intermittently. There were three low responders and in all three the inhibitor is now absent.

2.6 Use of blood products
For each patient recruited the total amount of clotting factor concentrate, cryoprecipitate or fresh frozen plasma used over seven years (1980-1986 inclusive) was recorded and a mean annual dose calculated and expressed as units of replacement therapy used per annum.

One unit of clotting factor activity was defined as the amount present in an international standard provided by the World Health Organization (WHO) and distributed by the National Institute of Biological Standards and Controls (London UK). The amount of clotting factor activity provided by the Scottish National Blood Transfusion Service (SNBTS) had been calibrated against the WHO standard.

Commercial factor VIII clotting concentrates had been purchased from several different manufacturers. It was assumed that the labelled activity was correct, although prior to 1982 discrepancies between labelled and actual activity had been noted (276, 277).

The units to indicate activity in activated prothrombin complex concentrates were those defined by the manufacturer. At this centre FEIBA was used during the study, the units had been assayed by measuring the
shortening of the partial thromboplastin time of a substrate inhibitor plasma by the manufacturer.

For cryoprecipitate the regional blood transfusion service had assumed that each plasma donation contained 100 iu of factor VIIIC activity.

The median mean annual dose of replacement therapy used per annum over the seven years was 9000 iu (IQR 1400 to 41,000). The median mean annual dose of replacement therapy used by factor VIIIC deficient patients was 8778.5 iu (IQR 1627 to 46728) and by factor IX patients: median 9500iu (IQR 1000 to 38,000) (p = 0.7).

2.6.1 Cryoprecipitate and fresh frozen plasma
All cryoprecipitate and fresh frozen plasma used was obtained from local donations of whole blood by the SNBTS.

2.6.2 Factor VIII concentrates
Figure 2.1.1 shows the use of factor VIII concentrates by this centre during the study period. Prior to 1984 a proportion of the concentrate used was fractionated from plasma donations obtained in the USA. The remainder was fractionated by the SNBTS from local donations, mean donor pool size was 1,0000 donations.

2.6.3 Factor IX concentrate (Prothrombin complex concentrates)
FIGURE 2.1.1a TOTAL AMOUNT OF FACTOR VIII CONTAINING MATERIAL USED AT THE WEST OF SCOTLAND ADULT HAEMOPHILIA UNIT.
FIGURE 2.1.1b TOTAL AMOUNT OF PROTHROMBIN COMPLEX CONCENTRATE USED

LOG 10 UNITS

1000000
100000
10000


YEAR

□ NHS FACTOR IX
□ FEIBA
All factor IX deficient patients requiring treatment received a concentrate fractionated from plasma donations obtained by the SNBTS (mean donor pool size 10,000 donations).

2.5.6 Activated Prothrombin Complex
Patients with high titre factor VIIIC inhibitor were usually treated with a commercially fractionated activated prothrombin complex concentrate (FEIBA Immuno, Austria).

2.5.5 Porcine factor VIII
After 1984 some patients were treated with a polyelectrolyte purified porcine factor VIII (Hyate C, Speywood Laboratories)

2.7 Liver Disease
Infection with hepatitis B and non-A, non B (NANB) viruses is common in haemophiliacs treated with clotting factor concentrates. Persistent infection with both types of virus results in chronic liver disease. Chronic liver disease due to hepatitis B is seen only in individuals who cannot clear antigen (72). No such markers are available for NANB and the severity of the liver disease can only be accurately assessed histologically. One study showed that a rise in serum immunoglobulin IgG is associated with progression to cirrhosis, however there was a considerable overlap, limiting its clinical utility in individual patients
As liver biopsy is a potentially hazardous procedure (2) and does not currently influence management, it was not performed in this centre. A clinical grading of liver disease severity was devised, based on clinical and biochemical findings since 1977. For each patient all available results of serum ALT were graphed and patients classified using the algorithm shown, Figure 2.7.1. The minimum number of serum ALT measurements per year for all patients was two.

Six grades of liver disease were identified:

**Grade 1:** Clinical and laboratory evidence of chronic liver disease in the absence of any other known cause. Clinical features sought included specifically hepatosplenomegaly indicative of portal hypertension, if clinically present this was confirmed by a radio-nucleotide liver and spleen scan. In patients who had presented with clinical features of upper gastrointestinal bleeding, endoscopic evidence of oesophageal varices was sought. Splenomegaly in the absence of hepatomegaly was not included. Ten patients were included in this group, their clinical features are shown in Table 2.7.1.

**Grade 2:** No clinical evidence of chronic liver disease but serum ALT persistently elevated above 150% of the upper limit of the laboratory normal (ALT>75IU/L) during the six years of prospective observation.
ALGORITHM USED TO CLASSIFY LIVER DISEASE

133 PTS.
> 2 ALT LEVELS
PER ANNUM

1

84
3

70
4

34
grade 3

36

26
grade 2

14
grade 4

22
grade 5

27
grade 6

49

2
1. Patients were divided into those with abnormal and normal serum ALT during the study.

2. Case notes of patients with normal ALT were reviewed and further divided into those with abnormal (Grade V) and normal (Grade VI) ALT prior to the study.

3. Patients with abnormal ALT were divided into those who developed NANB hepatitis during the study - Grade 4.

4. The remaining 70 patients were subdivided into those with persistently abnormal serum ALT (n=36) and those with intermittently abnormal serum ALT, (n=34), Grade 3.

5. Patients with persistently abnormal serum ALT were further subdivided into those with portal hypertension, (n=10) Grade 1 and those without, (n=26) Grade 2.
Table 2.7.1 Clinical features of patients with grade 1 liver disease.

<table>
<thead>
<tr>
<th>Clinical Features</th>
<th>Number of Patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hepatosplenomegaly</td>
<td>10</td>
</tr>
<tr>
<td>Decompensated cirrhosis</td>
<td>3</td>
</tr>
<tr>
<td>Hepatic encephalopathy</td>
<td>2</td>
</tr>
<tr>
<td>Oesophageal varices</td>
<td>3</td>
</tr>
</tbody>
</table>

10 patients had grade 1 liver disease. All three patients with oesophageal varices have had variceal bleeding. The two patients with hepatic encephalopathy have died, in one, investigation (hepatic ultrasound) indicated the presence of tumour deposits.
Grade 3: Patients who had no clinical evidence of chronic liver disease but in whom the serum ALT fluctuated from above 75IU/L to normal.

Grade 4: Patients infected with NANB hepatitis during the prospective period of study.

Grade 5: Patients with normal serum ALT during the previous six years but with biochemical evidence suggestive of NANB hepatitis prior to 1980.

Grade 6: Patients with normal serum ALT during the period of observation and no previous clinical or biochemical evidence of hepatitis.

Table 2.7.2 shows the characteristics of the patients with different grades of liver disease and Table 2.7.1 the clinical features of liver disease noted in patients with grade 1 liver disease.

2.8 Hepatitis B Infection

Hepatitis B surface antibody (AusAb, Abbott Diagnostics Division) was checked in all patients in 1981/2 and in 1987/8. Hepatitis B surface antigen was measured at each clinic visit (Auszyme Abbott Diagnostics Division). During the study period one new hepatitis B infection occurred. At the end of the study 55 patients had evidence of previous hepatitis B infection, of these five were chronic virus carriers Table 2.8.1.
Table 2.7.2 Characteristics of patients in liver disease groups.

<table>
<thead>
<tr>
<th>Grade of liver disease</th>
<th>No of Pts</th>
<th>Age (yrs)</th>
<th>Severity of disease</th>
<th>Mean annual dose of cfc</th>
<th>Antigen</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10</td>
<td>31</td>
<td>0</td>
<td>17423.5</td>
<td>(1)</td>
</tr>
<tr>
<td></td>
<td>(1)</td>
<td>(28-43)</td>
<td>(0-0)</td>
<td>(600-65000)</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>26</td>
<td>33.5</td>
<td>0.5</td>
<td>19557.5</td>
<td>(7)</td>
</tr>
<tr>
<td></td>
<td>(7)</td>
<td>(25-46)</td>
<td>(0-5)</td>
<td>(1400-45000)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>33</td>
<td>30</td>
<td>0</td>
<td>16912</td>
<td>(5)</td>
</tr>
<tr>
<td></td>
<td>(5)</td>
<td>(23-38)</td>
<td>(0-5)</td>
<td>(7724-46000)</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>13</td>
<td>29</td>
<td>8</td>
<td>4000</td>
<td>(5)</td>
</tr>
<tr>
<td></td>
<td>(5)</td>
<td>(25-51)</td>
<td>(5-10)</td>
<td>(1160-6210)</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>21</td>
<td>35</td>
<td>0</td>
<td>36353</td>
<td>(3)</td>
</tr>
<tr>
<td></td>
<td>(3)</td>
<td>(21-45)</td>
<td>(0-1)</td>
<td>(7387-78698)</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>23</td>
<td>28</td>
<td>5</td>
<td>1437</td>
<td>(7)</td>
</tr>
<tr>
<td></td>
<td>(7)</td>
<td>(20-51)</td>
<td>(4-20)</td>
<td>(638-2986)</td>
<td></td>
</tr>
</tbody>
</table>

The medians and interquartile ranges are shown. There was no significant age difference. Haemophiliacs with grades 4 and 5 liver disease had a milder severity of haemophilia (p=0.001, Kruskal Wallis), these patients had also used significantly less factor concentrate per annum over the previous seven years (p=0.003, Kruskal Wallis).
Table 2.8.1 The numbers of patients with serum markers of hepatitis B infections.

<table>
<thead>
<tr>
<th></th>
<th>1981/2</th>
<th>1986/7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hepatitis B Ab</td>
<td>65</td>
<td>66</td>
</tr>
<tr>
<td>Hepatitis B Ag</td>
<td>4</td>
<td>5</td>
</tr>
</tbody>
</table>

5 patients were persistent carriers of hepatitis B infection.

All had factor VIIIC deficiency.
2.9 Materials and Methods
The details of materials and experimental methods used in clinical and laboratory investigations are reported in appropriate chapters.

2.10 Statistical Methods:
All data was analyzed using non parametric statistical methods (278).

2.10.1 Inter-Group Comparisons
The Mann-Whitney U test was used to analyse data between two independent groups. Only two tailed p values were considered significant. The Kruskal-Wallis one way analysis of variance test was used to examine for possible differences when there were more than two independent groups.

Fisher's exact test (two tailed) was used for comparisons between dichotomous data between groups.

2.10.2 Intra-group comparisons
The Wilcoxon rank sum test for paired data was used to analyse sequential continuous data obtained from single groups.

2.10.3 Correlations
The Spearman Rank correlation analysis was used to determine the association between continuous variables.
To quantify the relationship between two continuous variables regression analyses were used. Logistic stepwise regression was used to examine the relationship between several independent variables (either dichotomous or continuous) to a dichotomous dependent variable. Logistic regression rather than multiple regression was more suited to the analyses of the data for the following reasons:

1 A qualitative dependent variable could be used.
2 For a continuous dependent variable no prior assumption on its distribution was required.

The data was analyzed using a Statgraphics package version 2.1 (Statistical Graphics Corporation USA).
CHAPTER 3.1

DO CLOTTING FACTOR CONCENTRATES HAVE A DIRECT "CHEMICAL" EFFECT ON LYMPHOCYTE FUNCTION IN VITRO.

Summary:
Study objective: To compare the effects of intermediate purity factor VIII, factor IX and a highly purified factor VIII concentrate on lymphocyte activation, proliferation, and interleukin 2 (IL2) production in vitro.

Measurements: Peripheral blood mononuclear cells (PBMC) from normal donors, were stimulated to proliferate with specific and non-specific activators in the presence of clotting factor concentrates. The capacity of normal PBMC to produce IL-2 in the presence of clotting factor concentrates was evaluated using a bioassay.

Results: Intermediate purity factor VIII concentrate impaired PBMC activation and proliferation in a dose dependent manner. IL-2 production was also reduced. The highly purified factor VIII concentrate like factor IX had no such effects.

Conclusions: Intermediate purity factor VIII concentrates have a direct chemical immunosuppressive effect. This may lead to in vivo impaired cell mediated immunity and predisposed some haemophiliacs to HIV-1 infection.
3.1.1. INTRODUCTION

Haemophiliacs depend on transfusions of blood products, such as cryoprecipitate and clotting factor concentrates to treat bleeding episodes. The use of such blood products is associated with a high incidence of viral infections such as non A, non B agents (NANB), and the human immunodeficiency virus (HIV-1).

The frequent use of clotting factor concentrates may sensitize some haemophiliacs to allogenic proteins. For example, 10 to 15 percent of severe haemophiliacs develop IgG antibodies (inhibitors) to factor VIIIC (233), and up to 50 percent of haemophiliacs have antibodies to the Km and Gm determinants of IgG (245). Sensitization to C4, complement allotypes also frequently occurs(246). Haemophiliac serum also contains immune complexes and depending on the size of the immune complex, immune stimulation as well as suppression can result (247-251).

Moreover the use of blood and blood products is also known to be immunosuppressive. For example, transfusion of blood and cellular blood products are standard practice in preparation of uremic patients for transplantation. Oplez et al showed that renal allografts survived longer in patients with prior transfusions, this correlates with the degree of immunosuppression (279-281). Conversely, in bone marrow
transplantation pre-transfusion is associated with a poor outcome (282). Furthermore, use of blood during surgical resection of tumours results in an increased mortality and risk of metastatic seeding. This is independent of histological type or clinical grade of the tumour but depends on the type of blood product used (283-290). In controlled animal experiments infusions of allogenic plasma but not cellular blood products promote chemically induced sarcoma growth (291-292). In autoimmune diseases the replaced plasma during plasmapheresis shows a beneficial effect greater than would be expected from the removal of immune complexes alone and in patients with idiopathic thrombocytopenia purpura the possibility that an immunosuppressive factor is present in preparations of human gammaglobulin has been raised (293-300).

A single blood transfusion activates both cellular and humoral immune reactions. The beneficial effect in renal transplantation is mediated by both cellular suppressive and protective antibody responses (281). An unidentified component of plasma is implicated in the tumour promoting effects of transfusion (301-304).

A haemophiliac therefore can potentially develop impaired cell mediated immunity due to the viruses transmitted by blood products, but also by factor concentrates having a direct "chemical" effect on the immune system.
To examine the latter possibility the effects of clotting factor concentrates on lymphocyte function in vitro were investigated.

3.1.2 METHODS

Heparinised peripheral venous blood (20 units preservative free heparin/mls, Leo Laboratories) was obtained from healthy heterosexual, male non-haemophilic volunteers aged between 19 to 35 years.

Cell separation

Peripheral blood mononuclear cells (PBMC) were separated by density centrifugation of heparinised venous blood using a modification of the method originally described by Boyum (305). Blood was diluted in a 1:1 ratio with Eagles' Minimum Essential Medium (MEM) and layered on to a sodium metrizoate gradient (Lymphoprep, Nyegarrad and Co, Oslo, Norway) in 15mm plastic conical tubes (diameter 12mm) (Costar, Cambridge, Massachusetts, USA), in a 1:1 ratio. Following centrifugation at 400xg for 30 minutes, the mononuclear cell bands at the Lymphoprep/plasma interface were aspirated using 2mls plastic pipettes and pooled in plastic conical tubes. The cells were washed twice in MEM by centrifugation at 600xg for 5 mins, and the cell pellet resuspended in 0.9 mls of cell culture medium. A 10ul aliquot was removed, stained with 90ul of white cell staining fluid (2% glacial acetic acid, and a few grains of crystal violet in distilled water) and the number of cells in
10ul counted in a standard haemocytometer (Improved Neubauer). The cell suspension was placed on ice until used.

Factor concentrates
Heat treated and non heat treated intermediate purity factor concentrates VIII and IX (IPFC VIII/IX) were obtained from the Scottish National Blood Transfusion Service. Pure human factor VIIIC isolated from the factor VIII complex by a mouse monoclonal antibody to human factor VIIIvWF and stabilized with 5% human albumin (Monoclate, Armour Pharmaceutical Co [MC]) was compared to the IPFC preparations.

All materials were reconstituted according to manufacturers' directions, cell culture medium was used for subsequent dilutions of the reconstituted preparation.

Preparation of agents used to stimulate lymphocytes
Both the non specific T-cell mitogens, phytohaemagglutinin (PHA) (Sigma, Poole, UK) and concanavalin A (Con A) (Sigma, Poole, UK) and the specific soluble antigen (PPD) were used. Final concentrations of these stimulants for optimal lymphocyte proliferation had been previously standardized. PHA and Con-A were used in a concentration of 50ug/ml per 1x10^6 cells/mls respectively. PPD was used in a final
concentration of 0.5ug/ml and 5.0ug/ml per 1x10^6 cells/mls.

Functional Assays

Proliferative responses were measured by culturing PBMC in 96 well, round bottomed, micro-titre plates (Gibco, Paisley, UK). Each well contained PBMC at the above concentrations, mitogen or antigen and one of the following:

1. Non heat treated IPFC (VIII).
2. Non heat treated IPFC (VIII) standardized for citrate concentration.
3. Heat treated IPFC (VIII).
4. Pure factor VIII in albumin which had been heat treated (MC).
5. Non heat treated IPFC (IX).
6. No factor concentrate.
7. Factor concentrate in the absence of mitogen/antigen.

The clotting factor concentrates were used at the following concentrations: 0.5iu/mls, 0.05iu/mls, and 0.005iu/mls. These concentrations are similar to those achieved in vivo after factor concentrate infusion.

The cell culture medium was Rowell Park Memorial Institute medium (RPMI-1640) containing 10 % pooled human serum supplemented with 50iu/ml penicillin, 100ug/mls streptomycin, and 2mmol/l L glutamine (Flow Laboratories, Irvine, Scotland). All cultures were
performed in triplicate at $37^\circ$C in a 5\% CO$_2$ enriched humidified environment for 3 days. For mitogens and PPD stimulated cultures were maintained for 4 days.

Proliferative responses were measured by the incorporation of $[^3]$H thymidine after a four hour pulse with 1uG of $[^3]$H thymidine (for PPD cultures cells were pulsed for 18 hr). Cultures were harvested on an automatic cell harvester (Skatron, Flow Laboratories, Irvine, UK) by suction through glass fibre paper. The paper was washed with 5\% methanol and trisodium acetic acid, allowed to dry and transferred to scintillation vials. After addition of scintillation fluid, (toluene [Proanalyar analytical May & Baker] 2,5 diphenyloxazole [Scintillation grade Packard]), incorporated $[^3]$H thymidine was measured on a Beta counter (LKB, Croydon, UK).

In further experiments PBMC in culture medium were pre-incubated with IPFC (VIII) and washed prior to addition of PHA or with PHA prior to addition of IPFC (VIII) at $37^\circ$C in a 5\% CO$_2$ enriched humidified atmosphere.

**Effect of factor concentrates on an interleukin-2 (IL-2) dependent cell line**

$1 \times 10^6$ Jurkat cells (an IL-2 dependent lymphoblastoid cell line) were incubated in the presence and absence of heat treated IPFC (VIII), or MC at the above concentrations,
proliferative response was measured after 2 days by measuring amount of [\(^3\)H] thymidine incorporated.

**Cell viability**

1\(\times\)10\(^6\) cells/ml in cell culture medium and IPFC (VIII) at a concentration of 5iu/ml were maintained for three days in a humidified atmosphere supplemented with 5% CO\(_2\) at 37°C and cell viability assessed as for counting experiments except cells were stained with Trypan blue, 100 cells were counted and the number of stained cells (dead) subtracted to give a percent viability.

**IL-2 secretion and assay**

1\(\times\)10\(^6\) PBMC from normal were incubated in complete RPMI/2% fetal calf serum (FCS) with or without factor VIII concentrates as described for functional assays in the presence of PHA (2 ug/ml). After 48 hours, the supernatant was separated from the cells by centrifugation at 500xg, and stored at -20°C until assayed.

Supernatant IL-2 activity was measured in a standard biological assay using the HT\(_2\)A cells (Cetus Corp, California, USA).

**IL-2 assay**

A bioassay was used to measure supernatant IL-2 production, using an IL-2 dependent cell line and measuring the increase in cell proliferation (306).

Cloned, murine, T-helper cells (HT2 cell line) (Cetus Corp., California, USA) maintained in RPMI 1640
containing 5% heat inactivated FCS supplemented with exogenous IL-2 for continued growth. The cell line was subcultured at a cell density of $4 \times 10^4$ cells/mls and fed with 20 units/ml human recombinant IL-2 (rIL-2) (BCL) every 48hrs in RPMI 1640/10% FCS and $2 \times 10^{-5}$ mercaptoethanol (complete medium).

48hrs after feeding with IL-2, the cells (HT2) were washed twice by centrifugation in RPMI 1640 and viability assessed by Trypan blue exclusion. $1.5 \times 10^5$ HT2 cells were resuspended in complete medium. 100ul of the cell suspension was dispensed into flat bottomed 96 well plates (Nunc. Gibco Ltd, Paisley, UK) to which serial solutions (100ul) of the supernatant of varying concentrations of standard (rIL-2)) were added in triplicate. After approximately 24hrs incubation, the plates were pulsed with $\text{I}u\text{C}^\text{3H}$ thymidine (20ul of 50uG/ml per well) and incubated for a further 4 hours. The plates were harvested onto glass fibre filters using a cell harvester (Skatron, Flow Labs, Irvine, UK) and the radioactivity incorporated into DNA determined by liquid scintillation counting.

Serial two-fold dilutions of 100u/ml recombinant IL-2 final concentration was used to obtain a standard curve.

Analysis of results
The results were expressed as units/mls IL-2 (u/ml) by first subjecting the data to probit analysis (to
determine the relationship between the frequency of occurrence of specific events and the dose that is responsible for the induction of those events). The results were determined at the 50% maximal \(^{3}\text{H}\) thymidine incorporation end point of our laboratory standard.

3.1.3 RESULTS

Lymphocyte proliferation in the presence of intermediate purity factor VIII concentrate.

PHA stimulates all T-cell subsets, whereas Con-A is a specific T-suppressor cell mitogen and PPD stimulates a distinct functional subset of T-helper lymphocytes that can be identified with a Leu 8 antibody (307,308). Figure 3.1.1 shows that non heat treated IPFC (VIII) at concentrations, (0.005-0.5 units/ml) significantly impaired lymphocyte proliferation. The effect was independent of the stimulus used for proliferation. Therefore in subsequent experiments only PHA was used. Figure 3.1.1 also shows that the effect of IPFC (VIII) was dose dependent and Table 3.1.2 shows that IPFC (VIII) did not shift the maximum proliferative response.

The impaired proliferation could be due to (a) a direct effect of IPFC (VIII) on lymphocytes; (b) binding of IPFC (VIII) to PHA; (c) non specific binding of IPFC (VIII) to cells; or (d) a cytotoxic effect of IPFC (VIII) on lymphocytes.
Figure 3.1.1 The Effect of IPFC (VIII) on Lymphocyte Transformation

Counts Per Minute

Log Dose Factor (VIII)
Figure 3.1.1. Median values for the three stimulants are shown. Significant impairment of lymphocyte proliferation occurred at all concentrations.
Table 3.1.1 The effect of IPFC (VIII) on lymphocyte transformation.

<table>
<thead>
<tr>
<th>Stimulant</th>
<th>Dose (units/ml)</th>
<th>0</th>
<th>0.005</th>
<th>0.05</th>
<th>0.5</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>PHA</td>
<td></td>
<td>48230</td>
<td>280068</td>
<td>18287</td>
<td>5018</td>
<td>90.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(24787-</td>
<td>(19170-</td>
<td>(11192-</td>
<td>(3049-</td>
<td>(51-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>79618)</td>
<td>33035)</td>
<td>26237)</td>
<td>16038</td>
<td>143</td>
</tr>
<tr>
<td>CON-A</td>
<td></td>
<td>29152</td>
<td>30291</td>
<td>18396</td>
<td>3090</td>
<td>135</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(23190-</td>
<td>(23465-</td>
<td>(12676-</td>
<td>(3049-</td>
<td>(104-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>33366)</td>
<td>33035)</td>
<td>19664)</td>
<td>5212</td>
<td>143</td>
</tr>
<tr>
<td>PPD</td>
<td></td>
<td>22551</td>
<td>20858</td>
<td>11192</td>
<td>10357</td>
<td>52</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(1202-</td>
<td>(13768-</td>
<td>(4140-</td>
<td>(4824-</td>
<td>(46-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>74538)</td>
<td>29278)</td>
<td>17978)</td>
<td>25144</td>
<td>66.6</td>
</tr>
</tbody>
</table>

The median and interquartile ranges for lymphocyte proliferation in counts per minute are shown. At >0.05 units/ml IPFC (VIII) significantly impaired lymphocyte proliferation compared to baseline (p<0.05). No inhibition was observed at 0.005 units/mls.
Table 3.1.2  Kinetics of IPFC (VIII) inhibition of lymphocyte proliferation.

<table>
<thead>
<tr>
<th>Length of culture (hrs)</th>
<th>PHA</th>
<th>PHA + (IPFC VIII)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td><strong>Median</strong></td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>Interquartile Range</strong></td>
</tr>
<tr>
<td>24</td>
<td>83.65</td>
<td>28.65</td>
</tr>
<tr>
<td></td>
<td>(68.3-121.65)</td>
<td>(24.3-34.4)</td>
</tr>
<tr>
<td>48</td>
<td>12783</td>
<td>1170.5</td>
</tr>
<tr>
<td></td>
<td>(10815.9-14543.2)</td>
<td>(984-1336)</td>
</tr>
<tr>
<td>72</td>
<td>36830</td>
<td>1232</td>
</tr>
<tr>
<td></td>
<td>(34086-38369)</td>
<td>(970.5-1583)</td>
</tr>
<tr>
<td>120</td>
<td>17673</td>
<td>1100</td>
</tr>
<tr>
<td></td>
<td>(14714.5-22908.5)</td>
<td>(1080-1243)</td>
</tr>
<tr>
<td>168</td>
<td>4188.5</td>
<td>1963</td>
</tr>
<tr>
<td></td>
<td>(2575-5788.5)</td>
<td>(1642-2573)</td>
</tr>
</tbody>
</table>

The median and interquartile ranges of cell proliferation in counts per minute are shown of 7 experiments. IPFC (VIII) and PHA were added at time 0 and cells harvested at the times shown. IPFC (VIII) significantly impaired lymphocyte proliferation at all times (p<0.05). IPFC (VIII) was used at a concentration of 0.5 units/mls.
Cell viability after pre-incubation with IPFC (VIII) for 36 hours or in its absence showed IPFC (VIII) did not have a direct cytotoxic effect as judged by Trypan blue exclusion, Table 3.1.3.

To exclude binding of IPFC (VIII) with lymphocytes, PBMC were incubated with IPFC (VIII) for 12 hours, washed and then stimulated with PHA. Figure 3.1.2 shows that significant suppression still occurred. Pre-stimulation with PHA and then adding IPFC (VIII) did not impair cell proliferation, Figure 3.1.3.

In further experiments the possibility that Tris/0.02M trisodium citrate, the buffer used in preparation of IPFC (VIII) was excluded as the cause of inhibition. Figure 3.1.4 and table 3.1.4 shows effects of IPFC (VIII) were independent of citrate content.

A comparison between IPFC, heat treated IPFC (VIII) and MC

Figure 3.1.5 and Table 3.1.5 shows the effect of a dry heat treated IPFC (VIII) was similar to a non heat-treated preparation at all concentrations. MC had no significant effect on lymphocyte proliferation in equivalent concentrations to heat treated IPFC (VIII), Figure 3.1.5. At 0.5 units/ml MC caused less inhibition than IPFC (VIII).

Effect of IPFC (IX)
FIGURE 3.1.2 PREINCUBATION OF LYMPHOCYTES WITH IPFC (VIII)

Counts Per Minute

Time (min)

0
10
20
30

40000
30000
20000
10000

Control
Figure 3.1.2. Median values are shown. PBMC were preincubated with IPFC (VIII) and stimulated with PHA at times shown. 0 values indicate proliferation in absence of IPFC (VIII). IPFC (VIII) used at 0.5 units/ml. Significant impairment occurred at all times (p<0.05).
Table 3.1.3  The effect of IPFC (VIII) on cell viability.

<table>
<thead>
<tr>
<th></th>
<th>Cell viability (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBMC</td>
<td>85 (63-90)</td>
</tr>
<tr>
<td>PBMC + IPFC (VIII)</td>
<td>86 (56-98)</td>
</tr>
</tbody>
</table>

The medians and interquartile ranges of percent viable cells after 36 hours incubation in the absence and presence of IPFC (VIII) are shown. There was no significant difference. A total of six experiments were performed.
FIGURE 3.1.3 TIME COURSE OF LYMPHOCYTE PROLIFERATION

Counts Per Minute

IPFC (VIII)

Control

Time of IPFC (VIII) addition (hours)
Figure 3.1.3. Median values are shown. PBMC preincubated with PHA and 0.05 units/ml IPFC (VIII) added at times shown. No inhibition occurred after time 0.
FIGURE 3.1.4 CITRATE IONS DO NOT AFFECT LYMPHOCYTE PROLIFERATION
Figure 3.1.4. Median values are shown. IPFC (VIII) reconstituted according to manufacturers instructions, all subsequent dilutions made with RPMI with added citrate ions. There was no significant difference.
A t doses >0.05 units/mls both IFP (VIII) impaired lymphocyte proliferation. Monoclate however had no effect except at doses >0.5 units/mls. The effect at 0.5 units/mls was significantly compared to IFP (VIII) p<0.05.

The experimental lymphocyte proliferation was compared to basal stimulation in the absence of concentrate. All values were compared to basal stimulation. The median and interquartile ranges are shown of ten experiments. *p<0.02.

Table 3.1.4 Comparison of different Factor VIII concentrates.

<table>
<thead>
<tr>
<th>Concentrate</th>
<th>IFP (VIII) (Heat)</th>
<th>IFP (VIII) (Non Heat)</th>
<th>IFP (VIII) (Heat)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentrate</td>
<td>IFP (VIII) (Heat)</td>
<td>IFP (VIII) (Non Heat)</td>
<td>IFP (VIII) (Heat)</td>
</tr>
<tr>
<td>0.5</td>
<td>1.49</td>
<td>1.0</td>
<td>0.05</td>
</tr>
<tr>
<td>0.14</td>
<td>1.4</td>
<td>1.0</td>
<td>0.05</td>
</tr>
<tr>
<td>0.05</td>
<td>1.4</td>
<td>1.0</td>
<td>0.05</td>
</tr>
<tr>
<td>0.05</td>
<td>1.4</td>
<td>1.0</td>
<td>0.05</td>
</tr>
<tr>
<td>0.05</td>
<td>1.4</td>
<td>1.0</td>
<td>0.05</td>
</tr>
<tr>
<td>0.05</td>
<td>1.4</td>
<td>1.0</td>
<td>0.05</td>
</tr>
<tr>
<td>0.05</td>
<td>1.4</td>
<td>1.0</td>
<td>0.05</td>
</tr>
<tr>
<td>0.05</td>
<td>1.4</td>
<td>1.0</td>
<td>0.05</td>
</tr>
<tr>
<td>0.05</td>
<td>1.4</td>
<td>1.0</td>
<td>0.05</td>
</tr>
<tr>
<td>0.05</td>
<td>1.4</td>
<td>1.0</td>
<td>0.05</td>
</tr>
<tr>
<td>0.05</td>
<td>1.4</td>
<td>1.0</td>
<td>0.05</td>
</tr>
</tbody>
</table>
Figure 3.1.5 A COMPARISION OF IPFC(VIII) AND HIGHLY PURIFIED FACTOR CONCENTRATES

Counts Per Minute

Log Dose of Factor Concentrate
Figure 3.1.5. Median values are shown. Dry heated non-heated IPFC (VIII) impaired PBMC proliferation with PHA. MC at equivalent concentrations did not impair PBMC proliferation except at >0.5 units/ml. Values compared to basal proliferation in absence of concentrate, less inhibition occurred with MC at 0.5 units/ml compared to IPFC (VIII) (p=0.02).
IPFC(IX) caused no inhibition of lymphocyte proliferation, Figure 3.1.6.

The Effect of clotting factor concentrates on IL-2 production.

One mechanism by which IPFC (VIII) may impair lymphocyte proliferation is by reducing IL-2 production or affecting the IL-2 response. This series of experiments shows effects of factor VIII concentrates on IL-2 production. Figure 3.1.7 shows that IPFC (VIII) but not MC had a dose dependent effect on proliferative capacity of Jurkat cells.

The ability of IPFC (VIII) to reduce IL-2 production was further investigated by examining the potential of IPFC (VIII) and MC to reduce IL-2 secretion from PHA stimulated lymphocytes, Figure 3.1.8. IPFC (VIII) significantly reduced IL-2 production whereas MC had no significant effect.

3.1.4 DISCUSSION

Cell mediated immunity, as measured in vivo by skin testing and in vitro by counting T-cell subsets has been frequently reported to be impaired in haemophiliacs treated with clotting factor concentrates (310-316). This could be due to human immunodeficiency virus-1 (HIV-1) infection, or to other viruses (eg NANB viruses). Alternatively IPFC (VIII) in the absence of these
FIGURE 3.1.6 IPFC (IX) - EFFECT ON LYMPHOCYTE TRANSFORMATION

Counts Per Minute

Log Dose (Units/ml)
Figure 3.1.6. Median values are shown. IPPC (IX) dry heated impaired PBMC proliferation at 5 units/ml only.
FIGURE 3.1.7 THE RESPONSE OF JURKAT CELLS CULTURED IN THE PRESENCE OF IPFC (VIII) OR MONOCLOATE

- Monoclate
- IPFC(VIII)
- Diluent

Counts per minute vs. Dose (units/ml)

Diluent 1:1000 1:100 1:10 neat
Figure 3.1.7. Median values are shown. Jurkat cells cultured in presence of IPFC (VIII) or MC at concentrations shown. IPFC (VIII) caused significant impairment of proliferation at all concentrations.
Figure 3.1.8 Effect of IPFC (VIII) and Monoclate on IL-2 Production

![Graph showing the effect of IPFC (VIII) and Monoclate on IL-2 production.](image-url)
Figure 3.1.8. Median values for percent maximum IL-2 activity are shown. IPFC (VIII) impaired IL-2 production from PBMC at all concentrations.
infections may directly "chemically" impair immune responses. Despite availability of tests to identify individuals infected with HIV-1 it has remained uncertain if treatment with IPFC (VIII) in the absence of HIV-1 and NANB infection is immunosuppressive.

The present study showed that IPFC (VIII) had the potential to modulate T-lymphocyte function. The experiments showed that intermediate purity factor VIII concentrates inhibited specific and non-specific T-lymphocyte proliferation in vitro, in a dose dependent manner. This inhibition was not due to an alteration of the time course of the proliferative response to PHA. Neither was it due to a non-specific cytotoxic effect as measured by trypan blue exclusion, Table 3.1.2.

Although in the present study only one batch of IPFC (VIII) was used, others have reported similar findings, suggesting that the impaired response was not peculiar to the batch investigated (316-319).

These observations raise two main questions: firstly, what component of IPFC(VIII) mediated this effect, and secondly what is the mechanism? The active component of IPFC (VIII) did not appear to be the citrate buffer (Figure 3.1.4). The proteins contained within IPFC (VIII) include: immunoglobulins, fibrinogen/fibronectin and the factor VIII complex. Using a very pure factor VIIIC concentrate and factor IX concentrate, no
inhibition of proliferative response was seen at replacement concentrations (Figure 3.1.6/7). This suggested that some "contaminant" of IPFC (VIII) is responsible. Others have shown that purified fibrinogen and fibronectin at equivalent concentrations had no effect (317). It has been suggested that this effect may be mediated by a small molecular weight inhibitor which can be dialysed (317, 320); this has not been confirmed by others (318,321). Interestingly it has been shown that immunoglobulin preparations have a similar inhibitory effect (322). In more recent studies Sandilands et al have shown that intravenous immunoglobulin contains FcY receptor antibodies, which are known to be immunosuppressive (323). Such antibodies are also present in IPFC (VIII) (324). Their presence in IPFC (IX) and MC has not yet been investigated.

On activation T cells secrete IL-2 which mediates continued proliferation of T cells by an autocrine mechanism (325). Figure 3.1.7 shows that IPFC (VIII) reduced proliferative capacity of Jurkat cells, an IL-2 dependent cell line and impaired the secretion of IL-2 from PHA stimulated lymphocytes, Figure 3.1.8. IPFC (VIII) did not affect lymphocyte proliferation once PBMC had been activated and stimulated to proliferate (Figure 3.1.4). This suggested that IPFC (VIII) affected activation of lymphocytes. The possibility that IPFC (VIII) bound PHA was excluded (Figure 3.1.3). It is
therefore possible that IPFC (VIII) blocks post-receptor-binding events.

An alternative explanation may be interaction of a component or component of IPFC (VIII) with non-T cells such as macrophages, generating inhibitory signals (or factors). In this regard it has been shown that IPFC impairs monocyte function (326). This effect was reproduced by using immunoglobulin complexes and was mediated by a high molecular weight fraction which contained immune complexes or IgG aggregates (326).

The finding that IPFC (VIII) inhibited lymphocyte function in vitro may explain impaired cell mediated immunity in vivo in haemophiliacs in the absence of HIV-1 infection. The clinical significance is not clear. Whilst there are reports of a higher incidence of tuberculosis and anecdotal instances of lymphomas, the exact prevalence of these observations needs to be substantiated and the effects of viruses other than HIV-1 excluded in non HIV-1 infected haemophiliacs (327-329).

It is not known if such abnormalities may have predisposed haemophiliacs to HIV-1 infection. In this study factor IX concentrates did not impair lymphocyte proliferation Figure 3.1.7, this may explain differences in the prevalence of HIV-1 infection between factor VIIIC and factor IX deficient haemophiliacs. The relative risk for HIV-1 infection in factor IX deficient patients
is four times less than patients with factor VIIIC deficiency (Chapter 4.2). However, HIV-1 preferentially partitions into cryoprecipitate during plasma fractionation (330).

In conclusion this study showed that IPFC (VIII) impaired lymphocyte function in a dose dependent manner. This finding raises the interesting possibility that use of factor concentrates in the absence of HIV-1 contamination is potentially immunosuppressive.
IMMUNOSUPPRESSION FOLLOWING REPLACEMENT THERAPY IN HAEMOPHILIA - A ROLE FOR LYMPHOCYTE FC GAMMA-RECEPTOR (FCY) BLOCKING ANTIBODIES.

Summary

Study objective: Do treated haemophiliacs have FcY-receptor blocking antibodies.

Study design: Open, cross-sectional comparison.

Study population: Multitransfused uraemic patients. Haemophiliacs treated with clotting factor concentrates. Homosexuals with and without AIDS.

Measurements: IgG antilymphocyte antibodies that block the FcY receptor were measured. The ability of haemophilic IgG to inhibit T-cell transformation was determined.

Results: 67 percent of haemophiliacs had IgG antilymphocyte antibodies levels were higher than in normal controls but similar to multitransfused uraemic patients. Homosexuals with AIDS had the highest levels.

Conclusion: Treated haemophiliacs had IgG FcY receptor antibodies that inhibit T-cell transformation. Depression of cell mediated immunity by these antibodies may have predisposed haemophiliacs to HIV-1 infection.
3.2.1. INTRODUCTION

Haemophiliacs may develop immunodeficiency from the viruses transmitted by clotting factor concentrates or due to alloantigens contained within such preparations. In the previous section it was found that intermediate purity factor VIII concentrates impaired lymphocyte function in vitro. An alternative mechanism may be an immune response to alloantigens present as contaminants in clotting factor concentrates. Such immunosuppression may have predisposed some haemophiliacs to HIV-1 infection (331).

The beneficial effect of pre-transplant blood transfusions on renal allograft survival is well established (279, 280). It has been proposed that the transfusion effect is due to production of immunosuppressive, anti-lymphocyte antibodies (304). Macleod et al showed that following transfusion, antibodies which had the capacity to block FcY-receptors (FcYR) on B lymphocytes are induced. The presence of such antibodies correlates with subsequent renal allograft survival (304).

Haemophiliacs depend on frequent transfusions of blood products and may develop such antibodies in response to alloantigens present as contaminants in such products (332). This study attempted to detect FcYR blocking
antibodies in the sera of multitransfused uraemic patients, haemophiliacs, and homosexuals with and without the acquired immunodeficiency syndrome (AIDS).

In haemophiliacs presence of FcγR blocking antibody was correlated with the mean annual dose of clotting factor concentrate used, and HIV-1 status. Furthermore the capacity of IgG fractionated from haemophilic serum to inhibit T-cell proliferation was investigated.

3.2.2. PATIENTS AND METHODS

Patients and Controls:
Serum was obtained from the following groups: (a) 17 heterosexual normal subjects with no previous history of blood transfusion or pregnancy, (b) 54 uraemic patients on long term dialysis, all of whom had been previously transfused (c) 39 haemophiliacs, 30 had factor VIIIIC deficiency and 9 factor IX deficient patients, (d) 15 homosexuals, of whom none had received blood transfusions. Six of this group had AIDS manifest as an opportunistic infection in all, 3 also had Kaposi's sarcoma (serum from uraemic, homosexuals and AIDS patients were provided by: Dr M Forwell, Western Infirmary, Glasgow, and Dr J A Harvey, Southern General Hospital, Glasgow, respectively).

The case notes of haemophiliacs were reviewed and the following additional information obtained:

(i) Mean annual dose of clotting factor concentrate
used over the previous seven years.

(ii) HIV-1 status - HIV-1 antibody was detected using a commercial enzyme linked immunosorbent assay (Wellcozyme).

(iii) Age.

Serum IgG Preparations

IgG was obtained from serum by diethylaminoethyl-cellulose cellulose (DE52 - Whatman). Both serum and DE52 were equilibrated with 10mM phosphate buffer at pH 7.8 prior to chromatography. IgG preparations were dialyzed against phosphate buffered saline (PBS), pH 7.2, for 18hrs at 4°C and ultracentrifuged (105,000xg for 1hr) prior to use. 500ul aliquots were stored at -20°C till used. The preparations were used at a concentration of 1mg/ml in PBS. The purity of the preparations was assessed by immunoelectrophoresis and no other class of IgG was detected by radial immunodiffusion. The monomeric nature of these IgG preparations was confirmed by polyacrylamide gel electrophoresis and by gel filtration (SephacrylS300 - Pharmacia).

Anti-Lymphocyte Antibodies - The EA-rosette Inhibition Assay

1x10^6 monocyte - depleted normal peripheral blood lymphocytes were resuspended in 120ul IgG preparation, or in PBS alone as a control. Following incubation at 37°C for 30 minutes, cells were washed twice in 14, Hanks
Essential Medium (HEM) and mixed with an equal volume of chicken erythrocytes (E) which had been pre-sensitized with an equal volume of rabbit IgG class antibody (A). The tubes were then centrifuged at 200xg for 5 minutes, EA rosette pellets were resuspended and fixed in 3% glutaraldehyde. Cells were resuspended in 0.75% trypan blue and inspected under sealed cover slips. Results were expressed as the percentage inhibition of EA rosette formation produced by IgG compared with PBS control.

Inhibition of T-cell transformation

Normal human peripheral blood mononuclear cells (PBMC) were separated (Chapter 3.1.2) and resuspended in RPMI 1640 culture medians with 10% heat inactivated autologous serum and antibiotics (Chapter 3.1.2) at a concentration of 4x10^6 cells, 50ul of PBMC (2x10^5 cells), 50ul of IgG (1mg/ml in PBS) and 10ul of phytohaemagglutinin (PHA-110ug/mls). They were then cultured in flat bottomed 96 well microtitre plates. The cells were incubated at 37°C for 66hrs in a humidified atmosphere of 5% CO2 in air. The cells were pulsed with radiolabelled thymidine and harvested as previously described (Chapter 3.1.2).

3.2.3 RESULTS

Figure 3.2.1 shows that IgG from haemophiliacs had significantly higher percent inhibition of EA rosette formation compared to controls. The values were independent of HIV-1 antibody status, (Figure 3.2.2). There was no correlation with age (r=.15, p=0.21) or mean annual dose of clotting factor concentrate used.
Figure 3.2.1 IgG CLASS ANTI LYMPHOCYTE ANTIBODIES BY EA ROSETTE INHIBITION
Figure 3.2.1 Haemophiliac IgG caused significantly greater inhibition of EA rosette formation compared to normals.
FIGURE 3.2.2 IgG ANTI LYMPHOCYTE ANTIBODIES IN HAEMOPHILIACS WITH AND WITHOUT HIV-1 ANTIBODY

PERCENT EA ROSETTE INHIBITION

HIV-1 STATUS

+VE

-VE

0

100

90

80

70

60

50

40

30

20

10

0
Figure 3.2.2. There was no significant difference in median EA rosette inhibition between HIV-1 antibody positive or negative haemophiliacs.
Figure 3.2.3 shows effect of IgG from haemophiliacs on T-lymphocyte transformation.

3.2.4. DISCUSSION
This study showed that up to 66 percent of haemophiliacs have antilymphocyte, FcYR-blocking antibodies. The level of the antibody was not related to HIV-1 status nor the mean annual dose of clotting factor concentrate used. It would therefore seem that antibody production in these patients may be determined by the immune response to the repertoire of alloantigen(s) to which the individual is exposed rather than the total antigen load. Forwell et al (1986) have also shown that the FcYR-blocking antibody cross-reacts with spermatozoa, and this may explain the high levels found in homosexuals who receive anal spermatozoa (333). Rectal insemination into rabbits produces IgG reactive with lymphocytes and a reduced responsiveness to T cell dependent antigens (334).

An immunosuppressive role for FcYR-blocking antibodies is suggested by the previously described correlation between the occurrence of these antibodies and renal allograft survival (304). The present study showed that FcYR-blocking antibodies can suppress T-cell proliferation, as assessed by inhibition of PHA transformation, thus providing further evidence that these antibodies are immunosuppressive. Although the precise mode of action is not known it would seem likely that these antibodies react with lymphocyte membrane alloantigens associated
FIGURE 3.2.3 INHIBITION OF PHA BLASTOGENESIS BY HAEMOPHILIC SERUM
Figure 3.2.3. Haemophiliac IgG resulted in significant inhibition of proliferation of PHA induced blastogenesis of normal PBMC.
with FcYR and indirectly trigger release of non-antigen-specific soluble suppressor factors; a mechanism generally thought to be initiated by direct binding of immune complexes to the FcY-receptor itself. Although aggregated IgG could produce such an effect in vitro it would seem unlikely that aggregates account for these findings, since IgG from normals and patients were prepared in parallel using an identical technique which involves an ultracentrifugation step designed to remove any aggregated or complexed IgG. Furthermore, the monomeric nature of the IgG used in this study was confirmed by gel filtration. FcYR-blocking and inhibition of PHA blastogenesis would therefore appear to be due to a genuine antibody effect and not to non-specific binding of IgG to the FcYR. This does not however preclude the possibility that antigen:antibody complexes may also be present in the serum of alloantigen stimulated patients and which may also contribute to triggering of non-antigen-specific suppressor cell mechanisms.

In conclusion, lymphocyte (FcYR-blocking) antibodies were demonstrated in haemophiliacs, as previously shown in other groups of patients exposed to alloantigens (333). These antibodies may have the capacity to stimulate release of soluble suppressor factors which in turn inhibit T-cell activation. If this occurs in vivo, then depressed cell mediated immunity (CMI) may result. Although depressed CMI is desirable where prolonged
allograft survival is required, such antibodies may pre-
dispose to infection. The occurrence of these antibodies in patients known to be at risk for the development of AIDS suggests that the FcγR-blocking antibodies may play a role in the progression of HIV-1 infection to AIDS. The presence of these antibodies in all 6 homosexual patients with clinically diagnosed AIDS would support such a hypothesis.
CHAPTER 4.1

The Epidemiology of HIV infection in West of Scotland Haemophiliacs.

Summary

Study Objective: To study the epidemiology of HIV antibody in adult haemophiliacs. To compare available HIV-1 ELISA antibody tests.

Study design: Open cross-sectional with retrospective review.

Study population: All haemophiliacs who had received a blood product from the West of Scotland Adult Haemophilia Centre since 1980.

Measurements: Serum HIV-1 antibody by Type I and Type II ELISA antibody tests. Positive results were confirmed by western blotting and immunofluorescence.

Results: The prevalence of HIV-1 antibody was 17 percent. The peak annual incidence for HIV-1 antibody occurred in 1983 and antibody was first detected in 1981.

Conclusions: Both commercial Type I and Type II ELISA for HIV-1 antibody were comparable in sensitivity and specificity. The prevalence of HIV-1 antibody in this cohort (17 percent) was lower than the U.K. national average of 39 percent in haemophiliacs treated since 1980 (369).
4.1.1. INTRODUCTION

The acquired immune deficiency syndrome (AIDS) is characterised by a progressive lymphopenia, predominantly of T helper/inducer lymphocytes, which renders the individual susceptible to a variety of opportunistic infections and malignancies. AIDS itself is a clinical diagnosis and is but one manifestation of a spectrum of clinical and subclinical immunological disorders caused by the human immunodeficiency virus-1 (HIV-1), also known as human T cell lymphotropic virus III, lymphadenopathy virus or AIDS related virus (335-338).

Conclusive evidence that HIV-1 has a primary aerological role in AIDS includes: in vitro the virus specifically infects and depletes lymphocytes that express the CD4 antigen producing a T-helper/inducer (T-h, CD4+ve) lymphopenia (339, 340) - an invariable finding in AIDS patients (341-345). HIV-1 can be frequently isolated from patients with AIDS and AIDS related syndromes (336). Virtually all such individuals have serological evidence (serum IgG antibody) of exposure to the virus (346, 347). Finally inoculation of HIV-1 into humans results in AIDS as seen in transfusion - recipient pairs (348-350).

HIV-1 infected individuals can be identified indirectly by serological tests, or by identifying viral proteins in serum, alternatively HIV-1 can be isolated from stimulated peripheral blood mononuclear cells. HIV-1
isolation is time consuming procedure, requires considerable laboratory expertise and containment facilities, it is therefore expensive and results show considerable inter-laboratory variation. Serological tests are cheaper, less labour intensive and are both sensitive and specific for HIV-1. The principles of the available serological tests are shown in Figure 4.1.1.

The enzyme linked immunosorbent assay (ELISA) tests are the most frequently used tests to screen serum samples for HIV-1 antibody. False positive results have been reported in multiparous women, multiply transfused individuals such as thalassaemia patients, and others whose medical history may have led them to react non-specifically with HIV-1 antigens (351-353).

Confirmatory tests are therefore necessary in ELISA positive samples, examples include Western blotting (WB), immunofluorescent (IF) tests and radio-immunoprecipitation assays (352,352). False negative results have been infrequently reported with WB (354, 355). Occasional false positive results have been reported with WB (356, 357).

AIDS was first reported in haemophiliacs in 1982 (273). Subsequently immune aberrations similar to those found in AIDS patients were noted in clinically well haemophiliacs treated with clotting factor concentrates (311, 312).
Figure 4.1.1 Solid Phase methods for the detection of anti-HIV

Type

1. Ag + specimen + anti hu IgG/enzyme + substrate

2. Ag +
   
   {specimen

   {anti HIV/enzyme + substrate

3. anti hu IgG + specimen + Ag + anti HIV/enzyme + substrate

4. Ag + specimen + Ag/enzyme + substrate

KEY

solid phase surface

Ag = HIV antigen

hu = human
Figure 4.1.1 The four types of HIV-1 antibody assay.
* HIV-1 antibody.
West of Scotland similar findings were reported (316). In the present study, presence of serum IgG antibody was sought as a marker of HIV-1 infection to determine the epidemiology of HIV-1 infection in a well-defined cohort of haemophiliacs, treated with clotting factor concentrates or plasma or cryoprecipitate. The sensitivity and specificity of available ELISA tests was compared to WB and IF tests which were taken as the gold standards for presence of HIV-1 antibody.

4.1.2 METHODS

Patients

All patients with haemophilia who had been treated with a blood product at the West of Scotland Adult Haemophilia Centre since 1980 were included in this study; additional selection criteria for entry into the study are given in Chapter 2. Of the 133 patients entered 100 were factor VIII deficient, and 33 had factor IX deficiency). The median age was 30 years (Interquartile range [IQR] 23 to 44). At the time of this study no patient had AIDS or clinical features suggestive of HIV-1 infection.

Methods

Serum samples collected from this cohort between 1977 and 1987 were assayed for HIV-1 antibody. All samples were tested for HIV-1 antibody using three different ELISA tests. The initial screening was done in 1984 using a Type I non commercial ELISA test. In this assay disrupted
whole virus was the substrate (HTLV III - H9) (358). Samples were run in duplicate and a known negative control was run eight times on each microtitre plate, the results for each being averaged. Samples were compared with negative control results through the ratio of the two values (346).

In 1985, the 1984 serum samples and the most recent specimen (1985) from all patients were tested on two different types of ELISA: a competitive inhibition assay (Type 2), Wellcozyme (Wellcome Diagnostics, Beckenham, Kent) and another Type 1, direct assay (Abbott Laboratories, Chicago, Illinois, USA). The principles of these tests are as follows:

**Direct HIV-1 ELISA (Type I - Abbott Laboratories)**

HIV-1 propagated in culture is the substrate for this assay. Viral antigens are obtained by treating propagated virus with a detergent and sonication. Antigen is then coated onto a solid phase: in this test beads are used. The coated beads are incubated with a diluted specimen and control sera. If HIV-1 antibody is present it binds to antigen on the solid phase and can be detected by goat antibody to human IgG conjugated to horseradish peroxidase. Bound conjugate can be detected by a colour reaction using o-Phenylene-diamine. The colour reaction in a specimen containing antibody is directly proportional to amount of antibody.
Competitive inhibition HIV-1 ELISA (Type II - Wellcozyme)

Chemically inactivated HIV-1 antigen is captured onto purified anti-HIV-1 previously immobilized onto a solid phase, in this case microwells. Test samples and control sera are incubated in separate wells with anti-HIV-1 chemically conjugated to the enzyme horseradish peroxidase (the conjugate). Competition for binding to the immobilized antigen occurs between antibodies to HIV-1 in the sample or control sera and conjugate; HIV-1 will block binding of conjugate. After washing wells to remove the samples and excess conjugate the enzyme remaining bound to the wells is visualized using 3,3',5,5' tetramethylbenzadine iodine and hydrogen peroxide to give a yellow reaction after termination of the reaction with sulphuric acid.

The amount of conjugate, and hence colour, remaining in the wells is inversely related to concentration of HIV-1 antibody in the sample.

Western Blotting (Dupont)

HIV-1 is propagated in cell culture and inactive antigen obtained using detergent and sonication. The HIV-1 antigen polypeptides are fractionated according to molecular weight by electrophoresis using polyacrylamide slab gel in the presence of sodium dodecyl sulphate. The separated polypeptides are then transferred from gel to nitrocellulose by electrophoretic blotting.
Nitrocellulose strips containing HIV-1 specific protein are then reacted with test serum specimens. The antigen/antibody complex is visualized using a goat anti-human immunoglobulin biotin conjugate, an avidin-horseradish peroxidase conjugate, and 4-chloro-1-napthol substrate.

4.1.3 RESULTS

Comparison of tests

Table 4.1.1 gives the results of each of the three ELISA tests for samples obtained during 1984 and 1985. One sample was weakly positive by the non-commercial Type I assay but was negative in the second Type I test and the Type II ELISA. The 1985 and all subsequent samples from this patient have remained negative.

All positive samples (n=42) in any test were confirmed by WB and IF. Only the weakly positive sample in the first type I direct ELISA was negative by WB; all positive samples had at least two envelope protein antibodies and one core protein antibody present. Figure 4.1.1 shows a typical positive WB result. Similar results were obtained by the IF test, (Table 4.1.2). Sera from 50 persistently ELISA HIV-1 antibody negative samples were tested by WB; none were positive.

No false positive reactions were obtained using the two commercial ELISA tests, (Table 4.1.2). Both had a high
Table 4.1.1. The results of ELISA serological tests to detect anti IgG HIV-1 antibody.

<table>
<thead>
<tr>
<th>Year</th>
<th>Type 1 (NC)</th>
<th>Type 1 (C)</th>
<th>Type II</th>
</tr>
</thead>
<tbody>
<tr>
<td>1984</td>
<td>20/133 (15%)</td>
<td>19/133 (14%)</td>
<td>19/133 (14%)</td>
</tr>
<tr>
<td>1985</td>
<td>ND</td>
<td>22/133 (16.5%)</td>
<td>22/133 (16.5%)</td>
</tr>
</tbody>
</table>

The Type I and II commercial ELISA tests for HIV-1 antibody gave similar results. One sample was positive in the non-commercial ELISA and negative in both commercial assays.

ND = Not done
NC = Non commercial
C = Commercial
STRIP ASSAY RESULT

REACTIVE PROFILE

- gp160
- gp120
- p64
- p55/p53
- gp41
- p31
- p24
- p15
Figure 4.1.1. A positive Western blot. To establish a diagnosis requires envelope, polymerase and group antigen encoded products. gp160 = envelope glycoprotein precursor; gp120 = outer envelope glycoprotein; p64 = reverse transcriptase component; p55/p53 = gag protein precursor; gp41 = transmembrane envelope glycoprotein; p31 = endonuclease; p24/p15 = Core gag protein.
Table 4.1.2  Results of Western blots and immunofluorescence test in samples positive in any ELISA.

<table>
<thead>
<tr>
<th>Test</th>
<th>Number tested</th>
<th>Number positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Western blot</td>
<td>45</td>
<td>44</td>
</tr>
<tr>
<td>Immunofluorescence</td>
<td>45</td>
<td>44</td>
</tr>
</tbody>
</table>

The one sample that was positive in the non-commercial ELISA was negative by both Western blotting and immunofluorescence.
sensitivity and specificity using WB and IF as the standard, (sensitivity = 100%, and specificity = 100%).

Incidence and prevalence of HIV-1 infection

Figure 4.1.3 shows the cumulative annual incidence of HIV-1 infection during this study (1983-1987) using the competitive ELISA test. Three seroconversions occurred in 1985 after the introduction of heat treated factor concentrate; in one patient seroconversion occurred after receipt of heat treated factor concentrate. The details of this patient are given at the end of this section. The prevalence of HIV-1 infection at the end of the study was 16.5 percent.

Stored, retrospective, sera from all HIV-1 antibody positive patients were obtained and tested by ELISA and WB, (Figure 4.1.4) 14 samples from 6 patients between 1974 and 1980 were available, none were positive. The first positive sample was found in 1981. Patients (n=14) in whom a negative sample was available the mid-point between the last negative and the first positive was taken as the date of seroconversion. No negative sample was available in 8 patients; in these patients the date of the first positive sample was taken as the date of infection.

Seroconversions after the introduction of heat treated factor concentrates.
FIGURE 4.1.3 CUMULATIVE ANNUAL INCIDENCE OF HIV-1 ANTIBODY.
Figure 4.1.3. A Kaplan Meier analysis of cumulative HIV-1 antibody positive results.
Table 4.1.4. Retrospective serum antibody results for HIV-1.

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>4</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>5</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>6</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>7</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>8</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>9</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>10</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>11</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>12</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>13</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>14</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>15</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>16</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>17</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>18</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>19</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>20</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>21</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>22</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>
Figure 4.1.4.  - = negative; + = positive; blank = no serum.
At this Centre, all patients were commenced on heat treated factor VIII concentrate December 1984. One seroconversion occurred in 1985; his treatment history is given below:

Patient 1
Age = 42
Factor VIIIC deficient; level < 1iu/dl
No inhibitor

<table>
<thead>
<tr>
<th>Year</th>
<th>Number of units used</th>
<th>Source of concentrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>1980</td>
<td>18,180</td>
<td>SNBTS</td>
</tr>
<tr>
<td>1981</td>
<td>13,720</td>
<td>SNBTS</td>
</tr>
<tr>
<td>1982</td>
<td>37,980</td>
<td>SNBTS</td>
</tr>
<tr>
<td>1983</td>
<td>83,630</td>
<td>SNBTS</td>
</tr>
<tr>
<td>1984</td>
<td>150,640</td>
<td>SNBTS</td>
</tr>
<tr>
<td>1985</td>
<td>173,830</td>
<td>SNBTS</td>
</tr>
</tbody>
</table>

HIV-1 serology (ELISA, Wellcozyme, Western Blot [Dupont])

<table>
<thead>
<tr>
<th>Date</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>20.03.83</td>
<td>-ve</td>
</tr>
<tr>
<td>09.09.83</td>
<td>-ve</td>
</tr>
<tr>
<td>05.10.84</td>
<td>-ve</td>
</tr>
<tr>
<td>25.10.85</td>
<td>+ve</td>
</tr>
</tbody>
</table>
The type of factor VIII concentrate used in 1984 and 1985 was of intermediate purity. He had received twenty different batches, of which ten were heat treated.

Twenty-nine HIV-1 antibody negative patients had been treated with the same batches, none were HIV-1 antibody positive. In the absence of any other seroconversions no one batch could be implicated.

4.1.4 DISCUSSION

The primary objective of this study was to evaluate the ability of available ELISA tests to detect HIV-1 antibody in haemophiliacs. This was specifically done in haemophiliacs because effects of previous transfusions, high frequency of immune complexes and effects of chronic liver disease on the performance of ELISA tests had not been established. The two commercial ELISA tests were found to be similar in their sensitivity and specificity to detect HIV-1 antibody in haemophiliacs. The initial Type 1 ELISA used was sensitive but not specific. The results of the commercial tests in these haemophiliacs are comparable to those reported in other UK high risk groups (359). Both tests were comparable in cost and required equivalent amounts of time for optimal usage. Therefore, for the purposes of screening haemophiliacs for HIV-1 antibody either test can be used.
Initially two confirmatory tests were used, however WB is now the preferred method. This method has the additional advantage of providing a discriminatory picture of the antibody response. The major disadvantages of WB are: the procedure is complex, slow and expensive. Furthermore, the interpretation of samples that react to only core proteins can be difficult. In individuals not at risk for HIV-1 infection, this may indicate: either an infection with some as yet unidentified virus immunologically related to the known human retroviruses, or an early phase of HIV-1 infection.

The major advantage of the IF method is that the procedure requires less time than WB, however, the conventional IF requires the additional use of a fluorescent microscope and expertise in the interpretation of marginally positive results (352). An alternative to IF and WB is the radioimmuno-precipitation assay (RIPA) (352). Although RIPA is an accurate method of demonstrating HIV-1 antibodies, it has remained a specialist procedure.

Using the described methods, the prevalence of HIV-1 infection in this cohort was 17 percent. The peak annual incidence of HIV-1 infection occurred in 1984, and antibody was first detected in 1981. The prevalence of infection is lower than that reported in haemophiliacs from the USA where the overall prevalence among haemophilia A patients has been approximately 70% and for
haemophilia B patients 35 percent (360) and other parts of Europe (361) but comparable to Eastern Scotland (221). This may reflect regional differences in amount of treatment given, contamination of the donated plasma pool with HIV-1 or variations in host susceptibility to HIV-1 infection (see Section 4.2).

HIV-1 infection in this cohort occurred later than in USA haemophiliacs (362, 363) but at a time similar to other UK haemophiliac patients (364). Although the peak incidence of HIV-1 infection occurred at the same time as in the East of Scotland (221), initial infections occurred earlier. The explanation may be different sources of clotting factor concentrates used. In the West of Scotland prior to 1984 factor concentrate fractionated from plasma donations in the USA and Scotland was used, whereas in Eastern Scotland only locally donated plasma fractions were used.

HIV-1 seroconversions in patients receiving heat-treated factor concentrates have been reported (257). The patient reported in this section had received non-heated treated factor concentrate as well as that treated with dry heat at 72°C for 24 hours. He had no other risk factors for HIV-1 infection. However, it is difficult to be certain that infection occurred due to a failure of heat treatment - a negative serum sample was not available prior to receipt of heat treated concentrate. Furthermore, the appearance of antibody may take up to 14
weeks in haemophiliacs and in homosexuals an even longer duration has been noted (221, 365). Twenty-nine other HIV-1 antibody negative haemophiliacs received the same batches, none seroconverted. There are several possibilities to explain infection (a) it occurred from non-heat treated concentrate; (b) occurred from heat-treated concentrate, due to either (1), (2), (3): 1. the size of the HIV-1 inoculum may have been larger in this patient; 2. he had a constitutional predisposition to HIV-1 infection; 3. previous treatment with factor concentrate had primed his immune system to allow HIV-1 infection to establish itself.

In vitro studies suggest that adequate heat treatment should have eliminated the risk of further HIV-1 infections occurring in haemophiliacs (Chapter 1). Nevertheless, 18 seroconversions have been well documented, 14 occurred prior to donor screening for HIV-1 antibody or with concentrates dry heated at temperatures of 60-68°C for 30-72 hours. All commercially manufactured dry heated intermediate purity concentrates have been withdrawn (257). Several alternatives to dry heat have been suggested. HIV-1 seroconversions have not been reported with pasteurized concentrate, however the numbers of patients treated is small (257). Prince et al have reported that treatment with detergent and sodium cholate inactivates HIV-1 (267). Clinical studies with such preparations are in progress (270).
In the West of Scotland measures instituted to prevent further HIV-1 infection in haemophiliacs via transfusion include heat treatment of all clotting factor concentrates. Further, all blood donors are provided with information about risk factors associated with HIV-1 infection and high risk individuals are requested to defer donations, and all donations are screened for HIV-1 antibody. HIV-1 infection has occurred after the use of screened blood donations. The estimated odds are 1:40,000 (366). Assays for HIV-1 antigen or other tests that detect antibodies to recombinant HIV-1 antigens are at present being considered for screening blood donations so that the "window period" from acquiring infection to manifesting detectable serum IgG antibody is covered (367, 368). A more recent development has been the ability to detect integrated HIV-1 provirus (369). The method is based on the polymerase chain reaction (PCR), in which selected DNA sequences are amplified several thousandfold using primer base pairs, in this case from multiple regions of the HIV-1 genome. The efficacy of this method is not yet known.

In conclusion, this study showed that the available ELISA screening tests were capable of detecting HIV-1 antibody in haemophiliacs. Using such tests it was shown that HIV-1 infection first occurred in 1981; the peak annual incidence of HIV-1 infection occurred in 1984; and the
prevalence was 16.5%. The steps taken to prevent further HIV-1 infections occurring in haemophiliacs have been discussed.
CHAPTER 4.2

Risk Factors for HIV-1 infection in haemophilia.

Summary

Study objective: To determine treatment- and host-related risk factors for HIV-1 infection in haemophilia.

Study population: 133 treated haemophiliacs in whom the HIV-1 antibody status was known.

Measurements: Mean annual dose of clotting factor concentrate used, source of concentrate, type of concentrate used, age, hepatitis B serology, severity of liver disease.

Results: HIV-1 antibody positive patients had used:
(a) more concentrate per annum
(b) more commercial concentrate
(c) were significantly younger than seronegative patients
Hepatitis B markers (surface antibody and antigen) occurred more frequently in seropositive patients. Severity of liver disease was similar.

Conclusions: The mean annual dose; source and type of clotting factor concentrate used were independent risk factors for HIV-1 infection.
4.2.1 INTRODUCTION

The prevalence of HIV-1 infection in severe haemophiliacs from the USA is as high as 90 percent and a similar high prevalence has also been found in Europe (360, 361). In the UK the overall HIV-1 antibody prevalence is 38.8 percent (370). One explanation for the geographical differences may be variations in sampling policies, however within the UK a uniform sampling method was applied. Despite this a varying regional prevalence was seen.

Ludlam et al have reported that exposure to an infected batch of factor concentrate did not invariably result in infection (221). Such resistance to infection has also been noted in homosexual men (222). Studies of animal retroviral infections show similar differences in susceptibility to infection (371). Failure of the virus to establish infection may be due to: (a) the relative virulence of the virus strain; (b) the inherent sensitivity of the host cell to virus replication; (c) genetic determinants or (d) levels of the antiviral immune response. The latter possibility has been raised by the occasional finding of asymptomatic individuals who have been HIV-1 seropositive becoming seronegative (223). The role of genetic factors in susceptibility to HIV-1 infection remains controversial. Steel et al showed a trend for histocompatibility locus DR-3 and susceptibility to HIV-1 infection (372). Eales et al reported an
association with group specific complement (vitamin D binding factor) but this initial claim has been retracted (373). Inherited partial C4 deficiency has also been suggested as a risk factor for infection (374).

An alternative explanation may be the frequency of exposure to HIV-1. In homosexuals a history of frequent sex with an individual in whom AIDS subsequently developed was independently associated with subsequent AIDS (375). In haemophiliacs exposure to HIV-1 depends on the degree of contamination of the donor plasma pool, as well as the amount of treatment given.

A proportion (8 percent) of the total factor VIII concentrate used at prior to 1984 was imported from the USA, (Figure 2.1.1). In this cohort HIV-1 antibody was first detected in 1981 and the peak annual incidence of infection occurred in 1983 (Chapter 4.1.1). The use of factor VIII concentrate imported from USA may therefore have been one predisposing factor to infection.

The influence of age and liver disease (due to hepatitis B and non-A, non-B) in predisposing to HIV-1 infection is not known. Chronic liver disease can be associated with laboratory abnormalities suggestive of immunodeficiency (376).

The primary aim of the present study was to identify treatment related risk factors for HIV-1 infection. The
mean annual total dose of clotting factor concentrate received, the type and source of clotting factor used by HIV-1 antibody positive and negative patients was compared. Age and severity of liver disease was also considered.

4.2.2 METHODS

Patients
Selection criteria and demographic characteristics of the study population have been previously described, (Chapter 2.1). Briefly, 133 adult haemophiliacs who had received a blood product from the West of Scotland Adult Haemophilia Centre since 1980 were studied. Of these 100 patients were factor VIII deficient and 33 factor IX deficient.

Since December 1984 no patient at this centre had been treated with non-heat treated clotting factor concentrate imported from the USA. The prevalence for HIV-1 antibody in 1984 was 14 percent, only one patient with factor IX deficiency was known to have HIV-1 antibody.

Methods
The case notes of all treated patients were reviewed and the following information recorded:

1. Total amount of clotting factor concentrate used from 1980-1984, inclusive.

2. The type and source of clotting factor concentrate
3. In those patients in whom a negative serum sample HIV-1 antibody was available, batch numbers of clotting factor concentrate used between last negative and first positive serum sample were recorded. The names of all seronegative patients who had received these batches and number of infusions were noted.

The criteria used to classify for severity of liver disease are given in chapter 2.

HIV-1 Serology

The presence of HIV-1 antibody was detected using a competitive inhibition ELISA (Wellcozyme, Wellcome Diagnostics, Beckenham, Kent) and confirmed by Western blotting (Dupont).

4.2.3 RESULTS

The peak annual incidence of HIV-1 infection in this cohort occurred in 1984, (Figure 4.1.3).

Host associated risk factors for HIV-1 infection

Table 4.2.1 shows the age, previous hepatitis B infection, and the severity of liver disease prior to the peak period of HIV-1 infection (1983). The median age of HIV-1 antibody positive patients was significantly lower than seronegative patients. Hepatitis B surface
Table 4.2.1  A comparison of the characteristics of HIV-1 antibody positive and negative patients.

<table>
<thead>
<tr>
<th>HIV status</th>
<th>-VE</th>
<th>+VE</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>114</td>
<td>19</td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>32</td>
<td>21</td>
<td>0.001</td>
</tr>
<tr>
<td>(25-45)</td>
<td>(18-35)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean Annual Dose of clotting factor concentrate</td>
<td>6503</td>
<td>80,000</td>
<td>0.0008</td>
</tr>
<tr>
<td>Liver disease severity</td>
<td>3.5</td>
<td>3</td>
<td>0.06</td>
</tr>
<tr>
<td>(2-5)</td>
<td>(2-3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hepatitis B surface antibody</td>
<td>44 (39%)</td>
<td>12 (63%)</td>
<td></td>
</tr>
<tr>
<td>Hepatitis B surface antigen</td>
<td>1</td>
<td>5</td>
<td></td>
</tr>
</tbody>
</table>

The medians and interquartile ranges are shown. HIV-1 antibody positive patients were significantly younger, and had used more clotting factor concentrate than seronegative patients. More HIV-1 positive patients had hepatitis B antibody than seronegative patients. (p=0.01, Fisher's Exact Test).
antigen and antibody was more frequent in HIV-1 antibody positive patients.

**Treatment related risk factors for HIV-1 infection**

**Type of treatment**

The most striking difference was the low incidence of HIV-1 infection in factor IX deficient patients (6 percent) compared to factor VIII deficient patients (20 percent) \( (p = 0.05, \text{Fisher's Exact Test}) \). The relative risk for HIV-1 infection in factor VIII deficient patients was 3.3 times greater than in factor IX deficient haemophiliacs. Table 4.2.2 shows there were no other significant differences between factor VIII and IX deficient patients.

Between 1980 and 1985, eight factor VIII deficient patients had been treated with cryoprecipitate alone; none had HIV-1 antibody. Ten factor VIII deficient patients had received both cryoprecipitate and factor concentrate, none had serological evidence of HIV-1 infection. No patient seroconverted while receiving activated prothrombin complex concentrates.

**Amount of clotting factor concentrate used**

Patients with HIV-1 antibody had used significantly more clotting factor concentrate than those without HIV-1 antibody, Table 4.2.3.

**Source of treatment**
Table 4.2.2  A comparison of Factor VIII and Factor IX deficient haemophiliacs in 1984.

<table>
<thead>
<tr>
<th>Type of haemophilia</th>
<th>VIII</th>
<th>IX</th>
<th>pvalue</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of patients</td>
<td>100</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>HIV antibody</td>
<td>18</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Mean annual dose of clotting factor concentrate</td>
<td>8778.5 (1627-46782)</td>
<td>9500 (1000-38000)</td>
<td>0.7</td>
</tr>
<tr>
<td>Severity of liver disease</td>
<td>3 (2-5)</td>
<td>4 (2-5)</td>
<td>0.5</td>
</tr>
<tr>
<td>Age</td>
<td>32 (23-45.5)</td>
<td>27 (23-29)</td>
<td>0.09</td>
</tr>
</tbody>
</table>

The medians and interquartile ranges are shown. There were significantly more HIV-1 antibody positive patients with factor VIII deficiency than factor IX deficient patients, (p=0.05, Fisher's Exact Test). There were no other significant differences between the two groups.
Table 4.2.3  A comparison of HIV-1 antibody positive and negative patients.

<table>
<thead>
<tr>
<th>HIV Status</th>
<th>+VE</th>
<th>-VE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean annual (total) dose of clotting factor concentrate</td>
<td>78016.5</td>
<td>6210</td>
</tr>
<tr>
<td></td>
<td>(50,000-88,371)</td>
<td>(900-26,000)</td>
</tr>
<tr>
<td>Total dose of local concentrate</td>
<td>95485</td>
<td>15282.5</td>
</tr>
<tr>
<td></td>
<td>(0-289,138)</td>
<td>(1730-59,184)</td>
</tr>
<tr>
<td>Total dose of commercial concentrate</td>
<td>76,473</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>(8387-41,3070)</td>
<td>(0-1765)</td>
</tr>
</tbody>
</table>

Severity of haemophilia

<table>
<thead>
<tr>
<th>Grade</th>
<th>Prevalence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mild</td>
<td>&gt;10 iu/dl</td>
</tr>
<tr>
<td>Moderate</td>
<td>&gt;5 &lt;10 iu/dl</td>
</tr>
<tr>
<td>Severe</td>
<td>&lt;5 iu/dl</td>
</tr>
</tbody>
</table>

The median and interquartile ranges are shown. HIV-1 antibody positive patients had used more clotting factor concentrate (total), similar amounts of concentrate fractionated from local plasma donations but more concentrate prepared from USA plasma donations. There were significantly more HIV-1 antibody positive haemophiliacs with a severe deficiency.

Seropositive patients had used significantly more commercial concentrate (p=0.001, Fisher's Exact Test).
Table 4.2.4 shows the number of patients treated with local and/or commercial concentrate. Patients who acquired HIV-1 infection prior to 1985 had used more factor VIII concentrate prepared from USA plasma donations than HIV-1 antibody negative subjects, Table 4.2.4. There were no statistical differences in the two groups for use of local products, Table 4.2.4.

**HIV-1 infection with local blood products**

Of those patients who had seroconverted prior to 1985 only one had never received factor VIII concentrate fractionated in the USA. He had no other risk factors for HIV-1 infection. His infection occurred between 1.7.82 and 12.12.83 (Patient 1). Two other patients had subsequently seroconverted while receiving clotting factor concentrates fractionated from local plasma donations; both were infected in 1985. The batches of clotting factor concentrate used between the first positive and 6 months prior to the last negative HIV-1 test were noted for patient 1 and all other patients treated with the same batches identified.

The index patient had used a total of nine different batches. Table 4.2.5 shows the number of infusions received by patients of each batch. Only one batch had been used by both the index patient and another HIV-1 antibody positive patient. This second patient had received this batch prior to his first antibody positive sample and the last negative serum specimen. He had not been treated with commercial clotting factor concentrate.
Table 4.2.4 The association between source of concentrate and HIV-1 status.

<table>
<thead>
<tr>
<th>Source of Concentrate</th>
<th>HIV Status</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+VE</td>
</tr>
<tr>
<td>Local</td>
<td>1</td>
</tr>
<tr>
<td>Commercial only</td>
<td>0</td>
</tr>
<tr>
<td>Local and commercial</td>
<td>18</td>
</tr>
<tr>
<td>Not treated</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>19</td>
</tr>
</tbody>
</table>

The number of patients who had received clotting factors according to the source of the donated plasma, commercial indicates clotting factor concentrates fractionated from plasma donations in the USA. Significantly more positive patients had been treated with both local and commercial concentrate. (p=0.001, Fisher's Exact Test)
Table 4.2.5 The number of infusions of each implicated batch used by treated patients.

<table>
<thead>
<tr>
<th>Last</th>
<th>First</th>
<th>HIV+ve</th>
<th>BATCH NUMBER</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>-ve</td>
<td>378</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+ve</td>
<td></td>
</tr>
<tr>
<td>Index</td>
<td>+</td>
<td>7/82</td>
<td>12/83</td>
</tr>
<tr>
<td>MK</td>
<td>+</td>
<td>8/81</td>
<td>11/82</td>
</tr>
<tr>
<td>AC</td>
<td>+</td>
<td>5/82</td>
<td>1/84</td>
</tr>
<tr>
<td>IC</td>
<td>+</td>
<td>82</td>
<td>31</td>
</tr>
<tr>
<td>NS</td>
<td>+</td>
<td>80</td>
<td>81</td>
</tr>
<tr>
<td>HM</td>
<td>+</td>
<td>81</td>
<td>83</td>
</tr>
<tr>
<td>AD</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AF</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DG</td>
<td>-</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>DH</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DI</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MJ</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>JMcA</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>JMcK</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>WMcK</td>
<td>-</td>
<td></td>
<td>20</td>
</tr>
<tr>
<td>JMcS</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>JM</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>McE</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TM</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>JQ</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SV</td>
<td>-</td>
<td></td>
<td>20</td>
</tr>
<tr>
<td>PW</td>
<td>-</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

A total of sixty six patients had been treated during 1982 and 1983 at this centre. 21 patients had received one of the batches used by the index patient. It is not known whether batch 692, the most likely batch, had been distributed elsewhere in Scotland.
It is however difficult to be sure if this batch can be implicated.

**Regression Analysis**

A multiple regression analysis showed that HIV-1 antibody status showed an independent direct correlation with the mean annual dose of clotting factor concentrate \( (r=0.64, \ p=.0001) \), and amount of commercial concentrate used \( (r=0.64, \ p=0.001) \). A poorer correlation was seen with mean annual dose of local concentrate \( (p=0.24, \ p=0.004) \). Age showed a weaker indirect correlation \( (p=-0.3, \ p=0.002) \).

To clarify the influence of age in acquiring HIV-1 infection a stepwise variable selection analysis in which age was the dependent variable and mean annual dose of total concentrate and total dose of local and commercial concentrate independent variables, only use of commercial concentrate was retained in the equation on both forward and backward selection \( (r=-0.7) \).

**Liver Disease**

In a stepwise variable selection analysis in which HIV-1 antibody status was the dependent variable and the independent variables were severity of liver disease and mean annual dose of clotting factor concentrate used, liver disease was not a significant contributory factor but mean annual dose of clotting factor concentrate used was \( (r=-0.62, \ p=0.04) \).
Significantly more HIV-1 antibody positive patients had hepatitis B surface antibody (Table 4.2.1), the presence of hepatitis B surface antibody was independent of HIV-1 infection but was related to mean annual dose of clotting factor concentrate used ($r = -0.62, p = 0.002$, stepwise variable selection analysis). More HIV-1 antibody haemophiliacs had persistent hepatitis B surface antigen, Table 4.2.1.

The results of the present study show that mean annual dose of clotting factor concentrate, type and source of factor concentrate were independent risk factors for HIV-1 infection.

The relative risk of acquiring HIV-1 infection from factor VIII clotting factor concentrates was four times higher than factor IX concentrates. Similar findings have been reported by others (360, 370) with one exception (361). In France a 43% incidence of HIV-1 antibody was found in patients treated with locally (french) fractionated factor IX concentrates whereas prevalence of HIV-1 antibody in factor VIII deficient treated with locally prepared concentrates was 34 percent. This may be due to differences in fractionation methods. In most countries the first step in the fractionation of pooled plasma to prepare clotting factor concentrates is cryoprecipitation. The prothrombin group of plasma proteins fractionates into
the supernatant. In vitro studies of plasma seeded with HIV-1 have shown that 90 percent partitions into the cryoprecipitate fraction (330). This preferential partitioning of HIV-1 may explain the lower incidence of HIV-1 infection in factor IX deficient haemophiliacs.

An alternative explanation may be that differences in host susceptibility exist between factor VIIIC and factor IX deficient haemophiliacs. In a previous study it was shown that factor IX concentrates did not suppress mitogen induced lymphocyte proliferation in vitro (Chapter 3, section 3.1). It is therefore possible that factor IX deficient patients are not as predisposed to HIV-1 infection as factor VIIIC deficient patients.

The finding of a higher prevalence of HIV-1 infection in those patients who had used large quantities of factor VIII concentrates is in keeping with similar studies (221, 360). However, it remains undetermined whether the immune aberrations associated with clotting factor concentrate predisposed to HIV-1 infection, or if the more frequent use of clotting factor concentrate increased the risk of exposure to an infected batch.

A strong statistical association was also found between the use of concentrate prepared from plasma donations obtained in the USA and HIV-1 infection. This finding has been subsequently confirmed by others (361, 370, 377, 378).
However it was also found that one patient with HIV-1 infection had never been exposed to USA clotting factor concentrate, this would suggest that the HIV-1 infection was present in the UK donor population as early as 1982. Ludlam et al similarly found that locally fractionated clotting factor concentrates transmit HIV-1 infection (221). In both the present study and Ludlam's report exposure to a potentially infected batch was not invariably associated with seroconversion. It is not known if false negative results were obtained using serological tests. An alternative explanation may be (a) innate immune reactions and the initial cell mediated immune response to HIV-1 cleared the virus, or (b) that such individuals have a reduced ability to mount an antibody response either because of a intrinsic B cell defect or abnormal T cell control of B cell function. In this regard recent studies have shown that individuals exposed to HIV-1 but without serological evidence of infection have HIV-1 provirus integrated into their genome (379, 380).

In the present study patients with HIV-1 antibody were significantly younger, (Table 4.2.1), this was probably due to the more frequent use of commercial clotting factor concentrates in younger patients rather than a true age related predisposition to HIV-1 infection.
Significantly more HIV-1 antibody positive patients had previous evidence of hepatitis B infection. This was due to a higher mean annual dose of clotting factor concentrate used rather than a feature of HIV-1 infection.

A higher frequency of hepatitis B surface antigen carriage in HIV-1 antibody positive patients was clear, because of the small numbers of HIV-1 antibody negative hepatitis B surface antigen carriers no firm conclusions can be made. There are, however, several possibilities: first, this may be another aspect of the more frequent use of commercial clotting factor concentrate; second, immune impairment due to HIV-1 may have contributed to failure to clear the hepatitis B virus and lastly, persistent immune stimulation due to hepatitis B may have primed the immune system to allow HIV-1 to establish infection.

Other suggested factors in susceptibility to HIV-1 infection in haemophilia include a genetic predisposition. To date only the influence of HLA-DR phenotype has been examined and no statistically significant association was found (371). This has been confirmed by others (381-382).

In conclusion, the amount, source and type of clotting factor concentrate were independent risk factors for HIV-1 infection. These risk factors can be reduced by donor
screening for HIV-1 antibody, heat treatment of factor concentrates and by restricting the number of different batches that a patient is exposed to.
CHAPTER 4.3

The ability to isolate HIV-1. Is it governed by the anti-viral immune response?

Summary

Study objective: To attempt HIV-1 isolates from antibody positive patients.

Study population: 10 HIV-1 antibody positive patients.

Measurements: Pro-virus expression indicated by syncitium formation in stimulated peripheral blood mononuclear cells co-cultured with C8166 cells, a HIV-1 permissive cell line.

Results: Pro-virus expression was obtained in only one patient in two of three sequential cultures. The failure to obtain a third isolate coincided with a dramatic increase in circulating CD8+ve T-lymphocytes. Electronmicrographs from the first isolate showed replication deficiency viral particles.

Conclusions: The ability to isolate HIV-1 may be determined by anti-viral immune responses. Some of the clinical features of HIV-1 infection may be due to replication deficient viral particles.
4.3.1 INTRODUCTION

The ability to isolate human immunodeficiency virus (HIV-1) from antibody positive patients has several advantages. Serological tests can be confirmed, and if HIV-1 could be isolated from haemophiliacs with HIV-1 antibody, it would argue against passive immunization with viral proteins as has been suggested (383). Identifying the virus in cultures may give an insight into mechanisms of provirus expression in-vivo and yield prognostic information (219). In animal retroviral infections such as feline acquired immunodeficiency virus infection, replication deficient viruses are frequently isolated from fresh tissues but remain pathogenetic (384). By analogy such variants may exist during the course of HIV-1 infection. Furthermore, the sequence of envelope proteins could be determined in isolates and frequency of mutations over time studied. Such information would be of potential value in determining the host-virus relationship.

The aim of this study was to obtain serial HIV-1 isolates from seropositive patients.

4.3.2 METHODS

Patients

Heparinised venous blood was obtained from 10 HIV-1 antibody positive haemophiliacs. Included in the present study were two patients who had clinically significant
HIV-1 related disease: one patient had AIDS related complex and one patient recurrent oral candidiasis.

**Cell separation**

Peripheral blood mononuclear cells (PBMC) were separated by density centrifugation on sodium metriazoate as previously described (Chapter 3.1.2). The cells were resuspended in RPMI-1640 with 15 percent heat inactivated fetal calf serum and supplemented with L-glutamine and antibiotics (Chapter 3.1).

$5 \times 10^5 \text{ to } 1 \times 10^6$ PBMC cells/ml were cultured in the presence of $5 \mu g/mls$ phytohaemagglutinin (PHA) (final concentrations) and $3 \times 10^5$ C8166 cells (a hybrid cell line obtained by fusing human umbilical cord blood lymphocytes with a HTLV-1 transformed cell line obtained from patients with T cell leukaemia and lymphoma (385). All cultures were performed in $80cm^3$ tissue culture flasks. PHA stimulated patient PBMC in the absence of C8166 cells, (Costar, Cambridge, Massachusetts) and C8166 cells in the presence or absence of PHA, served as controls.

**Virus identification**

The cultures were examined daily for 7 days for syncitia, indicating provirus expression. The presence of syncitia was confirmed by an independent observer. Electronmicrographs were obtained in cultures which showed large numbers of syncitia. The presence of HIV-1 was confirmed by immunofluorescence testing.
4.3.4 Results

Syncitium formation was noted in 5 of the 10 cultures. No syncitia were seen in the control cultures. In only one patient was the presence of HIV-1 confirmed in syncitia by immunofluorescence. The presence of virus particles in culture was confirmed by electronmicroscopy.

Virus isolation in this patient was attempted on 2 further occasions after six and twelve weeks. At week six large syncitia were again seen, provirus expression was not seen at week 12.

Figure 4.3.1 shows the results of T cell subset counts at the times HIV-1 isolation was attempted in this patient. It is interesting that at week 12 when no virus was obtained in culture there had been a dramatic increase in the number of circulating T-suppressor/cytotoxic cells, (CD8+ve T-cells), but the numbers of T-helper/inducer cells had not changed.

4.3.4 DISCUSSION

In the present study HIV-1 was isolated from one of ten patients. The failure to isolate virus in remaining patients however does not imply that these patients were not infected with the whole virus. This may have been due to other factors, for instance it is well established that HIV-1 isolation is more difficult in asymptomatic
FIGURE 4.3.1 T CELL SUBSETS AT TIMES OF HIV-1 ISOLATION

CELL COUNT

NO ISOLATE

ISOLATE

CD 8+ve cells

CD4 +ve cells

DATE

12/87  1/88  2/88
individuals compared to those with AIDS (224). This variation in HIV-1 replication in cultured cells appears to mirror the increase of viral p25 antigen in the plasma of individuals as they advance in disease (386). In this study one patient had detectable p25 antigen in serum, virus however was not isolated.

The most interesting observation was in the patient in whom isolates were obtained in two of three occasions. The failure to isolate virus on the third occasion may have been due to the dramatic increase in the number of T-suppressor/cytotoxic cells at week 12. In this regard it has been reported from other laboratories that the ability to isolate virus from asymptomatic individuals is increased when CD8+ve T-cells are selectively removed from the blood sample (224). Combined, these findings suggest that cell mediated immune response to HIV-1 may be important in the control of HIV-1 infection in vivo.

Electronmicrographs of the HIV-1 isolate of this patient showed that some of the released viral particles were defective. Similar findings have been reported from an isolate obtained from the lymph node of a HIV-1 antibody positive patient with persistent generalized lymphadenopathy (387). The clinical significance of this finding is not clear. Similar findings have been reported in the recently described syndrome of feline acquired immunodeficiency syndrome (FAIDS) which is also due to retroviral infection (384). It has been
considered that in FAIDS such viral particles may be pathogenic. Future studies of fresh tissue in HIV-1 positive patients are indicated to confirm this finding.

In conclusion the results of this study confirms previous reports that HIV-1 isolation in cell culture is not invariable, this however does not indicate that these individuals were not infected with the whole virus (224). The anecdotal observation that isolates are more difficult to obtain in the presence of increased CD8+ve T-cells merits further investigation.
CHAPTER 4.4

Human T-cell lymphotropic virus-1 (HTLV-1) is not implicated in the T-cell subset aberration in haemophilia.

Summary

Study objective: To determine the prevalence of HTLV-1 infection in haemophilia.

Study population: 133 haemophiliacs who had received both locally and commercially fractionated clotting factor concentrates.

Measurements: Serum HTLV-1 antibody measured by a commercial ELISA.

Results: No patient had serological evidence of HTLV-1 infection.

Conclusions: HTLV-1 is not transmitted by clotting factor concentrates.
4.4.1 INTRODUCTION

The human immunodeficiency virus-1 (HIV-1) is a retrovirus which specifically infects T-lymphocytes of CD4+ve phenotype. Another lymphotropic retrovirus that is known to be transmitted by blood is Human T-cell lymphotropic virus-1 (HTLV-1) (388). The virus is endemic in some areas of Japan, Africa and the Caribbean, the south eastern United States and South America (389-391). In the UK, HTLV-1 antibodies are found particularly in individuals of West Indian origin (392). Both viruses are CD4+ve T-cells tropic (393). Unlike HIV-1, HTLV-I is a cell transforming virus-1 (393). Clinically, HTLV-I is implicated in the aetiology of certain T-cell cutaneous, lymphomas, tropical spastic paraparesis and other myelopathies (394-396).

Increasingly, dual infections with both HTLV-I and HIV-1 have been recognized in intravenous drug abusers (397). The influence of HTLV-I on the course of HIV-1 related immunodeficiency has not been clearly established.

Like HIV-1, HTLV-I can be transmitted by blood and blood products, sexual contact, from mother to fetus, and by sharing of contaminated needles by drug abusers (397, 398).

Reduced numbers of CD4+ve T-cells have been reported in HIV-1 antibody negative individuals (399). It was important to exclude the possibility that HTLV-1 may be
causing these changes. In the present study HTLV-1 antibody was sought in a well defined cohort of treated haemophiliacs.

4.4.2. METHODS

Patients
All haemophiliacs recruited were enrolled in 1983; details of patient selection and evaluation are given in detail elsewhere (chapter 2). Briefly, all factor VIII or IX deficient haemophiliacs who had received a blood product from the West of Scotland Adult Haemophilia Centre between 1980 and 1985 were included. Serum obtained during 1984 was used. Heat-treated factor concentrates have been used at this centre since 1985, and like HIV-1, HTLV-1 is heat sensitive.

4.4.3 Methods and patients
The presence or absence of antibody to HTLV-1 was assessed using a commercial ELISA (Dupont).

4.4.4 Results
No patient was found to have HTLV-1 antibody.

4.4.5 Discussion
The results of this study indicate that HTLV-1 cannot be implicated in the CD4 T-cell lymphopenia in HIV-1 antibody negative haemophiliacs.
The absence of HTLV-1 antibody in this group of haemophiliacs is in keeping with previous reports that HTLV-1 infection is absent in haemophiliacs (400, 401). To my knowledge there is only one haemophiliac with HTLV-1 antibody in Scotland, this individual is of West Indian origin and it has been considered unlikely that he acquired infection via blood products (402).

Despite the negative findings it is difficult to be certain that HTLV-1 is not transmitted by clotting factor concentrates because first: HTLV-1 antibodies are directed against envelope glycoproteins which may not have been detected in the assay used; second: the sample size may have been inadequate if prevalence of the virus is low; third: the factor concentrate used was derived mainly from local plasma donations from an area not known to be endemic for HTLV-1.

Transmission of HTLV-1 by blood products has recently been reported in a group of patients with leukaemia in the USA (403). None had clinical findings to suggest an illness related to HTLV-1 infection or HIV-1 infection. The occurrence of these seroconversions confirms transmission of HTLV-1 by blood in areas of presumed low HTLV-1 prevalence. It is however not clear if testing blood donations for HTLV-1 antibody should be instituted in areas where HTLV-1 infection is not endemic.
CHAPTER 5.1

SERUM HIV-1 ANTIGENAEMIA - CLINICAL AND SEROLOGICAL CORRELATIONS

SUMMARY

Study objective: To determine frequency of serum HIV-1 antigenaemia and its relationship to clinical and serological parameters.

Study design: Open, cross-sectional with retrospective review.

Study population: 22 haemophiliacs with serum HIV-1 antibody and 20 without.

Measurements: Serum HIV-1 antigen levels and western blot profile.

Results: HIV-1 antigen was found in 4 of 22 (18%) HIV-1 antibody positive patients and in none of seronegative patients. Absence of antibody to core proteins was associated with serum HIV-1 antigenaemia. Patients with serum antigen had an increased frequency of clinical disease.

Conclusions: Serum HIV-1 antigenaemia may be a useful additional marker of disease progression.
5.1.1 INTRODUCTION

Human immunodeficiency virus-1 (HIV-1) specifically infects and depletes lymphocytes that express the CD4 molecule (T-helper; T-h, CD4+ve) (339-345). The natural history of infection is characterised by a progressive depletion of CD4+ve lymphocytes, impairing the host's ability to mount an effective immune response, predisposing to opportunistic infections and malignancies (344). Infection however, does not preclude an immune response to the virus particularly in the early stages of infection.

Exposure to HIV-1 is followed by a well-characterised humoral immune response (404). Serum IgG antibodies to all the structural and functional gene products can be identified by Western blotting. At present there is not entire agreement in the sequence of serological responses, current opinion using competitive immunoassays employing recombinant-DNA produced HIV envelope proteins as antigen indicate that envelope protein, gp41, appears earlier than antibody to the core protein, p24 (404). When p24 antibody is present in antigen excess, serum antigenaemia is less frequently found (404). In general, in individuals without significant clinical immunodeficiency increasing titres of antibody using ELISA tests to all viral proteins are found (375). Symptomatic immunodeficiency is often associated with loss of p24 antibody as assessed by competitive ELISA and
Western blotting and may be associated with a reappearance of serum antigen (405).

The aim of this study was to determine the frequency of HIV-1 antigenaemia in serum, its relationship to core antibodies and symptoms.

5.1.2. PATIENTS AND METHODS

Patients

Stored serum samples from haemophiliacs treated with clotting factor concentrates since 1980 at the West of Scotland Adult Haemophilia Centre were used. The criteria for selecting patients have been previously described (Chapter 2).

Serum was tested for HIV-1 antibody by ELISA (Wellcozyme) and confirmed by Western blotting (Chapter 4). In addition serum levels of HIV-1 antigen were measured in all HIV-1 antibody positive patients. HIV-1 serum antigen levels were also measured in 20 HIV-1 antibody negative patients. These patients were specifically chosen because:

1. Mean annual dose of clotting factor used was ≥40,000iu per annum.

2. They had been exposed to batches of clotting factor concentrate fractionated by the Scottish National Blood
Transfusion service which could have resulted in HIV-1 infection (Chapter 4.1 and 4.2).

HIV ANTIGEN DETECTION
Serum samples were assayed for HIV-1 antigen by a solid phase immunoassay (Abbott laboratories). 200ul of serum was incubated overnight at room temperature with a human anti HIV-1 coated bead. The beads were washed with distilled water and rabbit anti HIV-1 IgG was added and incubated for four hours at 45°C. The beads were washed as before and then incubated for two hours at 45°C with goat anti-rabbit IgG conjugated to horseradish peroxidase. After a final wash the beads were transferred to tubes and 0-phenylenediamine was added and allowed to react for 30 minutes at room temperature in the dark. The reaction was stopped with 1ml 1N sulphuric acid and absorbance at 429nm was read. Results were read as positive when the optical density was >0.050 plus the mean of 5 replicates of normal human serum.

5.1.3 RESULTS
HIV-1 Antibody
22 (16.5%) of the 133 treated haemophiliacs were HIV-1 antibody positive by ELISA and Western blotting. On Western blotting all patients had HIV-1 envelope protein antibodies. Antibody to core proteins was absent in 4 patients.

HIV-1 Antigen
None of the twenty HIV-1 antibody negative patients had serum HIV-1 antigen. Four (18%) of the 22 HIV-1 antibody positive patients had serum antigen. Table 5.1.1 shows the results of retrospective samples from these patients.

Sequential Western blots in these 4 patients showed that the presence of serum antigen is often associated with the absence of core antibodies, (Figs 5.1.1-4), Figure 5.1.4 shows that in this patient loss of p24 antibody preceded the appearance of serum antigen and that in his most recent sample antibody to p24 has reappeared. In patients without serum antigen all bands to HIV-1 were present on Western blotting.

Clinical Outcome

Full details of clinical outcome are given in Chapter 5. Briefly; one patient has recurrent oral candidiasis (Ag=-ve; p24Ab=+ve); one patient died of AIDS, ie. HIV encephalopathy and salmonella septicaemia, he had thrombocytopenia prior to the onset of this illness (Ag=+ve; p24Ab=-ve); two patients have AIDS related complex (in one Ag=+ve; p24ab=+ve).

5.1.4 DISCUSSION

The findings in the present study that 4 (18%) of 22 HIV-1 antibody positive patients had persistent antigenaemia after a median of 24 months (range: 12-37) is in keeping with previous findings in seropositive haemophiliacs.
### Table 5.1.1  Temporal pattern of serum antigen to antibody.

<table>
<thead>
<tr>
<th>YEAR</th>
<th>81</th>
<th>82</th>
<th>83</th>
<th>84</th>
<th>85</th>
<th>86</th>
<th>87</th>
<th>88</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient No</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Ab</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Ag</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>Ab</td>
<td>-</td>
<td>-</td>
<td>N/A</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Ag</td>
<td>-</td>
<td>N/A</td>
<td>N/A</td>
<td>WEAK+</td>
<td>-/-</td>
<td>+/-</td>
<td>-/+/-</td>
</tr>
<tr>
<td>3</td>
<td>Ab</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Ag</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+/+</td>
</tr>
<tr>
<td>4</td>
<td>Ab</td>
<td>N/A</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>
|   | Ag | N/A | -/- | N/A | - | +/+ | +/+/- | +/+/+ | +
Figure 5.1.1. Western blot from a patient found to have declining p24 antibody and no p15 antibody and declining p55 reactivity. Reactivity to gp120 persisted. Lane 1 = first positive serum sample.
Figure 5.1.2. Western blot from a patient with persistent p24, p55, p15 reactivity but declining p31. Lane 1 = first positive specimen.
Figure 5.1.3. Western blot from a patient with persistent p24, p55, p31 but absent p15. This patient's first positive serum sample occurred in 1984, patient no. 15 in Figure 4.1.4.
Figure 5.1.4. Patient no. 3 in Figure 4.1.4. Lane 1 = 1st positive p24 present but p55 and p15 absent. Lanes 2-6 loss of p24 and lane 6 resurgence of p24 antibody.
Allain et al reported a 23 percent prevalence (22 of 96 patients) after a median period of 34 months (of HIV-1 antibody presence, and Simmonds et al reported a 12.5 percent incidence (2 of 18 patients) after 2 years follow-up (405, 406). There was no significant difference between the three studies (Fishers Exact Test).

Three patterns of antigenaemia are reported during the course of HIV-1 infection. First, is occurrence of antigenaemia immediately after infection and prior to presence of anti p24 antibody, levels of antigen fall when p24 antibody is present in antigen excess. In the second pattern antigenaemia persists after infection despite presence of anti p24 antibody. Third, is resurgence of antigen occurring at variable times after infection (405).

Patients with the second and third pattern of antigenaemia have clinically more severe immunodeficiency (405). It has also been found that antigenaemia is a better predictor of disease progression than the absolute CD4+ve T-lymphocyte count (405). The clinical utility of this test however requires to be confirmed.

The association of antigenaemia and absent anti p24 antibody was noted in 3 of the 4 patients in this study. In the other patient antigenaemia was associated with reduced or absent levels of anti p15 antibody (Figs
In one patient absent anti p55 antibody was noted in the face of undetectable antigenaemia. Similar findings have been reported by others (405). The implications of these findings are not clear. There are several possibilities; first antigen may have been complexed to antibody. Antigen has however not been detected in precipitated immune complexes (407). Second, antigenaemia reflects enhanced proviral expression and the depletion of antibodies is a consequence of this. In support of this is the finding that individuals with antigenaemia often have increased titres of envelope antibodies and it is unlikely that reduced antibody production should manifest in a decline of one specific antibody (405). Lastly, the converse may be true that the decline in p24 antibody response allowed enhanced HIV-1 provirus expression to continue unchecked. The anecdotal finding in this study that one patient (Fig 5.1.3) had a reappearance of p24 antibody would suggest that this may occur.

In conclusion the frequency of HIV-1 antigenaemia in HIV-1 Ab positive patients in this study was found to be 18 percent. It was also found that the patients with antigenaemia had absent anti-core antibodies. The presence of antigenaemia was more frequently associated with HIV-1 related disease manifestations, although the number of patients were not sufficient to allow any definite conclusions. The frequency of antigenaemia

5.1.2-5).
increased with the duration of HIV-1 antibody positivity but occurred at random in individual patients.
CHAPTER 5.2
THE ANTIBODY RESPONSE TO HIV-1 INCLUDES A PROTECTIVE ANTIBODY DEPENDENT CELL MEDIATED CYTOTOXICITY (ADCC) RESPONSE TO THE ENVELOPE PROTEINS.

SUMMARY
Study Objective: To determine ADCC responses to HIV-1 envelope glycoprotein.

Study Design: Open, cross-sectional.

Study Population: 21 HIV-1 antibody positive and 42 seronegative serum samples from haemophiliacs.

Measurements:
(i) Percent ADCC lysis of an episomally transfected cell line with HIV-1 gp120.
(ii) T-cell subsets.
(iii) Serum IgG.
(iv) Clinical outcome.

Results: HIV-1 antibody positive patients mediated greater ADCC lysis of transfected cells. There was a correlation with CD4+ve cell count ($r=0.51$, $p=0.04$) and age of infection ($r=-0.5$, $p=0.05$).

Conclusions: The antibody response to HIV-1 includes an ADCC response to gp120 which correlates with the patients CD4+ve T-cell count.
5.2.1 INTRODUCTION

Long term follow-up studies of HIV-1 antibody positive patients indicate that between 1% to >12% with an average of around 5-7% per annum progress to AIDS (1). Progression to AIDS is more rapid in infants and the elderly (1). Predictors of progression include a reduced number of CD4+ve T-cells, an increased number of CD8+ve T-cells, a low level of HIV-1 antibody and a resurgence of viral antigen (375, 405).

In established infection the protective role of HIV-1 antibody as measured by enzyme linked immunoassay or Western blotting is not clear. The presence of HIV-1 antibody may play a role in limiting the rate of decline of CD4+ve T-cells but is not effective in totally eliminating established infection (375). Neutralizing IgG antibody demonstrates a limited capacity to inhibit viral infection in vitro but its protective role in vivo is not known (408-411). In animal retroviral infections antibody dependent and cellular mechanisms are important in protecting against disease (412 - 414). Antibody dependent cell mediated cytotoxicity (ADCC) has been proposed as a potential method of tumour surveillance and in control of initial cell to cell spread viral infections in vivo. The ADCC process is initiated by binding of antibody directed to a target cell antigen and mediated by non-immune leucocytes bearing surface Fc receptors for IgG binding to antibody sensitized target cells (415).
ADCC against the p24 protein of HIV-1 has been previously demonstrated in infected individuals (416, 417). Levels were found to be higher in asymptomatic HIV-1 antibody positive individuals than AIDS patients. The ADCC process against envelope proteins may be important in controlling the cell to cell spread of HIV-1.

The present study was designed to determine first whether sera from HIV-1 antibody infection mediate ADCC against cells transfected with the gp120 gene of HIV-1 and secondly whether this correlates with the degree of immunodeficiency determined by counting CD4+ve T-cell numbers.

5.2.2 PATIENTS AND METHODS

The most recent serum specimen obtained between January - June 1988 of 70 haemophiliac patients was used. These patients were part of a prospective cohort of 133 haemophiliacs, who had been treated with clotting factor concentrates since 1980, and were being closely monitored for signs of immunodeficiency. Included in this study were 21 of the 22 patients known to be HIV-1 antibody positive by ELISA (Wellcozyme) and Western blotting.

T-cell subsets
T-cells subsets were counted using monoclonal antibodies as described in Chapter 8.2, in all HIV-1 antibody positive patients and in 27 of seronegative patients.

Serum IgG
Serum IgG levels were measured in all patients by laser nephelometry.

Cell lines
To examine ADCC against gp120 protein of HIV-1, COS cells (418) were transfected (419) episomally with the gp120 plasmid, and used as target cells. COS cells were subcultured at $1.5 \times 10^6$ cells per 80cm$^2$ flask at 37°C in a 5% CO$_2$ humidified atmosphere, in Dulbecco’s minimum essential medium supplemented with antibiotics and glutamine. After 24 hours cells were harvested and washed twice in phosphate buffered saline.

The cells were transfected with 20ug of DNA plasmid of HIV-1 envelope protein (provided by Dr R Jarrett) using 5mls serum free medium and 500ul of 100x DEAE-dextran. The transfection mixture was incubated with cells for 4 hours, medium was removed and replaced with 2.5mls of serum free medium containing 50mM HEPES buffer and 10% glycerol. After 2 mins the transfection mixture was replaced with Dulbecco’s minimum essential medium/10% fetal calf serum.

Assay for ADCC
Transfected and non-transfected COS cells were washed and radiolabelled in suspension at a concentration of $2 \times 10^6$ cells in Dulbecco's minimum essential medium containing 10% fetal calf serum and 100 µCi/ml of $[^{51}\text{Cr}]$ sodium chromate for 60 mins at 37°C. The cells were washed, resuspended in DMEM at a concentration of $1 \times 10^5$ cells/ml and dispensed in 100µl aliquots into wells of flat bottomed 96 well microtitre plates.

In preliminary studies, a 1:10 serum dilution was optimal for mediating ADCC. Numerically coded sera by an independent observer (Dr E Follet) were heat inactivated at 55°C for 30 mins and 20µl of a 1:10 dilution added to appropriate test wells containing target cells. Each sample was tested in triplicate. Sera and target cells were incubated for 30 minutes at 37°C.

Peripheral blood mononuclear cells obtained by density centrifugation from a healthy HIV-1 antibody negative donor were suspended in DMEM at a concentration of $3 \times 10^6$ cells and added in 100µl aliquots to wells containing target cells. After a 4hr incubation at 37°C in 5% CO$_2$ in air at 100% humidity, supernatants were harvested and counted in a gamma counter.
Percent lysis was determined by the equation

\[
\text{Percent lysis} = \frac{A-B}{C-B} \times 100
\]

A = mean cpm in supernatant from wells containing antibody-coated target cells plus effector cells.

B = mean cpm in supernatant from wells containing non-antibody-coated target cells.

C = mean total cpm of target cells added to each well in the presence of detergent, Nonidet P40 (BDH Chemicals Ltd, Poole, England).

Effect of sera to mediate ADCC against non-transfected COS cells was also determined.

5.2.3 RESULTS

Twenty one sera from HIV-1 antibody positive haemophiliacs and 49 sera from HIV-1 antibody negative were tested to determine ADCC against COS cells transfected episomally with the gp120 gene. Table 5.2.1 shows the characteristics of patients studied.
Table 5.2.1 Characteristics of patients studied.

<table>
<thead>
<tr>
<th>HIV Status</th>
<th>+ve</th>
<th>-ve</th>
<th>pvalue</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number studied</td>
<td>21</td>
<td>49</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>23.5 (19-35)</td>
<td>29.5 (24.5-37.3)</td>
<td>0.04</td>
</tr>
<tr>
<td>Mean annual dose of clotting factor concentrate used (units/annum)</td>
<td>780,000 (50,000-88,371)</td>
<td>21,163 (7,962-40,500)</td>
<td>0.001</td>
</tr>
<tr>
<td>Severity of liver disease</td>
<td>3 (2-3)</td>
<td>3 (2-3)</td>
<td>0.9</td>
</tr>
<tr>
<td>CD4+ve T-cell count (cells/mm3)</td>
<td>525.5 (373-648)</td>
<td>560 (472-789)</td>
<td>0.13</td>
</tr>
<tr>
<td>CD8+ve T-cell count (cells/mm3)</td>
<td>595.5 (456-890)</td>
<td>355 (236-340)</td>
<td>0.005</td>
</tr>
</tbody>
</table>

Medians and interquartile ranges are shown.
None of the HIV-1 antibody positive patients had AIDS. One patient had recurrent oral candidiasis and one patient AIDS related complex.

ADCC

Figure 5.2.1 shows that sera from HIV-1 antibody positive patients mediated significantly greater percent ADCC lysis of transfected cells than sera from seronegative patients (p=0.05). There was no difference between the two groups for percent ADCC lysis of non-transfected cells (p=0.08).

For HIV-1 antibody positive patients percent ADCC lysis of transfected cells did not correlate with the duration of infection (r=.13, p=0.6) but showed a moderate inverse but significant correlation with the age of infection (r=-.5, p=0.05). Furthermore in this group there was a correlation with the CD4+ve T-cell count (r=.51, p=0.04) and no correlation with the CD8+ve T-lymphocyte count (r=.14, p=.36). There was no correlation with the IgG level (r=.1, p=.43).

In seronegative patients there was no correlation with age (r=0.04, p=0.82); CD4+ve T-cell count (r=-0.00, p=0.9) serum IgG level (r=-0.1, p=0.93) and ADCC against transfected COS cells. However, a moderate direct correlation was seen with CD8+ve T cell count (r=0.41, p=0.3).
5.2.4 DISCUSSION

Longterm follow-up studies of HIV-1 seropositive individuals indicate that up to a third remain symptom free for at least seven years (1). Defining variables that predict latency should provide valuable information for the development of anti-viral strategies. Potential determinants of the course of HIV-1 infection include: levels of the antiviral immune response, inherent sensitivity of the host cell to virus replication and relative virulence of the virus strain.

Several findings indicate that the antiviral immune response may be an important variable. First, in rare cases asymptomatic HIV-1 seropositive individuals have become seronegative. In some the presence of a latent HIV-1 infection in peripheral blood mononuclear cells could be detected by the polymerase chain reaction - in others no virus could be detected (223). Second, cytotoxic T-lymphocytes that react with cells expressing HIV-1 proteins have been noted in infected individuals but the clinical importance of this antiviral response is not known (420, 421). Lastly, in vitro virus yield in HIV-1 asymptomatic individuals is greater when the subset of CD8+ve T-lymphocytes is removed from the blood sample (224).

The results of the present study show that sera from HIV-1 antibody positive haemophiliacs mediate ADCC against
the major envelope glycoprotein of HIV-1. Furthermore,
correlation was seen between the ADCC response and the
CD4+ve T-lymphocyte count. No correlation was seen
between the ADCC response and the duration of infection.
Thus, in addition to permitting the measurement of a
specific immune response against the major envelope
protein, the results of this ADCC assay showed an inverse
correlation between progression of immunodeficiency as
measured by the CD4+ve T-lymphocyte count and the
presence of serum antibodies which can mediate ADCC.

In HIV-1 seronegative patients ADCC was observed in 27
sera above the lower quartile of HIV-1 antibody positive
patients (Figure 5.2.1). There could be several reasons:
it is considered unlikely that this was due to non­
specific reactivity against COS cells. It may be that
these patients had been exposed to HIV-1 and that the
innate and specific immune responses cleared the virus
prior to establishing infection. It was noteworthy that
in HIV-1 antibody negative patients there was some
correlation with CD8+ve T-cell count. It may therefore
be that these individuals have a specific subset of
CD8+ve T-cells that has either suppressed HIV-1 antibody
production or alternatively virus infected cells have
been cleared by a specific cell dependent immune
response. Viral isolation studies in such patients or
detection of the virus by the polymerase chain reaction
may clarify these findings.
In conclusion the findings of this study indicate that the ADCC response in HIV-1 antibody positive patients may be one determinant in controlling HIV-1 disease progression. It is however, not clear whether this is a consequence of the CD4+ve T-cell depletion or a precondition. It was also found that a substantial number of HIV-1 seronegative haemophiliacs had serum ADCC activity, this may be an indicator of successful elimination of HIV-1 infected cells.
CHAPTER 6.1

HAEMOPHILIC MORTALITY: THE IMPACT OF HIV-1 INFECTION.

SUMMARY

Study objective: To determine changes in mortality data in UK haemophiliacs between 1977 and 1986.

Study design: Retrospective review.

Study population: UK Haemophiliacs (Factor VIIIC and IX deficient) registered at the Oxford Haemophilia Centre.

Measurements: Annual death rate per 1000 haemophiliacs. Age specific death rate from all causes per 1000 haemophiliacs. Annual death rate from specific causes per 1000 haemophiliacs.

Results: The annual death rate increased from 4.5 in 1977 to 10.6 in 1986. This was due to more patients over the age of 49 years dying. HIV-1 related deaths showed a six fold increase since 1983. Liver disease accounted for 12 percent of all deaths and showed a five fold increase since 1984.

Conclusions: Infection related deaths including HIV-1 related disease but not hepatitis had exceeded bleeding as the prime cause of death by 1986.
6.1.1 INTRODUCTION

Over the past two decades the therapeutic approach to control haemophiliac bleeding has changed considerably. The widespread availability of clotting factor concentrates and the institution of home treatment programmes allowed haemophiliacs to achieve a near normal lifestyle and life expectancy was similar to the general population (3, 150-154). The early use of clotting factor concentrates also appears to delay onset of severe joint disease (155). The chief disadvantages of therapy include risk of acquiring human immunodeficiency virus-1 (HIV-1) infection and hepatitis due to the non-A, non-B (NANB) viruses and hepatitis B.

Studies of stored sera in the UK show that HIV-1 antibody was first detected in 1980 and the peak annual incidence of antibody occurred in 1982/3 (364). The incubation period of HIV - 1 infection and the onset of the acquired immunodeficiency syndrome (AIDS) is not yet established. The estimated average incubation period has been disconcertingly close to the time span over which the data are available, suggesting that the average could lengthen as more information accumulates. A recent analysis of 512 cases in which dates of transfusion and diagnosis of AIDS were known suggests that the mean and median incubation periods are both between 7 and 8 years in patients older than 12 years (422). A similar estimate is reported for homosexual men in a cohort study in which the dates of seroconversion were known (1).
The average is somewhat less in children (<12 years) and the elderly (>60 years) (422, 424). Estimates of the fraction of those infected with HIV-1 who will go on to develop AIDS have also increased with time. Current evidence suggests that 30 - 75 percent of infected individuals will have progressed to AIDS in six years (425). Once AIDS is diagnosed the mean life expectancy is one year (426, 427).

Based on data available the number of UK HIV-1 related deaths in haemophilia should show a progressive increase from 1983/4 with a peak in 1988/9 and a gradual decline, if no effective therapy is found.

In this study the causes of death in haemophilia have been reviewed in the UK during two time periods 1977 - 1981 and 1982 - 1986. The primary aim was to determine the extent to which HIV-1 infection had affected mortality in haemophilia. A secondary aim was to determine if any deaths could be attributed to effects of recurrent alloantigen stimulation.

6.1.2 METHODS

Death certificates listing haemophilia A or B as a primary or secondary cause death were obtained for the United Kingdom for 1977 - 1986. The information was collated by Ms R Spooner who first, confirmed individuals for whom death certificates were available were haemophiliacs registered at the Oxford haemophilia
Centre (OHC) and second that the death had been reported to the OHC. Additional reports of deaths were obtained from the Oxford Haemophilia Centre.

Deaths were classified according to a modified International Classification of Disease (ICD) coding for the primary cause of death:

1. Haemorrhage or presumed haemorrhagic such as cerebrovascular accidents, gastrointestinal bleeding.
2. Infectious diseases including pneumonia and HIV-1 related deaths.
3. Hepatic including hepatitis and cirrhosis.
5. Cardiovascular and pulmonary disease excluding pneumonias.
6. Misadventure which included accidents and suicide.
7. Miscellaneous included known medical conditions but not associated with haemophilia.
8. Not known.

For each year annual death rate for all causes per 1000 haemophiliacs was calculated using the formula:

\[
\frac{\text{Total number of deaths during a year}}{\text{Number of persons with haemophilia in the population during that year}} \times 1000
\]

In addition annual age specific death rates from all causes for each time period was calculated:
6.1.3 RESULTS

Table 6.1.1 is a comparison of the number of deaths obtained for each year from death certificates and those reported to the OHC, because the initial request for death certificates provided little additional information only deaths reported to the OHC were used for further analysis. This gave the additional advantage that total number of patients with haemophilia for each year was known.

A total of 329 deaths were reported to the OHC during the 10 years of these 127 occurred between 1977 - 1981 and 202 between 1982 - 1986. Table 6.1.2 shows the number of deaths in haemophilia A and B patients, there were significantly more deaths in factor VIIIC deficient patients.

Annual Death Rate

Figure 6.1.1 shows the annual death rate per 1000 haemophiliacs for each year studied. A sharp increase in the annual death rate was noted from 1983 to 1986,
Table 6.1.1 Comparison of number of deaths obtained from death certificates and those reported to Oxford Haemophilia Centre.

<table>
<thead>
<tr>
<th>Year</th>
<th>Death Certificate</th>
<th>OHC</th>
<th>Data Available</th>
</tr>
</thead>
<tbody>
<tr>
<td>1977</td>
<td>18</td>
<td>19</td>
<td>19</td>
</tr>
<tr>
<td>1978</td>
<td>23</td>
<td>28</td>
<td>23</td>
</tr>
<tr>
<td>1979</td>
<td>20</td>
<td>20</td>
<td>16</td>
</tr>
<tr>
<td>1980</td>
<td>23</td>
<td>35</td>
<td>23</td>
</tr>
<tr>
<td>1981</td>
<td>19</td>
<td>25</td>
<td>19</td>
</tr>
<tr>
<td>1982</td>
<td>23</td>
<td>27</td>
<td>23</td>
</tr>
<tr>
<td>1983</td>
<td>32</td>
<td>34</td>
<td>32</td>
</tr>
<tr>
<td>1984</td>
<td>30</td>
<td>31</td>
<td>30</td>
</tr>
<tr>
<td>1985</td>
<td>0</td>
<td>46</td>
<td>46</td>
</tr>
<tr>
<td>1986</td>
<td>0</td>
<td>64</td>
<td>64</td>
</tr>
</tbody>
</table>

Further data for analysis could only be obtained from OHC directors annual report. Access to any other data kept at OHC was not allowed.

OHC = no. of deaths recorded at Oxford Haemophilia Centre by Ms R Spooner on behalf of directors.
Table 6.1.2  Number of deaths in haemophilia A and B patients.

<table>
<thead>
<tr>
<th>TYPE OF HAEMOPHILIA</th>
<th>VIIIC</th>
<th>IX</th>
<th>TOTAL</th>
</tr>
</thead>
<tbody>
<tr>
<td>1977-81</td>
<td>98</td>
<td>29</td>
<td>127</td>
</tr>
<tr>
<td>1982-86</td>
<td>187</td>
<td>15</td>
<td>202</td>
</tr>
<tr>
<td>TOTAL</td>
<td>285</td>
<td>44</td>
<td>329</td>
</tr>
</tbody>
</table>

Table 6.1.2 shows that there had been a significant increase in number of deaths in haemophilia A patients (p=0.001, Fishers Exact Test) during 1982-86 compared to 1977-81.
FIGURE 6.1.1 ANNUAL DEATH RATE /1000 IN HAEMOPHILIACS STUDIED
Figure 6.1.1. An increase in the annual death rate occurred in 1984 continuing to 1986, this was due to more deaths in haemophilia A patients.
Table 6.1.2. The increased trend was due to more haemophilia A patients dying.

Age Specific Mortality

The age specific mortality per 1000 haemophiliacs was calculated for 1980 and 1985 and are shown in Figure 6.1.2. Age specific mortality showed a 34 percent increase for patients aged between 50-59 years; a 54 percent increase for patients aged 60-69 years and 104 percent increase for patients over 70 years of age. Figure 6.1.3 shows the age distribution in 1980 and 1985 for factor VIIIC deficient patients. Table 6.1.3 compares the number of patients above 50 years registered at OHC in 1980 and 1985, there were significantly more patients over the age of 50 years registered in 1985.

Causes of Death

The cause of death was known for 65 percent of all deaths. Of deaths for which cause was not known, 40 occurred between 1977 - 1981 and 21 between 1982 - 1986 (p = 0.02), Fishers Exact Test).

The total number of deaths in for each category for the ten years is shown in Table 6.1.4. Thirty two percent of all deaths were due to bleeding. Bleeding accounted for 40 percent of deaths in 1977 - 1981, of these 60 percent were due to intra cerebral bleeds. During 1982 - 1986, 28 percent of all deaths were due to bleeding (p = 0.05,
FIGURE 6.1.2 AGE SPECIFIC MORTALITY IN HAEMOPHILIA

AGE GROUP

AGE SPECIFIC MORTALITY

1985

1980
Figure 6.1.2. Age specific mortality showed a significant increase in those aged over 50 years. No changes were seen in those aged less than 50 years comparing 1980 and 1985 figures.
Figure 6.1.3 AGE DISTRIBUTION OF HAEMOPHILIA PATIENTS

NUMBER OF PATIENTS

1200
1100
1000
900
800
700
600
500
400
300
200
100
0

<10 <19 <29 <39 <49 <59 <69 N/K

AGE
Figure 6.1.3. Data obtained from Annual Directors reports and reproduced with permission. In both 1980 and 1985 age distribution was similar except there were patients over 50 years in 1985.
Table 6.1.3  Numbers of registered patients over 50 years with factor VIIIC deficiency.

<table>
<thead>
<tr>
<th>AGE GROUP (years)</th>
<th>Year</th>
<th>( \leq 49 )</th>
<th>( \geq 50 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>1980</td>
<td>2224</td>
<td>314</td>
<td></td>
</tr>
<tr>
<td>1985</td>
<td>3881</td>
<td>1131</td>
<td></td>
</tr>
</tbody>
</table>

These were significantly more patients over the age of fifty years registered in 1985 than 1980 (p=0.001, Fishers Exact Test).
Table 6.1.4 Causes of death.

<table>
<thead>
<tr>
<th>Category</th>
<th>Number of Deaths</th>
</tr>
</thead>
<tbody>
<tr>
<td>Haemorrhagic</td>
<td>104 (31.6)</td>
</tr>
<tr>
<td>Infectious</td>
<td>59 (17.9)</td>
</tr>
<tr>
<td>Hepatic</td>
<td>13 (3.9)</td>
</tr>
<tr>
<td>Malignancy</td>
<td>36 (10.9)</td>
</tr>
<tr>
<td>Cardiorespiratory</td>
<td>29 (8.8)</td>
</tr>
<tr>
<td>Misadventure</td>
<td>32 (9.7)</td>
</tr>
<tr>
<td>Miscellaneous</td>
<td>12 (3.6)</td>
</tr>
<tr>
<td>Not known</td>
<td>44 (13.4)</td>
</tr>
<tr>
<td><strong>TOTAL</strong></td>
<td><strong>329</strong></td>
</tr>
</tbody>
</table>

The causes of death in each category are shown, the numbers in brackets are the percentage number of deaths.
Fishers Exact Test) of these 78 percent were due to intracerebral bleeding, (p=0.005, Fishers Exact Test).

Infections including HIV-1 related deaths accounted for 18 percent of all deaths. Figure 6.1.4 shows the annual mortality per 1000 haemophiliacs in this group. From 1982 there was a progressive increase in infection related deaths. Annual mortality rate for HIV-1 related deaths is shown in Figure 6.1.4, the first HIV-1 related death was reported in 1983 and by 1986 there was a six fold increase in HIV-1 related deaths. In 1986 deaths due to HIV-1 exceeded deaths due to intracerebral bleeding.

Liver disease accounted for 12 deaths all but one of these occurred between 1982 - 1986. The overall relative risk of dying from liver disease was 4 times less than due to bleeding. Cancer related deaths were the next most common cause of mortality accounting for 11 percent of all deaths. There were 14 deaths due to cancer in the first five years (14 percent) and 22 between 1982 - 1986 (p = 0.53, Fishers Exact Test).

6.2.3 DISCUSSION

The results of this study show dramatic changes both in annual mortality rate and in causes of death in patients with haemophilia. First, there was a significant increase in annual death rate between 1977 and 1986, in 1977 the annual death rate per 1000 haemophiliacs was 4.5 per 1000
Figure 6.1.4 ANNUAL DEATH RATE /1000 DUE TO INFECTION INCLUDING HIV - 1 INFECTION
Figure 6.1.4. Annual mortality per 1000 due to infections including HIV-1 but excluding hepatitis are shown.
Figure 6.1.5 ANNUAL MORTALITY FROM THE TWO MOST COMMON CAUSES OF DEATH

- HAEMORRHAGE
- INFECTION
- HIV-1

YEAR

ANNUAL DEATH RATES

Figure 6.1.5. Annual death rate for haemorrhagic and infections are shown. Infection excludes HIV-1 and hepatitis. HIV-1 deaths are shown separately.
haemophiliacs, in 1986 this had increased to 10.6 (p=0.02, Fishers exact test). Second, causes of death had changed; at the end of this study infection including HIV-1 related deaths were the commonest cause of mortality and although liver disease accounted for only 4 percent of all deaths mortality due to liver disease showed a six fold increase over ten years. Third, a comparison of the age specific mortality in 1980 and 1985 showed that increased annual death rate was due to more haemophiliacs over the age 49 years and particularly patients over the age of 69 years dying, in 1985.

Bleeding related deaths accounted for 32 percent of all deaths of these 70 percent were due to intra cerebral bleeds. An apparent increase in the annual mortality rate due to intracerebral bleeding from 0.4 per 1000 in 1977 to 2.4 per 1000 haemophiliacs in 1986 was seen. This was due to an increase in the number of intracerebral bleeds and may be due to the increased numbers of patients over the age of 50 years, Table 6.1.2.

Infection related deaths showed a progressive increase from 1982 onwards. There were significantly more infection related deaths during the 1982 - 1986 (23 percent) compared to 1977 - 1981 (5 percent) (p=0.001, Fishers Exact Test). Sixty four of the deaths were found to be HIV-1 related. The first HIV-1 related death was recorded in 1983 and by 1986 there had been a six
fold increase. In 1986 34 percent of all deaths were due to an HIV-1 related illness compared to 25 percent of deaths due to bleeding the relative risk of dying from an HIV-1 related illness was 1.4 times greater than that from bleeding.

Of HIV-1 related deaths for which age was available (35 percent) all had occurred in individuals above the age of 29 years of these 58 percent were above the age of 49 years. This is in keeping with reports that show the rate of CD4+ve T-lymphocyte depletion is greater in older infected individuals and is independent of duration of HIV-1 infection (Chapter 8.2).

The increase in infection related deaths during the second five years cannot be entirely explained. It is possible that they were not recognized as HIV-1 related or were not reported as such. However, Aronson has also noted an increase in pneumonia related deaths in haemophiliacs in the USA prior to the AIDS epidemic (428). Although it is difficult to be sure, it is possible that the impaired ability to mount a cell mediated immune response in the absence of HIV-1 infection was a contributory factor in these deaths. The cancer related deaths could not be attributed to such a defect using the data available, although cancers associated with immunodeficiency have been reported in the absence of HIV-1 infection (329,429).
Liver disease accounted for only 12 deaths (4 percent), of these all but one occurred between 1982-1986. Since 1984 there had been a five fold increase in the number of deaths related to liver disease. From the data available (50 percent of liver disease related deaths) death was more common in individuals over the age of 49 years. This is in keeping with the data available from the non haemophiliac transfusion related cases of NANB hepatitis, that older individuals have a poorer outcome (176, 178).

In conclusion the results of this study show that HIV-1 related disease is now the major cause of mortality in haemophilia. Furthermore the major initial impact of HIV-1 related disease has been in older individuals than children. A increased incidence of infection related deaths was noted but the interpretation of this is difficult. Liver disease was found to be increasing cause of mortality.
CHAPTER 6.2

DOES TREATMENT WITH CLOTTING FACTOR CONCENTRATES PREDISPOSE TO CLINICAL IMMUNODEFICIENCY IN THE ABSENCE OF HIV-1 INFECTION?

SUMMARY

Study objective: To determine if the in vitro immunosuppression associated with clotting factor concentrates in the absence of HIV-1 antibody is associated with clinical immunodeficiency.

Study design: Retrospective case note review.

Study population: 80 factor VIIIC and 31 factor IX deficient haemophiliacs regularly treated with clotting factor concentrates.

Results: 5 percent of factor VIIIC deficient haemophiliacs had disease that could be attributed to immunodeficiency. No factor IX deficient patient had clinical cell mediated immunodeficiency.

Conclusions: Treatment with factor VIII containing clotting factor concentrates is associated with a higher than expected incidence of clinical immunodeficiency in the absence of HIV-1 antibody.
The treatment of haemophilia is associated with a high risk of acquiring blood borne viral infections. The risk of acquiring HIV-1 infection depends on the amount, type, source and the number of infusions with an infected batch of clotting factor concentrate (Chapter 4). Non A, non B hepatitis infection is an invariable complication of treatment. Up to 25 percent of infected patients may develop histological evidence of progressive liver disease manifest as cirrhosis or chronic active hepatitis (2). More recently concern has been expressed on the effects on cell mediated immunity of recurrent extraneous protein load that frequently treated patients are exposed to. In the previous chapter (Chapter 6.1) it was found that infections including HIV-1 related deaths were the second most frequent cause of death in haemophiliacs.

In vitro factor VIII concentrates impair lymphocyte activation and proliferation and the ability to produce interleukin 2 is impaired (3). In vivo clotting factor concentrates suppress cell mediated immunity as assessed by skin testing (Chapter 7). This was found to be independent of HIV-1 antibody status and severity of liver disease. A moderate direct correlation was noted with the degree of impairment of response to a new antigen, dinitrochlorobenzene and mean annual dose of
clotting factor concentrate. The clinical significance of these findings was not clear.

Significant impairment of cell mediated immunity from any cause predisposes the individual to intracellular infections and tumours. The tumours most commonly associated with impaired cell mediated immunity include non-Hodgkins lymphomas and epithelial carcinomas. The use of blood products is known to be immunosuppressive in non-haemophiliacs (279-304). This is seen particularly in the context of transplantation, pre-transfusion of blood has a beneficial effect on renal allograft survival but a detrimental effect in humans and animals with bone marrow transplants (279, 280, 282).

The aim of the present study was to determine whether haemophiliacs studied as part of this thesis had developed disease suggestive of significant clinical immunodeficiency in the absence of HIV-1 infection.

6.2.2 PATIENTS AND METHODS

The criteria for recruiting patients into this study have been previously described (Chapter 2). The case notes of all patients were reviewed and any episodes of unexplained infection during the period 1980 - 1988 were recorded. The causes of death were verified by case note review.
HIV-1 antibody status of all patients had been previously established by an ELISA method (Wellcozyme) and confirmed by Western blotting.

6.2.3 RESULTS

Mortality: Ten patients had died during the study. Of these four had died prior to 1982. The causes of death are listed in Table 6.2.1. One patient had died of AIDS, details of his case history are given in Chapter 6.3.2. In only one patient could death be attributed to probable immunodeficiency in the absence of HIV-1 infection. Details of his case history are given below.

Case report
A 50 year old patient deficient in factor VIII (<0.01 u/ml) was admitted with haemarthrosis of the right knee. He was taking Ibuprofen 1200mg daily and dihydrocodeine for chronic haemophiliac arthritis. He had received 75,000 units of factor VIII concentrate in the previous four years. His bleeding episode was treated with transfusion of factor VIII concentrate, analgesia and physiotherapy. He was negative for antibody to HIV-1 by both enzyme linked immunosorbent assay (ELISA) and Western blotting. Tests for antibodies to human T-cell lymphotropic virus type I also yielded negative results. Intradermal infection of both purified protein derivative and dinitrochlorobenzene yielded no
Table 6.2.1 Causes of death in patients prospectively studied.

<table>
<thead>
<tr>
<th>Cause of death</th>
<th>Number of patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Haemorrhagic</td>
<td>2 (1)</td>
</tr>
<tr>
<td>Infection (including HIV-1 related)</td>
<td>2</td>
</tr>
<tr>
<td>Hepatic</td>
<td>2</td>
</tr>
<tr>
<td>Malignancy</td>
<td>2</td>
</tr>
<tr>
<td>Cardiorespiratory</td>
<td>1</td>
</tr>
<tr>
<td>Misadventure</td>
<td>1</td>
</tr>
<tr>
<td><strong>TOTAL</strong></td>
<td><strong>10</strong></td>
</tr>
</tbody>
</table>

The numbers in bracket are inhibitor patients.

Both haemorrhagic deaths were intracerebral bleeds. One patient died of AIDS and one of gram negative septicaemia after rupture of a colonic diverticulum.
reaction. The T lymphocyte ratio was 0.36 (T-helper cells 0.7x10⁹/l and T suppressor cells 1.925x10⁹/l).

Two weeks after admission he had a haematemesis and his blood pressure fell to 80/50mmHg. Three units of blood and 3000 units of factor VIII concentrate were transfused. Endoscopy showed superficial gastric ulcers; Ibuprofen was stopped, and he was treated with factor concentrate and Ranitidine.

Though there was no further gastric bleeding, his renal function deteriorated, having been normal on admission. Serum urea concentration on the day of the haematemesis was 32.3mmol/l and creatinine 180umol/l; a week later creatinine had risen to 370umol/l. He was initially presumed to have acute tubular necrosis, and fluid overload was corrected with diuretics. Renal ultrasonography showed both kidneys to be enlarged. Management was complicated by chest infections, and on one occasion he suffered septicaemia and cardiopulmonary arrest, from which he was resuscitated. Fourteen days after his haematemesis serum creatinine concentration was 900umol/l. Renal biopsy was not performed because of the risk of bleeding: an inhibitor to factor VIII was detected six weeks after the onset of renal failure. Prednisolone 40mg was then started, with a transient improvement in renal function (creatinine concentration 450umol/l). His condition deteriorated, and he was
considered unsuitable for dialysis. He died of bronchopneumonia and septicaemia due to Escherichia coli.

At necropsy both kidneys were enlarged (250g) with uniform pale cut surfaces. The corticomedullary junction was poorly defined, and there was no evidence of scarring or haemorrhage. Microscopy showed a diffuse, mainly cortical infiltrate of large lymphoid cells, which were pleomorphic with convoluted nuclei and numerous mitoses. Results of immunocytochemical staining with common leucocyte antigen were positive. Fresh tissue was not available to define the lymphocyte infiltrate in more detail. There was no evidence of tumour infiltrate in the rest of the organs examined. A postmortem diagnosis of extra-nodal non-Hodgkin's lymphoma was made.

Two patients had died secondary to intracerebral haemorrhage. One was known to have treated essential hypertension prior to death, the other was a heavy cigarette smoker and had a high titre of factor VIIIC inhibitor.

Two patients died secondary to severe liver disease. Both had previous serological evidence of hepatitis B infection but had cleared hepatitis B surface antigen and had biochemical evidence of NANB hepatitis. One patient had prior history of heavy alcohol abuse and this was presumed to be the major factor in precipitating liver failure. Liver ultrasound in the second patient showed
evidence of neoplastic deposits suggestive of a primary liver tumour. Postmortem permission was refused in both.

Infections
Of the 111 HIV-1 antibody negative patients 2 had been treated for pulmonary tuberculosis, one in 1979 and the other in 1986. In one other patient a florid positive reaction to the purified protein derivative of M. tuberculosis was noted in studies of cell mediated immunity, but no active infection was identified.

One patient developed a panophthalmic infection of his left eye, no organism was isolated and his illness settled after intensive parenteral antibiotic therapy. He has had no other infective illness.

Another patient presented with a history of night sweats and general malaise. Blood cultures grew a Staph Aureus. There was no echocardiographic evidence of infective endocarditis, his initial symptoms responded to intravenous antibiotics. He subsequently again developed symptoms and increasing pain in his left knee joint which had been previously replaced. Joint aspiration grew Staph. aureus. He was diagnosed to have a infected knee arthroplasty which was surgically removed. Culture of the removed prosthesis was negative.
Three months later he was readmitted with a pyrexia, splenomegaly and night sweats. Blood cultures grew a Serratia organism. He was treated with intravenous gentamicin and cefotaxime. Recovery was complicated by frequent recrudescentes of pyrexia. Gallium scanning showed increased tracer accumulation in the right lung. He developed a profound neutropenia presumed to be due to antibiotic therapy, recovery was complicated by probable systemic candidiasis which resolved without treatment.

Although he has had a further two episodes of pyrexia no route of infection has been identified yet. There was circumstantial evidence to suggest illicit intravenous drug abuse which may have been a contributory factor.

The last patient presented with a history of night sweats and weight loss of greater than ten percent of his previous body weight. Investigations showed activated circulating lymphocytes but no serological evidence of a viral infection was obtained. His symptoms have persisted although there has been no further weight loss.

6.1.4 DISCUSSION
In the present prospective clinical study, it was found that five percent of factor VIIIC deficient haemophiliacs in the absence of HIV-1 antibody had clinical disease possibly indicative of impaired cell mediated immunity. The absence of any disease in factor IX deficient haemophiliacs may be because the cohort was
not large enough, alternatively it may be due to the lack of effect of factor IX concentrates on lymphocyte activation and proliferation (Chapter 3.1).

Aronson has reported in a retrospective study of haemophilia related deaths that more patients than expected had died of pulmonary infections in the USA prior to the AIDS epidemic (328, 428). Furthermore, the incidence of primary tuberculosis in haemophiliacs inadvertently exposed to case of open tuberculosis was similar to that noted in children receiving cytotoxic therapy for tumors (327). The occurrence of primary tuberculosis in haemophiliacs correlated with the amount of clotting factor concentrate infused in the preceding year but was independent of the HIV-1 status of the patients.

The presentation, site and the nature of the lymphoma noted in one of the patients in this study is rare and not previously noted in HIV-1 antibody negative haemophiliacs. In a review of all haemophilia related deaths in the UK between 1977 - 1986 one other non Hodgkin's and a Hodgkin's lymphoma were listed as the cause of death in 1984, the relationship to HIV-1 infection remains undetermined (Chapter 6.1). Interestingly, in Italy one patient with non endemic Kaposi sarcoma had been reported in the absence of HIV-1 antibody by both ELISA and Western blotting (429).
In conclusion this prospective study showed that HIV-1 antibody negative factor VIIIC deficient haemophiliacs had a higher than expected incidence of clinical disease possibly indicative of impaired cell mediated immunity. Further studies of the nature of this defect and its relationship to clotting factor concentrate use are indicated.
CHAPTER 6.3

HIV - 1 RELATED DISEASE IN HAEMOPHILIACS AT THE WEST OF SCOTLAND HAEMOPHILIA CENTRE.

SUMMARY

Study Objective: To describe the spectrum of HIV - 1 related disease in haemophiliac studied. To evaluate and compare the Center for Disease Control (CDC) and Walter Reed (WR) HIV - 1 disease stagings.

Study design: Retrospective review

Study population: HIV - 1 antibody positive haemophiliacs.

Results: Manifestations of HIV - 1 related disease included: thrombocytopenia - 3 patients; lymphadenopathy - 3 patients; bacterial infections - 2 patients; AIDS related complex - 3 patients; opportunistic infection - 3 patients. There was a moderate correlation in CDC and WR disease classifications in December 1986 and January 1988. WR classification showed a correlation with age. WR classification was able to detect change.

Conclusions: HIV - 1 related disease is associated with a spectrum of disease including bacterial infections and thrombocytopenia, these may be due to B cell abnormalities. The WR classification was found to be sensitive in detecting change.
6.3.1 INTRODUCTION

Infection with the human immunodeficiency virus-1 (HIV-1) is classically associated with an impaired ability of the host to mount a cell mediated immune response. Clinically this is manifest by the occurrence of opportunistic infections and tumours (430, 431). The infections most frequently associated with end stage HIV-1 induced immunosuppression are listed in Table 6.3.1. The majority of infections result from endogenous reactivation of a previously acquired infection, for example Mycobacterium tuberculosis and herpes zoster (432). Infections are rarely single and concurrent or consecutive infections with different organisms are common (432). Fungal and parasitic infections depend on the prevalence of asymptomatic infection with these pathogens in the local population (432). More recently certain bacterial infections have been recognized as HIV-1 associated diseases (432); the association may be partly due to defects in B cell function described in persons with HIV-1 infection (Chapter 11).

Until recently the Centre for Disease Control (CDC) in addition to Kaposi's sarcoma included primary central nervous system non-Hodgkin and high grade lymphoma occurring at all sites but excluded intermediate-grade non-Hodgkin lymphoma at non-central nervous system sites and all cases of Hodgkin disease from diagnostic criteria (430, 431). Nonetheless, an increasing number of reports of diffuse aggressive B cell non-Hodgkin lymphoma of
Table 6.3.1. Infections associated with HIV-1 infection.

<table>
<thead>
<tr>
<th>Organisms</th>
<th>Clinical Manifestations</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Protozoa</strong></td>
<td></td>
</tr>
<tr>
<td>Pneumocystis carinii</td>
<td>Pneumonia (Common)</td>
</tr>
<tr>
<td>Toxoplasma</td>
<td>Encephalitis (Infrequent)</td>
</tr>
<tr>
<td>Cryptosporidium</td>
<td>Retino-choroiditis (Infrequent)</td>
</tr>
<tr>
<td>Isopora belli</td>
<td>Enteritis (Infrequent)</td>
</tr>
<tr>
<td><strong>Fungi</strong></td>
<td></td>
</tr>
<tr>
<td>Candida sp.</td>
<td>Stomatitis (Common)</td>
</tr>
<tr>
<td>Cryptococcus neoformans</td>
<td>Meningitis (Common)</td>
</tr>
<tr>
<td>Histoplasma capsulatum</td>
<td>Dissemination (Common)</td>
</tr>
<tr>
<td>Coccioides immitis</td>
<td>Dissemination (Infrequent)</td>
</tr>
<tr>
<td><strong>Bacteria</strong></td>
<td></td>
</tr>
<tr>
<td>Mycobacterium tuberculosis</td>
<td>Pneumonia (Common)</td>
</tr>
<tr>
<td>Mycobacterium avium-intracellulare</td>
<td>Dissemination (Infrequent)</td>
</tr>
<tr>
<td>Mycobacterium kansasii</td>
<td>Dissemination (Infrequent)</td>
</tr>
<tr>
<td>Streptococcus pneumoniae (esp. pediatric)</td>
<td>Upper respiratory (Infrequent)</td>
</tr>
<tr>
<td>Salmonella sp.</td>
<td>Sepsis (Common)</td>
</tr>
<tr>
<td>Treponema pallidum</td>
<td>Diarrhoea (Common)</td>
</tr>
<tr>
<td><strong>Viruses</strong></td>
<td></td>
</tr>
<tr>
<td>Cytomegalovirus</td>
<td>Retino-choroiditis (Common)</td>
</tr>
<tr>
<td>Herpes Simplex</td>
<td>Pneumonia (Common)</td>
</tr>
<tr>
<td>Herpes Zoster</td>
<td>Colitis (Infrequent)</td>
</tr>
<tr>
<td>Epstein-Barr</td>
<td>Mucotaneous (mouth, digit, rectum) (Infrequent)</td>
</tr>
<tr>
<td>Jacob - Creutzfeld</td>
<td>Dermatomal skin (Infrequent)</td>
</tr>
<tr>
<td></td>
<td>Hairy leukoplakia (Infrequent)</td>
</tr>
<tr>
<td></td>
<td>Progressive multifocal leuko-encephalopathy (Infrequent)</td>
</tr>
</tbody>
</table>
intermediate as well as high grade histopathological types and of atypical, aggressive Hodgkin's disease occurring in AIDS risk group populations have appeared in the literature (433-445). The activation with respect to Hodgkin's disease is not clear particularly as Hodgkin's disease normally also occurs in the same age group as persons at risk of HIV-1 infection.

More recently pro-viral HIV-1 DNA has been identified in brain tissue, endothelial and epithelial cells suggesting that HIV-1 has the potential to cause disease in many organ systems independent of the effects of immune system (208-211). Host responses to HIV-1 infection may also contribute to pathological and immunological consequences of infection. However, progressive immunodeficiency remains the most serious outcome of HIV-1 infection.

The aim of this study was first, to describe the pattern of HIV-1 related illness seen in this population and second to evaluate the clinical utility of two recently proposed HIV-1 related disease classifications to detect change.

6.3.2 PATIENTS AND METHODS

The case notes of all HIV-1 antibody positive patients were reviewed and details of any clinical illness recorded. All patients had been regularly reviewed since 1981 and from 1983 all patients were specifically asked
about symptoms that indicated HIV-1 related disease at each clinic visit. The weight of patients was also recorded.

In December 1986 and January 1988 all patients were staged according to the Walter Reed Classification and the criteria proposed by the Centre for Disease Control classification for HIV-1 infection (446, 447).

**HIV-1 antibody status**

HIV-1 antibody status had been previously established by ELISA (Wellcozyme) and confirmed by Western Blotting (Chapter 4). All retrospective sera available were tested to determine duration of infection (Chapter 4.2).

**HIV-1 serum antigen**

Serum antigen was measured as described in Chapter 5.

**T-cell subsets**

T-helper/inducer (CD4+ve) lymphocytes were enumerated as described in Chapter 8.1.2.

### 6.3.3 RESULTS

**HIV-1 related disease:**

**Acute infection**

No patient had symptoms attributable to acute HIV-1 infection. However in one patient generalised lymphadenopathy coincided with the date when he was first
noted to be HIV-1 antibody positive. Enlarged, painless, mobile lymph nodes were noted bilaterally in the submental, supraclavicular and axillary areas. There was no associated skin rash or splenomegaly.

On subsequent examination four weeks later lymphadenopathy had regressed. Investigations showed: positive IgG anti-HIV-1 antibody by ELISA; his serum reacted to the following HIV-1 proteins on Western blotting: gp160, 120 and 41 and p64, 55, 31, 24 and 15.

Thrombocytopenia
3 patients had developed significant thrombocytopenia; in one this preceded onset of subsequent bacterial infections. No bleeding related to thrombocytopenia occurred. In two patients trephine bone marrow aspirates showed plentiful megakaryocytes with budding suggesting peripheral destruction. Only one patient was noted to have persistent splenomegaly. Anti-platelet antibodies were not measured.

The platelet counts in these patients are shown in Figure 6.3.1 (a-c).

Lymphadenopathy
No patient could be classified to have persistent generalised lymphadenopathy as defined by the CDC. However 3 patients had intermittent cervical and axillary
FIGURE 6.3.1 PLATELET COUNT IN THROMBOCYTOPENIC PATIENTS.

PATIENT 1

DATE
FIGURE 6.3.1B PLATELET COUNT IN THROMBOCYTOPENIC PATIENTS

PATIENT 2

DATE
FIGURE 6.3.1C PLATELET COUNT IN THROMBOCYTOPENIC PATIENTS

PATIENT 3

DATE

PLATELET COUNT

400
300
200
100
0
lymphadenopathy. During the episodes of lymphadenopathy there were no associated systemic symptoms.

**Bacterial infections**

Two patients had recurrent bacterial infections: their case histories are given below:

**Case 1**

Factor VIIIC level: <0.01 iu/ml. Inhibitor = absent
First positive serum HIV-1 antibody - 5/81
Last negative serum HIV-1 antibody - 3/82

In January 1987 he was noted to have clinical splenomegaly, no hepatomegaly and weight loss of eight kilograms. Platelet count was noted to be low 18,000/mm³ (described in thrombocytopenia section) and serum HIV-1 antigen positive. He was diagnosed to have thrombocytopenia secondary to HIV-1 infection. There was no associated bleeding attributable to the thrombocytopenia.

In March 1987 he was admitted with a pyrexia of unknown origin and treated with intravenous cefotaxime and subsequently oral erythromycin. While in hospital he was inadvertently frequently exposed to a patient who was a salmonella carrier. He subsequently developed enlarged right posterior cervical lymphadenopathy - open biopsy and histology showed reactive changes. Subsequent cultures of the unhealed surgical incision grew
salmonella and he developed a salmonella septicaemia. Despite intensive supportive and specific therapy his clinical condition deteriorated manifest as HIV-1 related encephalopathy, a painful peripheral neuritis and cachexia.

He died of an acute respiratory arrest.

Case 2
Factor IX < 2iu/dl. No inhibitor.
Date of first positive serum HIV-1 antibody = 4/82.
No negative specimens available.

In January 1986 he presented with a 3 day history of a febrile illness. On examination signs consistent with a left basal consolidation were present. He had small shotty nodes in both axillae but only one in the left axilla was >1cm. X-rays confirmed a left basal pneumonia. Emergency sputum gram stain showed gram positive cocci, he was presumed to have pneumococcal pneumonia and treated with benzyl penicillin. Recovery was complicated with an infected herpes simplex lesion on the left lower lip.

Further enquiry revealed that he had a previous right sided pneumonia complicated by an effusion in January 1985 while visiting relatives in Manchester. Aspiration of the effusion was negative for mycobacterium
tuberculosis and cytology did not reveal any neoplastic cells.

In March 1986 he presented with a 2 week history of an abscess in his left flank. Wound swabs showed a coagulase positive Staph Aureus sensitive to Erythromycin and Flucloxacillin. The abscess was drained under general anaesthetic. Recovery was uneventful.

In January 1988 he was again admitted with a pyrexial illness. Chest x-ray showed patchy right basal consolidation and sputum culture grew Haemophilus Influenzae sensitive to Ampicillin. After 5 days he developed a generalised erythematous rash presumed to be due to Ampicillin. Further recovery was uneventful.

Three patients were classified to have AIDS related complex (ARC).

Patient 1
Factor VIIIC level <0.01iu/ml. Inhibitor = absent
First positive serum HIV-1 antibody - 5/82
Last negative serum HIV-1 antibody - 1/84

In July 1987 the patient complained of persistent throat discomfort on wakening. On examination there was no palpable lymphadenopathy but he was noted to have bilateral enlargement of his tonsils. There was no evidence of oropharyngeal candidiasis.
A bilateral tonsillectomy was performed, his post-operative course was uneventful. Histology showed: expanded T-cell zones but preserved tonsillar architecture and prominent lymphoid follicles. These findings were consistent with reactive tonsillar enlargement.

Culture of tonsils grew only oral commensals. Peripheral blood T-cell phenotyping showed a reduced CD4+ve cell count, CD4+ve T-cells = 304 cells/mm³. In January 1988 he was noted to have lost greater than ten percent of his weight and he complained of night sweats, fatigue and malaise. Serum was positive for HIV-1 antigen and the number of CD4+ve T-cells had declined to 227 cells/mm³.

Patient 2
Factor VIIIIC <0.01iu/ml. Inhibitor = 13 Bethesda units/mls.
First positive serum HIV-1 antibody = 10/85
Last negative serum HIV-1 antibody = 12/85

In December 1988 he complained of lethargy and one upper respiratory tract infection. Clinical examination for stigmata of HIV-1 related disease was negative.

Investigations showed:

- HIV-1 antigen serum = positive
- Total WBC = 2030 cells/mm³
- CD4+ve T-cell count = 122 cells/ml³
CD8+ve T-cell count = 238 cells/mm³

He was reluctant to commence azidothymidine.

Four months later he was noted to have: facial and scalp seborrhoeic dermatitis. Oral hairy leukoplakia on both tongue margins. Candida intertrigo of groin, scrotum and anus.

T-cell phenotyping showed:

CD4+ve T-cell count = 52 cells/mm³
CD8+ve T-cell count = 150 cells/mm³

He was commenced on nebulized pentamidine 300mg twice a week and 200mg azidothymidine four hourly.

After four weeks of therapy he developed a normochromic anaemia (haemoglobin 8.1g/dl) due to bleeding and was transfused 3 units of whole blood.

By August 1988 the seborrhoeic dermatitis had improved and candida intertrigo resolved. Oral hairy leukoplakia had also significantly improved.

Patient 3

Factor VIIIC level <0.01 iu/mls. Inhibitor = absent
First positive serum for HIV - 1 antibody: 8/84

No negative serum samples were available.
Relevant previous medical history included significant congenital mental retardation.

In January 1988 he complained of sweats, a cough productive of purulent sputum of 3 days duration. He was noted to have: bilateral enlarged tonsils, shotty cervical and right axillary lymphadenopathy and signs of right basal pulmonary consolidation. Chest x-ray confirmed the clinical findings. No bacterial or opportunistic organisms were grown from sputum cultures. He was treated empirically with oral erythromycin and had an uneventful recovery. On further questioning he admitted to lethargy, occasional night sweats and weight loss.

Further investigation showed:

- Serum HIV-1 antigen = negative
- Total WBC = 3792 cells/mm$^3$
- CD4+ve T-cell count = 263 cells/mm$^3$
- CD8+ve T-cell count = 136 cells/mm$^3$

He was classified as having possible early AIDS related complex. In view of resolution of his symptoms azidothymidine therapy has not yet been commenced.

HIV-1 related disease attributable to immunosuppression.

Opportunistic infections

One patient had recurrent oral candidiasis. His case history is given below:
The patient is a caucasian born in the West of Scotland. Circulating factor level is <1iu/dl. He was first noted to have HIV-1 antibody in October 1985, and his last antibody negative sample was in October 1984. His serum was negative for HIV-1 p24 antigen on both occasions. On questioning he denied any symptoms that may have been attributable to acute HIV-1 infection.

In May 1987 he complained of white patches on the undersurface of his tongue. Swabs grew candida albicans. There were no symptoms to suggest oropharyngeal candidiasis. On examination he had intermittent cervical lymphadenopathy and tinea pedis of recent onset which was resistant to local treatment. T-cell phenotyping in 1988 showed a markedly reduced CD4+ve T-lymphocyte count 200 cells/mm³ and 382 cells/mm³ CD8+ve T-lymphocytes.

His most recent serum sample remains reactive to all viral protein on Western blotting and HIV-1 p24 antigen is absent from serum.

Disease classification

Tables 6.3.2 and 6.3.3 show the patients classified according to the CDC and Walter Reed Classification. There was a moderate correlation between the two methods at both time points, \((r=0.42, p=0.05\) for December 1986; \(r=0.51, p=0.01\) for January 1988). For the CDC classification there was no correlation with age \((r=0.02,\)
Table 6.3. Walter Reed Classification of patients.

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>1986</th>
<th>1988</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>3B</td>
</tr>
<tr>
<td>4</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>5</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>6</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>7</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>8</td>
<td>3</td>
<td>3B</td>
</tr>
<tr>
<td>9</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>10</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>11</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>12</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>13</td>
<td>1</td>
<td>6</td>
</tr>
<tr>
<td>14</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>15</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>16</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>17</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>18</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>19</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>20</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>21</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>22</td>
<td>3</td>
<td>3</td>
</tr>
</tbody>
</table>

A significant difference was noted using this classification - it therefore appears to be sensitive.
Table 6.3.2. Centre for Disease Control Classification of patients studied.

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>YEAR 1986</th>
<th>YEAR 1988</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>3</td>
<td>2</td>
<td>IV A</td>
</tr>
<tr>
<td>4</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>5</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>6</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>7</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>8</td>
<td>2</td>
<td>IV</td>
</tr>
<tr>
<td>9</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>10</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>11</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>12</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>13</td>
<td>2</td>
<td>IV C2</td>
</tr>
<tr>
<td>14</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>15</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>16</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>17</td>
<td>2</td>
<td>IV C2</td>
</tr>
<tr>
<td>18</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>19</td>
<td>IV C2</td>
<td>IV C2</td>
</tr>
<tr>
<td>20</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>21</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>22</td>
<td>2</td>
<td>2</td>
</tr>
</tbody>
</table>

No significant change in progression of disease was noted using this classification.
p=0.91, Dec 1986; r=0.31, p=0.14, Jan 1988), duration of infection (r=0.25, p=0.25 Dec 1986; r=0.28, p=0.19) or age of infection (r=-0.02, p=0.91, Dec 1986; r=0.31, p=0.14 Jan 1988). The Walter Reed classification in January 1988 showed a moderate correlation with age (r=0.49, p=0.02) but no correlation with age was seen for the December 1986 staging (r=0.30, p=0.18). There was no correlation with the duration of infection at either time point (r=0.07, p=0.7 Dec 1986; r=-0.09, p=0.6 Jan 1988). Age of infection however showed a moderate correlation with disease staging in Dec 1988 (r=0.45, p=0.04) but not with the initial staging (r=0.24, p=0.26).

There was no significant change in the CDC classification at the two time points (p=0.5) however the Walter Reed classification was able to detect change (p=0.03), Table 6.3.3.

6.3.4 DISCUSSION

The low prevalence of HIV-1 antibody and relatively short duration of infection in this cohort have not allowed any detailed information to be culled from the clinical spectrum of disease observed. However, some interesting observations were made. First, more disease directly attributable to HIV-1 rather than immunodeficiency was observed, this occurred in 27 percent of patients. Three patients developed thrombocytopenia and three ARC. The occurrence of thrombocytopenia appeared to be a random
event and was only associated with serum p24 antigen in one of three patients. In these patients thrombocytopenia was not related to CD4+ve T-cell depletion and in two it could be attributed to peripheral platelet consumption. The most interesting observation was that platelet count recovered without further treatment. In previous studies aggressive treatment that may potentially lead to progression of HIV-1 related disease had been recommended in haemophiliacs (448-455). The mechanism of thrombocytopenia are unknown but it is frequently associated with anti-platelet antibodies (456). It may therefore be that the thrombocytopenia is due to the B cell abnormalities that occur in patients with HIV-1 infection (Chapter 11).

Second, of the three patients with ARC in only one was serum p24 antigen absent. It may therefore be that in the two patients with serum antigen that symptoms and laboratory findings are a manifestation of declining cell mediated immunodeficiency and it is a matter of time before they develop an opportunistic infection.

Third, three patients (14 percent) developed infections that could be attributed to HIV-1. The two patients with bacterial infections had recurrent episodes. In one patient occurrence of a bacterial infection led to a progressive, rapid depletion of his CD4+ve T-cell count. In both patients occurrence of bacterial infections was not due to a significant depletion of the number of
CD4+ve T-cells. As with thrombocytopenia it may be that the susceptibility of certain patients to bacterial infections is due to a B cell defect independent of the decline in cell mediated immunity.

The evaluation of the two disease classifications showed that the Walter Reed staging was more sensitive in detecting change. This may be because this system is based on CD4+ve T-cell depletion. Cutaneous tests of cell mediated immunity were not included in this study because haemophiliacs may be anergic in the absence of HIV-1 infection (Chapter 7). Although the CDC disease classification allows for a stratification of patients according to the CD4+ve T-cell count no substaging has yet been proposed.

The major deficiency in both classifications was that no numerical weighing of certain disease manifestations was provided so that progression could be statistically compared. Both classifications were, however, simple to use.

In conclusion, 36 percent of patients in this cohort had developed significant HIV-1 related disease. The correlation between age and the Walter Reed staging suggested that HIV-1 disease progression is a function of age and not duration of HIV-1 infection.
CHAPTER 7.1

IMPAIRED CELL MEDIATED IMMUNITY IN HIV-1 ANTIBODY NEGATIVE HAEMOPHILIACS.

SUMMARY

Study objective: To assess in vivo cell mediated immunity in haemophiliacs with and without HIV-1 antibody.

Study design: Open, cross-sectional study.

Study population: Severe treated haemophiliacs in whom HIV-1 antibody status was known.

Measurements: The cutaneous inflammatory response to a new antigen - dinitrochlorobenzene (DNCB); mean annual dose of clotting factor concentrate used and severity of liver disease.

Main results: The DNCB response was depressed in haemophiliacs. This was independent of HIV-1 antibody status. Response correlated with mean annual dose of clotting factor concentrate used in seronegative patients. Severity of liver disease showed no correlation with DNCB score in seronegative patients.

Conclusions: Treated haemophiliacs in the absence of HIV-1 antibody have impaired cell mediated immunity in vivo.
7.1.1 INTRODUCTION

A severe haemophiliac requires regular treatment with clotting factor concentrates to control or prevent bleeding episodes. The clotting factor concentrates are fractionated from pooled plasma donations (Chapter 1). A single haemophiliac may be exposed to alloantigens from as many as 10,000 different donors per batch of clotting factor concentrate used. Exposure to such a large allogenic antigen load can both stimulate as well as directly, impair immune responses (Chapter 3.1). The use of blood products also carries an increased risk of hepatitis B, the non-A non-B (NANB) hepatitis viruses, and HIV-1 infection.

In chapter 3.1 it was shown that intermediate purity factor VIII concentrate impaired the ability of peripheral blood mononuclear cells to proliferate in response to soluble antigen and phytohaemagglutinin. The capacity of phytohaemagglutinin stimulated peripheral blood mononuclear cells to produce interleukin-2 was also reduced in a dose dependent manner. Haemophiliac patients also had high circulating levels of Fc Y receptor antibody, an immunosuppressive protein (Chapter 3.2). The significance of these findings is unclear.

The aim of this study was to determine if haemophiliacs treated with clotting factor concentrates are immunosuppressed in vivo. The dinitrochlorobenzene skin test is considered to be the best single test of cell
mediated immunity in vivo and measures the ability of an individual to respond to a new antigen (457, 458). The dinitrochlorobenzene score was compared to the mean annual dose of clotting factor concentrate used and the HIV-1 antibody status.

7.1.2 METHODS

Patients
Thirty five patients with moderate or severe haemophilia (factor VIII or IX level <5iu/dl) were studied. Included were 14 of the 22 patients known to be seropositive for antibody to HIV-1. Patients were examined for features of disease related to HIV-1. Case records were examined for clotting factor concentrate use over the previous seven years, and mean annual exposure to clotting factor calculated.

The presence of significant liver disease was scored as described in Chapter 2. Previous exposure to hepatitis B virus as assessed by antigen and antibody testing at routine clinic visits was noted (Chapter 2.2).

Dinitrochlorobenzene testing
A modified dinitrochlorobenzene test was performed in each case (281). Patients were sensitized to dinitrochlorobenzene (2000ug dissolved in 0.1ml acetone) on the dominant forearm. After 14 days the patient was rechallenged with five doses of dinitrochlorobenzene (30, 15, 7.5, 3.7 and 1ug), the dinitrochlorobenzene being
dried onto 1cm diameter felt pads (A1 test patches, Astra Chemicals, Watford, UK) and applied on the opposite forearm. The reaction was assessed after 48 hours as follows: 0, no reaction or erythema only; 1, erythema and induration confined to the patch; 2, erythema and induration extending beyond the patch; 3, as for 2, plus blistering. The maximum possible score was 15.

Serological testing
Blood was withdrawn for IgG HIV-1 antibody (ELISA, Wellcozyme) and screened for other viruses including measles, cytomegalovirus, the Herpes group including Epstein-Barr virus; IgM antibody was sought. HIV-1 antibody status was confirmed by Western blotting.

Controls
The normal range for the DNCB response had been previously established for male volunteers from the West of Scotland using the same methods and scoring system (281). Historical controls were used because previous experience indicated that an intact immune system produced severe adverse reactions even in the absence of previous inadvertent sensitization. The control group were comparable to haemophiliacs for age.

7.1.3 RESULTS
Two patients had appreciable cervical lymphadenopathy but no other features of disease related to HIV-1. One patient had persistent mild thrombocytopenia, the lowest
FIGURE 7.1.1 DNBCB SCORE

HIV - 1 STATUS
Figure 7.1.1. The response in HIV-1 +ve patients was lower than controls (p<0.05) but did not differ from HIV-1 -ve patients (p=0.07). There was a significant difference between HIV-1 -ve and controls (p<0.05).
recorded count being $96 \times 10^9/\ell$. No other patients had clinical features of HIV-1 related disease.

**Serological results**

Fourteen patients were seropositive for antibody to HIV-1. No patient had circulating IgM antibody to other viruses. Previous exposure to hepatitis B virus and presence of transaminasaemia at clinic visits was similar in the patients HIV-1 positive and negative (data not shown).

**Dinitrochlorobenzene testing**

The median score in normal subjects was 9 (IQR 7-11). All the patients in the present study had a score below 9 (Figure 7.1.1). The median score in patients was 3 (IQR 0-5): this was significantly different from normal ($p<0.001$). In patients positive for HIV-1 the median score was 0 (IQR 0-3): this was significantly different from normal ($p<0.001$). Among the three patients with features of disease related to HIV-1 the score was 0 in the patient with thrombocytopenia and 0 and 4 in the two patients with lymphadenopathy. In seronegative patients median score was 3 (IQR 0-5): this was significantly different from normal ($p<0.001$) but not significantly different from the score in the seropositive patients.
Consumption of clotting factor

The median annual consumption of clotting factor over six years in all patients was 48,564 units/annum (IQR 21,878-83,000). The median consumption in seropositive patients was 73,360 units/annum (IQR 48,564-100,000) and in seronegative patients 38,000 units/annum (IQR 21,349-65,224). This difference was not significant (p=0.07).

An inverse correlation was noted between increasing usage of clotting factor and decreasing score on dinitrochlorobenzene testing for all patients (Figure 7.1.2) (r=-0.3, p<0.05). In seronegative patients with a score less than normal (<4) median consumption of clotting factor was 40,000 units/annum (IQR 24,120-65,224): this was significantly more than in seronegative patients with a response in the normal IQR (median 23,183 units/annum, IQR 21,107-59,052; p<0.02). An inverse correlation was noted in all seronegative patients between consumption of clotting factor and score on dinitrochlorobenzene testing (r=-0.4, p<0.05), but the correlation in seropositive patients was not significant (r=0.08); there was no significant difference, however, between correlations in seropositive and seronegative groups.

Liver disease

In all patients grade of liver disease showed a poor correlation with dinitrochlorobenzene score (r=-0.12, p=0.42). In seropositive patients there was a significant inverse correlation (r=-0.59, p=0.05) but correlation was poor in seronegative patients (r=-0.009, p=0.9).
FIGURE 7.1.2 THE MEAN ANNUAL DOSE OF TREATMENT USED AND DNCB SCORE

MEAN ANNUAL DOSE OF CLOTTING FACTOR CONCENTRATE
Figure 7.1.2. Correlation between mean annual dose of clotting factor concentrate and DNCB score in all patients was $r=0.4$ ($p=0.05$).
7.1.4 DISCUSSION

The response to dinitrochlorobenzene evaluates both the afferent and efferent arms of the immune response; it is therefore a measure of a patient's current cell mediated immune state (457, 458). The results showed that all patients with moderate to severe haemophilia treated with factor concentrate had a response below the median normal value, and in 20 of 35 patients studied response was on or below the lower limit of the normal range (that is, ≤4). In addition, response was impaired in patients seronegative for antibody to HIV-1.

In patients negative for HIV-1 antibody response to dinitrochlorobenzene was inversely related to consumption of clotting factor (Fig 7.1.2). In particular, patients who had a subnormal response had used significantly more factor concentrate than patients with a response in the normal range. This association of consumption of clotting factor with reduced cell mediated immunity may be due to transmission of viral infections, to a direct chemical effect of concentrate, or to an indirect effect mediated via the immune system.

Concomitant serological studies excluded the possibility of a coincidental viral infection. Infection with HIV-1 in the absence of antibody was considered to be unlikely, and no patient in the seronegative group had features of disease related to HIV-1. In our patient population HIV-1 seroconversion occurred during 1980-5, and in a previous study it was found that seroconversion was associated with use of imported American concentrate.
No further seroconversions have been recorded in the patients studied. In addition, isolates of virus from seronegative patients are exceptional (354, 355). It therefore appears that the depression in cell mediated immunity is probably due to either a direct effect or an immunological consequence of infusion of factor concentrate.

A previous study of patients reported high circulating levels of FcY receptor antibody (Chapter 3.2). This antilymphocyte antibody develops after repeated blood transfusions, and its presence correlated directly with survival of renal allografts. The FcY receptor is an important immunoregulatory protein, and blocking of this receptor with antibody or immune complexes stimulates a non-specific T suppressor cell response which could additionally inhibit macrophage activation or T-helper function (459). Excessive activity of T suppressor cells may therefore account for the abnormalities seen in seronegative patients on dinitrochlorobenzene testing. In the 14 seropositive patients the score on dinitrochlorobenzene testing was significantly below normal (p<0.001), and 11 patients had a score below the normal range (Figure 7.1.1). The score did not differ significantly from that in seronegative patients. No significant correlation in seropositive patients between the score and consumption of clotting factor could be shown. Both these negative findings, however, may have been due to the small numbers in this group.
correlation with the grade of liver disease was seen in this group.

In summary therefore, the results of the present study suggest that cell mediated immunity is decreased in patients with severe haemophilia treated with clotting factor concentrates and that this is related to the amount of clotting factor concentrate used. Whether this abnormality is partly related to risk of infection with HIV-1 or to its sequelae, or both, is not yet known. Although no significant difference in the score on dinitrochlorobenzene testing was observed between seronegative and seropositive patients, infection with HIV-1 in the seropositive patients was fairly recent. Further studies of the response to dinitrochlorobenzene in patients who have had infection with HIV-1 for a longer period may well show lower responses, as immunosuppression induced by HIV-1 may take many years to become firmly established.
CHAPTER 7.2

IMPAIRED CELL MEDIATED IMMUNITY IN HIV-1 ANTIBODY NEGATIVE HAEMOPHILIACS - IS IT DUE TO LIVER DISEASE?

SUMMARY

Study objective: To exclude the possibly that the impaired in vivo cell mediated immunity in haemophiliacs is due to liver disease.

Study design: Open, cross-sectional study.

Study population: Haemophiliacs without HIV-1 antibody and different grades of liver disease but matched for the mean annual dose of clotting factor used were studied.

Measurements: The cutaneous inflammatory response to 7 recall antigens was measured in addition to the mean annual dose of clotting factor concentrate used and severity of liver disease.

Results: HIV-1 antibody negative haemophiliacs had an impaired cutaneous response to recall antigens. The response showed a poor correlation with grade of liver disease.

Conclusions: Severity of liver disease in the absence of clinical cirrhosis does not influence in vivo cell mediated immune responses in haemophiliacs.
7.2.1 INTRODUCTION

Haemophiliacs have an impaired ability to mount an immune response to a new antigen as assessed by the dinitrochlorobenzene (DNCB) skin test (Chapter 7.1). It was previously shown that this is independent of the HIV-1 antibody status of the patient and that there is a moderate inverse correlation to the mean annual dose of clotting factor concentrate used, (Chapter 7.1). This may be due to the contaminating alloantigen load eliciting an immune response, a direct chemical effect of concentrate or a consequence of the viral infections transmitted via blood.

Viral infections can suppress immune reactions by a direct effect on the immune system as in HIV-1 infection, or by non-specifically suppressing immune reactions (\(\)). Alternatively, immunosuppression may arise due to the diseases caused by the virus, for example chronic liver disease due to hepatitis B virus or non-A, non-B (NANB) infection. Immune abnormalities have been described in patients with liver cirrhosis of diverse aetiologies (376).

In this study, the in vivo immune response was measured using standard recall antigens and the response compared to mean annual dose of clotting factor concentrate used and severity of liver disease in patients with and without HIV-1 antibody.
7.2.2 METHODS

Patients
Skin responses to seven recall antigens were tested from selected groups of haemophiliacs: patients known to have HIV-1 antibody but in the absence of HIV-1 related disease, HIV-1 antibody negative haemophiliacs with a persistently raised serum alanine aminotransferase above one and a half times the upper limit of the laboratory range, and those patients with persistently normal serum ALT over the preceding five years. The mean annual dose of clotting factor concentrate used over the previous five years was known for all patients and therefore patients with and without abnormal serum ALT levels were matched for the mean annual dose of clotting factor concentrate used.

Further details of the methods used to assess the severity of liver disease are given in Chapter 2.

Skin testing
The response to the following antigens was tested: tetanus, diphtheria, streptococcus, tuberculin, candida, trichophyton, proteus and a glycerine control, on the volar aspect of the non-dominant forearm, using a standardized commercially available test, Multitest-CMI (Institut Merieux, Lyons, France) (461).

The responses were read after 48 hours by measuring the diameter of indurated area in millimeter for each antigen. The results were expressed as a compound score, which was calculated by dividing the mean sum of the average diameter of the response to each antigen by the
number of antigens to which a reaction occurred. The mean total score in mm of all reactions and the total number of antigens to which patients responded to was also analyzed.

7.2.3 RESULTS

Clinical

32 patients were studied, included were 7 of the 22 known patients with HIV-1 infection. No HIV-1 antibody positive patient had clinical manifestations of cellular immunodeficiency.

Liver disease

Two patients had severe liver disease with evidence of portal hypertension; both had previous episodes of bleeding from oesophageal varices and one had episodes of jaundice. He continued to have ankle oedema due to low serum albumin during this study. Both patients failed to respond to any antigen and were excluded from the study.

Three of nine patients with persistently abnormal liver function tests had clinically detectable splenomegaly. Of the remaining 22 patients, 9 had intermittently abnormal ALT levels (grade 3), 10 had a normal serum ALT levels but previous evidence of NANB, 2 patients acquired NANB during the study and one patient had no previous evidence of NANB infection. All patients had serum hepatitis B antibody but none had circulating antigen.
Median score of liver disease in HIV-1 positive patients was not significantly different from HIV-1 antibody negative patients (p=0.07), Table 7.2.1.

RECALL SKIN RESPONSE

HIV-1 antibody status

The median total score in all haemophiliacs was 12mm (IQR 9 to 15) and the median number of antigens to which haemophiliacs responded was 3 (IQR 2 to 4) giving a median compound score of: 4.75 (IQR 4 to 6), Table 7.2.1. Patients positive for HIV-1 antibody had a median total score of 11mm (IQR 4 to 11); this was not significantly different from HIV-1 antibody negative haemophiliacs, median total score 13mm (IQR 9 to 15), p=0.15.

Effect of treatment on recall skin response

The median annual dose of clotting factor concentrate used by patients studied was: 39,000 units per annum (IQR: 5,855 to 61,905). HIV-1 antibody positive patients had used a median of 83,000 units annum (IQR 56,852 to 117,400) HIV-1 antibody negative patients, median 24,487 units annum (IQR 2,586 to 43,910), p=0.001.

Figure 7.2.1 shows the relationship between the mean annual dose of clotting factor concentrate used and the compound skin response. In all patients there was a poor inverse correlation (r=-0.23, p=0.09). In HIV-1 antibody positive patients, r=-0.7 (p=0.08) and in seronegative patients r =-0.22 (p=0.28).
Table 7.2.1. Results of skin testing in HIV-1 antibody positive and negative haemophiliacs.

<table>
<thead>
<tr>
<th>HIV antibody status</th>
<th>+ve</th>
<th>-ve</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of patients</td>
<td>7</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>21</td>
<td>30</td>
<td>0.07</td>
</tr>
<tr>
<td>Mean annual dose of treatment (units/annum)</td>
<td>83000 (56852-117400)</td>
<td>24487 (2586-43910)</td>
<td>0.001</td>
</tr>
<tr>
<td>Liver disease</td>
<td>2.5 (2-3)</td>
<td>4 (3-5)</td>
<td>0.07</td>
</tr>
<tr>
<td>No. of antigens</td>
<td>2 (1-3)</td>
<td>3 (2-4)</td>
<td>0.26</td>
</tr>
<tr>
<td>Total score</td>
<td>11 (4-11)</td>
<td>13 (9-15)</td>
<td>0.15</td>
</tr>
<tr>
<td>Compound skin score</td>
<td>3.5 (3-6)</td>
<td>5 (4-6)</td>
<td>0.09</td>
</tr>
</tbody>
</table>

Medians and interquartile ranges are shown. The only significant difference was that seropositive patients had used more clotting factor concentrate.
Figure 7.2.1 THE COMPOUND SKIN SCORE IN RELATION TO THE ANNUAL DOSE OF TREATMENT USED
Figure 7.2.1. There was no significant difference in compound skin score between the two groups, $p=0.15$).
Effect of liver disease on the recall skin response

In all patients there was a poor direct correlation with the severity of liver disease and the compound skin score ($r=0.23$, $p=0.25$) this was independent of the HIV-1 status, Fig 7.2.2.

7.2.4 DISCUSSION

The aim of this study was to determine if the impaired cell mediated immunity seen in HIV-1 antibody negative haemophiliacs was due to chronic liver disease or an effect of treatment with clotting factor concentrates. The recall skin response was used rather than DNCB. This test has several advantages; it is simple to use and more acceptable to patients. Furthermore, responses are standardized and there is no risk of sensitization.

This study confirmed that in vivo cell mediated immunity was impaired in haemophiliacs treated with clotting factor concentrates and was independent of HIV-1 status (Chapter 7.1). Previous infection with NANB viruses did not affect recall skin response. Furthermore, results showed that liver disease due to NANB hepatitis does not account for the impaired cell mediated immunity seen in haemophiliacs treated with clotting factor concentrates. However, in the presence of clinically severe liver disease in 2 patients the skin responses were impaired.

In previous studies it was shown that clotting factor concentrates have both a direct chemical effect on lymphocyte proliferation in vitro and that treatment
Figure 7.2. THE COMPOUND SKIN SCORE IN RELATION TO GRADE OF LIVER DISEASE
Figure 7.2.3. There was a poor correlation between compound skin score and mean annual dose of treatment ($r=0.23$, $p=0.09$).
also results in FcY receptor antibodies (Chapter 3.2). In this study and the dinitrochlorobenzene study (Chapter 7.1) only a moderate inverse correlation was shown between mean annual dose of clotting factor concentrate used and in vivo cell mediated immunity. Impaired skin responses may be due to an immune response to contaminating allogenic proteins contained in clotting factor concentrates, rather than due to the total dose. Alternatively, a component or components contained in clotting factor concentrates may modify function of T-helper/inducer cells. The results could also be explained by excessive T-suppressor cell activity. Treated haemophiliacs frequently have increased amounts of circulating immune complexes (247-251). The binding of such complexes to Fc receptors on T-suppressor may stimulate a suppressive signal, which could inhibit macrophage or T-helper/inducer cell function (459). In conclusion, the previous observation that haemophiliacs treated with clotting factor concentrates have impaired cell mediated immunity in vivo was confirmed. The impaired response was not due to NANB hepatitis. Further in vitro studies of cell mediated immunity in haemophiliacs are indicated to determine if this is a quantitative or qualitative defect.
IMPAIRED IN VIVO CELL MEDIATED IMMUNITY IN HIV-A ANTIBODY NEGATIVE HAEMOPHILIACS - FACT OR FICTION?

SUMMARY

Study Objective: Is there a functional defect in cell mediated immunity to a recall antigen in treated haemophiliacs.

Study design: Open, cross-sectional.

Study population: 13 patients with severe haemophilia of whom three had HIV-1 antibody.

Measurements: Histometric analysis of lymphocyte subsets in biopsies of cutaneous tuberculin reactions.

Results: The principle abnormality noted was absence of increasing CD4:CD8 ratio of diffusely infiltrating lymphocytes with depth.

Conclusions: Haemophiliacs appear to have a functional abnormality of lymphocytes in vivo.
7.3.1 INTRODUCTION

In the previous studies reported in this section it was shown that haemophiliacs treated with clotting factor concentrates had impaired cell mediated immunity in vivo as assessed by responses to dinitrochlorobenzene (DNCB) and recall antigens. In chapter 8.2 it is shown that HIV-1 antibody negative haemophiliacs do not have reduced total numbers of T-helper/inducer (CD4+ve) lymphocytes. It may therefore be that the impaired cell mediated immunity is due to a functional defect of CD4+ve T-cells or of a subpopulation of CD4+ve T-cells.

Gibbs et al have described a histometric method for assessment of immunocytochemical staining of lymphocyte and monocyte/macrophage subsets in tuberculin skin test reactions; this method allows discrimination of differences that cannot be readily separated on direct microscopic examination (462 - 465). In the present study this histometric method was used to determine if there was a functional defect in cell mediated immunity to a recall antigen in treated haemophiliacs.

7.3.2 MATERIALS AND METHODS

Patients

Thirteen patients with clinically severe haemophilia were studied: 3 had serum antibody to HIV-1 and the remaining 10 patients have been persistently seronegative for HIV-1 antibody. The mean annual dose of clotting factor concentrate used was calculated.
The subjects were tested by an intradermal injection of 0.1 ml of "new tuberculin" an ultrasonicated skin test antigen prepared from Mycobacterium tuberculosis. The injection site was examined 48hrs later and the response was recorded as (a) "negative" if no change was observed, (b) "+" if there was localized erythema, "++" if the epidermis was blistered over the indurated area. A heparinised venous blood sample was collected for enumeration of CD-4+ve and CD-8+ve T-lymphocytes as described in Chapter 8.2. Thereafter the skin test site was anesthetized by injection of 1% plain lignocaine and a biopsy removed with a 4mm skin punch under full aseptic conditions: the procedure was covered by injection of additional clotting factor concentrate and local pressure was maintained until a stable haematoma was formed. All biopsy sites healed normally and none was complicated by infection. Recurrent bleeding was encountered in two patients and this was controlled with clotting factor concentrate and local measures. The biopsy core was snap frozen with dry ice and sealed in aluminium foil. Immediately before sectioning, the tissue was cryopreserved in glycerol: 6-8um cryostat sections were cut for immunocytochemical staining. The major T lymphocyte subsets were localized with Leu-3a (anti-CD4) and Leu-2a (anti-CD8) and monocyte/macrophages with Leu-M3 murine monoclonal antibodies (Becton Dickinson, Sunnyvale, CA, USA); monoclonal antibodies to receptors for interleukin-2 (CD25) and transferrin.
(Becton Dickinson) were used to identify "activated" lymphocytes. The slides were stained with the peroxidase avidin-biotin complex (ABC kit, Vectastain, Sera-Lab Ltd, Crawley Down, Sussex) using diaminobenzidene as substrate.

Histometric counts were performed on large (20x16in) monochrome photographic prints (x166); the outline of perivascular and periappendicular foci was delineated using a felt pen to facilitate measurement of the areas of the foci and of intervening dermis with a semi-automated planimeter. The number of cells in the two compartments (the foci and the intervening dermis) were counted and each expressed as a density (number/mm²): in addition to measurements over the whole of the section, cell density in the diffuse infiltrate was measured separately in three successive 250mm layers into the dermis.

7.3.3 RESULTS

The intensity of changes seen on clinical examination of tuberculin tests (a long term recall response) correlated with that observed with DNCB (a short term recall response) in the previous study. Two of the three seropositive patients were negative in both tests, but the other patient gave a relatively strong reaction in both tests. The intensity of the tuberculin test seen on clinical examination showed no correlation with mean annual dose of clotting factor used or HIV-1 antibody status.
The microanatomical distribution of cellular infiltrate in the dermis at the site of the tuberculin test in the haemophilia patients was similar to that previously reported in normal subjects (463-465).

The focal infiltrates range in extent from 2.14% to 26.96% of the area of dermis - this is similar in intensity to that previously reported in other patient groups. The size of focal infiltrate was not related to clinical appearance and percentage focal infiltrate did not correlate with mean annual dose of clotting factor concentrate used or HIV-1 antibody status.

There was no difference in density of lymphocytes (CD4 + CD8) in the diffuse infiltrate and intervening dermis; values range from 20.2 to 137.6 cells/mm², except for one outlier with 674.6 cells/mm²: thus 12 of 13 patients had diffusely infiltrating lymphocyte densities similar to that reported in other patient groups. The measurements were not related to clinical appearances of the reaction, use of clotting factor concentrates, or HIV-1 antibody status.

The CD4:CD8 ratio of peripheral blood lymphocytes was within the normal range, but was generally lower in HIV-1 seropositive subjects. The CD4:CD8 ratio in the lymphocytes in the dermal focal inflammatory infiltrate was not significantly different from that of cells in peripheral blood.

However, the CD4:CD8 ratio in diffusely infiltrating lymphocytes overall and in three successive 250mm layers
into the dermis was significantly greater than that in either blood or focal lymphocytes \((p<0.05)\) in seronegative patients: the ratio of diffusely infiltrating lymphocytes in seropositive patients was only slightly increased. The \(CD4:CD8\) ratio of diffusely infiltrating lymphocytes did not show any significant change with increasing depth into the dermis, either in seropositive or seronegative subjects: in this respect the haemophiliacs differed from normal volunteers or patients with pulmonary tuberculosis or across the spectrum of leprosy \((p<0.01)\) studied previously (464).

The \(CD4:CD8\) ratio of all lymphocytes (cells bearing receptors for IL2 and transferrin) was positively correlated with lymphocyte density throughout the section (in both diffuse and focal infiltrates). It was noticeable that some of the clinically "negative" reactions "activated" cells were present in the infiltrates.

The density of monocyte/macrophages throughout the section (both focal and diffuse infiltrate) was 42.2 to 167.9 cells/mm\(^2\), this density was not correlated with the intensity of changes seen clinically nor with the mean annual dose of clotting factor concentrate used or HIV-1 antibody status. The overall density of macrophages was clearly related to that of the lymphocytes.

7.3.4 DISCUSSION

The microanatomical distribution of \(CD4\), \(CD8\) lymphocytes and monocyte/macrophages in tuberculin reactions in
haemophiliacs was similar to that previously reported in normal healthy controls and in patients with mycobacterial infections where there is active antigenic stimulation (eg. tuberculosis and leprosy, (464). The number of "activated" lymphocytes at the site of stimulation was related to the total density of lymphocytes in the infiltrate.

The mean annual dose of clotting factor concentrate used was not related to the major indicators of the immune response (focal infiltrate as a percentage of the dermis and the density of lymphocytes in the intervening dermis), but it was inversely related to CD4:CD8 ratio of lymphocytes throughout the section. This ratio is usually substantially higher than that in peripheral blood (466). Furthermore, in healthy subjects, the relative preponderance of CD4+ve cells in diffuse infiltrates is high (3 to 5 fold greater than peripheral blood) (466). In haemophiliacs studied there were fewer CD4+ve lymphocytes in diffuse infiltrates than in peripheral blood. Moreover tuberculin tests in haemophiliac patients did not show a gradient of increasing CD4:CD8 ratio of diffusely infiltrating lymphocytes with depth into dermis:in this respect haemophiliacs differed from healthy controls tested with the same antigen (466).

These findings suggest that there is a functional defect in T-cells from haemophiliac patients and this may be related to the increasing evidence of clinically significant immunosuppression reported in patients
treated with clotting factor concentrates in the absence of HIV-1 infection (327-329).
CHAPTER 8.1

THE ABSOLUTE LYMPHOCYTE COUNT IN HAEMOPHILIA

Summary

Study objective: To study prevalence, aetiology and outcome of lymphopenia in treated haemophiliacs.

Study design: Open, prospective longitudinal.

Study population: Haemophiliacs treated with clotting factor concentrate since 1980 in whom HIV-1 antibody status, mean annual dose of clotting factor concentrate used and severity of liver disease was known.

Measurements: Absolute lymphocyte count.

Results: The prevalence of lymphopenia was 29.4 percent, after 4 years observation the incidence did not change. Early HIV-1 infection was not associated with a lymphopenia. Two patterns were identified episodic (14.7 percent) and persistent (14.7 percent). Persistent lymphopenia correlated with mean annual dose of clotting factor concentrate. No association with liver disease was noted. 13 percent of persistently lymphopenic patients developed infections in the absence of HIV-1 antibody.
Conclusion: Treatment with clotting factor concentrates and not blood borne viral infection results in a persistent clinically significant lymphopenia.
8.1.1 INTRODUCTION

Severe adult haemophiliacs with reduced numbers of lymphocytes (lymphocyte count <1,200/mm³) have an eight times higher mortality than non-lymphopenic patients (467). Lymphopenia may arise due to several mechanisms: (a) the viruses transmitted by clotting factor concentrates; (non-A, non-B hepatitis, hepatitis B and human immunodeficiency virus-1), (b) clotting factor concentrates may have a direct chemical effect or (c) an immune mediated response against a leucocyte crossreacting alloantigen contained in clotting factor concentrates may be responsible (332).

HIV-1 selectively infects and depletes lymphocytes that express the CD4 molecule. The virus may also infect a T-helper/inducer (CD4⁺ve, T-h) precursor or stem cell, further contributing to the lymphopenia (468). Lymphopenia during NANB infection could be a consequence of chronic liver disease but may also be a direct or an immune mediated effect of the NANB viruses on lymphocytes.

The effect of clotting factor concentrates on the lymphocyte count in the absence of these infections is not known. Moreover, repeated exposure to contaminating allogenic leucocyte antigens contained in clotting factor concentrates may sensitise the individual to produce anti-lymphocyte antibodies (332). In this regard it was
shown in Chapter 3.2 that clotting factor concentrates induce non-cytotoxic anti lymphocyte antibodies. Alternatively, anti-leucocyte antibodies may be passively infused; in this context haemolysis due to passive infusion of red cell agglutinins in factor concentrates (230-232).

In the present study the frequency of lymphopenia and its outcome in treated haemophiliacs over four years was investigated. The lymphocyte count was compared between patients with and without HIV-1 antibody. In this context, the association between mean annual dose of clotting factor concentrate used and chronic liver disease on total lymphocyte count was examined.

8.1.2. METHODS

Patients

The patients in this study were part of a well defined, prospective, cohort to determine aetiological factors and frequency of immunodeficiency in treated haemophiliacs, (Chapter 2).

Lymphocyte count

Venous blood in potassium ethylenediaminetetraacetate (EDTA) was obtained from all patients annually or more frequently if clinically indicated. The total number of white blood cells were counted on a automated counter (Coulter S Plus), a differential white cell count was carried out on blood films stained with Wright's stain and the percent number of lymphocytes obtained.
HIV-1 Status

Prevalence and duration of HIV-1 infection in this population was known (Chapter 4) and clinical outcome accurately documented (Chapter 6).

Liver Disease

The serum alanine aminotransferase (ALT) (Techicon, autoanalyser) was measured on at least two occasions per annum on all patients. Patients were graded for severity of liver disease based on serum ALT levels and clinical features as described in Chapter 2.

8.1.3 RESULTS

Clinical outcome of HIV-1 infection and liver disease have been described in Chapters 6 and 2 respectively.

Lymphocyte count

The lymphocyte count at the start of the study was available on 102 (77 percent) patients and after four years on 121 (91 percent) patients. Thirty patients (29 percent) were lymphopenic (Total lymphocyte count < 1500 cells /mm$^3$) at the start of the study and after four years 42 patients (35 percent) were lymphopenic, (p=0.233 Fishers Exact Test).

Of the original 30 lymphopenic patients, 15 had a lymphopenia after four years. The count did not fall significantly in this group (p=0.59), Table 8.1.1. compares persistently lymphopenic patients to patients
TABLE 8.1.1 Differences between patients with and without lymphopenia at the start of the study.

<table>
<thead>
<tr>
<th></th>
<th>Persistently lymphopenic</th>
<th>Initial Lymphopenia</th>
<th>Normal lymphocyte count</th>
</tr>
</thead>
<tbody>
<tr>
<td>No of patients</td>
<td>15</td>
<td>15</td>
<td>72</td>
</tr>
<tr>
<td>Lymphocyte count (initial)</td>
<td>1070 (900-1220)</td>
<td>1170 (830-1320)</td>
<td>2236 (1820-2595)</td>
</tr>
<tr>
<td>Lymphocyte count (final)</td>
<td>1160 (930-1300)</td>
<td>1740 (1628-2660)</td>
<td>2112 (1400-2970)</td>
</tr>
<tr>
<td>Mean annual dose of clotting factor (units/year)</td>
<td>18760 (1980-36353)</td>
<td>26000 (4260-83000)</td>
<td>7724 (1160-43910)</td>
</tr>
<tr>
<td>Age (years)</td>
<td>35 (29-45)</td>
<td>29 (21-37)</td>
<td>31 (23-44)</td>
</tr>
<tr>
<td>Grade of liver disease</td>
<td>3 (3-5)</td>
<td>3 (3-5)</td>
<td>3 (2-5)</td>
</tr>
<tr>
<td>HIV-1 antibody</td>
<td>0</td>
<td>3</td>
<td>16</td>
</tr>
</tbody>
</table>

The median and interquartile ranges for persistently lymphopenic, initial lymphopenia and non-lymphopenic patients are shown. There was no significant difference in the age, mean annual dose of clotting factor concentrate used or grade of liver disease.
who recovered from the lymphopenia and non-lymphopenic patients.

Two persistently lymphopenic patients acquired infections, one patient had an unexplained panophthalmic infection of his left eye and the other pulmonary tuberculosis. One patient had a florid reaction to intradermal purified protein derivative of Mycobacterium tuberculosis (PPD) but had no clinical evidence of tuberculosis. All three patients remained HIV-1 antibody negative. Patient age showed no correlation with lymphocyte count, \( r = -0.12, p = 0.17 \).

**HIV-1 Antibody status**

In four HIV-1 antibody positive patients the initial lymphocyte count was not available, the final count was available on 21 positive patients. Figures 8.1.1 and 2 shows lymphocyte counts in HIV-1 antibody positive and negative patients. Twenty eight percent of HIV-1 antibody positive patients were lymphopenic at the start of the study, after four years 24 percent had a lymphopenia, \( p = 0.395 \), Fishers Exact Test). The frequency of lymphopenia on the initial or final counts did not differ between HIV-1 antibody positive and antibody negative patients, Figure 8.1.1, \( p = 0.285 \), Fishers Exact Test).
Figure 8.1.1 THE INITIAL LYMPHOCYTE COUNT IN HIV-1 ANTIBODY POSITIVE AND NEGATIVE HAEMOPHILIACS
Figure 8.1.1. 30 patients were lymphopenic, there was no significant difference in counts between HIV-1 +ve and HIV-1 -ve patients.
Figure 8.1.2 THE LYMPHOCYTE COUNT AT THE END OF THE STUDY

HIV-1 Status

Absolute Lymphocyte Count (cells/mm³)
Figure 8.2.2. 42 patients were lymphopenic at the end of the study. There was no significant difference between HIV-1 +ve and -ve patients. Final values did not differ from initial values in either group.
In HIV-1 antibody positive patients there was no correlation between age and lymphocyte count, \((r=-0.18, p=0.42)\), or duration of infection \((r=0.20, p=0.34)\).

Four HIV-1 antibody positive patients were lymphopenic at the start of the study, none were symptomatic. In all four, the lymphocyte count was in the normal range at the end of the study. Five patients were lymphopenic at the end of the study, none had symptoms of HIV-1 related disease.

Liver Disease
There was no correlation between lymphocyte count and grade of liver disease, \((r=0.08, p=0.366)\), Table 8.1.2. Moreover, no correlation was seen with the lymphocyte count and grade of liver disease in persistently lymphopenic patients, \((r=0.06, p=0.53)\).

Clotting factor concentrate use
There was no correlation between the initial lymphocyte count and the mean annual dose of clotting factor concentrate used \((r=0.05, p=0.52)\) in all patients. Mean annual dose of clotting factor concentrate used accounted for 5 percent of the variance of lymphopenia at the end of the study \((r=0.23, p=0.05)\). In persistently lymphopenic patients \((n=15)\), mean annual dose of clotting factor concentrate used showed a highly, significant correlation with final lymphocyte count, \((r=0.64, p=0.02)\).
TABLE 8.1.2. Liver disease, HIV-1 status and the total lymphocyte count.

<table>
<thead>
<tr>
<th>Grade</th>
<th>All</th>
<th>HIV-1+ve</th>
<th>HIV-1-ve</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Median (IQR)</td>
<td>Median (IQR)</td>
<td>Median (IQR)</td>
</tr>
<tr>
<td>1</td>
<td>1380 (9)</td>
<td>1768 (1)</td>
<td>1290 (8)</td>
</tr>
<tr>
<td></td>
<td>(810-1690)</td>
<td>(740-1605)</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>1800 (25)</td>
<td>1664 (5)</td>
<td>1900 (20)</td>
</tr>
<tr>
<td></td>
<td>(1508-2970)</td>
<td>(1508-2142)</td>
<td>(1495-2995)</td>
</tr>
<tr>
<td>3</td>
<td>1836 (29)</td>
<td>1836 (9)</td>
<td>2110 (20)</td>
</tr>
<tr>
<td></td>
<td>(1200-2980)</td>
<td>(1628-2397)</td>
<td>(1165-3235)</td>
</tr>
<tr>
<td>4</td>
<td>1340 (11)</td>
<td>1316 (1)</td>
<td>1420 (10)</td>
</tr>
<tr>
<td></td>
<td>(1200-2140)</td>
<td></td>
<td>(1200-2140)</td>
</tr>
<tr>
<td>5</td>
<td>2340 (21)</td>
<td>2600 (3)</td>
<td>2185 (18)</td>
</tr>
<tr>
<td></td>
<td>(1470-2890)</td>
<td>(2397-2640)</td>
<td>(1200-2900)</td>
</tr>
<tr>
<td>6</td>
<td>1855 (22)</td>
<td>1820 (1)</td>
<td>1890 (21)</td>
</tr>
<tr>
<td></td>
<td>(1240-2900)</td>
<td></td>
<td>(1240-2900)</td>
</tr>
</tbody>
</table>

TABLE 8.1.2 shows the medians and interquartile range of the most recent lymphocyte count according to grade of liver disease and HIV-1 status. The numbers in brackets are the number of patients in each group. There was no significant difference in the lymphocyte count between different grades of liver disease (Kruskal-Wallis Analysis of Variance).
8.1.4 DISCUSSION

In this study, 30 of 102 haemophiliacs had an initial lymphopenia and 42 of 121 had a reduced lymphocyte count after four years. In half of the patients with an initial lymphopenia (15 patients) the lymphopenia persisted. These findings are similar to those reported by Eyster et al (468).

None of the persistently lymphopenic patients had HIV-1 antibody. Furthermore it was found that HIV-1 infection was not associated with a higher prevalence of lymphopenia. It may however be that as CD4+ve cell lymphocyte count declines further in HIV-1 antibody positive patients that this will be reflected in total lymphocyte count.

In contrast to the findings of Eyster et al, who reported an association between lymphopenia and liver disease this was not found in the present study (468).

The discrepancy between the results of this study and Eyster's retrospective study could be due to differences in the patient population and methodology. Further, in Eyster's study no account was taken of the amount of clotting factor concentrate used or HIV-1 status. The association with liver disease was based on four deaths due to cirrhosis, of these one had abused alcohol and
another had a hepatic carcinoma and was known to be a chronic hepatitis B surface antigen carrier.

In this study only 3 of the 15 patients with persistent lymphopenia had chronically elevated serum ALT activity above 150% of the upper limit of laboratory normal. The most frequent grade of liver disease seen in persistently lymphopenic patients was an intermittent rise in serum ALT Grade 3. In a previous report such a pattern of ALT activity has been attributed to an allergic/chemical effect of clotting factor concentrate on the liver (182). It is therefore possible that the lymphocyte count may be reduced in a similar manner in the patients studied.

There was no correlation between the total lymphocyte count and mean annual dose of clotting factor concentrate used in all patients, in persistently lymphopenic patients the amount of treatment used accounted for 40 percent of the reduced count. In such patients lymphopenia was considered unlikely to be a consequence of passive infusion of anti-leucocyte antibodies but would be in keeping with an endogenous, persistent lymphocytotoxic antibody. Further studies to determine if such antibodies are present are indicated.

The clinical significance of the persistent lymphopenia is not clear, however, infections occurred in 2 patients from this group. Others have reported a higher attack rate of tuberculosis in haemophiliacs and in a review of
haemophilia deaths in the USA prior to the AIDS epidemic a higher than expected incidence of deaths due to pneumonias was noted (327, 328).

The prevalence of non-persistent lymphopenia was 14.7%, of these, 20 percent HIV-1 antibody positive. As in the persistently lymphopenic group there was no correlation with grade of liver disease. There was however, a weak association with mean annual dose of clotting factor concentrate used. It may be that lymphopenia is due to passive infusion of antilymphocyte antibodies or a direct chemical effect of clotting factor concentrates. Lymphopenia in this group of patients was not associated with complications.

The total lymphocyte count represents a heterogenous population, the phenotypes can be identified by monoclonal antibodies. Studies using such markers may be more useful in identifying lymphocyte subpopulation that are reduced. Such studies may also explain why HIV-1 antibody positive patients despite biological effects of HIV-1 showed no fall in total lymphocyte count. Such investigations should prove to be more useful in providing prognostic information particularly in HIV-1 antibody positive patients.

In conclusion, persistent lymphopenia occurs in up to 15 percent of haemophiliacs treated with clotting factor concentrates. It does not appear to be due to HIV-1
infection or liver disease, but an association with mean annual dose of clotting factor concentrate used was seen. The presence of a persistent lymphopenia was found to be associated with infections, in the absence of HIV-1 antibody.
CHAPTER 8.2

THE IMMUNOREGLATORY RATIO IN HAEMOPILIACS

SUMMARY

Study objective: i To determine the prevalence of changes in the immunoregulator ratio in treated haemophiliacs. ii To identify aetiological factors.

Study design: Open cross-sectional

Study Population: 63 haemophiliacs grouped according to HIV-1 status; liver disease and mean annual dose of clotting factor concentrate used.

Measurements: CD4+ve and CD8+ve T cell counts in peripheral blood.

Results: The prevalence of CD4+ve lymphopenia was 44% and CD8+ve lymphopenia was 12%. 28% had CD8+ve lymphocytosis. HIV-1 infection was associated with a significantly lower CD4+ve count. No other associations were noted.

Conclusions: HIV-1 infection in haemophiliacs is associated with a reduced CD4+ve lymphocyte count.
8.2.1 INTRODUCTION

The risk of infection with the human immunodeficiency virus - 1 (HIV -1) in haemophiliacs is related to the mean annual amount of clotting factor concentrate used, the number of exposures to an infected batch (the size of the innoculum) (Ludlam et al 1986).

HIV -1 infection is frequently associated with a reduced number of T-helper inducer (CD 4 +ve,T-h) cells, and suppressor T lymphocytes (CD 8 +ve, T-s) cells have been reported to be increase, decreased, as well as normal. Changes in the immuno-regulatory ratio have also been frequently reported in HIV - 1 antibody negative haemophiliacs have been reported to be increased (Spira et al 1986) and CD 4 +ve lymphocytes reduced (Carr et al 1985). In one study those haemophiliac who seroconverted after exposure to a single infected batch of factor VIII concentrate tended to have fewer CD - 4 +ve lymphocytes compared to those who did not (Ludlam et al 1986).

The prevalence and cause of reduced CD - 4 +ve lymphocyte numbers is not known in haemophiliacs. In a previous study we showed that a reduced CD 4 +ve count was associated with all impaired cell mediated immune response in vivo , the response was independent of the HIV - 1 status and an inverse correlation with treatment
219

use was also seen (Madhok et al., 1986). However, treatment with clotting factor concentrates also predisposes to non-A, non-B viral infections and chronic liver disease.

In this study the absolute numbers of CD 4+ve and CD 8+ve lymphocytes in haemophiliac patients have been counted and compared independently to HIV-1 antibody status, liver diseases severity and mean annual dose of clotting factor concentrate used.

8.2.2 PATIENTS AND METHODS

Patients

In 1986, T cell subsets were enumerated in 60 of 133 haemophiliacs who formed part of a prospective study of a HIV-1 infection and immuno-competence in haemophilia. Included were 20 of the 22 patients known to be seropositive for HIV-1 antibody. All patients were examined for clinical features of HIV-1 related disease. Additional information obtained from case records included:

(i) Mean annual amount of treatment used by patients over the previous five years.

(ii) Severity of liver disease.
Further details of the methods have been previously given (Chapter 2).

MATERIALS

Cell-separation:
Heparinised venous blood was obtained and mononuclear cells (PBMC) separated by density centrifugation on a sodium metrizoate as previously described in Chapter 3.1.1

T-lymphocyte-subsets
T-lymphocyte subset populations were counted by indirect immunofluorescence on a fluoresceine activated cell sorter (FACS) (Becton Dickinson, Sunnyvale, California, USA) using commercially available monoclonal antibodies (OKT3u for T cells, OKT4 for T-helper cells and OKT8 for T-suppressor cells (Ortho Diagnostics, Raritan, New Jersey). The cells to be stained were resuspended in Hanks/10% fetal calf serum (FCS) and the cell concentration adjusted to 5x10^6/ml. 1x10^6 cells a 0.2mls aliquot) were stained with 20ul of each monoclonal antibody and incubated on ice for 45 minutes. After incubation, the cells were washed twice in Dulbecco's phosphase buffered saline/10% FCS by centrifugation at 400 x g for 10 minutes at 4°C.

The cells were then counterstained using 200uls at 1:250 dilution of fluroscein isothiocyante (FITC) - conjugated
goat-anti-mouse immunoglobulin. After 30 minutes incubation on ice, the cells were washed as previously and fixed with 1mls of 1% paraformaldehyde fixative solution and stored at 4°C till counted. For each patient a negative control for non-specific staining by the secondary antibody was also obtained, 1x10^6 cells were stained with counter mouse IgG instead of the monoclonal antibody and then stained with the secondary antibody as described.

The percentage positive cells stained with each monoclonal antibody were counted on a fluorescene activated cell sorter (Becton Dickinson, Sunnyvale California). The absolute count was obtained by multiplying the percent count with the total lymphocyte count.

8.2.3 RESULTS

T cell subsets were counted in 63 haemophiliacs of whom 21 were HIV-1 antibody positive. Table 8.2.1 compares the haemophiliacs studied and those patients who formed part of the prospective cohort but were not studied. The haemophiliacs studied were comparable to the control group for age.

Total T cell (CD-3, T-3) numbers
Table 8.2.1. A comparison of haemophiliacs in whom T cell subsets were counted and not counted.

<table>
<thead>
<tr>
<th>No. of patients</th>
<th>Patients</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Studied</td>
<td>Not studied</td>
<td>p value</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>27</td>
<td>37</td>
<td>0.001</td>
<td></td>
</tr>
<tr>
<td>Factor deficiency (iu/dl)</td>
<td>0</td>
<td>5</td>
<td>0.001</td>
<td></td>
</tr>
<tr>
<td>Mean annual dose of clotting factor concentrate used (units/annum)</td>
<td>27300</td>
<td>2663</td>
<td>0.001</td>
<td></td>
</tr>
<tr>
<td>Grade of liver disease</td>
<td>3</td>
<td>4</td>
<td>0.28</td>
<td></td>
</tr>
<tr>
<td>HIV-1 antibody positive</td>
<td>21</td>
<td>2</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Median and interquartile ranges are shown.

Studied haemophiliacs had a more severe deficiency, used more clotting factor concentrate over the previous seven years and were younger than patients not studied.
Table 8.2.2 and Figure 8.2.1 show the total CD-3+ve cell count in haemophiliacs, it did not differ from controls, p=0.6.

**HIV-1 antibody status**

No difference was seen when HIV-1 antibody positive haemophiliacs were compared to seronegative patients (p=0.93) or controls (p=0.7). Seronegative patients did not differ from the control group (p=0.5).

Despite the absence of any statistical difference in the median counts, five patients (8 percent) had a CD-3+ve count below the normal range. All were HIV-1 antibody negative and had abnormal liver function tests, Figure 8.2.1.

**Effect of Liver disease**

There was a poor correlation with grade of liver disease and total CD3+ve T cell count for the whole group (r=0.31 p=0.015). In HIV-1 antibody positive patients, the correlation was poor and not statistically significant (r=0.23 p=0.32) and in HIV-1 antibody negative patients the relationship was significant but remained poor, (r=0.31, p=0.4).

**Effect of Mean annual dose of clotting factor concentrate used**

There was no correlation between CD3+ve T-cell count and mean annual dose of clotting factor concentrate used in
Table 8.2.2. T cell subsets in haemophiliacs and controls.

<table>
<thead>
<tr>
<th>T cells (cells/mm³)</th>
<th>Control</th>
<th>All</th>
<th>+</th>
<th>-</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD-3+ve</td>
<td>987</td>
<td>832</td>
<td>1285</td>
<td>1071</td>
</tr>
<tr>
<td></td>
<td>(767-1500)</td>
<td>(697-978)</td>
<td>(972-1486)</td>
<td>(816-1971)</td>
</tr>
<tr>
<td>CD-4+ve</td>
<td>765</td>
<td>593</td>
<td>525.5</td>
<td>622</td>
</tr>
<tr>
<td></td>
<td>(634-879)</td>
<td>(453-814)</td>
<td>(373-648)</td>
<td>(483-1003)</td>
</tr>
<tr>
<td>CD-8+ve</td>
<td>532</td>
<td>472</td>
<td>484</td>
<td>415</td>
</tr>
</tbody>
</table>

Median and interquartile ranges are shown.

CD-3+ve T cells - Haemophiliacs did not differ from controls HIV-1 Ab+ve vs HIV-1 Ab-ve p=0.93

CD-4+ve T cells - Haemophiliacs had fewer cells p=0.008 HIV-1 Ab+ve vs HIV-1 Ab-ve p=0.02

CD-8+ve T cells - Haemophiliacs did not differ from controls HIV-1 Ab+ve vs HIV-1 Ab-ve p=0.35
Figure 8.2.1 THE TOTAL T-CELL (CD-3 +VE) COUNT

CD-3 Positive Cell Count (cells/mm3)

HIV-1 Status
Figure 8.2.1. CD-3+ve T cell count in either group did not differ from control values.
either HIV-1 positive patients \((r=0.06, \ p=0.78)\) or HIV-1 negative patients \((r=0.10, \ p=0.49)\).

The CD4+ve T-cell count

Haemophiliacs as a group had fewer numbers of CD4+ve T-cells than controls \((p=0.008)\), Table 8.2.2.

HIV-1 antibody status

Table 8.2.2, and Figure 8.2.2 shows that HIV-1 antibody positive haemophiliacs had a significantly lower CD4+ve T-cell count compared to seronegative patients \((p=0.02)\) and controls \((p=0.007)\). There was no significant difference in the CD4+ve T-cell count between HIV-1 negative haemophiliacs and controls \((p=0.17)\).

The Effect of Liver Disease

Patients with severe liver disease (Grades 1 and 2) and previous evidence of NANB hepatitis had reduced numbers of CD4+ve T cell lymphocytes, compared to patients with no previous exposure to NANB (Grade 6). In both HIV-1 antibody positive and negative patients there was a poor correlation with the severity of liver disease \(r=0.32 (p=0.18)\) and \(r=0.32 (p=0.03)\) respectively.

The effect of the mean annual dose of clotting factor concentrate used

The mean annual dose of clotting factor concentrate used showed a poor correlation with the CD4+ve T-cell count \((r=-0.26, p=0.04)\). In HIV-1 antibody positive patients
FIGURE 8.2.2 THE T-HELPER CELL (CD-4 +VE) COUNT IN HIV-1 ANTIBODY POSITIVE AND NEGATIVE PATIENTS
Figure 8.2.2. HIV-1 +ve patients had a significantly lower CD-4+ve T cell count compared to HIV-1 -ve patients (p=0.02) and controls (p=0.007). HIV-1 -ve patients did not differ from controls (p=0.17).
mean annual dose of clotting factor concentrate used did not influence CD4+ve T-cell count (r=0.1, p=0.65) whereas in HIV-1 antibody negative patients a poor but significant inverse correlation remained (r=-0.2, p=0.05).

Correlation Analysis
To determine the extent to which mean annual dose of clotting factor concentrate used, liver disease severity and HIV-1 status influenced CD4+ve T-cell count, a stepwise logistic regression analysis was performed. The above variables were independent variables and the CD4+ve cell count was the dependent variable. The analysis was weighted for patients age. Both liver disease, r=0.680 (p=0.0001) and HIV-1 status r=0.807 (p=0.03) remained significantly associated. The mean annual dose of clotting factor concentrate used no longer remained a significantly associated influence on the CD4+ve T-cell count.

CD8+ve T-cell count
Figure 8.2.3 and Table 8.2.1 show the CD8+ve T-cell count in haemophiliacs and controls. There were no significant differences observed in the median CD8+ve T-cell counts. However, 28 percent of haemophiliacs had a CD8+ve T-cell count above the upper limit of the laboratory normal, Figure 8.2.3. Forty-five percent of HIV-1 antibody positive patients had a raised CD8+ve T-cell count and 20 percent of HIV-1 negative patients,
Figure 8.2.3 THE T SUPPRESSOR /CYTOTOXIC (CD8+VE) CELL COUNT IN HIV-1 ANTIBODY POSITIVE & NEGATIVE HAEMOPHILIACS.
Figure 8.2.3. There was no significant difference in counts between HIV-1 +ve and controls. HIV-1 -ve patients did not differ from controls.
Twenty percent of patients had a CD-8+ve count below the normal range, none of these patients had HIV-1 antibody, and all but one had abnormal serum ALT levels.

The Effect of Liver Disease

No correlation was noted between the CD8+ve count and the grade of liver disease in either HIV-1 antibody positive or negative patients.

The Effect of Mean Annual Dose of Clotting Factor Concentrate Used

There was no correlation between the mean annual dose of clotting factor concentrate used and the absolute CD8+ve count in HIV-1 antibody positive patients (r=-0.08, P=0.7) or seronegative haemophiliacs (r=-0.07, P=0.6).

The CD4+ve T-cell count in patients with increased CD8+ve count

Nine HIV-1 antibody positive patients (45 percent) had a CD8+ve count above the normal range, only two of these patients had a CD4+ve T-cell count below the normal range. Figure 8.2.4 shows that those patients with a raised CD8+ve count had more CD4+ve T-cells than those patients with CD8+ve count in the normal range, (P=0.05, one tailed test).

8.2.4 DISCUSSION
FIGURE 8.2.4 THE CD 8+VE COUNT IN HIV-1 ANTIBODY POSITIVE PATIENTS WITH REDUCED & NORMAL CD 4+VE COUNT
Figure 8.2.4. Patients with a normal CD-4+ve count had more CD-8+ve cells (p=0.05 one tailed test).
In chapter 6 haemophiliacs were found to have impaired cell mediated immunity in vivo, as assessed by skin testing to a new and recall antigens. A poor immune response to an antigen may be due to impaired T-cell help/induction or excessive T-cell suppression/cytotoxicity. In this study we have counted T cell subsets to determine if quantitative abnormalities could account for the previous observations.

The prevalence of CD4 lymphopenia in haemophiliacs was found to be 37 percent. In patients with HIV-1 antibody both the frequency of CD4 T-cell lymphopenia was increased and the total numbers were reduced compared to seronegative patients, Figure 8.2.2. These findings are similar to previous reports (469-485). It is well established that HIV-1 selectively infects and depletes cells that express the CD4 molecule. The mechanism of cytopathic effect however is not clear, in vitro cell to cell fusion and resulting syncitium formation due to the crosslinking of neighbouring CD4 molecules and the viral envelope protein gp120 is important in cytopathicity (486). However, syncita have not been seen in vivo (218).

The accumulation of integrated virus DNA is important in animal retroviral infections (487, 488) this has been considered unlikely in HIV-1 infections (489). Zagury et al postulated that expression of HIV-1 may trigger the
CD4+ve cell to begin terminal differentiation, and premature cell death (490).

Other mechanisms may include, enhanced immune clearance of CD4+ve T-cells due to free gp120 coating CD4+ve T-cells rendering them foreign (491). Alternatively HIV-1 infected cells may alter HLA Class II phenotype and therefore enhance immune clearance (492).

In HIV antibody negative haemophiliacs 38 percent had reduced CD4+ve cell counts. The reasons for this are not clear, there was a poor correlation between the CD4+ve cell count and the mean annual dose of clotting factor concentrate used and liver disease severity.

There are two possibilities, first the CD4+ve T cell set represents a functionally heterogenous population which can be subdivided phenotypically with the use of the Leu 8 monoclonal antibody (307, 308). CD4+ve, Leu8-ve T cells are potent helpers of B cell responses whereas CD4+ve Leu 8-ve T cells have a minor role in B cell responses and are the prime responders in antigen induced proliferative assays and in the mixed autologous reaction. They may also induce CD8+ve T cell suppressor function (307, 308). A better association may have been seen if these phenotypes had been selectively counted. Second, the immunosuppression that occurs with clotting factor concentrates is presumed to be due to the contaminating alloantigens and not factor VIIIC (Chapter 3.1).
present study the amount of clotting factor activity infused was measured, to accurately quantify the amount of extraneous protein previously infused was not possible.

A poorly responsive immune system can result in poor helper induction or excessive suppressor influences. In this study we found that 29 percent of haemophiliacs had increased numbers of CD8+ve T-cells, and this occurred more frequently in HIV-1 antibody positive patients (45 percent), Figure 8.2.3. The CD8+ve T-cell population can also be further subdivided into phenotypic subsets that are associated with suppressor versus cytotoxic function by using two-colour immunofluorescence (493, 494). The increased number in HIV-1 antibody positive patients is due to CD8+ve T cells that do not express Leu 15 antigen (495) associated with cytotoxic precursor and effector functions.

In this study it was found that those individuals with HIV-1 antibody and increased CD8+ve T cells had more CD4+ve T-cells compared to patients with normal numbers of CD8+ve T-cells, Figure 8.2.3. This suggests that the cell mediated cytotoxic immune response to HIV-1 may be one determinant in the depletion of CD4+ve T-cells.

In HIV-1 antibody negative haemophiliacs, 21 percent had an increased CD8+ve T-cell count. No correlation between numbers of CD8+ve T-cells and mean annual dose of
clotting factor concentrate used or the grade of liver disease was found. However, it is possible that abnormal suppressive signals are responsible for the impaired in vivo response to antigen. In Chapter 3.2 it was found that haemophiliacs have increased amounts of Fc Y receptor antibody and that such antibodies can impair lymphocyte activation and transformation in vitro. The presence of such antibodies are implicated in the transfusion associated immunosuppressive effect observed in uraemic patients who have received a renal allograft (304). It is therefore possible that immunosuppression in haemophiliacs without HIV-1 antibody is due to qualitative rather than quantitative changes in the CD8+ve cell population.

In conclusion it was found that HIV-1 antibody positive and negative haemophiliacs have abnormalities in T-cell subsets. These are more frequent and quantitatively greater in HIV-1 antibody positive patients. In HIV-1 antibody negative patients no consistent abnormality was found. There was no association between cell counts and severity of liver disease or use of clotting factor concentrate.
CHAPTER 8.3

COFACTORS IN THE CD4+VE T-CELL DECLINE IN HIV-1 INFECTED HAEMOPHILIACS

SUMMARY

Study objective: (i) To measure the decline in CD4+ve lymphocytes. (ii) To identify cofactors that may influence this decline.

Study design: Open, prospective longitudinal.

Study population: Haemophiliacs with and without HIV-1 antibody.

Measurements: T-cell subsets.

Results: HIV-1 infected patients showed a significant decline in CD4 cell count and a significant increase in CD8 count. No changes were observed in seronegative patients. In seropositive patients age inversely correlated with CD4 count, and this was shown to be due to fewer CD8 cells.

Conclusions: Older HIV-1 Ab+ve patients had a more rapid decline in CD4+ve T-cells. Liver disease or the mean
annual dose of clotting factor concentrate infused was not associated with the CD4+ve T-cell count.
8.3.1 INTRODUCTION

The human immunodeficiency virus (HIV-1), infects, replicates in and depletes cells of the CD-4 phenotype (T-helper/inducer T-h; CD4+ve) (335-345). The progressive, numerical depletion of CD4+ve cells predisposes the host to opportunistic infections and malignancies: the acquired immunodeficiency syndrome (AIDS). In HIV-1 infected patients the latency period for AIDS shows considerable variation between individuals, furthermore differences between risk groups in outcome of HIV-1 infection have been observed (1). This may be a function of the duration of infection or alternatively host/environmental factors may be determinants of outcome.

In vitro, lymphocyte activation with mitogens, soluble antigen, and other viruses is a prerequisite to pro-virus expression (335-338, 496, 497). It has been suggested that repeated antigenic stimulation by infectious agents or alloantigens may be important cofactors in the latency period of infection (490). Physiological stimuli enhancing pro-virus expression in vitro include granulocyte macrophage colony stimulating factor and interleukin-3, Y interferon had the opposite effect (498-501). This is in keeping with clinical observations, for instance: in male homosexuals the numbers of sexually transmitted diseases is one co-factor in disease progression (375). Similarly in
intravenous drug users the rate of CD 4 +ve cell loss correlates with the continued abuse of illicit drugs (502). Possible co-factors in haemophiliacs include influence of pre-existing liver disease due to NANB infection or recurrent allogenic stimulation from the repeated use of blood products. The relationship between these factors and the CD 4 +ve T-cell count has not been investigated.

In the present study the numbers of CD4+ve and CD8+ve T-lymphocytes have been prospectively counted in a cohort of treated haemophiliacs with and without HIV-1 antibody over four years. The effects of age, chronic liver disease and mean annual dose of clotting factor concentrate used on the T-cell subset numbers were investigated.

8.3.2 METHODS

Patients

In 1983, all factor VIII and IX deficient haemophiliacs who had been treated with a blood product at the West of Scotland Adult Haemophilia Centre were enrolled into a prospective study to evaluate the relationship between impaired cell mediated immunity to AIDS in haemophilia. The aims of the study and the characteristics of the cohort have been described (Chapter 2).
Subjects selected for sequential counting of T cell subsets were chosen on the basis of being HIV-1 antibody positive in 1984 and 1986, and selected seronegative treated patients matched for the severity of haemophilia were the control group. Serum from all treated patients was tested for HIV-1 antibody (ELISA, Wellcozyme) and the following additional information was obtained.

(i) Age of HIV-1 infection.
(ii) Duration of HIV-1 infection (Chapter 4).
(iii) Severity of liver disease (Chapter 2).
(iv) Mean annual dose of clotting factor concentrate used (Chapter 2).
(v) Clinical status (Chapter 6).

T-lymphocyte subset counting
Lymphocyte subpopulations were stained using monoclonal antibody markers for T-helper/inducer (T-h, CD4+ve) using OKT4 antibody and T suppressor/cytotoxic (T-s, CD8+ve) using OKT8 antibody and counted on a fluorescence activated cell sorter, as previously described (Chapter 8.2.2).

8.3.3. RESULTS
T-cell subsets were counted in 1984 and 1986 in 29 haemophiliacs, of these 11 were HIV-1 antibody positive. Furthermore in all HIV-1 antibody positive patients (n=21) patients' T-cell subsets were counted in 1988. T-
cell counts prior to seroconversion were not available in the three patients who were infected in 1985.

Helper/Inducer (CD4+ve, T-h) cell count

Table 8.3.1 shows that both groups (HIV-1 antibody positive and negative) were comparable for the absolute CD4+ve T-cell count in 1984, by 1986 median count in HIV-1 antibody positive patients had dropped significantly and a further decline had occurred by 1988, Figure 8.3.1. Whereas in seronegative patients there was no significant change, Table 8.3.2.

Symptomatic patients had the lowest CD4+ve T-cell counts (see Chapter 6).

Factors affecting the CD4+ve T-cell count in HIV-1 antibody positive haemophiliacs.

The grade of liver disease and the mean annual dose of clotting factor concentrate (1984-1988) used did not correlate with the CD4+ve T-cell count, \( r=0.31 \) \( (p=0.18) \) for liver disease grade and \( r=-0.11 \) \( (p=0.62) \) for the mean annual dose of clotting factor concentrate used. However, age \( (r=-0.5, p=0.02) \) and the age of infection \( (r=-0.5, p=0.01) \) did inversely influence the CD4+ve T cell count, but duration of infection did not \( (r=-0.02, p=0.01) \).

Table 8.3.3 shows differences between those patients with a CD4+ve T cell count below the normal range and those
Table 8.3.1. Sequential T cell subsets in HIV-1 seropositive patients.

<table>
<thead>
<tr>
<th>Year</th>
<th>CD4+ve</th>
<th>CD8+ve</th>
</tr>
</thead>
<tbody>
<tr>
<td>1984</td>
<td>692 (597-905)</td>
<td>484 (437-550)</td>
</tr>
<tr>
<td>1986</td>
<td>525.5 (373-648)</td>
<td>595.5 (436-890)</td>
</tr>
<tr>
<td>1988</td>
<td>356 (253-651)</td>
<td>470.5 (377-600)</td>
</tr>
</tbody>
</table>

Medians and interquartile ranges are shown.

CD4+ve count: 1984 vs 1986, p=0.02  
1984 vs 1988, p=0.04  
1986 vs 1988, p=0.3

CD8+ve count: 1984 vs 1986, p=0.05  
1984 vs 1988, p=0.5  
1986 vs 1988, p=0.1
FIGURE 8.3.1 SEQUENTIAL CD-4 CELL COUNT IN HIV-1 ANTIBODY POSITIVE PATIENTS
Figure 8.3.1. There was a significant fall in CD-4+ve T-cell count between 1984 and 1986 (p=0.003) and a further decline had occurred between 1986 and 1988 (p=0.05 one tailed test).
Table 8.3.2. Sequential T cell subsets in HIV-1 antibody negative patients.

<table>
<thead>
<tr>
<th>Year</th>
<th>T cell subsets (cells/mm$^3$)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CD4+ve</td>
<td>CD8+ve</td>
<td></td>
</tr>
<tr>
<td>1984</td>
<td>622</td>
<td>717.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(483-1003)</td>
<td>(553-847)</td>
<td></td>
</tr>
<tr>
<td>1986</td>
<td>415</td>
<td>569</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(302-662)</td>
<td>(378-699)</td>
<td></td>
</tr>
</tbody>
</table>

Medians and interquartile ranges are shown.

There was no significant differences.
Table 8.3.3. A comparison of patients with low and normal CD4+ve count (1986).

<table>
<thead>
<tr>
<th>CD-4 cell count</th>
<th>Low</th>
<th>Normal</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>29</td>
<td>19</td>
<td>0.004</td>
</tr>
<tr>
<td></td>
<td>(22-39)</td>
<td>(17-20)</td>
<td></td>
</tr>
<tr>
<td>Mean annual dose of treatment (units/annum)</td>
<td>77335</td>
<td>78698</td>
<td>0.84</td>
</tr>
<tr>
<td></td>
<td>(56852-100000)</td>
<td>(50000-83000)</td>
<td></td>
</tr>
<tr>
<td>Liver disease grade</td>
<td>3</td>
<td>3</td>
<td>0.78</td>
</tr>
<tr>
<td></td>
<td>(2-3)</td>
<td>(2.5-4)</td>
<td></td>
</tr>
<tr>
<td>Duration of HIV-1 infection</td>
<td>48</td>
<td>54</td>
<td>0.2</td>
</tr>
<tr>
<td></td>
<td>(35-60)</td>
<td>(50-61)</td>
<td></td>
</tr>
</tbody>
</table>

Medians and interquartile ranges are shown. Patients with a low CD-4 count were older but had a similar duration of infection to patients with a normal count.
with a normal CD-4+ve T cell count, patients with a count below the normal range were significantly older.

T-suppressor/cytotoxic (T-s, CD8+ve) cell count

Table 8.3.1 and Figure 8.3.2 show sequential measurements of the CD8+ve T-cell count in seropositive haemophiliacs. In seropositive patients there was a significant increase in the CD8+ve T-cell count, (p=0.05). The CD8+ve T-cell count did not significantly change in the seronegative group, Table 8.3.2 (p=0.09).

HIV-1 antibody positive patients with the CD4+ve T-cell count in the normal range had significantly more CD8+ve T-cells, Table 8.3.4.

Correlation Analysis

When CD8+ve T-cell count, age, mean annual dose of clotting factor concentrate used, and liver disease were taken as the independent variables in a stepwise logistic regression analysis with CD4+ve T-cell count as the dependent variable and correcting for the duration of infection, only the CD8+ve T-cell count remained significantly associated with CD4+ve T-cell count, (r=0.44, p=0.03) on both backward and forward selection.

8.3.4 DISCUSSION

Sequential measurements of T-cell subsets in haemophiliacs showed a significant decline in CD4+ve T-
FIGURE 8.3.2 SEQUENTIAL T SUPPRESSOR / CYTOTOXIC (CD 8 +VE) COUNT IN HIV - 1 ANTIBODY POSITIVE HAEMOPHILIACS
Figure 8.3.2. A significant increase in CD-8+ve T cell count occurred between 1986 and 1988 (p=0.04 one tailed test).
Table 8.3.4. The CD8+ve cell count in haemophiliacs (HIV-1 Ab+ve) with low and normal CD4+ve cell count in 1986 and 1988.

<table>
<thead>
<tr>
<th>CD4 count (cells/mm³)</th>
<th>Normal</th>
<th>Low</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD8+ve count (cells/mm³)</td>
<td>828 (640-950)</td>
<td>472 (414-824)</td>
</tr>
<tr>
<td></td>
<td>732.5 (515-1071)</td>
<td>411.5 (277-502)</td>
</tr>
</tbody>
</table>

Medians and interquartile ranges are shown.

Patients with a normal CD4 count had significantly more CD8+ve cells, p=0.04 in 1986.

This was seen in the 1988 counts, p=0.01.
cell count in HIV-1 antibody positive patients and an increase in CD8+ve count over four years. This was in contrast to HIV-1 negative patients in whom neither count significantly changed. In HIV-1 antibody positive patients CD4+ve T-cell count was significantly lower in older patients. Furthermore this prospective study confirms a previous observation Chapter 8.2.3 that a low normal CD4+ve T-cell count is associated with a lower CD8+ve T-cell count.

A progressive decline in CD4+ve T-cell count in HIV-1 infected patients with and without haemophilia has been a consistent finding (344). However it remains unexplained why certain patients have a more rapid decline than others. Eyster et al noted that patients with a more rapid decline were older when infected with HIV-1, in this study both age of infection and patient age but not duration of infection influenced the CD4+ve T-cell count (503). A stepwise logistic regression analysis showed that age was the main determining factor. Older patients did not have more severe liver disease and neither had they used more factor concentrate per annum. No correlation between age and CD4-ve T-cell count in HIV-1 negative patients was noted.

How age influences CD4+ve cell count decline is not known. There was however, a moderate inverse correlation with age and CD8+ve T-cell numbers in HIV-1 antibody positive patients. A more detailed analysis
showed that CD8+ve count and not age has a more important influence on the CD4+ve T cell count suggesting that older patients may mount a poorer T-cell cytotoxic, suppressive immune response against HIV-1.

This observation is in keeping with studies in which viral isolation has been attempted, removal of CD8+ve T-cells is associated with more success in viral isolation and provides a higher yield of virus (224). HIV-1 replication in these in vitro studies was inhibited by a soluble suppressive factor secreted by CD8+ve cells and not a cell mediated cytotoxic mechanism (224).

It has been previously shown that once the CD4+ve T-cell count falls below 200 cells/mm$^3$ there is a 25 percent chance of developing AIDS in the subsequent year and this doubles for the second year (503). At present it is not clear whether all such "high risk" individuals should be treated with azidothymidine (Zidovudine, Wellcome): controlled trials are in progress.

Extrapolating from in vitro observations in (Chapter 3.1), recurrent alloantigen load from treatment with clotting factor concentrates could be another cofactor. A poor correlation was found between mean annual dose of clotting factor concentrate used and the CD4+ve T-cell count. It may be that number of infusions or total protein load received would be a better measure of
alloantigen load rather than mean annual dose of clotting factor concentrate.

In HIV-1 antibody positive homosexuals, an increased number of sexually transmitted infections is a risk factor of subsequent AIDS (375). In haemophiliacs concommitant infections include hepatitis B and NANB hepatitis. In both, chronic liver disease arises due to viral persistence because of immune defects and their activity can be presumed from the resulting liver disease (171). Using a clinical and laboratory based grading of liver disease severity it was found that in HIV-1 positive patients, chronic liver disease made only a minor contribution to depletion in CD4+ve T-cell numbers.

In conclusion, a progressive decline in CD4+ve T-cell numbers during the course of HIV-1 infection was seen. Increasing age appears to influence quantitative, suppressive/cytotoxic response to HIV-1 and is one contributing factor in depletion of CD4+ve T-cell numbers. Presence of chronic liver disease and mean annual dose of clotting factor concentrate used did not contribute to CD4+ve T-cell lymphopenia in haemophilia.
CHAPTER 9.1

LYMPHOCYTE ACTIVATION AND PROLIFERATION IN HAEMOPHILIA -
THE EFFECT OF HIV-1 INFECTION, LIVER DISEASE AND
TREATMENT.

SUMMARY

Study objective: (i) To compare lymphocyte function in
vitro in HIV-1 antibody positive and negative
haemophiliacs. (ii) To determine whether chronic NANB
hepatitis or treatment with clotting factor concentrates
impairs lymphocyte function in vitro.

Study design: Open, cross-sectional.

Study population: 40 haemophiliacs grouped according to
serum HIV-1 antibody status, liver disease severity and
mean annual dose of clotting factor concentrate.

Measurements: Lymphocyte activation and proliferation in
response to the non-specific T-cell mitogen
phytohaemagglutinin (PHA) and to purified protein
derivative (PPD) of M. tuberculosis, a specific soluble
stimulator of T-cells.

Results: HIV-1 antibody positive haemophiliacs had
suppressed responses to PHA, proliferation correlated
with CD8+ve T-cell count and age of infection. Seronegative patients showed normal responses to PHA. PPD responses were impaired independent of HIV-1 antibody status. Seropositive patients had a more impaired response than seronegative patients. In seropositive patients PPD response showed a moderate inverse correlation with CD4+ve T-cell count.

Conclusions: HIV-1 infection results in a selective defect of CD4+ve T-cell function which is partly accounted for by CD4+ve T-cell depletion. In seronegative patients a selective defect of CD4+ve T-cell function also occurs but this was not due to quantitative changes. In seronegative patients this could not be accounted for by liver disease or mean annual dose of treatment used.
9.1.1 INTRODUCTION

Human immunodeficiency virus-1 (HIV-1) infection results in a progressive decline in the host's ability to mount an immune response (344). Clinically, immunodeficiency results in recurrent opportunistic infections and 'opportunistic' tumours or AIDS. In AIDS both quantitative and qualitative changes occur: CD4+ve T-cell numbers are reduced and CD8+ve T-cell counts may be increased, normal or reduced (207,375). Qualitative changes include an impaired proliferative response of peripheral blood mononuclear cells to non-specific stimulators of cell activation and proliferation - mitogens and specific soluble antigens such as tetanus toxoid or purified protein derivative of M. Tuberculosis (PPD) (345, 504). It is not known if such functional changes are due to a reduced ability to recognize antigen, inadequate effector mechanisms or both. Similar quantitative and qualitative changes occur in HIV-1 antibody positive in the absence of AIDS (207,344). In Chapter 8.2 it was found that 38 percent of HIV-1 antibody negative haemophiliacs had quantitative changes suggestive of immunodeficiency. Such changes could not be entirely accounted for by chronic non-A, non-B hepatitis or mean annual dose of clotting factor concentrate used. Furthermore, in vivo skin response to a new antigen dinitrochlorobenzene was impaired to an extent similar to HIV-1 antibody positive haemophiliacs (Chapter 7.1 and 7.2). In seronegative patients a moderate significant
correlation with mean annual dose of clotting factor concentrate was noted. Moreover, factor VIII concentrates suppressed activation and proliferation of cultured lymphocytes to both mitogens and antigen in vitro in a dose dependent manner (Chapter 3). In the absence of clinical immunodeficiency haemophiliac lymphocytes have an impaired proliferative response to alloantigens (505). It is however not clear if such findings are due to (a) liver disease, (b) an effect of contaminating alloantigens contained in clotting factor concentrates, or (c) due to latent HIV-1 infection. Alternatively, cellular abnormalities could be due to a suppressive factor present in haemophiliac serum.

In this study, immunocompetence in haemophiliacs was evaluated by measuring proliferative responses of peripheral blood mononuclear cells to specific and non specific T-cell activators. Presence of HIV-1 infection, liver disease and treatment was assessed independently. In addition the effect of haemophiliac serum on lymphocyte proliferation was measured.

9.1.2 METHODS

Patients

All haemophiliacs included in this study were part of a prospective cohort, in whom the mechanism and outcome of impaired cell mediated was being evaluated.
Forty haemophiliacs were studied and were grouped according to HIV-1 antibody status. Patients were further subdivided according to severity of liver disease. The method used to subdivide for liver disease has been previously described (Chapter 2.2). Age-matched, healthy, male, volunteers with no risk factors for HIV-1 infection were used as controls.

Lymphocyte preparation:

Peripheral blood mononuclear cells (PBMC) were separated from heparinised venous blood by sodium metrizoate density centrifugation, (Chapter 3.1.2). The PBMC band was removed and washed twice in Eagle's minimum essential medium and PBMC resuspended at $1 \times 10^6$ PBMC/mls in RPMI 1640 and 10 % pooled human serum supplemented with glutamine, streptomycin and gentamicin (see Chapter 3.1.2 for further details).

Lymphocyte activation and proliferation

Proliferative responses were measured in 96 well, round bottomed microtitre plates (Nunc. Gibco, Paisley, UK). Each well contained 100ul of PBMC in culture medium; and one of the following: phytohaemagglutinin (PHA) (Sigma, Poole, UK) or antigen, purified protein derivative of M. Tuberculosis (PPD) (State Serum Institute, Copenhagen, Denmark); 5ug of PHA per milliliter and PPD 5ug/mls were used. For PHA the cell concentration was adjusted to
1x10^5 cells/ml and for PPD cultures 1x10^6 cells/ml in 100ul of culture medium were used. All cultures were performed in triplicate at 37°C in a humidified atmosphere supplemented with 5% CO₂.

Proliferative responses to PHA were measured on day 3 and PPD on day 5 by measuring incorporation of [³H] thymidine after a 4hr pulse (19 hours for PPD) with 1µCi of [³H] thymidine (Amersham, UK). The cells were harvested as previously described (Chapter 3.1.2).

Effect of haemophiliac serum on normal lymphocyte proliferation

The PHA proliferative response was measured as described above except lymphocytes from a single normal donor were used and instead of using pooled normal serum in cell culture medium, serum from individual haemophiliacs was substituted.

T-cell subsets

CD4+ve and CD8+ve T-cells were counted as previously described (Chapter 8.2.2).

Expression of results

Results were expressed as corrected counts per minute (CPM). The mean of each triplicate of results obtained
in the absence of mitogen (or antigen) ie. spontaneous incorporation of $^3$H-Thymidine is subtracted from mean triplicate response in the presence of stimulus.

9.1.3 RESULTS

The 40 patients studied were comparable to the control group for age. Table 9.1.1 shows that those haemophiliacs not studied did not differ from study patients except in having a milder severity of haemophilia and a lower mean annual dose of clotting factor concentrate used.

HIV-1 Status

12 of the known 22 HIV-1 antibody positive patients were included in this study. There was no difference in mean annual dose of clotting factor concentrate used, age or clinical symptoms between those studied and not studied (Table 9.1.2), however studied patients had significantly reduced numbers of CD4+ve T-lymphocytes, but this was not known prior to the study. None of the study patients had a previous opportunistic infection, two patients had previous intermittent lymphadenopathy and one had thrombocytopenia (lowest recorded count = 50,000/mm$^3$). Table 9.1.2 compares HIV-1 antibody positive and negative patients studied.

PHA stimulated response
Table 9.1.1. A comparison of haemophiliacs in whom in vitro lymphocyte function was studied and not studied.

<table>
<thead>
<tr>
<th>PATIENTS</th>
<th>Not studied</th>
<th>Studied</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of patients</td>
<td>93</td>
<td>40</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>32 (24-46)</td>
<td>30 (21.5-39)</td>
<td>.25</td>
</tr>
<tr>
<td>Factor Deficiency (i.v./dl)</td>
<td>5 (0-10)</td>
<td>0 (0-5)</td>
<td>.002</td>
</tr>
<tr>
<td>Mean annual dose of clotting factor concentrate used (units/annum)</td>
<td>5920 (854-30000)</td>
<td>25892.5 (7409-61683)</td>
<td>.0004</td>
</tr>
<tr>
<td>Grade of liver disease</td>
<td>3 (2-5)</td>
<td>3 (2-5)</td>
<td>.24</td>
</tr>
<tr>
<td>HIV-1 antibody positive</td>
<td>10</td>
<td>12</td>
<td></td>
</tr>
</tbody>
</table>
Table 9.1.2  A comparison of haemophiliacs studied divided according to HIV-1 antibody status.

<table>
<thead>
<tr>
<th>HIV-1 ANTIBODY STATUS</th>
<th>-ve</th>
<th>+ve</th>
<th>+ve</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>(not studied)</td>
</tr>
<tr>
<td>No. of patients</td>
<td>28</td>
<td>12</td>
<td>10</td>
</tr>
<tr>
<td>Age (years)</td>
<td>31</td>
<td>21.5</td>
<td>26</td>
</tr>
<tr>
<td></td>
<td>(26.5-41.5)</td>
<td>(19-35)</td>
<td>(18-38)</td>
</tr>
<tr>
<td>Mean annual dose</td>
<td>18834</td>
<td>61683</td>
<td>79349</td>
</tr>
<tr>
<td>of clotting factor concentrate (units/annum)</td>
<td>(4675-41112)</td>
<td>(40435-101568)</td>
<td>(6961-84756)</td>
</tr>
<tr>
<td>Liver disease</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>(2-5)</td>
<td>(2-3)</td>
<td>(3-4)</td>
</tr>
<tr>
<td>CD4+ve T-cell count</td>
<td>606</td>
<td>399</td>
<td>679.5+</td>
</tr>
<tr>
<td>(cell/mm³)</td>
<td>(482-833)</td>
<td>(333-588)</td>
<td>(483.3-758.5)</td>
</tr>
<tr>
<td>CD8+ve T-cell count</td>
<td>343</td>
<td>668</td>
<td>594</td>
</tr>
<tr>
<td>(cells/mm³)</td>
<td>(266-479)</td>
<td>(471-903)</td>
<td>(428-826)</td>
</tr>
<tr>
<td>PHA stimulation</td>
<td>43167</td>
<td>21358.5*</td>
<td></td>
</tr>
<tr>
<td>(cpm)</td>
<td>(34895-53565)</td>
<td>(18972-45312)</td>
<td></td>
</tr>
<tr>
<td>PPD stimulation</td>
<td>7094</td>
<td>3662**</td>
<td></td>
</tr>
<tr>
<td>(cpm)</td>
<td>(1821-43557)</td>
<td>(892-6291)</td>
<td></td>
</tr>
</tbody>
</table>

Medians and interquartile ranges are shown of patients studied according to HIV-1 antibody status. Also included is a comparison of HIV-1 antibody patients not studied.
It was found that the HIV-1 antibody patients studied had reduced numbers of CD4+ve T-cells compared to studied positive patients, patients were however selected without prior knowledge of CD4+ve T-cell count. HIV-1 antibody negative patients studied differed significantly from positive patients in CD4 and CD8 cell numbers but were comparable for age and severity of liver disease. HIV-1 antibody patients had used significantly more treatment than seronegative patients.

+ p=0.04  
* p=0.002  
** p=0.03
Table 9.1.2 shows that corrected PHA stimulated values in HIV-1 antibody positive patients were significantly lower than in seronegative patients. Figure 9.1.1 shows that 58 percent of HIV-1 antibody positive patients had a PHA response below the lower quartile of the normal range, this was similar to the numbers of HIV-1 antibody negative patients with a low normal response, 43 percent (p=0.29, Fishers Exact Test).

Table 9.1.3 shows that HIV-1 antibody positive patients with a response above the lower quartile of the control group had significantly more CD8+ve T-lymphocytes (p=0.05). Patients with a reduced response were also significantly older (p=0.05) but had used similar amounts of clotting factor concentrate per annum to normal responders. There was no correlation between PHA response and severity of liver disease (r=0.28, p=0.39) mean annual dose of clotting factor concentrate used (r=0.35, p=0.28), duration of infection (r=0.35, p=0.23) but there was a strong inverse correlation with age of infection (r=-0.69, p=0.02). PHA induced proliferation showed no correlation with CD4+ve T-cell count (r=0.25, p=0.43) but there was a good correlation with CD8+ve T-cell count (r=0.56, p=0.07), in a simple regression analysis - multiplicative model.

Figure 9.1.1 shows that there was no difference in median proliferative response between HIV-1 antibody negative patients and controls, but 43 percent of
FIGURE 9.1.1 PHA INDUCED LYMPHOCYTE ACTIVATION AND PROLIFERATION

Counts Per Minute

0 20000 40000 60000 80000

Control +ve -ve

HIV - 1 Status
Figure 9.1.1. PHA induced proliferation in seropositive haemophiliacs was significantly lower than controls (p=0.001). Seronegative patients did not differ from controls (p=0.2). 43 percent of seronegative haemophiliacs had a response below the lower quartile of the control population.
Table 9.1.3  Differences between HIV antibody positive patients with a low normal response and a normal response to PHA.

<table>
<thead>
<tr>
<th>PHA RESPONSE</th>
<th>Low 7</th>
<th>Normal 5</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4+ve (cells/mm$^3$)</td>
<td>371 (321-573)</td>
<td>537 (375-602)</td>
<td>.37</td>
</tr>
<tr>
<td>CD8+ve (cells/mm$^3$)</td>
<td>530 (345-785)</td>
<td>909 (897-950)</td>
<td>.05</td>
</tr>
<tr>
<td>Age (years)</td>
<td>35 (22-39)</td>
<td>19 (19-21)</td>
<td>.05</td>
</tr>
<tr>
<td>Liver disease severity</td>
<td>3 (2-3)</td>
<td>3 (2.5-4)</td>
<td>.54</td>
</tr>
<tr>
<td>Mean annual dose of clotting factor concentrate (units/annum)</td>
<td>64336 (30870-103136)</td>
<td>58810 (500000-82164)</td>
<td>.7</td>
</tr>
<tr>
<td>PPD (CPM)</td>
<td>3416.5 (1527-7843)</td>
<td>3741.5 (892-6291)</td>
<td>.9</td>
</tr>
</tbody>
</table>

Medians and interquartile ranges are shown. HIV-1 antibody positive patients with a low normal response to PHA were older and had reduced numbers of CD8+ve T-cells compared to patients with normal responses.
patients had a low normal response, i.e. below the lower quartile of the normal range.

Table 9.1.4 compares these two groups, no significant differences were noted. No correlation was seen with severity of liver disease \((r = -0.28, p = 0.14)\), mean annual dose of treatment used \((r = 0.08, p = 0.66)\) or age \((r = -0.15, p = 0.42)\) and proliferation of PBMC with PHA. There was no correlation with PHA response and CD4 T-cell count \((r = 0.07, p = 0.7)\) or CD8 T-cell count \((r = -0.21, p = 0.32)\) in a simple regression analysis (multiplicative model).

**PPD stimulated response.**

The PPD induced proliferative response was significantly impaired in haemophiliacs, \((p = 0.001)\) Figure 9.1.3. HIV-1 antibody positive haemophiliacs had an impaired response compared to controls \((p = 0.007)\) and HIV-1 antibody negative patients \((p = 0.02)\), Table 9.1.2. As shown in Figure 9.1.2, seronegative patients had a significantly lower response compared to controls, \((p = 0.01)\).

In HIV-1 antibody positive patients there was a moderate inverse correlation with age but this did not achieve statistical significance \((r = -0.45, p = 0.17)\). Interestingly, there was no correlation with age of infection \((r = -0.09, p = 0.74)\) or duration of infection \((r = 0.48, p = 0.09)\). There was no correlation with mean annual dose of clotting factor concentrate used \((r = 0.46, p = 0.16)\) and grade of liver disease \((r = 0.45, p = 0.17)\). In
Table 9.1.4  A comparison of the characteristics of HIV-1 antibody negative haemophiliacs with a low normal and normal response to PHA.

<table>
<thead>
<tr>
<th>PHA RESPONSE</th>
<th>Low</th>
<th>Normal</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>31.5 (23-44.5)</td>
<td>30.5</td>
<td>.76</td>
</tr>
<tr>
<td>Liver disease severity</td>
<td>5 (3-5.5)</td>
<td>3 (1.3-4.5)</td>
<td>.09</td>
</tr>
<tr>
<td>Mean annual dose of clotting factor concentrate used (units/annum)</td>
<td>15834 (1812-41112)</td>
<td>15670.5 (6782-47290)</td>
<td>.56</td>
</tr>
<tr>
<td>CD4+ve cells (cells/mm³)</td>
<td>649.5 (472-777)</td>
<td>590.5 (491-877)</td>
<td>.9</td>
</tr>
<tr>
<td>CD8+ve cells (cells/mm³)</td>
<td>355 (300-725)</td>
<td>328 (212-452)</td>
<td>.5</td>
</tr>
<tr>
<td>PPD (cpm)</td>
<td>4352 (1480-22137)</td>
<td>12394 (2964-62174)</td>
<td>.15</td>
</tr>
</tbody>
</table>

The median and interquartile ranges are shown. There were no significant differences between the two groups.
FIGURE 9.1.2 THE PROLIFERATIVE RESPONSE TO PPD IN HAEMOPHILIACS
Figure 9.1.2. PPD induced proliferation was significantly lower in seropositive patients (p=0.007) and seronegative patients (p=0.01) compared to controls.
seronegative patients no correlations were seen, liver
disease (r=0.27, p=0.16) mean annual dose of treatment
(r=0.19, p=0.32) or age (r=0.12, p=0.12).

Impaired proliferative PPD responses could be due to
quantitative or qualitative change in the functional
subset of CD4+ve T-lymphocytes that recognize antigen
(CD4+ve, Leu 8+ve). In HIV-1 antibody positive patients a
moderate inverse correlation was noted between
proliferative capacity to PPD and CD4+ve T-cell numbers,
r=-0.43 (p=0.15), suggesting (a) a quantitative defect
(b) a functional impairment or (c) presence of
suppressive cytotoxic factors impairing response. There
was no correlation with CD8+ve T-cell count (r=0.12,
p=0.16).

In HIV-1 antibody negative patients there was no
correlation with either CD4+ve T-cell count (r=-0.02,
p=0.9) or CD8+ve T-cell numbers (r=-0.08, p=0.7) and PPD
induced lymphocyte proliferation.

The Effect of Haemophiliac Serum on lymphocyte activation
Haemophiliac serum had no effect on PHA or PPD stimulated
PBMC (normals) proliferation.

9.1.4. DISCUSSION

PHA is a non-specific activator of T lymphocyte
proliferation (506). An impaired response to PHA could
be due to: reduced numbers of T-cells, or a shift in
proportion of T cell phenotypes. For instance, if there were more CD8+ve T-cells, proliferation would result in suppressive/cytotoxic ratios that may dampen overall response. Alternatively, pre-stimulation, ie. in vivo stimulation would exhaust cells and result in impaired activation and proliferation, or there may be a functional defect.

In this study, HIV-1 antibody positive haemophiliacs had an impaired proliferative response to PHA compared to controls and seronegative haemophiliacs, Figure 9.1.1. Both HIV-1 antibody positive and seronegative haemophiliacs had a similar severity of liver disease but seropositive patients had used more clotting factor concentrate than seronegative patients. A reduced proliferative response to PHA was one of the first laboratory abnormalities reported in patients with AIDS and is a characteristic of HIV-1 infection regardless of the individual's risk group (345). It is therefore unlikely that impaired activation and proliferation is an effect of clotting factor concentrates. In support of this it was found that in HIV-1 positive patients there was no correlation between proliferative response and mean annual dose of factor concentrate used, Table 9.1.3.

It has previously been suggested that impaired PHA response in HIV-1 infection is a consequence of CD4+ve T-cell depletion (345). However, recent studies of CD4+ve T-cell enriched lymphocyte populations show that
to PHA in HIV-1 infection is similar to normals (504). In the present study patients with an impaired response had similar numbers of CD4+ve T-cells but CD8+ve T-cell was reduced compared to patients with a response in the normal range, Table 9.1.3. It was also found that CD8+ve T-cell count showed a significant correlation with PHA response in a multiplicative regression analysis. An inverse correlation with age and age of infection but not with duration of infection was also found.

Furthermore, using PPD it was found that quantitative CD4+ve T-cell differences between low and normal PHA responders were due to a functional impairment or a selective depletion of CD4+ve T-cells that selectively proliferate in response to soluble antigen. Similar findings have been reported in AIDS patients belonging to other risk groups (504). The data from this study further shows this is an early manifestation of HIV-1 infection occurs in the absence of clinical immunodeficiency and is influenced inversely by patient age. The latter finding may be a determinant of the immune response to HIV-1 infection (Chapter 5.2).

Such a defect may be due to (a) a failure in recognition of antigen presented by macrophages, (b) an impaired ability to produce interleukin 2 or (c) an excessive cytotoxic/suppressive response to HIV-1, so that cells expressing HIV-1 are destroyed. Identical twin studies, one of whom had AIDS and the other was HIV-1 negative
showed that the interaction of macrophages and CD4+ve T-cells is normal in HIV-1 infection (507). Furthermore, HIV-1 infected cells express normal amounts of IL-2 messenger RNA (508). The functional defect to soluble antigen therefore cannot be entirely explained on CD4+ve T-cell depletion, macrophage interaction or IL-2 production but could be due to (a) HIV-1 blocking the CD4 receptor, (b) a non-infectious manifestation of HIV-1 due to replication defective viral particles or (c) due to the immune response to HIV-1.

In HIV-1 negative haemophiliacs there was no statistical difference in PHA response compared to normals but a low response to PHA occurred as frequently as in HIV-1 antibody positive patients, Figure 9.1.1. An impaired response to soluble antigen (PPD), suggested a functional defect or quantitative depletion in the subset of CD-4+ve T-cells that proliferate in response to antigen may be the prime abnormality.

In a previous study, (Chapter 3.1) clotting factor concentrates impaired the proliferative response of normal lymphocytes to PHA and PPD in vitro. Others have shown that clotting factor concentrates impair macrophage function in vitro (326). In vivo treatment with factor concentrate induce FcY receptor antibodies, this blocking antibody impaired lymphocyte activation and proliferation has the ability to stimulate CD8+ve T-cells to generate a suppressive response (459). Alternatively, it may be due
to an impaired ability to produce the T-cell trophic factor interleukin 2 (see Chapter 10).

In conclusion, in this study it was found that haemophiliacs have an abnormally low response to PPD; this was independent of HIV-1 infection. In HIV-1 antibody positive patients in addition to reduced numbers of CD4+ve T-cells, there may be a functional defect or a selective quantitative reduction in CD4+ve T-cells that preferentially proliferate in response to soluble antigen. The impaired PHA response in seropositive patients was not due to reduced CD4+ve, T-cells but due to proportionally more CD8+ve, T-cells. An inverse correlation with patient age and age of HIV-1 infection, but not with duration of infection was a contributory factor.

In HIV-1 antibody negative patients impaired PPD response showed no correlation with CD4+ve or CD8+ve T-cell counts suggesting a functional defect in the subset of T-cells that selectively respond to antigen in vitro. No association with mean annual dose of clotting factor concentrate used or liver disease was observed. Haemophilic serum did not contain suppressive/cytotoxic factors.
CHAPTER 9.2

THE EFFECTS OF NON-INFECTIONOUS HIV-1 LYSATE ON LYMPHOCYTE FUNCTION.

SUMMARY

Study objective: Can HIV-1 impair lymphocyte function in the absence of productive infection.

Study design: Open.

Study population: Normal volunteers with no risk factors for HIV-1 infection.

Measurements: Lymphocyte transformation in response to PHA and PPD in the presence of an HIV-1 lysate. IL-2 production was also measured.

Results: HIV - 1 lysate inhibited PPD induced transformation and IL-2 production in a dose dependent manner. PHA induced transformation was impaired at high concentrations but stimulated at low concentrations.

Conclusions: HIV-1 proteins impair lymphocyte function in the absence of productive infection.
9.2.1 INTRODUCTION

In the previous studies it was found that human immunodeficiency virus - 1 (HIV-1) results in a progressive decline of CD4+ve T-cell (Chapter 7.1 & 7.2) and in abnormalities of lymphocyte function independent of CD-4+ve T-cell depletion (Chapter 8.2).

The mechanism remains unexplained. The use of fluoresinated antibodies against viral encoded proteins and in-situ hybridization techniques to detect cells expressing viral proteins, show that 1 in $10^5$ cells in the peripheral blood of HIV-1 infected individuals are expressing virus at any time (217). A larger number of cells may however be latently infected. The depletion of infected cells is a direct cytopathic effect of HIV-1 (486, 489, 490). However CD4+ve T-cell depletion does not parallel onset of clinical immunodeficiency, and other mechanisms must therefore be postulated (467). In feline AIDS it has been shown that non-infectious virus particles may be implicated in the pathogenesis of disease (384). In Chapter 4.3 it was found that such replication deficient particles are present in HIV-1 culture. In the present study therefore effects of a non-infectious HIV-1 lysate on lymphocyte function was investigated.
9.2.2 METHODS

Cell separation and culture:

Peripheral blood mononuclear cells (PBMC) were isolated from 8 normal healthy volunteers as previously described and resuspended in cell culture medium Chapter 3.1.1). HIV-1 lysate was a kind gift from Dr R C Gallo. The protein content of HIV-1 lysate was 9mg/ml and was diluted in tissue culture medium to give a reference stock solution at 1mg/ml.

Triplicate PHA and PPD stimulation assays were used as previously (Chapter 9.1.2) and to each culture 100ul of HIV-1 lysate was added at final concentrations of 1:50; 1:100; 1:250; 1:500; 1:1000 of reference stock solution and cultured for 3 days for PHA and 4 days for PPD. Cells were harvested and incorporated $[^3]H$] thymidine counted in a liquid scintillation counter, as previously described, Chapter 3.1.2. The mean of each triplicate was expressed as a percentage of the maximal response in the absence of HIV-1 lysate.

The effect of HIV-1 lysate on interleukin 2 (IL-2) production was investigated, using methods similar to those described in Chapter 3.2.2.

Briefly, PBMC in tissue culture medium were adjusted to $1x10^6$/mls, stimulated with PHA 2ug/ml (final concentration) and 0.5mls of each concentration of HIV-1
lysate. Supernatant was harvested after 48 hours and assayed for IL-2 activity in a bioassay (Chapter 3.1.2). The IL-2 levels were expressed as a percentage of amount produced in the absence of HIV-1.

9.2.3 RESULTS

Figure 9.2.1 shows that at low concentrations probably similar to those found in serum, in the absence of biochemically detectable antigenaemia, HIV-1 stimulated lymphocytes, activated and proliferated in response to PHA. At higher concentrations HIV-1 inhibited the response. HIV-1 inhibited PPD induced lymphocyte proliferation at all concentrations, Figure 9.2.2. HIV-1 inhibited IL-2 production in a dose dependent manner, Figure 9.2.3.

9.2.3 DISCUSSION

The results of the present study show that non-infectious HIV-1 lysates inhibit PHA and PPD induced proliferation of lymphocytes in vitro. These findings are similar to those reported by Pahwa et al (509). Furthermore, in this study it was found that HIV-1 proteins inhibit interleukin-2 production in vitro in a dose dependent manner. One other observation in this study was that at similar concentrations (1:300) of HIV-1 increased PHA induced proliferation occurred whereas PPD driven
Figure 9.2.1. PBMC from normal volunteers were stimulated with PHA in the presence of HIV-1 lysate. At low concentrations (≤1:250) HIV-1 enhanced proliferation but at >1:100 HIV-1 inhibited proliferation.
FIGURE 9.2.1 PHA RESPONSE OF PBMC CULTURED IN THE PRESENCE OF HIV-1 LYSATE

Percent Maximum Inhibition

Dilution of HIV-1 Lysate

1:1000 1:500 1:250 1:100 1:50
FIGURE 9.2.2 PPD RESPONSE OF PBMC IN THE PRESENCE OF HIV-1 LYSATE.
Figure 9.2.2. PBMC from normal volunteers were stimulated in the presence of HIV-2 lysate. A dose dependent inhibition was seen.
FIGURE 9.2.3 IL-2 PRODUCTION FROM PBMC IN THE PRESENCE OF HIV-1 LYSATE

Dilution of HIV-1 Lysate

Percent Maximum Response
Figure 9.2.3. PBMC from normal volunteers were stimulated to produce IL-2 in the presence of HIV-1 lysate a dose dependent inhibition of IL-2 production was seen.
activation and proliferation was inhibited, Figure 9.2.1. This is in keeping with findings in Chapter 9.1 that HIV-1 antibody patients had a selective defect of T-cells that are prime responders to antigen in-vitro. Alternatively, HIV-1 may stimulate CD8+ve, T-cells at low concentrations.

The implications of these findings are that HIV-1 proteins are immunosuppressive in the absence of productive HIV-1 infection. The binding of external viral envelope protein (gp120) to its receptor the CD4 molecule may result in cell-cell fusion and giant cell or syncitia formation with subsequent cell lysis (496). Alternatively, it may be that binding of HIV-1 to its receptor prohibits the T-cell to enter the cell cycle.

Mitogens such as phorbol myristate have no effect on inositol lipid hydrolysis but activate protein kinase C directly (510). Antigen binding to T-cells induces inositol lipid hydrolysis, mobilizes intracellular calcium and causes the translocation and activation of protein kinase C (510). It is therefore possible that binding of HIV-1 to the CD4 molecule inhibits protein kinase C.

In conclusion in this study it was found that HIV-1 proteins inhibit T-cell proliferation in vitro, therefore part of the clinical immunosuppression seen during the course of HIV-1 infection may be mediated by
replication deficient viral particles or free gp120 in serum.
CHAPTER 9.3

CONCANAVALIN A INDUCED LYMPHOCYTE PROLIFERATION IN HAEMOPHILIACS.

SUMMARY

Study objective: To determine if there is a function defect of haemophilic lymphocytes that selectively proliferate in response to Concanavalin A (Con A).

Study design: Open, cross-sectional.

Study population: 41 haemophiliacs with (n=13) and without HIV-1 antibody.

Measurements: Con A induced lymphocyte transformation.

Results: All haemophiliacs had a normal Con A response.

Conclusions: The primary functional defect in HIV-1 antibody positive haemophiliacs is in the subset of T-cells that express CD4 antigen.
9.3.1 INTRODUCTION

Haemophiliacs have an impaired immune response to a new antigen in-vivo, and this is independent of the patients HIV-1 status (Chapter 7.1). This may be due to an impaired ability to recognize and respond to an antigen, alternatively excessive T-suppressor/cytotoxic cell activity may be responsible. Although, in Chapter 8.2.3 it was found that haemophiliacs do not have increased numbers of CD8+ve T-cells, others have reported an increased count (469). In a previous study it was also shown that haemophiliacs have increased amounts of FcY receptor antibody (Chapter 3.2). This antibody may have the capacity to stimulate release of suppressive substances, and it has been suggested that the beneficial effects of blood transfusion on renal allograft survival may be partly mediated by such a mechanism (304).

In this study the proliferative capacity of peripheral blood mononuclear cells to concanavalin-A (Con-A) was measured, as a indicator of CD-8+ve T-cell activity. Con A is a specific mitogen for this subset of lymphocytes (506). The Con-A response was compared between HIV-1 antibody positive and negative patients.

9.3.2 PATIENTS AND METHODS

Heparinised venous blood was obtained from 41 haemophiliacs, who were taking part in a prospective
study of immunodeficiency in haemophiliacs. The selection criteria for inclusion are given in Chapter 2.

Cell separation and culture:

Peripheral blood mononuclear cells (PBMC) were obtained by density centrifugation of heparinised venous blood (see Chapter 3.1.2). Washed PBMC were resuspended in cell culture medium, RPMI 1640 supplemented with antibiotics and essential amino acids, (Chapter 3.1.2). $5 \times 10^5$ PBMC were cultured in triplicate in the presence and absence of Con-A (final concentration $50 \mu g/ml$), at $37^\circ C$ in a humidified atmosphere and air enriched with $5\%$ CO$_2$. All cultures were performed in triplicate.

Four hours prior to harvesting, the cells were pulsed with $3[H]$ thymidine. At 72 hours cells were harvested onto glass fibre discs and incorporated radioactivity was counted on a liquid scintillation counter (as previously described in Chapter 3.1.2).

Results were expressed as described in Chapter 9.1.3.

T-cell subsets:

T-cell subsets were counted in PBMC as previously described (Chapter 8.2.2).

9.3.3 RESULTS

There was no significant difference in the median age of patients and controls.

HIV-1 status
Thirteen of 41 haemophiliacs studied had HIV-1 antibody. Median duration of infection was 53 months (interquartile range (IQR) 43 to 60). Four of the HIV-1 antibody positive patients had detectable levels of free serum HIV-1 antigen (Chapter 4.1.2). No patient had AIDS, two patients had a low platelet count, no other manifestations of HIV-1 infection were noted. Ages of HIV-1 antibody positive and negative patients were comparable, Table 9.3.1.

Con A induced lymphocyte proliferation:

Figure 9.3.1 and Table 9.3.1 shows that Con A responses corrected for background counts per minute (cpm) between patients and controls was similar (p=0.5). There was no difference in median cpm between HIV-1 positive and negative patients (p=0.5). HIV-1 antibody negative patients had a response similar to controls (p=0.7). Nineteen haemophiliacs (46 percent) had a Con A response below the lower quartile of the normal range (Figure 9.3.1). Of these 6 were HIV-1 antibody positive (46 percent of all HIV-1 antibody positive) and 13 were HIV-1 antibody negative (46 percent of all HIV-1 antibody negative, (p=0.6, Fishers Exact Test). Four of six HIV-1 antibody positive patients with a response below the lower quartile of the normal range had serum HIV-1 serum antigen, three had symptoms of HIV-1 infection whereas none of the high responders had antigen or significant clinical symptoms, (p=0.02 Fishers Exact Test), Figure 9.3.1.
FIGURE 9.3.1 THE CONCANAVLIN A PROLIFERATIVE RESPONSE IN HAEMOPHILIACS
Figure 9.3.1. There was no significant difference in proliferative response between HIV-1 +ve and HIV-1 -ve haemophiliacs compared to controls.
Table 9.3.1  The characteristics of patients in whom the Con-A response was measured.

<table>
<thead>
<tr>
<th>HIV Status</th>
<th>Positive</th>
<th>Negative</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of patients</td>
<td>13</td>
<td>28</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>22 (19-35)</td>
<td>31 (26.5-41.5)</td>
<td>.14</td>
</tr>
<tr>
<td>Haemophilia severity (i.u./dl)</td>
<td>0 (0-0)</td>
<td>0 (0-7)</td>
<td>.03</td>
</tr>
<tr>
<td>Grade of liver disease</td>
<td>3 (2-3)</td>
<td>3 (2-5)</td>
<td>.28</td>
</tr>
<tr>
<td>Mean annual dose of clotting factor concentrate used (units/annum)</td>
<td>64556 (50000-10000)</td>
<td>15834 (4675-41117)</td>
<td>.001</td>
</tr>
<tr>
<td>CD4+ve (cells/mm³)</td>
<td>423 (345-573)</td>
<td>606 (482-833)</td>
<td>.01</td>
</tr>
<tr>
<td>CD8+ve (cells/mm³)</td>
<td>551 (470-897)</td>
<td>343 (266-479)</td>
<td>.004</td>
</tr>
</tbody>
</table>

Medians and interquartile ranges are shown. HIV-1 antibody positive patients were comparable to seronegative patients for age and severity of liver disease. However, HIV-1 antibody positive patients had a more severe deficiency of haemophilia, had used more clotting factor concentrate and significantly fewer CD4+ve T-cells but more CD8+ve T-cell.
Table 9.3.2  A comparison of HIV-1 antibody positive patients with a low normal and normal Con-A response.

<table>
<thead>
<tr>
<th>HIV-1 antibody positive</th>
<th>pvalue</th>
</tr>
</thead>
<tbody>
<tr>
<td>Con A Response</td>
<td>low</td>
</tr>
<tr>
<td>Number of patients</td>
<td>6</td>
</tr>
<tr>
<td>HIV serum antigen</td>
<td>4</td>
</tr>
<tr>
<td>(no. of patients)</td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>35</td>
</tr>
<tr>
<td>(years)</td>
<td>(19-49)</td>
</tr>
<tr>
<td>Mean annual dose</td>
<td>68072.5</td>
</tr>
<tr>
<td>of clotting factor</td>
<td>(308870-103136)</td>
</tr>
<tr>
<td>concentrate used</td>
<td></td>
</tr>
<tr>
<td>(units/annum)</td>
<td></td>
</tr>
<tr>
<td>Grade of liver</td>
<td>3</td>
</tr>
<tr>
<td>disease</td>
<td>(3-3)</td>
</tr>
<tr>
<td>CD4+ve</td>
<td>543.5</td>
</tr>
<tr>
<td>(cells/mm³)</td>
<td>(423-602)</td>
</tr>
<tr>
<td>CD8+ve</td>
<td>668</td>
</tr>
<tr>
<td>(cells/mm³)</td>
<td>(472-883)</td>
</tr>
</tbody>
</table>

Medians and interquartile ranges are shown, there were no significant differences between the two groups.
Table 9.3.3  A comparison of HIV-1 antibody negative patients with a low normal and normal Con-A response.

<table>
<thead>
<tr>
<th>HIV-1 antibody negative p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Con A response</td>
</tr>
<tr>
<td>Number of patients</td>
</tr>
<tr>
<td>Age (years)</td>
</tr>
<tr>
<td>Mean annual dose of clotting factor concentrate used (units/annum)</td>
</tr>
<tr>
<td>Grade of liver disease (1.5-5)</td>
</tr>
<tr>
<td>CD4+ve (cells/mm^3)</td>
</tr>
<tr>
<td>CD8+ve (cells/mm^3)</td>
</tr>
</tbody>
</table>

Medians and interquartile ranges are shown. Patients with a low normal response were older than patients with a normal response.
Con A responses in seropositive patients showed no correlation with age ($r=-0.25$, $p=0.37$), or duration of infection ($r=0.22$, $p=0.44$). In seronegative patients there was a good inverse correlation with Con A response and age, ($r=-0.61$, $p=0.001$).

Liver disease

There was no correlation noted between Con A response and clinical grade of liver disease in HIV-1 antibody positive ($r=-0.17$, $p=0.35$).

Mean annual amount of factor concentrate

In neither HIV-1 antibody positive ($r=-0.15$, $p=0.6$) or negative ($r=0.06$, $p=0.7$) patients did mean annual dose of clotting factor concentrate used have an effect on Con A response.

9.3.4 DISCUSSION

In the present study proliferative responses of lymphocytes from haemophiliacs to Con A was normal, and this was independent of the patients' HIV-1 status. This is in contrast to effects of PHA and PPD which are impaired confirming that the primary defect in HIV-1 antibody positive patients is in the subset of T cells that express the CD4 phenotype (344).

Despite the absence of a difference in Con A response between HIV-1 positive patients and controls some
interesting trends were seen. Patients with serum HIV-1 antigen had a low normal response compared to those without serum antigen. This suggests that suppressive/cytotoxic response may be another factor in determining disease progression. It may be as duration of infection increases in this cohort that the Con A responses in patients with antigen will fall further.

As in HIV-1 antibody positive patients seronegative patients had normal Con A responses. In a previous study it was found that haemophiliacs had high levels of FcY receptor antibody (Chapter 3.2), 12 of these patients were also included in this study. Two patients had normal FcYR levels, both had responses below the lower quartile of the normal range and only one of 10 patients with raised FcY receptor antibody levels had a low response. Additional studies to confirm this anecdotal observation are indicated. In seronegative patients the Con A response showed a significant inverse correlation with age.

In conclusion in this study there was no evidence to show that the Con-A response in haemophiliacs is impaired.
INTERLEUKIN-2 LEVELS IN THE ABSENCE AND PRESENCE OF HIV-1 ANTIBODY IN HAEMOPHILIA.

SUMMARY

Study objective: To compare the capacity to produce interleukin-2 (IL-2) in haemophiliacs with and without HIV-1 antibody and determine whether impaired production was due to functional or quantitative abnormalities.

Study design: Open, cross-sectional.

Study population: Haemophiliacs were selected from a well defined treated cohort on the basis of HIV-1 antibody status, mean annual dose of clotting factor used and liver disease severity.

Measurements: Capacity to produce IL-2 from peripheral blood mononuclear cells (PBMC), T-cell subsets and proliferation of PBMC to purified protein derivative (PPD) of mycobacterium tuberculosis were measured.
Results: The reduced capacity to produce IL-2 was independent of HIV-1 antibody status. The mean annual dose of clotting factor concentrate used and liver disease severity did not correlate with IL-2 levels. In seropositive patients reduced levels correlated with PPD responses. No correlations were seen in seronegative patients.

Conclusions: The reduced capacity to produce IL-2 in HIV-1 seronegative haemophiliac was due to functional defects. In HIV-1 positive patients it may be due to a functional or a quantitative defect in the CD4+ve cell subsets that are prime responders to soluble antigens.
The human immunodeficiency virus-1 (HIV-1) primarily infects and depletes T-helper/inducer lymphocytes (T-h, CD4+ve) (339, 340, 345). The T cell receptor on CD4+ve lymphocytes recognises processed antigen, presented by macrophages in the context of major histocompatibility (MHC) class II molecules (511-513). In response to antigen and interleukin-1 secreted by macrophages, CD-4+ve cells produce the lymphokine interleukin 2 (IL-2) (514). IL-2 results in clonal proliferation of CD4+ve T-cells expressing high affinity IL-2 receptors and induces both antibody dependent and independent effector immune responses (514). A reduced IL-2 production may therefore be one consequence of HIV-1 infection.

In vitro HIV-1 infected cell lines show that IL-2 gene transcription is intact and normal levels of IL-2 are produced using purified CD4+ve lymphocyte preparations from AIDS patients (508, 504). Conversely, using unfractionated peripheral blood mononuclear cells capacity to produce IL-2 is reduced and the reduced proliferative capacity of phytohaemagglutinin stimulated peripheral blood mononuclear cells from AIDS patients can be partially restored by addition of exogenous IL-2 (515-519). The reduced capacity to produce IL-2 appears to be independent of CD4+ve cell numbers suggesting an additional defect in the immune system in AIDS patients.
It is not clear whether this is (a) a non-infectious effect of HIV-1 as shown in Chapter 9.2, (b) due to the immune response to HIV-1 or (c) due to opportunistic infections and tumors that occur in AIDS. Borzy et al reported reduced IL-2 production in HIV-1 infected, asymptomatic, homosexuals suggesting that this is independent of secondary infections that occur during the course of HIV-1 infection (519). No studies have yet been reported in haemophiliacs in whom the immunological environment is unique.

Haemophiliacs frequently have concomitant non-A, non-B (NANB) hepatitis and hepatitis B infection; both can potentially alter immune function both quantitatively and qualitatively. In addition haemophiliacs are frequently challenged with a large contaminating alloantigen load which may be potentially deleterious. In Chapter 3.1 it was found that factor VIII concentrate is directly immunosuppressive, and reduced IL-2 production.
In the present study, capacity to produce IL-2 was measured in haemophiliacs with HIV-1 infection in the absence of present or previous opportunistic infections. Specifically unfractionated peripheral blood mononuclear cells were used to reproduce the \textit{in vitro} intercellular relationships. Furthermore, values were correlated to immune status by measuring peripheral blood T-cell subsets. The proliferative response of peripheral blood mononuclear cells to soluble antigen was measured to determine if there is a functional defect in CD-4+ve cells to produce IL-2 \textit{in vivo}.

10.1.2 METHODS

Patients

The study population consisted of 46 haemophiliacs regularly treated with clotting factor concentrates who were participating in a prospective study of HIV-1 related disease and immune abnormalities. Selection criteria for entry into the prospective cohort are listed in Chapter 2. Included were 16 of the 22 patients known to be HIV-1 antibody positive. Serum HIV-1 antibody negative patients were selected on the basis of NANB related liver disease severity and mean annual dose of clotting factor concentrate used. Criteria for grading NANB liver disease have been previously stated (Chapter 2).
Cell preparation

Peripheral blood mononuclear cells (PBMC) separated from heparinised venous blood by density centrifugation over sodium metrizoate (Chapter 3.1) were resuspended in complete RPMI/2% heat inactivated fetal calf serum (FCS), and the cell concentration adjusted to 1×10⁶ cells/mls.

IL-2 supernate production

IL-2 supernatant was produced by culturing PBMC in 24 well flat bottomed tissue culture plates (Costar, Cambridge, Massachusetts, USA). Each well contained 1ml of cell suspension (1×10⁶ cells) and phytohaemagglutinin (PHA) at a final concentration of 2μg/mls (Wellcome grade) or no mitogen.

The cultures were incubated at 37°C in a humidified atmosphere supplemented with 5% CO₂. After 48 hrs supernatants were recovered by centrifugation and stored at -20°C until assayed.

Supernatant IL-2 was measured as described in Chapter 3.1 (306).

T-cell Subsets

T-cell subsets were counted as previously (Chapter 9.2.2).
Proliferative response to soluble antigen

The ability of PBMC to proliferate in response to purified protein derivative (PPD) of M. Tuberculosis was measured (Chapter 9.1.2).

10.1.3 RESULTS

The ability to produce IL-2 was measured in 46 haemophiliacs. Median patient age was 30.5 years (interquartile range (IQR) 23-40 years), this was comparable to the control group. 45 patients had factor VIIIIC deficiency and only one patient had factor IX deficiency. Of these 32 had factor levels ≤5iu/dl, 11 had levels of between 5-10iu/dl and 2 had factor levels >10iu/dl.

Table 10.1.1 shows that HIV-1 antibody positive and negative patients were comparable for age, factor deficiency, and grade of liver disease. However, HIV-1 antibody patients had used significantly more clotting factor concentrate per annum (p=0.002). There was no correlation between patients age and IL-2 production (r=0.12, p=0.39).

HIV-1 status

Sixteen patients were HIV-1 antibody positive. Median duration of infection was 53 months (IQR: 43 - 60). Of these three had serum HIV-1 antigen. None had AIDS, one
Table 10.1.1 A comparision of patients in whom IL-2 levels were measured.

<table>
<thead>
<tr>
<th>HIV-1 Status</th>
<th>All</th>
<th>Antibody +ve</th>
<th>Antibody -ve</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of patients</td>
<td>46</td>
<td>16</td>
<td>30</td>
</tr>
<tr>
<td>Age (years)</td>
<td>30.5</td>
<td>27.5</td>
<td>31</td>
</tr>
<tr>
<td>(23-40)</td>
<td>(21-38.5)</td>
<td>(26-43)</td>
<td></td>
</tr>
<tr>
<td>Factor deficiency (iu/dl)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>(0-5)</td>
<td>(0-0)</td>
<td>(0-9)</td>
<td></td>
</tr>
<tr>
<td>Mean annual dose (iu/annum)</td>
<td>29084</td>
<td>73472.5</td>
<td>10324.5</td>
</tr>
<tr>
<td>(6605-75880)</td>
<td>(49282-101568)</td>
<td>(40000)</td>
<td></td>
</tr>
<tr>
<td>Grade of liver disease</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>(2-5)</td>
<td>(2-3)</td>
<td>(2-5)</td>
<td></td>
</tr>
</tbody>
</table>

Table 10.1.1 shows medians and interquartile ranges. HIV-1 antibody positive patients did not differ from antibody negative patients except in mean annual dose of clotting factor concentrate used, p=0.002.
patient had previous recurrent non-opportunistic bacterial infections but was infection free at the time of study, and one had intermittent thrombocytopenia which had not required treatment.

Neither HIV-1 antigen positive patients or symptomatic patients could be identified from the amount of IL-2 produced (Figure 10.1.1). There was no correlation with the duration of infection, \( r = -0.2 \) (\( p = 0.5 \)).

**IL-2 production:**

**Effect of HIV-1 infection.**

Figure 10.1.1 shows HIV-1 antibody positive patients produced less IL-2 than controls, (\( p = 0.002 \)) and seronegative patients (\( p = 0.04 \)). Seronegative patients also produced less IL-2 than controls (\( p = 0.05 \)). Twenty eight (61 percent) haemophiliacs had IL-2 levels below the lower quartile of the normal range (18.6iu/dl), of these 11 were HIV-1 antibody positive (69 percent of HIV-1 antibody positive patients studied) and 17 were HIV-1 antibody negative (57 percent of HIV-1 negative patients studied), (\( p = 0.225 \) Fishers Exact Test).

**Effect of liver disease:**

The capacity to produce IL-2 was not related to grade of liver disease for the whole group, (\( r = 0.007 \) \( p = 0.916 \)), however, in HIV-1 antibody positive patients there was a moderate correlation, (\( r = 0.53, \) \( p = 0.04 \)) and in HIV-1
FIGURE 10.1.1 INTERLEUKIN 2 PRODUCTION IN TREATED HAEMOPHILIACS
Figure 10.1.1. Seropositive haemophiliacs produced less IL-2 compared to controls (p<0.05) and HIV-1 -ve patients (p<0.05). HIV-1 -ve patients produced less IL-2 than controls (p<0.05).
negative patients there was a poor inverse correlation \(r=-0.24, \ p=0.2\).

The effect of mean annual dose of clotting factor concentrate used:
Mean annual dose of clotting factor concentrate used did not affect the amount of IL-2 produced, in HIV-1 antibody positive patients, \(r=0.22\) (\(p=0.38\)) and in HIV-1 antibody negative patients, \(r=0.22\) (\(p=0.23\)).

Mechanism of reduced IL-2 production:
A reduced in-vitro capacity to produce IL-2, could be due to (a) CD4 T-cell depletion, (b) a functional defect of CD4 \(^+\)ve T-cells as measured by stimulating lymphocytes with PPD (see chapter 9.3) or (c) an effect of T-suppressor /cytotoxic lymphocytes (CD8 \(^+\)ve; T-s).

In HIV-1 antibody positive patients IL-2 production showed a poor correlation with CD4\(^+\)ve T-cell count, \(r=0.3, \ p=0.2\), a moderate correlation with CD8\(^+\)ve T-cell count \(r=0.5, \ p=0.05\) and a high correlation with PPD response \(r=0.6, \ p=0.04\).

To determine relative contributions of each a stepwise variable regression equation was used, in which IL-2 level was the independent variable, and CD8\(^+\)ve T-cell count and PPD values weighted for number of CD4\(^+\)ve T-cells the dependent variables. PPD stimulation values remained significantly associated with IL-2 values, \(r=0.61, \ p=0.04\).
p=0.002). On both forward and backward analysis CD-8+ve count was not retained in the regression equation.

In HIV-1 antibody negative patients as a group there was no correlation between ability to produce IL-2 and CD4+ve, (r=0.09, p=0.6) CD8+ve cell (r=0.001, p=0.9) counts or PPD stimulated values (r=.02, p=.1). This suggested a functional defect in the capacity to produce IL-2, but no correlation was seen with PPD response (r=0.3, p=0.2).

10.1.4 DISCUSSION

The most interesting observation in the present study was that haemophiliacs had a reduced capacity to produce IL-2; in vitro this was independent of HIV-1 antibody status. The finding that IL-2 production was reduced in HIV-1 infection has been reported by others (515-519), in this study it was found that this abnormality occurs early in the course of infection and in the absence of significant clinical immunodeficiency.

It was observed that those HIV-1 antibody positive individuals with the severest grade of liver disease also had reduced levels of IL-2. This was not the case in HIV-1 antibody negative patients and suggests that either HIV-1 infection alters the course of NANB liver disease or the converse may occur. It is presumed that HIV-1 induced immunosuppression allows NANB hepatitis to proceed unchecked. In such individuals treatment with
either recombinant IL-2 or recombinant gamma interferon may be desirable. These agents have been shown to: (a) gamma interferon improves histological outcome of NANB liver disease (520, 521), (b) suppress HIV-1 replication in vitro and (498-501) (c) gamma interferon is superior to recombinant IL-2 in the management of AIDS patients (522).

Furthermore this study shows that HIV-1 antibody positive subjects with reduced IL-2 production had impaired response to PPD. This implied that either the CD4+ve lymphocyte subset that responds to antigen in vitro is selectively depleted or functionally deficient. This observation is in keeping with the studies of Lane et al (504).

The finding that IL-2 production was reduced in HIV-1 negative patients was surprising but in keeping with previous observations of a reduced in vivo response to a new antigen, Chapter 7.1. The mechanism is not clear. The absence of a correlation with CD4+ve T-cells suggested a functional defect. The mechanism is probably different from HIV-1 positive patients as no correlation was seen with PPD stimulation. An alternative explanation would be that recurrent contaminating alloantigenic load from treatment with clotting factor concentrates temporarily exhausts the capacity of lymphocytes to produce IL-2. To test this hypothesis "resting" cells in vitro prior to stimulation would be necessary.
Interestingly in systemic lupus erythematosus resting cells prior to stimulation restored their capacity to produce IL-2 (533).

In conclusion, haemophiliacs with HIV-1 infection in the absence of clinical immunodeficiency have a reduced capacity to produce IL-2. Prospective studies in this cohort will allow the clinical utility of this test to be evaluated. The reduced IL-2 production appears to be due to a qualitative and quantitative defect.
CHAPTER 11.1

SERUM IMMUNOGLOBULIN LEVELS IN HAEMOPHILIA.

SUMMARY

Study objectives: To compare serum immunoglobulin levels in HIV-1 antibody positive and negative haemophiliaics.

Study design: Open, prospective, longitudinal study.

Study population: A well defined cohort of treated haemophiliaics in whom severity of liver disease, mean annual dose of clotting factor concentrate used HIV-1 status was known.

Measurements: Serum IgA, IgG and IgM measured by laser nephelometry.

Results: In a stepwise variable selection analysis serum IgG showed a good correlation ($r = 0.77$) with HIV-1 antibody status and a good correlation ($r = -0.57$) with grade of liver disease and no correlation mean annual dose of clotting factor concentrate used.

Conclusions: Raised levels of serum IgG in haemophiliaics are associated with the presence of HIV-1 antibody; in the absence of antibody a progressive rise indicates severe liver disease.
11.1.1 INTRODUCTION

Severe haemophiliacs require regular treatment with clotting factor concentrates. The concentrates are derived from pooled plasma donations and contain a large contaminating alloantigen load. Sensitization to specific alloantigens results in auto-antibodies and raised levels of plasma immunoglobulins are a frequent finding (233, 245, 246). Hypergammaglobulinaemia could occur due to infections transmitted by clotting factor concentrates. For instance hypergammaglobulinaemia is a frequent finding during the course of HIV-1 infection (344). Chronic liver disease due to non-A, non-B hepatitis (NANB) also results in raised gammaglobulin levels (376).

Hypergammaglobulinaemia in haemophiliacs has been reported both in the absence and presence of HIV-1 infection (186, 192, 399, 524). Earlier studies attributed raised immunoglobulin levels to alloantigen stimulation, more recently Hay et al reported a correlation between serum IgG levels in patients who progressed to liver cirrhosis after chronic NANB hepatitis, the possibility that HIV-1 infection could have masked their results was considered but not excluded (192). Raised serum IgG levels have also been reported by others but the effect of HIV-1 infection, liver disease and replacement therapy were not independently considered (525-528). The aim of this prospective study was twofold: (i) to establish the
prevalence and pattern of hypergammaglobulinaemia; and (ii) to investigate whether raised levels correlate with clotting factor concentrate use, liver disease or HIV-1 infection.

11.1.2 PATIENTS AND METHODS

Patients
Venous blood obtained in 1981/2 and 1986/7 and serum stored at -20°C from haemophiliacs attending the West of Scotland Adult Haemophilia Centre were used. Testing of serum samples of patients was restricted to those who fulfilled the criteria for entry into a prospective study of immunodeficiency in haemophiliacs (see Chapter 2.2).

The diagnosis, mean annual dose of clotting factor concentrate used and severity of liver disease was established (see Chapter 2). HIV-1 antibody status all patients was known and stored sera tested to obtain an estimate of the duration of HIV-1 infection (Chapter 4.1). At the time of this study no patient had clinical immunodeficiency.

Immunoglobulin measurement
Serum levels of IgA, IgG and IgM isotypes were measured by laser nephelometry.

In samples with an elevated level above 10 percent of the normal laboratory range of any isotype, the clonality of the rise was established by protein electrophoresis.
11.1.3 RESULTS

Clinical

Liver disease

The grade of liver disease and manifestations of HIV-1 infection are reported in detail in Chapter 2.1 and 6.2 respectively.

HIV-1 Status

Figure 4.1.1 shows the cumulative annual incidence of HIV-1 antibody, only one patient had HIV-1 infection at the outset. No patient had an opportunistic infection during the study.

Serum Immunoglobulin

Immunoglobulin levels were measured in stored serum samples in 106 (80%) patients at the start of the study and in 1986/7 in 130 (98%) patients.

At the start of the study one patient had all three isotypes elevated above 10 percent of the laboratory upper limit of normal, in two patients both IgG and IgA were raised and in another patient IgG and IgM were elevated. As shown in Figure 11.1.1, 24 patients (23 percent) had one isotype raised, 22 of these patients had a raised serum IgG and one of each had raised IgA or IgM.
FIGURE 11.1.1 SERUM IMMUNOGLOBULIN IMMUNOGLOBULIN LEVELS AT THE START OF THE STUDY.
Figure 11.1.1. Only one patient was HIV-1 antibody positive at the start of the study. The most frequently elevated isotype was IgG.
In 1986/87 seven patients had all three isotypes elevated, Figure 11.1.2, of these two were HIV-1 antibody positive. Nine had two isotypes elevated, of these six were HIV-1 antibody positive and 26 had one isotype elevated, 8 patients had HIV-1 antibody. In three of 26 patients with a single raised isotype, IgM was elevated, two had HIV-1 antibody; the remainder (23 patients) had a raised IgG of whom six had HIV-1 antibody. The single HIV-1 negative patient with an isolated raised IgM had normal levels on retesting.

HIV-1 Status
Table 11.1.1 shows serum immunoglobulin levels in patients with and without HIV-1 antibody. Of HIV-1 antibody positive patients in 1986/87 only one had antibody in 1981. There was no significant difference in initial immunoglobulin levels between those who subsequently developed HIV-1 antibody and those who did not, Table 11.1.1.

At the end of the study seropositive patients had significantly higher IgG (p=0.0001) and IgM levels (p=0.0001) but serum IgA was similar, Table 11.1.1. IgG and IgM levels increased significantly in seropositive patients, levels did not significantly change in seronegative patients, Table 11.1.1.

Liver Disease
FIGURE 11.1.2 SERUM IMMUNOGLOBULIN AT THE END OF STUDY.
Figure 11.1.2. Serum levels did not differ between seropositive and seronegative patients for IgM but were significantly higher for IgG \( (p=0.003) \) and IgM \( (p=0.03) \).
Table 11.1.1 Serum immunoglobulin levels.

<table>
<thead>
<tr>
<th></th>
<th>HIV-1 STATUS</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+ve</td>
<td>-ve</td>
</tr>
<tr>
<td>IgG (pre)</td>
<td>14.8</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>(13.4-17.4)</td>
<td>(10.8-15.2)</td>
</tr>
<tr>
<td>IgG (post)</td>
<td>19.1</td>
<td>13.2</td>
</tr>
<tr>
<td></td>
<td>(14-25.2)</td>
<td>(11.2-15.4)</td>
</tr>
<tr>
<td>IgA (pre)</td>
<td>2.2</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>(1.8-2.8)</td>
<td>(1.5-2.6)</td>
</tr>
<tr>
<td>IgA (post)</td>
<td>1.9</td>
<td>1.4</td>
</tr>
<tr>
<td></td>
<td>(1.7-2.4)</td>
<td>(1-2.2)</td>
</tr>
<tr>
<td>IgM (pre)</td>
<td>1.3</td>
<td>1.3</td>
</tr>
<tr>
<td></td>
<td>(1.1-2.2)</td>
<td>(1-7)</td>
</tr>
<tr>
<td>IgM (post)</td>
<td>2.8</td>
<td>1.4</td>
</tr>
<tr>
<td></td>
<td>(1.9-3.2)</td>
<td>(1-2.2)</td>
</tr>
</tbody>
</table>

Serum IgG, IgA and IgM levels were similar in both groups at the start of the study. At the end of the study IgG and IgM were significantly higher in seropositive patients, there had been a significant increase in IgG (p=0.003) and IgM (p=0.03) but not IgA (p=0.9). There was no significant change in the seronegative group.
In all patients there was a moderate inverse correlation between grade of liver disease and serum IgG ($r=-0.38$, $p=0.001$), Figure 11.1.3 and poor inverse correlations with serum IgA ($r=-0.20$, $p=0.02$) and for IgM ($r=-0.22$, $p=0.01$).

**Treatment Use**

Mean annual dose of clotting factor concentrate used showed a moderate direct correlation with serum IgG level ($r=0.40$, $p=0.0001$), no correlation with serum IgA level ($r=0.05$, $p=0.05$) and there was poor correlation with IgM ($r=0.24$, $p=0.005$).

To determine the relative influence of HIV-1 status, severity of liver disease and mean annual dose of clotting factor concentrate used on serum immunoglobulin levels separate stepwise variable regression analysis were used for each isotype. Dependent variables were serum IgG, IgA or IgM level, the independent variables included grade of liver disease and mean annual dose of clotting factor concentrate used, and HIV-1 antibody status.

Serum IgG showed the best correlation for coefficient estimates with HIV-1 status ($r=0.77$) and a good correlation with severity of liver disease ($r=-0.58$) and a poor correlation with mean annual dose of treatment used ($r=0.22$). For serum IgA, only severity of liver disease was retained in the analysis, ($r=-0.23$), and for
FIGURE 11.1.3 GRADE OF LIVER DISEASE AND SERUM IgG LEVEL IN SERONEGATIVE HAEMOPHILIACS
Figure 11.1.3. A good correlation was seen between grade of liver disease and serum IgG, there was however considerable overlap between groups.
IgM only HIV-1 status was retained ($r=0.57$). All correlation coefficient values were significant ($p<0.05$).

11.1.4 DISCUSSION

In most Centres, the incidence of HIV-1 infection especially in severe haemophiliacs is high. It has therefore been difficult to separate effects of HIV-1 infection from NANB associated chronic liver disease and that due to alloantigens contained within blood products used for treatment. The low prevalence of HIV-1 infection, availability of accurate medical records documenting total amount of clotting factor concentrate used and biochemical as well as clinical data to assess severity of liver disease allowed relative contributions of each variable to be assessed in this study. Moreover, in this cohort no HIV-1 antibody positive patient had clinical immunodeficiency, it was therefore possible to examine effects of HIV-1 on the immune system in the absence of an immune response to an opportunistic infection.

In the present study it was found that the most frequently elevated serum immunoglobulin isotype is IgG. A raised serum IgG level occurred more frequently in seropositive patients (59 percent) that HIV-1 seronegative patients (23 percent) ($p=0.001$, Fishers Exact Test). Seropositive patients had significantly higher levels than seronegative haemophiliacs. The importance of the findings of this study lie in the
analysis of the relevant contributions of alloantigen stimulation, HIV-1 infection and chronic liver disease. It was found that HIV-1 infection had a greater influence on serum IgG levels than liver disease and that the effect of treatment is in predisposing to infections, rather than directly contributing to hypergammaglobulinaemia.

The finding of polyclonal increase in serum IgG and IgM in HIV-1 antibody positive haemophiliacs, indicates (a) in vivo activation of B cells, (b) impaired T-cell control of B cells or (c) subclinical infections evoking an antibody response. The frequent finding of autoantibodies in patients with HIV-1 infection and absence of clinical symptoms in the face of compromised cell mediated immune system makes the latter possibility unlikely (344). In previous studies (Chapter 10) it was found that T-helper cell function is compromised in HIV-1 infection, it is therefore unlikely that increased levels of immunoglobulin are due to excessive T-cell help. The possibility that there is direct B cell stimulation during HIV-1 infection is considered in Chapter 11.2.

It was also found that (Table 11.1.1) HIV-1 infection results in a progressive increase in serum IgG, whereas in the absence of HIV-1 infection despite levels being frequently elevated there is no significant statistical increase over five years. The findings in the present study further show that in the absence of HIV-1 infection
up to 20 percent of the raised serum IgG level is accounted for by liver disease; Hay et al have shown that those individuals with histological evidence of chronic hepatitis who progress to develop liver disease have higher serum IgG levels (192). However, it is unlikely that serum IgG levels are likely to be of additional clinical help in predicting liver disease, first, as shown in Figure 11.1.3, there is considerable overlap in actual levels in patients with different grades of liver disease and second, levels do not significantly change over five years observation.

In conclusion, the results of this study show that IgG hypergammaglobulinaemia occurs in up to 31 percent of treated haemophiliacs. The significance of raised levels in HIV-1 antibody positive patients is not clear, it is possible that despite this quantitative increase, there is a qualitative abnormality in secreted immunoglobulins. In HIV-1 antibody negative patients raised levels are not due to recurrent alloantigen stimulation of B cells due to use of clotting factor concentrates - a moderate correlation was seen with severity of liver disease.
CHAPTER 11.2

B CELL FUNCTION IN HAEMOPHILIA.

SUMMARY

Study objectives: To compare B cell activation in HIV-1 antibody positive and negative haemophiliacs.

Study design: Open, cross-sectional study.

Study population: 58 haemophiliacs selected on the basis of HIV-1 antibody status, liver disease severity and mean annual dose of clotting factor concentrate used.

Measurements: Spontaneous and stimulated production of IgG and IgM in vitro.

Results: HIV-1 infection was associated with a decreased resting B cell pool and increased partially and fully activated cells. In seronegative patients there was a shift to a greater proportion of partially activated B cells in patients with severe liver disease. The remainder had normal B cell proportions.

Conclusions: B cell abnormalities in HIV-1 infection occur early in the course of infection. Liver disease and not clotting factor concentrate treatment causes B cell abnormalities in the absence of HIV-1 infection.
11.2.1 INTRODUCTION
Cell mediated immunity in vivo is frequently impaired in treated haemophiliacs both in the absence and presence of human immunodeficiency virus-1 (HIV-1) infection (Chapter 7). In Chapter 11.1 it was found that serum immunoglobulin levels are frequently elevated in haemophiliacs. In patients with HIV-1 infection serum levels of IgG and IgM are higher than seronegative patients, moreover in these patients there is a progressive increase in these isotypes over five years. In seronegative patients raised levels of IgG occur less frequently, do not substantially increase and are indicative of chronic liver disease. These findings imply polyclonal activation of B cells.

In normal individuals, the circulating B cell pool contains B cells at different stages of activation. Fully differentiated B cells spontaneously secrete immunoglobulin, partially activated B cells, when provided with adequate T-cell help, can differentiate into antibody secreting cells and resting B cells can be induced by a number of T independent B cell stimuli to undergo proliferation and to produce immunoglobulin (531).

Previous studies in haemophiliacs showed that the number of fully differentiated and partially activated B cells are increased (525, 526). These findings were noted in both HIV-1 antibody positive and negative patients. No
account, however, was taken of the resting B cell pool. In seronegative patients these observations were attributed to the used of clotting factor concentrates and no account was taken of chronic liver disease (525, 526). Although it is possible that clotting factor concentrates may have influenced T cell control of B cell activation no association with mean annual dose of clotting factor concentrate used and serum IgG was found in Chapter 11.1.

In this study B cell function in both HIV-1 antibody positive and negative patients was investigated. The effects of liver disease and allogenic antigen stimulation due to clotting factor concentrate use were considered independently. Furthermore, the possibility that there is an intrinsic B cell defect in haemophiliac patients was studied.

11.2.2 PATIENTS AND METHODS
58 factor VIIIC and IX deficient haemophiliacs attending the West of Scotland Adult Haemophilia Centre who had been treated with a blood product since 1980 were studied. All patients were examined for clinical features of HIV-1 related disease (Chapter 6.1) and chronic liver disease (Chapter 2). The case notes were reviewed and the following additional information recorded:
1. Mean annual dose of clotting factor concentrate used.

2. Grade of liver disease based on the classification given in Chapter 2.

3. HIV-1 antibody status and duration of infection (see Chapter 4).

Heterosexual males with no known risk factors for HIV-1 infection matched for age were used as controls.

Cell Preparation and Culture Conditions:

Peripheral blood mononuclear cells (PBMC) were obtained by density centrifugation of heparinised venous blood and washed, as previously described (Chapter 3.1.2). PBMC were cultured in RPMI 1640 medium supplemented with L glutamine, gentamicin, penicillin (Chapter 3.1.2) and 10 percent heat inactivated fetal calf serum. Duplication cultures of $1 \times 10^6$ PBMC in a final volume of 1ml culture medium were incubated in 24 well flat bottomed plates (Costar, Cambridge, Massachusetts, USA) for 7 days with: pokeweed mitogens (PWM), final concentration 1:500 (Gibco, Paisley, UK); formalin inactivated Staph. Aureus Cowan strain A (SACA), final concentration $10^7$ bacteria/ml, or 1 ml of culture medium. Cultures were incubated at $37^\circ C$ in air enriched with 5 % CO$_2$.

Supernatants were harvested after 7 days and stored at -70°C till assayed for IgG and IgM using a solid phase enzyme linked immunosorbent assay (522). For PWM
stimulated cultures the sum of IgG and IgM was compared. For SACA stimulated cultures, only supernatant IgM was measured as protein A from SACA binds covalently with IgG and therefore gives falsely low values (532).

T cell subsets
T-cell subsets were enumerated as previously (Chapter 8.2.2) on the same venous blood sample.

11.2.3 RESULTS
Of the 56 patients studied 45 were factor VIIIC deficient and 11 had factor IX deficiency. 45 patients had factor levels ≤5iu/dl, 7 patients had factor levels of between 5-10iu/dl and 4 had factor levels >10iu/dl. The median age of the whole group was 27.5 years (Interquartile range (IQR) 21 to 38). Table 11.2.1 compares patients studied and those not included in the study.

HIV-1 Antibody Status:
14 patients (25 percent) were HIV-1 antibody positive. Median duration of HIV-1 infection was 53 months (IQR 43 to 60). At the time of this study no patient had AIDS or other clinical manifestations of HIV-1 infection. Table 11.2.2 compares HIV-1 antibody positive and negative patients.

Liver Disease:
Table 11.2.2  A comparison of HIV-1 antibody positive and negative patients studied.

<table>
<thead>
<tr>
<th>HIV-1 STATUS</th>
<th>+ve</th>
<th>-ve</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of patients</td>
<td>14</td>
<td>42</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>21</td>
<td>30</td>
<td>0.009</td>
</tr>
<tr>
<td>(18-27)</td>
<td>(23.40)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean annual dose of treatment (units/annum)</td>
<td>10324.5</td>
<td>74154</td>
<td>0.001</td>
</tr>
<tr>
<td>(1141-4390)</td>
<td>(5000-83000)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver disease severity</td>
<td>3</td>
<td>3</td>
<td>0.1</td>
</tr>
<tr>
<td>(3-5)</td>
<td>(2-5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD4+ve cells/mm³</td>
<td>423</td>
<td>650</td>
<td>0.04</td>
</tr>
<tr>
<td>(345-633)</td>
<td>(451-951)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD8+ve cells/mm³</td>
<td>548</td>
<td>355</td>
<td>0.02</td>
</tr>
<tr>
<td>(442-883)</td>
<td>(266-588)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The median and interquartile ranges are shown. Seropositive patients were younger, had fewer CD4+ve cells and more CD8+ve cells than seronegative patients.
35 patients (63 percent) had abnormal liver function tests during the study. Of these, six had grade I liver disease: 11 patients had grade II and 14 grade III liver disease. Four patients had acquired NANB infection in the five years prior to this study (Grade IV). Of the remaining patients with normal serum ALT, 13 patients had previous NANB infection but during the study serum ALT was always below the upper limit of laboratory normal (Grade V). Eight had grade VI liver disease.

Of patients with HIV-1 infection, one had grade I liver disease, five grade II and 10 grade III. One HIV-1 antibody positive patient acquired NANB, in the previous five years. Three patients had grade V liver disease.

Two patients were hepatitis B surface antigen carriers, both were HIV-1 antibody positive. There was no difference in the grade of liver disease between HIV-1 antibody positive patients and HIV-1 antibody negative patients (p=0.16), Table 11.2.2.

Replacement Therapy with Clotting Factor Concentrates:
Table 11.2.2 shows that HIV-1 antibody positive patients had used significantly more clotting factor concentrate per annum compared to seronegative patients (p<0.001).

There was a poor correlation between mean annual dose of clotting factor concentrate used and grade of liver disease (r=-0.09, p=0.5).
IN-VITRO B CELL FUNCTION

Spontaneous immunoglobulin production:
Table 11.2.3 and Figure 11.2.1 shows that HIV-1 antibody positive haemophiliacs secreted more immunoglobulin compared to controls (p<0.05) and HIV-1 antibody negative patients (p<0.05). In HIV-1 antibody positive spontaneous total immunoglobulin production showed a good inverse correlation with CD4+ve T-cell count, (r=-0.52, p=0.04), there was no correlation with CD8+ve T-cell count, (r=-0.2, p=0.14). HIV-1 antibody negative patients did not secrete more immunoglobulin than controls, Figure 11.2.3. In seronegative patients there was no correlation with CD4+ve T-cell count (r=0.05, r=0.4) or CD8+ve T-cell count (r=0.06, r=0.5).

Stimulated immunoglobulin production:
Pokeweed Mitogen Stimulation:
Table 11.2.3 and Figure 11.2.2 shows that PBMC from HIV-1 antibody positive haemophiliacs could not be further stimulated to produce more immunoglobulin (p=0.57), whereas the control group produced significantly more immunoglobulin on stimulation (p=0.001). PBMC from HIV-1 negative patients could be further stimulated to produce more immunoglobulin (p=0.001). Some HIV-1 antibody negative patients, however, failed to secrete more immunoglobulin, all had grade I liver disease, Table 11.2.4. HIV-1 antibody negative patients with all other
Table 11.2.3 Basal and PWM stimulated immunoglobulin levels.

<table>
<thead>
<tr>
<th>IMMUNOGLOBULIN PRODUCED (ng/ml)</th>
<th>Basal</th>
<th>PWM</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>140 (124-166.5)</td>
<td>495 (424-635)</td>
<td>0.001</td>
</tr>
<tr>
<td>HIV-1 +ve</td>
<td>272 (100-472)</td>
<td>362.5 (193-485)</td>
<td>0.57</td>
</tr>
<tr>
<td>HIV-1 -ve</td>
<td>132 (93-120)</td>
<td>430 (267-313)</td>
<td>0.001</td>
</tr>
</tbody>
</table>

Median and interquartile ranges are shown. Basal levels were significantly higher in HIV-1 antibody positive patients. PWM stimulated values did not differ between the three groups. However, HIV-1 antibody positive patients could not be further stimulated to produce immunoglobulin with PWM.
FIGURE 11.21 BASAL IMMUNOGLOBULIN PRODUCTION

HIV -1 Status

IgG IgM

Controls +ve -ve
Figure 11.2.1. PBMC from seropositive haemophiliacs had higher basal immunoglobulin production than controls. Seronegative patients did not differ from controls, see table 11.2.3.
Table 11.2.4  

**In vitro** B cell function in HIV-1 negative patients according to severity of liver disease.

<table>
<thead>
<tr>
<th>Grade of liver disease</th>
<th>Basal</th>
<th>PWM</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>172</td>
<td>480.5</td>
<td>0.22</td>
</tr>
<tr>
<td></td>
<td>(96-226)</td>
<td>(420-522)</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>103</td>
<td>249.5</td>
<td>0.01</td>
</tr>
<tr>
<td></td>
<td>(61-138.5)</td>
<td>(161-313.5)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>143.5</td>
<td>521.5</td>
<td>0.01</td>
</tr>
<tr>
<td></td>
<td>(95-257)</td>
<td>(398-622)</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>188</td>
<td>395</td>
<td>0.2</td>
</tr>
<tr>
<td></td>
<td>(136-267)</td>
<td>(308-440)</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>163</td>
<td>477</td>
<td>0.005</td>
</tr>
<tr>
<td></td>
<td>(101-281)</td>
<td>(451-525)</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>73</td>
<td>318</td>
<td>0.04</td>
</tr>
<tr>
<td></td>
<td>(39-185)</td>
<td>(238-470)</td>
<td></td>
</tr>
</tbody>
</table>

Median and interquartile ranges are shown. No significant differences were seen between the groups in basal or stimulated values (Kruskal Wallis analysis of variance). However, patients with severe liver disease failed to secrete more immunoglobulin on PWM stimulation.
Figure 11.2.2  PWM STIMULATED IMMUNOGLOBULIN PRODUCTION

Total Ig Production (ng/l)

HIV -1 Status

Controls    +ve    -ve
11.2.2. PBMC from seropositive haemophiliacs could not be further stimulated to produce immunoglobulin with PWM. There was however a significant increase in both controls and seronegative patients, see table 11.2.3.
categories of liver disease could be stimulated to produce more immunoglobulin, Table 11.2.4.

SACA stimulation:
Table 11.2.5 shows basal and SACA stimulated values. SACA stimulation did not result in a significant increase in IgM values in HIV-1 antibody positive patients. In HIV-1 negative patients values increased significantly, (p=0.05). This was also true for the control group, (p=0.04).

11.2.4 DISCUSSION
The results of the present study show marked B cell abnormalities in haemophiliacs. In patients with HIV-1 infection these include: in vivo B cell activation, an impaired response to the T dependent B cell activator, PWM and a failure to respond to SACA, a T independent B cell activator. Similar results were found in some HIV-1 antibody negative patients but response to SACA was normal.

The findings in this cohort of asymptomatic HIV-1 antibody positive patients are consistent with marked polyclonal B cell activation in vivo. These findings are similar to those reported in patients with AIDS in whom numbers of resting B cells are decreased and circulating pool of partially and fully activated B cells increased (534). Furthermore, it was found that there was an inverse correlation between amount of immunoglobulin
Table 11.2.5 Basal and SACA stimulated values.

<table>
<thead>
<tr>
<th></th>
<th>Basal (IGm)</th>
<th>SACA</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>30 (25-38)</td>
<td>180 (123-210)</td>
<td>0.04</td>
</tr>
<tr>
<td>HIV-1+ve</td>
<td>71 (40-108)</td>
<td>140 (107-200)</td>
<td>0.5</td>
</tr>
<tr>
<td>HIV-1-ve</td>
<td>38 (19-64)</td>
<td>73 (47-109)</td>
<td>0.05</td>
</tr>
</tbody>
</table>

Median and Interquartile ranges are shown. Basal IgM production was higher in HIV-1 positive patients. SACA stimulation values did not differ, however, on comparing paired basal and SACA stimulated values HIV-1 antibody positive patients failed to produce more IgM.
secreted spontaneously and degree of cellular immunodeficiency as assessed by measuring numbers of circulating CD4+ve T-cells. Taken together these findings would imply viral transformation or stimulation of B cells.

In this regard, in vitro studies with various HIV-1 isolates have shown that HIV-1 can result in polyclonal B cell activation to an extent that is comparable to that seen with standard B cell activators (535,536). Alternatively, reactivation of a prior, latent B cell transforming virus may explain these findings. Both Epstein-Barr virus and the newly described B cell tropic virus, human herpes virus 6, may have produced such changes (537 - 540). Infection with both viruses is common and both have been implicated in the pathogenesis of lymphomas which can occur during the course of HIV-1 infection (435-445). The results of this study also show that the in vivo B cell activation during HIV-1 infection occurs in the absence of clinical immunodeficiency and is not restricted to paediatric age groups.

The impaired response to PWM in HIV-1 antibody positive patients in the present study is in keeping with previous reports and with the marked T-helper cell depletion that occurs during HIV-1 infection (534). However in seropositive patients there was also an impaired response to SACA implying an additional primary B cell defect. Similar findings have been reported in patients with AIDS
In this study an impaired SACA response was observed in four of nine patients studied. Prospective studies may indicate whether such an abnormality is invariable or occurs in selected patients who may be predisposed to develop a lympho-proliferative neoplasm.

The findings in HIV-1 antibody negative patients are particularly noteworthy. B cell abnormalities were noted only in patients with severe liver disease. These included in vivo B cell activation and an impaired response to PWM. This could be due to (a) a defect in T-cells providing excessive help, (b) impaired T-cell suppression or (c) a primary abnormality of B cells. As shown in Table 11.2.5, haemophiliacs without HIV-1 antibody secreted more immunoglobulin in response to SACA, it is therefore considered unlikely that there is a primary B cell defect. In Chapter 8.2 it was shown that HIV-1 antibody negative haemophiliacs do not have reduced numbers of T-helper cells, but interleukin 2 production was reduced (Chapter 10). Taken together, these results would indicate that the increased immunoglobulin levels in vivo (Chapter 11.1) and increased spontaneous immunoglobulin production ex in vivo could be due to decreased T-cell suppression in patients with severe liver disease.

In a previous study it was shown that numbers of circulating T-suppressor cells in HIV-1 negative haemophiliacs were in the normal range and therefore
decreased suppression may be due to a functional defect in this subpopulation (469). Alternatively it may be due to reduced numbers or function of the T-helper cell subpopulation that induce T-suppressor cells (307 - 309).

In conclusion, the findings of this study show that polyclonal B cell stimulation in vivo is independent of HIV-1 status in haemophiliacs. In seropositive patients the present data implies that most circulating B cells are fully or partially activated. This occurs in the absence of clinical immunodeficiency but is inversely related to CD4+ve T-cell depletion. The major clinical implication of this finding is that serological tests to indicate secondary infections may be difficult to interpret. Furthermore, B cell clones secreting autoantibodies may emerge.

In the majority of HIV-1 antibody negative patients the circulating pool appears to consist of normal proportions of resting, activated and fully differentiated B cells. However, in patients with sever liver disease there is a shift to a greater proportion of partially activated B cells compared to resting B cells. This could be due to stimulation from gut associated antigens exposed by the abnormal portal circulation, alternatively there may be decreased T-cell suppression of B cells.
REFERENCES


12. Takahaski N, Ortel TL and Putnam FW. Single chain structure of human caeruoplasmin: The complete


35. Griffith MJ, Reisner HM, Lundblad RL and Roberts HL. Measurement of human factor IXa activity in


67. Lindquist PA, Fujikawa K and Davie EG. Activation of bovine factor IX (Christmas factor) by factor XIa (activated plasma thromboplastin antecedent)

68. Byrne R, Amphlett GW and Castellino FJ. Metal ion specificity of the conversion of bovine factors IX, IX alpha, and IX\textsubscript{a} alpha to bovine IX\textsubscript{a} beta. J Biol Chem 1980; 255: 1430-1433.


70. Tuszynski GP. Kinetics of the factor XI\textsubscript{a} catalyzed activation of human blood coagulation factor IX. J Clin Invest 1984; 73: 1392-1395.


72. Bajaj SP. Co-operative Ca\textsuperscript{2+} binding to human factor IX. Effects of Ca\textsuperscript{2+} on the kinetic parameters of the activation of factor IX by factor XI\textsubscript{a}. J Biol Chem 1982; 257: 4127-4130.


76. Amphlett GW, Kinel W, Castellino FJ. The interaction of Ca\textsuperscript{2+} with human factor IX. Archives of Biochem and Biophys 1981; 208: 576-585.


97. Tanimoto M, Kajima T, Kamya T et al. DNA analysis of seven patients with hemophilia B who have anti-factor IX antibodies: relationship to clinical manifestations and evidence that the abnormal gene was inherited. J Lab Clin Med 1988; 112: 307-313.


100. Lane S. Haemorrhagic diathesis - successful transfusion of blood. Lancet 1840; i: 185-188.


156. Aledort L. Personal communication.


181. Craske J, Spooner RJD and Vandervelde EM. Evidence in factor of the existence of at least


235. Shapiro SS. The immunologic character of acquired inhibitors of antihemophilic globulin (factor VIII) and the kinetics of their interaction with factor VIII. J Clin Invest 1967; 46: 147-156.


247. Kazatchkine MD, Sultan Y, Burton Kee EJ. Circulating immune complexes anti-VIII antibodies


301. Hutchison IV an Morris PJ. The role of major and minor histocompatible antigens in active enhancement of rat kidney allograft survival by
blood transfusion. Transplantation 1986; 41: 166-70.


345. Salahuddin SZ, Markham PD, Popovic M et al. Isolation of infectious human T-cell
leukemia/lymphotopic virus type III (HTLVIII) from patient sith acquired immunodeficiency syndrome (AIDS) or AIDS-related complex (ARC) and from healthy carriers: A study of risk groups and tissue sources. Proc Natl Acad Sci, USA 1985; 82: 5530-5534.


355. Mayer KH, Stoddard AM, McCusker J et al. Human lymphotropic virus iii in high risk, antibody


402. Ludlam CA. Personal Communication.


431. Center for Disease Control. Revision of case definition of acquired immune deficiency syndrome. MMWR 1987; 36: Suppl: 1S-15S.


440. Kalter SP, Riggs SA, Cabanillas F et al. Aggressive non-Hodgkin's lymphomas in


462. Lowe JG. Personal Communication.


470. Luban NLC, Kelleher JF, Reaman GH. Altered distribution of lymphocyte-T subpopulations in


480. Beddall AC, Alrubei K, Williams MD, Hill FGH, Martin C, Flower AJE. HTLV-III, hemophilia and
巨大人体での遺伝子映像の形成についての研究は、巨視的な形態学的な観察から、細胞の分化や機能に影響を及ぼすことが示唆されています。今後の研究で、遺伝子レベルでの理解が進むことが期待されます。


500. Hamer M and Gilli JM. Synergistic activity of granulocyte-macrophage colony stimulating factor


532. Kelly BS, Levy JG and Sikova L. The use of the enzyme linked immunosorbent assay (ELISA) for the detection and quantification of specific antibody from cell cultures. Immunology 1979; 37: 45.


Clinical and laboratory evidence of immunodeficiency has been frequently reported in haemophiliacs treated with clotting factor concentrates. Potential causes include:

1. The viruses transmitted by clotting factor concentrates
2. A direct chemical effect of clotting factor concentrates on the immune system
3. Alternatively immunodeficiency may arise due to the frequent extraneous alloantigens infused.

Impairment of cell mediated immunity can be due to:

1. Impaired T helper/inducer cell activity this may be due to quantitative or qualitative changes.
2. Excessive T suppressor/cytotoxic cell activity either quantitative or qualitative.
3. Impaired function of monocytes/macrophages

The aims of this thesis are:

1. To establish presence of in vivo immunodeficiency in haemophiliacs.
2. To determine influences of each of the potential causative factors.
3. To study the immune response and effects of HIV-1 on the immune system in haemophiliacs.

The aims were achieved by:

1. Assessing the in vitro effects of clotting factor concentrates on peripheral blood mononuclear cells from immuno-competent individuals.
2. Establishing the extent of exposure to HIV-1 and determining the risk factors for HIV-1 infection.
4. In chapter 6 the impact of HIV-1 infection in haemophilia was determined and evidence for clinical immunodeficiency was sought in seronegative haemophiliacs.
5. In vivo cell mediated immunity was measured in seropositive and negative haemophiliacs (Chapter 8)
6. Quantitative changes in the immunoregulatory ratio were measured (chapter 8).
7. Measurements of qualitative changes of lymphocyte
function were made and the effects of HIV-1 proteins on the immune system in vitro were established.

8 The capacity to produce the lymphokine interleukin 2 was measured and reported in chapter 10.

9 B cell function is partly controlled by T cells and therefore assessed.