DETECTION OF ANTIBODY RESPONSES TO INFECTION WITH HERPES SIMPLEX VIRUS AND HUMAN IMMUNODEFICIENCY VIRUS

by: PETER SIMMONDS

Department of Bacteriology
University of Edinburgh
Teviot Place

EDINBURGH, EH8 9AG

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ABSTRACT

Antibody responses to infection with herpes simplex virus (HSV) and human immunodeficiency virus type 1 (HIV) formed the core of the work described in this thesis. Several techniques, including some developed during the course of the work, were used to study the targets of antibody elicited by infection. In particular, sera from patients taken around the time of seroconversion for antibody to HSV and HIV were tested by a range of assays in order to make a critical comparison of their sensitivities and specificities.

Immunoblotting (IB) and radioimmunoprecipitation (RIP) assays were used to study sera from patients with primary genital HSV infection of both types. The tests proved to have different sensitivities in that the IB assay detected antibody to a tegument protein in the acute phase sera, although predominant reactivity with a herpesvirus glycoprotein, gB, was found in the RIP assay. Furthermore, antibody at an earlier stage of infection was found in this latter assay. The discrepancy was investigated by the development of new tests for antibody. The first was a non-denaturing version of the IB assay. Despite the methodological similarities between this test and the conventional IB assay, essentially similar results to the RIP assay were obtained by this new method. This underlines the importance of denaturation of antigen in the performance of such tests. However, it was the poor solubility of capsid proteins and possibly certain glycoproteins in the non-denaturing detergents used in the modified IB and RIP assays that prevented the detection of antibody to a wider range of proteins. A further test, that was not dependent on the solubilisation and separation of proteins for specificity, was developed. In this assay, sera competed with monoclonal antibodies for

the latter's target antigen. Applying this method to the seroconversion study, antibody to a wide range of proteins was found in the acute phase sera, including reactivity to several proteins not detected in either the conventional IB or in the RIP and modified IB methods.

The performance of the IB assay for antibody to HIV was compared with that of several commercially available enzyme immunoassays. Although the results from the different tests were substantially in agreement, in some patients, antibody at an earlier stage in the seroconversion was detected by the IB method. Serological assays were also used to investigate the course of disease in a cohort of haemophiliacs. These patients had received a single batch of HIV-contaminated clotting factor, and had become infected. Since the time of exposure was known, and frequent sera were taken from all of the patients for other reasons, it was possible to reconstruct the seroconversion events by retrospective testing. In particular, it was possible to measure the time to seroconversion, and to estimate the duration of the antigenaemia that accompanied the primary infection. Follow-up studies of the same patients were carried out with a view to correlate serological measurements, such as loss of antibody to core proteins, and reappearance of antigenaemia, with clinical progression to AIDS, or AIDS-related complex. Although all of the patients who are unwell have lost antibody to core proteins, and the majority are antigenaemic, there remain several patients with similar findings but who have remained well. Further studies are needed to establish whether there is a difference in prognosis between these patients and the other group of well patients who have high levels of antibody to core proteins and who are uniformly negative for serum antigen.

CONTENTS

TITLE PAGE i

ABSTRACT ii

CONTENTS iv

DECLARATION vii

PUBLICATIONS & POSTERS viii

PREFACE x

INTRODUCTION

CHAPTER 1 1

Introduction 1
Clinical features of HSV infection 2
Biology of HSV 7
Structure of the HSV genome 7
Morphology 10
The structural proteins of HSV 11
HSV glycoproteins B 13
C 15
D 18
E 19
G 22
H 24
Other glycoproteins 25
Process of infection 27
Virus attachment 28
Virus fusion 29
Intracellular events 30
Egress 31
Regulation of HSV replication 33
Genome structure 33
Classes of regulatory proteins 34
Immediate early genes 35
Delayed early genes 37
Late genes 39

CHAPTER 2 42

Pathology 42
Latency 43
Host responses to HSV infection 45
Non-specific host defences to HSV 46
Specific immunity to HSV 47

CHAPTER 2 (cont'd)

Immune responses to acute infection 48
Immunological responses and latency 52
Vaccine development 54
Type-specific serological assays 59

CHAPTER 3 61

Discovery of HIV 62
Clinical features of HIV infection 63
CDC Classification 63
Biology of HIV 69
Process of infection 71
Regulation of gene expression 73
HIV structural proteins 76
Reverse transcriptase 76
Capsid proteins 77
Envelope proteins 78
Virus attachment 79

CHAPTER 4 82

Pathogenesis of HIV infection 82
Markers of infection 82
Exposure and seroconversion 83
Asymptomatic infection 88
Persistent generalised lymphadenopathy 92
ARC and AIDS 93
Theories of pathogenesis 96
Resistance to the immune system 97
Theories of immunosuppression 103
Summary 105

CHAPTER 5 106

Preliminary work 106
Aims 108

MATERIALS 111

Herpes simplex virus 111
Human immunodeficiency virus 112

METHODS 113

Herpes simplex virus 113
Human immunodeficiency virus 119

DEVELOPMENT OF METHODS 121

ELISA for HSV antibody 121
MABIA 122
Immunofluorescence assay 127
Immunoblotting 128
Radioimmunoprecipitation assay 130
Non-denaturing immunoblotting 131
Immunoblotting (HIV) 136
ELISA for antibody to HIV 137
Quantification of "env" and "core" antibody 138

RESULTS 140

HSV study groups 140
Targets of antibody in convalescent sera 143
Seroconversion studies 146
HIV study groups 153
Confirmation of positive sera 153
Seroconversion studies 159
The haemophiliac cohort 162
Primary infection 164
Follow-up period 166

DISCUSSION 172

Antibody to HSV 172
Immunoblotting 172
Radioimmunoprecipitation assay 175
Non-denaturing immunoblotting 180
Comparison of the tests for HSV antibody 185
MABIA 189
Detection of antibody during seroconversion by MABIA 194
Summary 196
Future work 197
Human immunodeficiency virus 201
Targets of antibody in convalescent sera 201
Confirmation of positive sera by IB 208
Seroconversion studies 212
Follow-up period 221
Serological markers and clinical progression 231
Future work 236

ACKNOWLEDGEMENTS 237

REFERENCES 239

ABBREVIATIONS 267

DECLARATION

The results in the thesis and its composition are solely the work of the author.

PUBLICATIONS

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PREFACE

The aim of the work described in this thesis was to investigate serological responses to viral infection. Both herpes simplex virus (HSV) and human immunodeficiency virus (HIV) cause chronic infections and have developed different methods for evading the immune system. HSV exhibits the phenomenon of latency followed by periodic recrudescence. The site of latent infection by HSV is the cell body of sensory neurones. Latency of HSV is maintained by poorly understood regulatory mechanisms that suspend the rapid lytic replicative cycle of HSV observed *in vitro*,. During reactivation, HSV exploits the remarkable morphology of nervous tissue by moving from the cell body along the axon to infect the site of innervation of the neurone. Pre-existing immunity, either cell-mediated or humoral, is often unable to prevent localised infection of the skin and shedding of virus. Neither specific immunity nor drug treatment with antiviral agents affect HSV in its latent phase, and this makes medical treatment of HSV a major challenge.

The method by which HIV infects individuals and persists despite demonstrable immunological responses is not known. Even more importantly, it is not known how or why certain individuals progress to AIDS from the asymptomatic stage. The site of infection of HIV is not entirely clear; at present it seems probable that there is a reservoir of infection in macrophages. It has also been suggested that HIV may be able to exhibit latent infection in such cells. In the absence of synthesis of viral proteins for long periods, infected macrophages may avoid immunological destruction and the mobility of these cells could result in the wide dissemination of HIV infection throughout the body. It would appear unlikely that a vaccine could

prevent AIDS from developing in those already infected with the virus, although it is entirely possible that it could prevent primary infection. Drug treatment of HIV infection shares similar problems to that of HSV infection; the currently used drug of choice only affects HIV during active virus replication, and appears unable to eradicate infection permanently.

Understanding the pathogenesis of infection by these two viruses, and the relationship they have with the immune system of the infected host is important for immunisation and medical treatment. Study of this subject involves clinical, immunological and virological data to build up a picture of the relationship between the viruses and their host.

A review of this background data is presented in chapters 1 to 4. The two viruses have been considered separately, although there are many important parallels and differences between them to make a comparison interesting. The large amount of previous published work about these two viruses, in particular, has necessitated a long introduction that does no more than summarise many of the areas of current research into the two viruses. Chapters 1 and 3 describe the clinical features of infection with HSV and HIV respectively, and then proceed with virological aspects, including the structure and composition of the two viruses, their replicative cycle and their interaction with the cells thay infect. Chapter 2 considers the pathogenesis of HSV infection, and then reviews in detail the immune response to primary infection, during reactivation, and during experimental immunisation of experimental animals. Chapter 4 reviews the laboratory markers of HIV infection and then considers several theories for the causation of disease.

Chapter 5 outlines preliminary work leading up to the project and the aims of the proposed work. The section "Development of Methods" has been included since a significant proportion of the work was developmental. The "Results" section that follows covers the application of such tests to patient study groups.

CHAPTER 1

INTRODUCTION

HSV causes a spectrum of disease in humans, from clinically inapparent to fatal conditions. First exposure to HSV leads to primary infection, so called because it occurs in the absence of any specific immunity and may be diagnosed by demonstrating a rise in titre of HSV antibody. Following primary infection, HSV becomes latent in the central nervous system but may periodically reactivate and lead to shedding of infectious virus. Such reactivations may be clinically inapparent. However, localised inflammation and vesicle formation may occur. This symptomatic reactivation is called a recrudescence. It may be distinguished from a primary infection, which it may resemble clinically, by serology. In the case of recrudescences, there is generally no rise in antibody titre.

The term "herpes" was used originally to describe the cutaneous manifestations of several diseases, but later was restricted to those with vesicle formation. As knowledege advanced, firstly the poxviruses and secondly, varicella-zoster virus were distinguished and described separately. The detection of neutralising antibodies in patients with regular recrudescences of HSV caused some confusion historically, since no other viruses were, at that time, known to show the phenomenon of latency. The discovery of the site of latency of HSV in sensory ganglia was established on the basis of results of experimental inoculation of HSV into animals, in whom involvement of the central nervous system was more apparent.

For many years it had been noted that genital HSV isolates differed from those from oral lesions in cytopathic effect during *in vitro* passaging. Schneweis, (1962) first established that the virus recoverable from genital herpes infections was serologically distinct from the virus causing oral lesions. The latter was called HSV type 1 (HSV-1), while HSV causing genital infection was called type 2 (HSV-2). HSV-2 infection can occur despite previous exposure to HSV-1. Such an infection is called an initial infection, to distinguish it from primary HSV-2 infection that occurs without prior exposure, and which is generally more severe. The association between the site of isolation and virus type is not absolute, and, in particular, a proportion of genital infections are caused by HSV-1. The proportion varies between studies, and appears to be influenced by patient selection (Smith *et al.*, 1973; Barton *et al.*, 1982; Nahmias & Roizman, 1973).

CLINICAL FEATURES OF HSV INFECTION IN MAN

The primary infection may pass entirely unnoticed, or more commonly may produce relatively trivial symptoms, and rarely may cause severe disease and systemic involvement. The recurrent infection is usually trivial, except in immunosuppressed patients. The most frequent sites of primary HSV-1 infection are the mouth and lips. Single or crops of vesicles on the gums, tongue or buccal membranes are observed (gingivostomatitis). Severity has been shown to be partially dependent on age. As the prevalence of childhood HSV infection falls, increasing numbers of older children or young adults are affected and they tend to have more severe symptoms. Severe infection may begin with a sore throat, malaise, fever (occasionally rigors), extensive generalised

lymphadenopathy, especially of the neck and rarely splenomegaly. Extensive ulceration of the gums, hard palate, soft palate and lips follows. The lesions are exceptionally painful and severely affected patients have problems swallowing. Lesions generally resolve within 10-14 days untreated.

Recrudescent HSV-1 infection normally affects the lips, skin around the mouth and around the nose. Recurrent gingivostomatitis is rare. Approximately a third of patients with evidence of past primary infection suffer from regular recrudescences. These are often associated with prior stimuli such as sunlight, emotional upset or fever. Recrudescences may be preceded by prodromal symptoms such as tingling, itching or hyperaesthesia. Each episode tends to occur in the same site. The average duration of lesions is around 10 days, although there may be secondary infection with *Staphylococcus aureus* which prolongs the resolution period. Healing is normally complete with no scarring, since only the superficial layers of the epidermis are involved. The time between recrudescences tends to increase with age. In some patients, there may be trigeminal neuralgia, and in others, progressive hypoaesthesia may occur at the site of the recrudescence.

Herpetic whitlow is an infection of the fingers caused by HSV. It is an occupational hazard of dentists and nurses, and tends to occur in those without prior exposure to HSV. Infection rarely occurs through intact skin. Primary infection in patients with eczema may be very much more severe, particularly if they are on concurrent steroid treatment. Spread of the virus is not effectively checked and there may be generalised lesions, particularly where the eczema is severe. Systemic symptoms, such as generalised lymphadenopathy, fever, rigors,

splenomegaly and hepatomegaly occur to varying degrees. Recurrent infection may also lead to widespread lesions, but systemic symptoms are less severe or absent. Severe systemic illness due to HSV occurs in immunosuppressed patients, in whom common sites of infection are the lungs and the liver. HSV pneumonia resembles that of other "atypical" virus lung infections, with marked consolidation. Liver infection may lead to transient elevation of aspartate transaminase and alkaline phosphatase levels, but jaundice is rare. Systemic involvement is thought to occur to a lesser extent in many immunologically normal patients with primary infection.

Primary infections of the eye are normally unilateral, giving rise to a follicular conjunctivitis. There may be regional lymphadenopathy and associated swelling of the eye. Resolution is relatively rapid (3-5 days), although patients may be left with corneal opacities from epithelial thickening. Recrudescent lesions are modified by prior immunity to HSV and follicular lesions are rarely seen. Instead, there may be dendritic ulceration of the cornea. Steroid treatment exacerbates the severity of the lesions. Frequent recrudescence may lead to progressive blindness.

HSV-1 may rarely cause a severe form of encephalitis. It is normally associated with primary infection; a history of recrudescence makes the diagnosis less likely. Headaches, personality change, and possibly fits rapidly followed by neck rigidity, vomiting and neurological deficits are signs suggestive of HSV encephalitis. Cerebral oedema precludes the routine testing of cerebrospinal fluid, but when done, shows normal glucose, raised protein and increased leucocytes, features typical of viral menigitis and encephalitis. Diagnosis was a primary concern in the managment of patients with suspected HSV

encephalitis when treatment with cytotoxic drugs was all that was available. With the advent of Acyclovir, a relatively non-toxic drug with marked efficacy against HSV, a case could be made for blind Acyclovir treatment for all patients with suspected viral encephalitis. The drug is very safe and its rapid use may reduce the risks associated with delayed treatment while the diagnosis is established by virus isolation from brain biopsy. The current poor prognosis (45% survival) may be confidently expected to improve with Acyclovir treatment.

Genital infection is closely associated with HSV-2 rather than HSV-1. It is generally transmitted by sexual intercourse. In the male, there are cutaneous lesions on the glans, in the coronal sulcus and on the shaft of the penis. Primary infection is normally accompanied by systemic symptoms and lesions at other sites of the body. In the female, there is often extensive and painful infection of the labia and vagina. Central nervous system involvement is more common in HSV-2 infection and may manifest itself as a transient aseptic meningitis. This is a benign and self-limiting condition, diagnosed by findings of raised white cells in the CSF. HSV-2 has rarely been isolated from meninges or CSF in this condition.

Recrudescent genital infection with HSV-2 is common, although the frequency and severity declines with time. Recurrence of HSV-1 genital infections is generally considered to be less frequent than HSV-2. The severity of genital primary and recrudescent infections may be sufficient to warrant treatment with Acyclovir. HSV infection may be acquired by a neonate on vaginal delivery during maternal primary or recrudescent infection. The prognosis of neonatal HSV infection is extremely poor, with up to 70% mortality; neurological damage is found

in a proportion of the survivors. Neonates are commonly unable to prevent spread of HSV and succumb to generalised disease involving many organs of the body. Caesarian section is advocated for mothers at term with evidence of genital infection. Two factors make primary maternal infection more serious than recrudescence. Firstly, the lesions are generally more widespread and infectious during primary infection. Secondly, the neonate will have acquired HSV antibody from a mother with recrudescent disease, and there is evidence that such antibody may be protective.

Patients with impaired immunity are more susceptible to complications arising from HSV infection. In particular, patients on long term immunosuppressive treatment following heart, bone marrow and renal transplants, and patients with advanced AIDS appear to be particularly at risk from fatal, overwhelming HSV infection. Data on the stimuli that lead to recrudescence and the increased severity of disease in newborn and immunosuppressed patients point to a central role for immunity in the pathogenesis of HSV infection. Normal immune reponses appear to be necessary for the maintenance of latency, and recrudescence in a proportion of patients can be regarded as a transient failure to contain the disease.

BIOLOGY OF HSV

Herpesviruses have been isolated from a wide range of vertebrate and invertebrate species of animals, as well as from yeasts and fungi. There are at least six distinct human herpesviruses; In addition to the familiar HSV types 1 and 2, varicella-zoster virus (VZV), human cytomegalovirus (HCMV) and Epstein-Barr virus (EBV), human B-lymphotropic virus (HBLV) has been recently discovered (Salahuddin *et al.*, (1986) and partially characterised. (Josephs *et al.*, 1986). Features found in all herpesviruses are a linear double stranded genome consisting of DNA with molecular weights (MWs) ranging from 80 to 160 million daltons; an icosahedral nucleocapsid comprising 162 capsomeres; and a membranous envelope that is acquired on budding through the nuclear membrane of the infected cell. Herpesviruses, particularly human herpesviruses, are the most intensively studied of all virus groups. Reasons for this include the implication of certain herpesviruses in oncogenesis, the ease with which most herpesviruses may be isolated and passaged *in vitro* , the phenomenon of latency exhibited by many members of the group and finally the desire to prevent the wide spectrum of medical and veterinary illnesses that they cause.

STRUCTURE OF THE HERPES SIMPLEX VIRUS GENOME

HSV is a double stranded DNA virus of relatively large size and functional complexity. The genome of HSV is linear and has a total length of approximately 150 000 base pairs. HSV is classified as an alpha herpesvirus, the criteria being broad host range, short repoductive cycle, rapid spread in tissue culture, destruction of

infected cells and the establishment of latency in neurones in sensory ganglia (Matthews, 1982). Beta herpesviruses, typified by CMV, have a restricted host range *in vitro* , long reproductive cycle and syncytial formation. Gamma herpesviruses, whose prototype is EBV, are characterised by growth in lymphoblastoid cells, and a slow and sometimes arrested growth cycle.

The genome of HSV can be described as comprising two covalently linked segements, the long and short (figure 1). The ends of the two components are flanked by two types of repeated sequence (Hayward *et al.*, 1975; Delius & Clement, 1976). To distinguish the these regions, the following nomenclature has been adopted. The U_L and U_S regions are the unique regions of the long and short segements respectively. TR_L and IR_L refer to the terminal and inverted (internal) repeats found at the ends of the long segment, and TR_S and IR_S are those of the short segment. The *b* region is found in TR_L and IR_L at the two ends of the long segment; the sequence of bases being inverted relative to each other. Likewise the short segment is flanked by inverted repeats of the *c* regions. These two regions are coding; thus while most proteins have only one gene, those proteins coded for within the *b* or *c* regions have two gene copies (diploid). Beyond the *b* and *c* regions, at the extreme ends of the genome and in the joint region between the long and short regions is the *a* region. This is a non-coding, heterogeneous sequence of DNA which varies greatly between HSV-1 and HSV-2 and even between strains of HSV-1 (intertypic and intratypic variation (Wagner & Summers, 1978). The *a* sequences at the ends of the genome have the same orientation, while the *a* sequence in the joint region is inverted relative to the other two. The *a* sequence at the end of the long sequence may have a variable number of tandem repeats, as may the *a* region in the joint region.

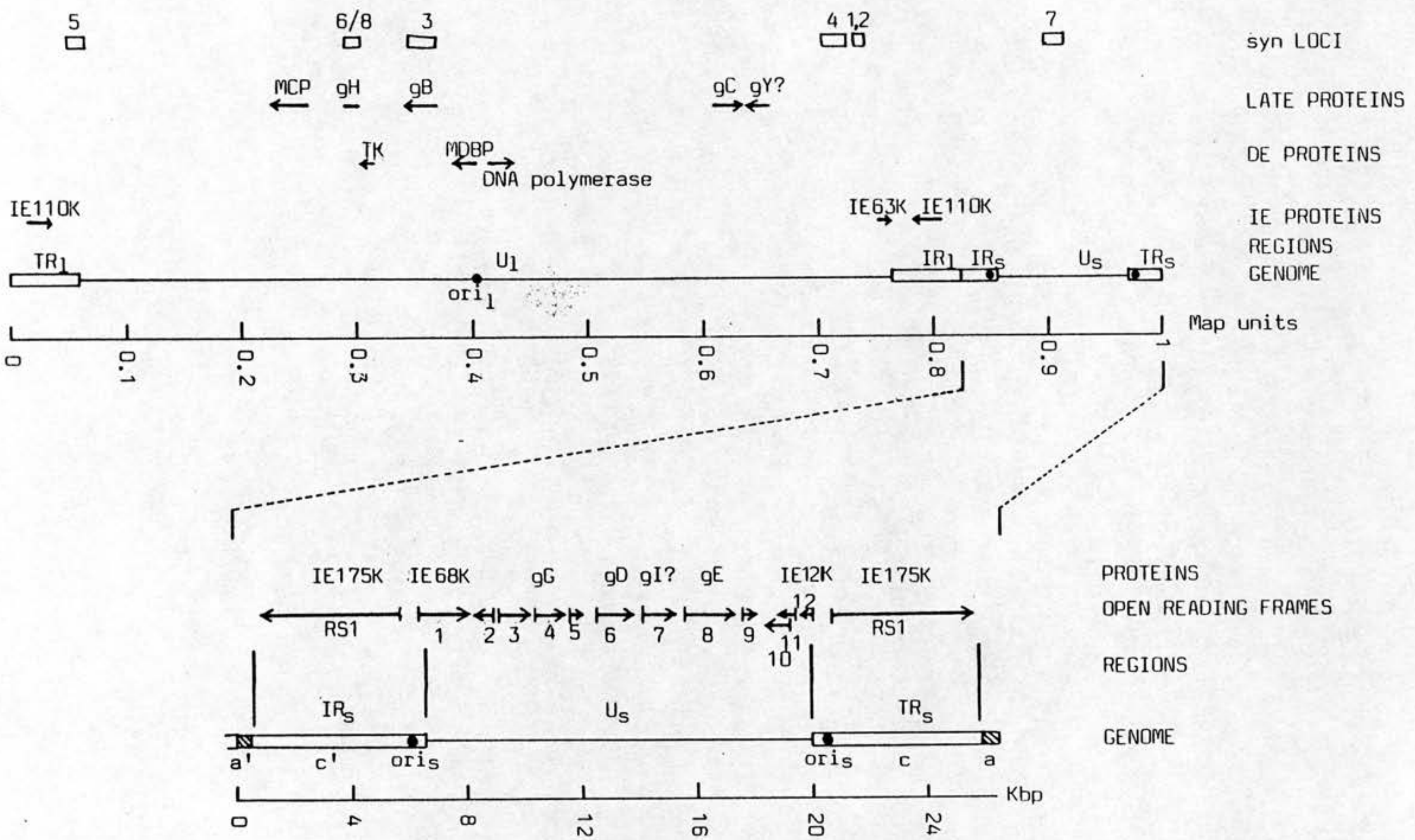

FIGURE 1: HSV-1 genome and location of genes. Upper diagram: Entire genome (after Wagner, (1984); Scaffer et al., (1984); Ruyechan et al. (1984)). Lower diagram: Short segment based on sequence data HSV-1 (McGeoch et al., 1986). See text for abbreviations.

This variation is strain dependent, although the number of copies of the *a* sequence in the two areas is not related. The ends of the *a* regions themselves have very short (20 base pair) inverted repeats.

Isomerisation is a term to describe the variable orientation of both U_L and U_S, the unique long and short regions. For each of the two orientations of the long segment, there are two possible orientations of the short segment, making 4 isomers altogether. There is currently no evidence that there is any biological preference for any of the possible isomers either *in vivo* or *in vitro* (Davison & Wilkie, 1983). Populations of wild type HSV appear to contain equal numbers of each of the four isomers. The length of restriction enzyme fragments that straddle the junction between unique and reiterated sequences depends on the orientation of the unique segments. This accounts for the previous observation of so-called half-molar and quarter-molar fragments on restriction enzyme analysis of HSV (Wilkie, 1976; Hayward *et al.*, 1975; Skare & Summers, 1977).

The significance of the internal repeats in the joint region is unclear. It is an evolutionarily conserved feature of many herpesviruses and is obviously involved at a fundamental level in the mechanism by which herpesviruses replicate. The *ab* and the *ac* regions undoubtedly provide sites for recombination, and this would provide a mechanism by which all four isomers could be produced. Whether recombination is responsible for isomerisation remains an open question. A notable feature of herpesviruses is the very wide range in the G+C content of the genomes; there is some correlation between G+C content and the alpha, beta and gamma groupings of herpesviruses. Typically, alpha herpesviruses such as HSV have avery high G+C content, while the gamma herpesviruses such as EBV have a low content,

around 35 to 50%. Furthermore, the repeated regions in the genome of HSV and other herpesviruses consistently have a 10-20% higher G+C content than the unique regions. The average G+C content of HSV-1 is 67% and HSV-2 is 69%, and the content in the repeated regions may be 85% or more. This is beyond the limit at which coding can be preserved by nucleotide substitution. The implications and possible reasons for this are discussed in Honess, (1984).

MORPHOLOGY

In its extracellular form, HSV has four distinct morphological elements. 1) an electron-opaque core, 2) an icosahedral capsid, 3) an amorphous tegument surrounding the core, and 4) a lipid envelope encircling all of the other elements. Viral DNA is found in the core, and is responsible for the electron-opacity. The DNA has a toroidal shape and surrounds a spindle of electron-dense material, probably protein (Furlong *et al.*, 1974). The diameter of the capsid is approximately 100 nm, and it has a precise geometric structure containing a fixed number of five- and six-sided elements arranged similarly to many virus groups (Wildy & Watson, 1963). The tegument is a poorly defined and variable structural feature of HSV. There appears to be fibrous material in this area which may be involved in attachment of the capsid to the outer envelope. The thickness of the tegument layer appears to be at least partially dependent on the cell line in which the virus is grown (McCombs *et al.*, 1971). The outer envelope consists of a typical cellular lipid bilayer, as it is acquired when the formed capsid buds through nuclear, cytoplasmic or vesicular membranes on egress from the cell. In common with cellular membranes, the envelope is sensitive to ether and detergents, a factor

that makes HSV very susceptible to inactivation experimentally (Spear & Roizman, 1972). Embedded in the envelope are a number of virally-coded glycoproteins that are the sites of antibody-mediated neutralisation and major antigenic determinants of the virus. HSV-1 and HSV-2, the two serotypes, are indistinguishable morphologically.

THE STRUCTURAL PROTEINS OF HSV

It has been variously estimated that the extracellular form of HSV has between 15 and 33 different polypeptides (reviewed in Spear & Roizman, 1980). Difficulties encountered when attempting to estimate the number arise from 1) the limited resolution that can be achieved by conventional methods of protein analysis; 2) different abundances of the various proteins so that minor species may be obscured; 3) contamination of purified virus by non-structural, or even cellular proteins and 4) the presence of precursor and mature forms of proteins with differing mobilities in the virion. The normal method of analysis of HSV is separation of proteins on a size basis by polyacrylamide gel electrophoresis (PAGE); resolution may be improved by 2-dimensional gel electrophoresis, where separation in the second dimension is by pKa on an ampholine gradient. Even more protein species may be detected when this method is used to analyse HSV. Partially glycosylated forms of glycoproteins are always present in HSV virions, and this causes some glycoproteins to appear as more than one band, thus complicating the analysis. There is surprisingly little knowledge about the composition of the capsid and the tegument, mainly because of their insolublility in detergents used to disaggregate the virus prior to analysis. The nomenclature for herpes proteins is based on three main systems. The first one is a numerical system in which the

bands that appear on PAGE analysis of HSV virions are numbered VP1 to VP24, with VP1 being the heaviest and VP24 being the lightest (Spear & Roizman, 1972). The second system is similar except that radiolabelled cell-lysate is used instead, in order that some of the non-structural proteins of HSV may be identified. The problems mentioned above have diminished the usefulness of both schemes. Newly discovered proteins needed a number and a suffix to distinguish them from their neighbours; proteins with precursor forms have been assigned more than one number; and finally, neither system gives information about homologies that exist between HSV-1 and -2 proteins. The third system, used for identifying glycoproteins, is very much more useful, since it attempts to give one alphabetic designation to each of the glycoproteins, with a prefix followed by the same letter for precursor forms. The same letters are used for homologous HSV-1 and -2 proteins, regardless of any difference in apparent MW between them. A policy of renaming glycoproteins has been adopted as precursor forms are identified (eg. gA to pgB) and as new homologies are detected (eg. gF to gC-2).

The capsid contains at least four proteins, of which the most abundant species is VP5, commonly called the major capsid protein (MCP) with about 800-1000 copies/capsid (Gibson & Roizman, 1972). Full HSV-1 capsids, ie. those containing DNA, appear to contain an extra protein, VP21 (Gibson & Roizman, 1974). An early report also described the association of another protein, variously described as VP22, ICP35, ICP37, ICP35 a-f, the 35 family, or p40, with full, but not empty capsids (Braun *et al.*, 1984). However, recent work has discounted this association, and localises p40 in the tegument and not the capsid (Rixon *et al.*, 1986). It is possible that there are several other tegument proteins, but they are normally so identified on the negative

grounds that they are found in purified virus, but not in capsid or in the envelope. Very little is known about the arrangement and function of these proteins, although presumably some may have roles in the attachment of the envelope to the capsid, in packaging of DNA and in release of viral DNA on infection of the cell (see below).

HSV GLYCOPROTEINS

The glycoproteins encoded by HSV are found in the viral envelope and have roles in the extracellular phase of the life cycle of HSV. In order to review the properties of the various glycoproteins, the following account will pay attention to the position of the gene encoding each glycoprotein in the HSV genome; its relation to other genes; the predicted size and structure of the translation product; glycosylation, and any properties that this may confer; homologies between equivalent HSV 1 and 2 glycoproteins and with other herpesviruses, and finally the functions attributable to each glycoprotein.

GLYCOPROTEIN B

Glycoprotein B is a major constituent of the viral envelope with a clearly defined role in attachment and penetration of the virus into the cell (see below). The gene encoding this protein is found in the U_L region of the genome at 0.37 map units. gB of both HSV 1 and HSV 2 has been sequenced (Bzik *et al.*, 1984; Bzik *et al.*, 1986); overall there is a high degree of nucleotide sequence homology (85%). Most of the changes are conservative, occuring at the third nucleotide of the

codon, and do not affect the final amino acid sequence (synonymous changes). The amino acid sequence can be predicted to produce a protein with typical internal, transmembrane and external portions. There are potentially 9 sites for N-linked oligosaccharide addition. Comparison of the outer portions of gB-1 and gB-2 indicates the presence of predominantly type-common potential antibody binding sites, although minor differences in secondary structure indicate the possibility of type-specific epitopes. The two proteins cross-react extensively in serological assays, as do the vast majority of gB monoclonals so far produced. Of all the herpes proteins, this is the most conserved, with extensive homologies with those of VZV and bovine mammilitis virus, both alpha viruses, and with more distant homology with equivalent proteins of HCMV and EBV (Davison & Scott, 1986; Pellett *et al.*, 1985). The genes for these homologous proteins lie in equivalent areas of the genome of the other herpesviruses. Furthermore, the proteins in other herpesviruses appear to play the same functional role in attachment and fusion. It is noteworthy that the two other glycoproteins encoded by the U_L region (gH-1 and gC) also have EBV and other herpesvirus homologies, while no homologies are found between EBV and any of the HSV glycoproteins encoded by the U_S region. It has been shown that antibody to HSV gB cross-reacts with those of equivalent proteins of other herpesviruses. This can be demonstrated unequivocally with bovine mammilitis virus, VZV and other alphaviruses, (Snowden & Halliburton, 1985; Snowden *et al.*, 1985) but the evidence for reactivity with EBV and HCMV is less convincing and requires confirmation (Balachandran *et al.*, 1987).

The predicted MW of gB from the sequence data is 100KDa. Experimentally, the MW is found to be around 118KDa, although there are several bands around this area corresponding to precursor

(intermediate stages of glycosylation) forms with slightly differing MWs (Spear, 1976). The apparent MW of gB in HSV infected ricin-resistant (hence lacking in O-linked glycosylation) CL6 cells is increased; this indicates that there are O-linked as well as N-linked oligosaccharides in this protein (Johnson & Spear, 1983). SDS-PAGE in milder, non-reducing conditions reveals the existence of higher MW forms of gB, probably the result of dimer formation (Snowden & Halliburton, 1985).

GLYCOPROTEIN C

Glycoprotein C of HSV 1 and 2 are encoded in the U_L region at position 0.63. Both genes have been sequenced and have been shown to share 64% nucleotide homology. (Frink *et al.*, 1983; Swain *et al.*, 1985). VZV has a homologous gene sequence, but with no recognised translation product (Davison & Scott, 1986). The predicted MWs of gC-1 and -2 are 55 and 51 KDa respectively. gC-1 has precursor forms of 85 and 110 KDa while the mature form of gC-1 is 130 KDa. The MW of gC-2 (formerly called gF) is 75KDa (via 66 and 68 KDa precursors; Spear, 1976). Endo-β-N-acetylglucosaminidase treatment of gC-2 removes all glycosylation, and results in a protein of MW 54 KDa, a good agreement with the sequence data. The large discrepancy between the predicted and observed MWs of gC-1 reflects its very high carbohydrate content, with at least 30KDa of sugars attached via 9 N-linked sites and extensive addition of O-linked oligosaccharides (Wenske & Courtney, 1983). gC-1 differs from gC-2 in having affinity for the *Helix pomatia* lectin (HPA), via an N-acetylgalactosamine residue (Olofsson *et al.*, 1981). HSV-1 grown in the presence of tunicamycin, which prevents N-linked glycosylation, produces gC with an apparent MW of

100KDa. This protein retains its binding ability with HPA but differs in antigenicity and other properties from the native form. It appears that gC-1 has two main antibody binding sites. Site I is found in a part of the protein with relatively little glycosylation, and is most likely to be of a peptide nature. Site II is found in the most heavily glycosylated part of the protein and is extremely sensitive to sialidase-periodate treatment, a process that removes terminal monosaccharides from oligosaccharides (Sjoeblom *et al.*, 1987). By a totally different method, (investigation of the sites of neutralisation of gC monoclonals), Marlin *et al.*, (1985) also found that there were two distinct antigenic sites.

There is little cross-reactivity serologically between gC-1 and gC-2, and this is probably because gC-2 lacks the immunodominant carbohydrate antigenic area of gC-1. gC-1 has been used as antigen in several type-specific assays in the past, although there was a degree of misunderstanding about the nature of gC-1 and its relationship to the glycoproteins of HSV-2. Suchanova *et al.*, (1984) used lectin from *helix pomatia* in columns to purify both HSV-1 and HSV-2 proteins with lectin binding properties. This proceedure purified gC-1 and was correctly identified. A HSV-2 protein of MW 130 KDa also showed an affinity for HPA and was assumed to be the HSV-2 counterpart of gC-1. It was originally called gC-2, whereas, in fact, the true counterpart did not bind to HPA and was then lurking under the name of gF. gC-1 and "gC-2" were used as antigens in a serological assay to determine type-specific antibodies in human sera in order to obtain epidemiological data about past exposure to the two viruses. "gC-2" was apparently type-specific while gC-1 showed some cross-reactivity. This data was very hard to interpret and the investigators concluded erroneously that HPA-purified gC-1 contained minor quantities of

contaminating cross-reactive proteins. Svennerholm *et al.*, (1984) used a similar assay system, and also found cross-reactivity. Attempts by other investigators to use gC-1 presumably failed for similar reasons. One feature of the carbohydrate antigen of gC-1 is that it is only present on the fully processed form of the protein. The antigenicity of immature gC-1 is presumably dominated by the site I area, which would, in view of the peptide sequence similarity, be expected to be partially cross-reactive. "gC-2" was later shown to be unrelated to gC-1 and was renamed gG. Unlike gC-1, this protein appears to be entirely type-specific, although a comparable reading frame was found in HSV-1 and a translation product (gG-1) subsequently identified (see below).

gC-1, but not gC-2 appears to have C3b binding activity (Friedman *et al.*, 1984), although the activity is not related to the higher carbohydrate content of gC-1. The functional significance of the activity is not known, although there has been extensive investigation of this and other possible roles of gC *in vitro* and *in vivo* . HSV deficient in gC production can be readily selected for by *in vitro* culture in the presence of gC-specific monoclonals (Marlin *et al.*, 1985). Such mutants may also arise spontaneously on extensive passaging. Deficient strains grow rapidly in culture, and several investigators have found that they have normal pathogenicity in experimental animals (Johnson *et al.*, 1986). The conclusion to be drawn from such work is that gC does not play an essential role in HSV infectivity. However, given that spontaneous mutants deficient in gC production may arise *in vitro* , it is a little surprising that no such mutants have ever been isolated from patients. Another criticsm of the above conclusion is that it is possible that the function of gC may be duplicated elsewhere in the genome. Sequence data has indicated

that there is a gene clearly homologous to that of gC immediately downstream in the U_L region which may have originated by tandem duplication (Marsden *et al.*, 1987). HSV may therefore be diploid for the function of gC.

GLYCOPROTEIN D

Glycoprotein D is produced by both HSV 1 and 2 and they are homologous in sequence, antigenicity and function. The overall nucleotide homology is 82% (Watson *et al.*, 1982; Watson, 1983), similar to that between gB-1 and -2. The gene for gD lies in the U_S region and has been called US6, a nomenclature based on numbering the open reading frames (ORFs) found on sequencing the U_S region of HSV 1 (McGeoch *et al.*, 1985). gD shows no homologies with proteins encoded by other herpesviruses. In view of the unrelatedness of the U_S of HSV and EBV and other non-neurotropic viruses, this is not surprising. However, the U_S of VZV, while largely colinear with that of HSV, has 6 rather than 12 ORF's, and US6 is one of the absent genes. The predicted MW of gD-1 and -2 is 43 KDa, and both proteins have three potential sites on the exterior part of the protein for N-linked glycosylation. The observed MW of gD of both serotypes is around 59 KDa (Spear, 1976), and both N-linked and O-linked glycosylation has been demonstrated (Johnson & Spear, 1983).

gD-1 and -2 cross-react serologically, although there is evidence for the presence type-specific as well as type-common epitopes. The protein has been shown to play an essential role in both the attachment and entry of the virus into the cell (see below). Antibodies to gD are readily neutralising, and this protein is

currently a strong candidate for a sub-unit vaccine against HSV infection. As a result, there have been numerous studies of its antigenic determinants, with distinctions made between conformational (resulting from tertiary and quaternary structure, and denaturation sensitive) and linear (secondary; denaturation resistant) epitopes (Eisenberg *et al.*, 1985b; Dietzschold *et al.*, 1984). Sites I, III, IV and VI are conformational epitopes which have been mapped to distinct areas of the external part of the protein. The localisation of this type of epitope was not exact, and there was evidence of cross-reactivity between these four epitopes. This suggests that the four areas are closely associated, despite the number of residues separating them. It is possible to build models of the quarternary structure of gD from this type of data. Several epitopes were linear; monoclonals reactive with these epitopes also reacted with synthetic oligopeptides of 10 to 20 amino acids in the same sequence. In contrast to conformational epitopes, linear epitopes were well localised. Single amino acid changes in a linear epitope abolished the reactivity of monoclonals. Where there was intertypic variation between gD-1 and gD-2 in a linear epitope, single amino acid substitutions could convert the epitope from HSV-1 to HSV-2 specificity and vice versa (Dietzschold, 1984).

GLYCOPROTEIN E

This protein is encoded by the U_S region. The HSV-1 gene has been identified as US8 and would produce a protein of 59 KDa on translation (McGeoch *et al.*, 1985). The protein would be predicted to have a typical membrane protein structure and two sites of N-linked glycosylation on the external portion of the protein. Sequence data

from the HSV-2 equivalent is not available although a homologous protein has been previously reported (Cassai *et al.*, 1975). There is sequence homology between US8 and an equivalent VZV gene potentially encoding a hitherto undetected 70 KDa protein (Davison & Scott, 1986). Like the other HSV glycoproteins, gE is processed extensively after translation. In addition to N- and O- linked glycosylation, there is evidence for extensive sulphation. gE differs from the other HSV glycoproteins in also being modified by the addition of fatty acids, as shown by pulse labelling with tritiated palmitate, a fatty acid residue. The modification was found to take place in the golgi apparatus and occured after N-linked glycosylation but before O-linked glycosylation (Johnson & Spear, 1983). This is further evidence for the multi-stage modifications of HSV glycoproteins; this has consequences for theories of egress of the HSV virion from the cell.

gE of HSV-1 has been found to have affinity for the Fc portion of immunoglobulin G (IgG; Baucke & Spear, 1979). gE was shown to be unnecessary for HSV growth in tissue culture, since a mutant that failed to produce several proteins, including gE was able to grow in a variety of cell lines (Longnecker & Roizman, 1986). This experiment is unlikely to have any relevance for the *in vivo* role of gE. If Fc binding is the role of gE, than *in vitro* passaging in the absence of antibody is unlikely to demonstrate it. gE has been shown to play some role in virus attachment to cells (see below). Baucke & Spear (1979) used IgG columns to purify gE from a detergent lysate of radiolabelled HSV-1 proteins prior to analysis. However, a recent report has shown that there is a second HSV protein with Fc binding properties. This protein could co-purify with gE-1 (Johnson & Feenstra, 1987), so the original studies may have to be reinterpreted. Notwithstanding this, the original report identified three distinct polypetides of MWs in

the range from 65 to 80 KDa. Partial proteolysis of these fragments indicated to the investigators that the three species were related, and it was assumed that the extra bands were precursors, yet with Fc binding activity. Johnson & Feenstra, (1987) showed that one of the bands (of MW 70 KDa) had distinct partial proteolysis profiles from the other two, and presented evidence that the so-named g70 complexed with gE-1 and that both gE-1 and g70 were required for Fc binding. No HSV-2 counterpart for g70 was found in these experiments, and it is possible that gE-2 may bind to Fc without the need to be complexed to another protein.

The gene for g70 was mapped in the U_S, close to the gene for gE-1 by intratypic recombinants. However this data is preliminary, and it is not impossible that g70 may in fact prove to be a cellular protein. g70 was shown to have an unglycosylated MW of 56 KDa. Looking at the U_S sequence of HSV-1, there are two ORF'S encoding what would appear to be so far undiscovered glycoproteins. US5 encodes a protein of 9K, with a transmembrane region and one external site of glycosylation. The authors have reservations about the existence of this gene, and it is too small to correspond to g70. US7 potentially encodes a protein of MW 41 KDa in its unprocessed form. Lee *et al.*, (1982) transcribed an mRNA from this gene and translated it *in vitro* (non-glycosylated) to a 55 KDa protein. This measurement was made by SDS-PAGE and the discrepancy could be attributed to its high proline content. It is therefore entirely possible that US7 does encode g70. The US7 gene has no homologies with any other HSV-1 gene in the U_S segment, thus discounting the possibility that it represents a tandem repeat of the gE gene, hence encoding a protein with similar properties to gE. There is clear homology, however, between US7 and a VZV gene encoding a hitherto undescribed 39 KDa protein. A report in press (Longnecker *et*

al., 1987) will present data on the characterisation of the gene product of US7, that they have named gI. Whether it is the same as g70 or not should shortly be revealed.

GLYCOPROTEIN G

Glycoprotein G of HSV-1 and HSV-2 are distinct proteins with very limited homology between them. Until the availabililty of sequence data there was no indication that they were related to each other, since they are of different size and there is no detectable serological cross-reactivity between them. gG-2 has a mature MW of 130 KDa in certain gel systems and was originally been named gC-2 (see above) on this basis (Spear, 1976). The gene for gG-2 lies in the U_S region and encodes a protein of MW 72 KDa in its unprocessed form (McGeoch *et al.*, 1987). There is no VZV or EBV counterpart to this protein. US4 in the U_S region of HSV-1 occupies an analogous place to the gene for gG-2, but encodes a much shorter protein, with homology confined to the internal carboxy terminus (McGeoch *et al.*, 1985).

There are only 4 sites for N-linked glycosylation on the external part of gG-2, but there is much evidence for extensive O-linkage. Serafini-Cessi *et al.*, (1985) showed gG-2 to have more O-linked oligosaccharides than any other HSV specified glycoprotein, including gC-1. As may be predicted, gG-2 has affinity for lectin from *helix pomatia* . gG-2 differs from other proteins in other aspects of its post-translational modifications. Several investigators have detected a proteolytic cleavage step on processing (Balachandran & Hutt-Fletcher, 1985; Su *et al.*, 1987) The most recent paper traced the processing of gG-2 from initial translation through a 104 KDa high

mannose intermediate form processed on the rough endoplasmic reticulum. Following this, the protein was cleaved into two fragment of size 31 and 72 KDa. Further processing by addition of O-linked oligosaccharides in the golgi apparatus converted the two fragments to proteins of 34 and 108 KDa respectively. The larger fragment is derived from the carboxy terminus and is incorporated into the mature virion. The smaller fragment is water soluble and apparently secreted into the tissue culture medium.

The mature form of gG-2 migrates anomalously on SDS-PAGE due to its high glycosylation. The use of gG-2 specific monoclonals led to agreement that the g92K protein of Marsden *et al.*, 1984, the 124K protein, named gG, of Roizman *et al.*, (1984) and the lectin binding 130 KDa protein of Suchanova *et al.*, (1984), Svennerholm *et al.*, (1984) and Olofsson *et al.*, (1986) were one and the same protein.

gG-1 remained undiscovered until the advent of specific monoclonals and sequence data of the U_S region, since it co-migrates with gD-1. Richman *et al.*, (1986) described the properties of an HSV-1 protein reactive with monoclonal antibody LP10. Although originally thought to be gD-1, the protein was found to label less efficiently with methionine than gD, to be less sensitive to tunicamycin and to map to a separate place in the U_S region. Frame *et al.*, (1986) produced an oligopeptide corresponding to a region of the US4 gene and used it to immunise a rabbit. The antiserum reacted with 56, 48 and 37 KDa glycosylated proteins of HSV-1. An antiserum prepared from an oligopeptide (McGeoch *et al.*, 1987), corresponding to an area of homology between US4 and gG-2 genes, was found to be reactive not only with gG-2, but also with the same HSV-1 glycoprotein as described by Frame *et al.*, (1986). It was then possible to name the

product of US4 as gG-1. US4 is much shorter than the gene for gG-2, encoding a protein of only 25 KDa. The external part of the protein has three N-linked glycosylation sites, and there is no evidence that it has affinity for *helix pomatia* lectin. Comparison of the US4 gene with the neighbouring US6 gene encoding gD-1, has shown some sequence homology, and it is postulated that the two genes may have arisen by tandem duplication. No data has yet been published on possible homologies between gG-2 and gD-2. There are no VZV or EBV homologues to gG.

No role has been ascribed to either gG-1 or gG-2, although data on pathogenicity in animals or man of gG deficient mutants is lacking. On a speculative note, it is interesting that both HSV-1 and -2 have proteins with extensive O-linked glycosylation and affinity for certain lectins. Since different proteins are involved, it is possible that there has been convergent evolution towards some essential function conferred by this type of processing. Theories about the origin of the two distinct types of HSV predict that there might be a selective advantage for HSV-2 to develop a type-specific immunodominant protein to circumvent pre-existing immunity to reinfection by HSV-1.

GLYCOPROTEIN H

This recently discovered protein has so far only been found in HSV-1. It is present in relatively smaller amounts than the other glycoproteins and required the use of a specific monoclonal for its detection. Showalter *et al.*, (1981) first described a 110 KDa protein, distinct from other HSV proteins reactive with monoclonal

52D. Buckmaster *et al.*, (1984) described a similar protein reactive with LP11. Subsequently the gene encoding the proteins has been found at 0.3 map units in the U_L region of the HSV-1 genome (McGeoch & Davidson, 1986; Gompels & Minson, 1986). The gene codes for a protein of MW 90 KDa with a predicted high arginine and proline content, 7 potential N-linked glycosylation sites, typical transmembrane hydrophobic areas and external hydrophilic loops corresponding to antibody binding domains. Little is known about its post-translational processing. The gene has homologies with EBV and VZV genes, in both cases situated in corresponding positions in the U_L segment and in the same relationship to neighbouring thymidine kinase genes.

Monoclonals reactive with gH-1 have been shown to have neutralising activity and to be able to block attachment of HSV to cell membranes. Whereas LP11 (anti-gH-1) inhibits plaque formation when added to tissue culture cells infected with HSV, neutralising monoclonal antibodies aginst gB and gD do not do so to the same extent. On this basis, Buckmaster *et al.*, 1984 and Gompels & Minson (1986) have postulated a role for gH-1 in maturation and exit functions (see below).

OTHER GLYCOPROTEINS

gY is a further HSV glycoprotein, but it is not readily detectable by conventional methods of protein analysis. Some evidence for the existence of gY was presented in work described by Palfreyman *et al.*, (1983), that used 2-dimensional electrophoresis to identify a glycoprotein that comigrated with gC-1 on the basis of molecular weight, but could be differentiated from it on the basis of charge.

This paper also showed the existence of several proteins of MW 50-65 KDa that also were not identified. Marsden *et al.*, (1987), in a report to be published, will present data that maps gY to a similar place in the genome as gC-1.

In the search for further HSV glycoproteins in the U_L region of the genome, the sequence data from VZV (Davison & Scott, 1987) may be of some use in view of the extensive homologies between the two viruses. It appears that the prototype orientation of the published VZV sequence is opposite to the conventional orientation used in illustrations of the genomic arrangement of HSV (including figure 1). Surprisingly, a total of 7 potential glycoproteins are found in the U_L region of VZV (UL 5, 14, 15, 31, 37, 39 and 50). Three have clear homologies: gB with UL31; gH-1 with UL37 and gC with UL14; the rest are uncharacterised in both VZV and HSV. It is possible that, after extensive rearrangement of the genome, some of the missing U_S genes encoding VZV glycoproteins have been relocated in the U_L region. However, a gene adjacent to UL15 (homologous to gC-1) in the VZV genome (UL16) codes for a glycoprotein. Perhaps this is the homologue of gY, in view of the recombination data of Marsden *et al.*, (1987) suggesting the close proximity of the two genes.

A broad area of HSV research, not discussed previously, concerns the mapping of genes responsible for the syncytial phenotype *in vitro*. On the assumption that mutants defective in normal glycoprotein production may induce syncytium formation, mapping of the defects may help to localise further glycoproteins. There are 8 loci involved in the syncytial phenotype (reviewed by Spear, 1985; see figure 1). Loci 3, 7 and 6/8 map within the genes encoding gB, gD and gH-1 respectively. Locus 1 maps within a gene encoding what would appear to

be a glycoprotein, with typical internal and external hydrophilic regions and a hydrophobic anchor region (Debroy *et al.*, 1985). Interestingly, this gene has a homologue in the VZV genome (UL5), also predicted to encode a glycoprotein, but so far uncharacterised. gC-1 deficient mutants commonly induce syncytium formation but the critical site of the mutation appears to lie to one side of the gC-1 gene (Honess *et al.*, 1980). This is further evidence that localises the position of gY.

There is recent evidence for intermolecular associations between the glycoproteins. By electronmicroscopy after immunogold labelling of the different glycoproteins, gB was found to be the main component of long thick spikes protruding from the envelope (Stannard *et al.*, 1987). It is probable that these are the spikes observed previously by conventional staining methods (Wildy & Watson, 1963). gC was associated with thinner shorter spikes. Future studies that link this structural observation with individual functions could provide information about the attachment of HSV to cellular membranes.

PROCESS OF INFECTION

Much of the evidence in the following section comes comes from ultramicroscopic observations of cultured cells infected with HSV *in vitro* . Typically, cells are fixed, stained, and examined by electron-microscopy. As well as introducing a number of possible artefacts, the method is also open to the criticism that the infected cells may not parallel the infection *in vivo* . Caution is required in generalising EM findings to the *in vivo* situation.

VIRUS ATTACHMENT

Most evidence points to a role for glycoproteins and possibly a non-glycosylated protein in virus absorption (Johnson *et al.*, 1984). In contrast to many viruses, including the beta and gamma herpesviruses, HSV has a very broad host range, and can productively infect a wide range of mammalian cells. This observation indicates that if there were a cellular receptor for HSV, it would need to be ubiquitous, and highly conserved between species. Virus absorption has been shown to be blocked by high concentrations of heparin (Hochberg & Becker, 1968), but little else is known about the cellular receptor for HSV. HSV absorption is not affected by inhibitors of phagocytosis, and EM evidence also points to direct fusion of the viral envelope with the cytoplasmic membrane (Morgan *et al.*, 1968). gE has readily detectable Fc binding activity; such binding is demonstrable on the cytoplasmic membrane after infection, indicating fusion of the viral envelope to the outer membrane of the cell (Para *et al.*, 1980) The latter two lines of evidence came from experiments that used high multiplicities of infection (moi's); this does not reflect the natural course of infection in humans so the data should be interpreted with caution.

Sarmiento *et al.*, (1979) proposed a role for gB in virus attachment by observing the properties of virosomes, artificial lipid membranes into which HSV-encoded membrane proteins have been inserted. Virosomes containing gB fused more readily with uninfected cell membranes than those without. Antibody to gB reduced the avidity with which virosomes bound to cell membranes (Johnson *et al.*, 1984). The experiments with virosomes also provided evidence for the role in viral attachment of VP14, a 78KDa protein, found in the membrane but not glycosylated.

Fuller and Spear (1985) investigated the blocking by antibody of attachment of radiolabelled HSV particles to cell membranes. Briefly, all polyclonal and some monoclonal antibodies to gB and gD prevented attachment as did high concentrations of Fc fractions of human IgG. This latter observation indicates a role for gE in attachment, since this glycoprotein has Fc receptor binding properties. The ability of the monoclonals to neutralise HSV was also measured. This did not correlate with the ability of the monoclonals to prevent attachment and the authors proposed that antibody may interfere with infection of the cell after the attachment process. The authors also proposed that the three implicated glycoproteins, and possibly others made up a viral attachment structure. The recent EM studies of HSV envelopes showing different glycoproteins associated with distinct morphological entities (Stannard *et al.*, 1987) does not concur with this hypothesis (see above).

VIRUS FUSION

Certain monoclonals to gD neutralise HSV but do not prevent attachment to uninfected cells. It is possible that gD plays a role in the next stage of the infection of the cell, that is penetration of the cellular membrane by fusion (Fuller & Spear, 1985). Temperature sensitive (*ts*) mutants deficient in gB production have also been shown to be unable to penetrate the cell membrane although they can attach to the cell (Sarmiento *et al.*, 1979; Little *et al.*, 1981).

INTRACELLULAR EVENTS

Cytoskeletal elements draw the capsid towards the nucleus. Capsids accumulate at nuclear pores and the viral DNA appears to be actively secreted into the nucleus by a component of the capsid or tegument (Batterson *et al.*, 1983). Changes in the appearance of the nucleus begin very soon after the release of viral DNA. The nucleolus, the site of ribosome synthesis enlarges and eventually disaggregates. Host chromosomes become marginated and later may break up. The nucleus eventually becomes distorted and invaginated to form reduplicated membranes. The first microscopic evidence of virus replication can be seen at about 7 to 9 hours after infection. Capsids self-assemble in the nucleus after synthesis of the component proteins in the cytoplasm. Viral DNA in synthesised in multi-unit lengths, and cleaved immediately before encapsidation. There is evidence for the involvement of p40 (VP22, ICP 35), a phosphorylated structural protein in this process. The original work suggested that this protein was a component of full but not empty capsids; furthermore, in the process it is modified to a form with a different elctrophoretic mobility (VP22a; Braun *et al.*, 1984). This work has been widely quoted, but more recent evidence with monoclonal antibodies has not confirmed these findings (Rixon *et al.*, 1986). The protein is phosphorylated and exists in multiple forms as determined by both one and two dimensional electrophoresis. It therefore has the attributes of a DNA binding protein, yet is unequivocally present in preparations of purified virus.

EGRESS

The route HSV takes on egress from the cell is disputed, even in the *in vitro* studies. There are at least three theories of egress. The first theory, (Morgan *et al.*, 1959) proposes that the capsid buds through the internal nuclear membrane, but then fuses with the extrnal membrane and enters the cytoplasm, losing its envelope. On reaching the external cytoplasmic membrane, HSV-encoded membrane proteins accumulate in areas through which the capsid buds. This theory accounts for the observation of large amounts of glycoprotein on the surface of infected cells, but does not account for the observation of glycoproteins present on the nuclear membrane. It is, of course, possible that there is inappropiate routing of glycoproteins, particularly in a damaged cell. This theory suffers from two problems. Firstly, it would seem unlikely that the viral envelope would be entirely devoid of cytoplasmic membrane proteins, and secondly, HSV has not been observed budding through the external nuclear membrane.

Schwartz & Roizman, (1969) postulated the formation of microtubules connecting the perinuclear space with the extracellular fluid. This entails the synthesis and post-translational processing of glycoproteins to take place before migration to the nucleus. While this is the simplest model of egress, it has certain problems. It predicts that the eventual viral envelope will be derived from the nuclear membrane, albeit modified by virally coded proteins. It seems unlikely, however, that all membrane proteins of cellular origin could be excluded during this modification process. However, to date, no cellular proteins have been found in the envelope of HSV or any of the other human herpesviruses, except as contaminants in the virus purification process. Another problem is that the proposed

microtubules have not been observed, although it could be argued that the frequent observation of enveloped HSV particles in what appear to be secretory vesicles are, in fact, microtubules cut transversely. The clearest evidence against this theory is the frequent observation of HSV apparently budding through membranes in the cytoplasm; this should not occur if the theory was correct.

The most convincing explanation for HSV egress (Johnson & Spear, 1982) proposes that the capsid buds into the perinuclear space, acquiring an envelope bearing partially glycosylated HSV glycoproteins. The virus then migrates to the golgi apparatus, the site of further processing. The glycoproteins would be embedded on newly produced membrane after processing, explaining the absence of cellular proteins in the mature virion. Enveloped virus would leave the cell by means of the well characterised and physiological secretory pathway associated with the golgi. HSV infected cells treated with monensin, an inhibitor of N-linked glycosylation, produced enveloped virus but failed to excrete it. Secretory vesicles containing several enveloped virus particles accumulated in the cytoplasm. This hypothesis accounts for many of the observations of egress in *in vitro* models of HSV replication. It remains to be seen whether this corresponds to the *in vivo* situation.

REGULATION OF HSV REPLICATION

HSV, in common with other herpes viruses, but unlike most other viruses encodes a very large number of non-structural proteins with presumed enzymatic and regulatory functions. Much of the evidence comes from studies of the effects of conditional lethal mutants of the genes encoding these proteins. It is beyond the scope of this review to present any more than a brief overview of some of the accumulating experimental evidence in this area.

GENOME STRUCTURE

The origins of DNA replication in HSV were determined in advance of sequence data. The evidence came from studies of defective interfering virus particles. This type of HSV may be obtained by multiple passage in cell culture at high moi's (Murray *et al.*, 1975). Virus prepared in this way comprises a minority of wild type virus that plays a helper role in the replication of the majority of defective viruses. The defective genomes are of the same length as wild type genomes (presumably regulated by packaging) but the majority of the coding capacity has been lost. Instead there are multiple tandem repeats of either an area in the middle of the long segment, or within the *c* region of the short segment. These so-called type I and type II repeats were predicted to encompass separate replication origins from the long and short segments, termed ori_L and ori_S (Ciufo & Hayward, 1981). They would have a replicative advantage over wild type virus; however, the latter would have to be retained in small numbers to act in a helper role to provide missing functions and structural proteins for the defective genome. Interestingly, it is very difficult to clone

fragments of ori_L. Any attempt to do so leads immediately to a 120 bp deletion. The reason for this deletion became appparent when the area was sequenced (Gray & Kaerner, 1984). there was a 144 bp palindromic sequence at the centre of ori_L; attempts to clone this failed because the sequence would form a self-annealed loop and would be excised. The ori_S region also revealed a palindromic sequence, but only 45 bp in length (Stow, 1982). There is extensive homology between ori_L and ori_S; ori_S has a small deletion beyond the palindromic area; if it were hypothetically restored, a 144 bp palindrome would result. Ori_S lies very close to IE175K, one of five immediate early proteins, but the only one to have a clearly defined role.

CLASSES OF REGULATORY PROTEINS

The replication of the structural proteins of HSV involves the activity of several non-structural proteins with enymatic activity to regulate transciption and translation. There are at least three classes of HSV genes, that are trascribed and translated at different times in the replicative cycle. Immediate early genes are those transcribed in the first hour of HSV DNA reaching the nucleus of the cell. Transcription is mediated by a cellular protein, RNA polymerase II, with maximum rates of synthesis 2-4 hours after infection (Preston & Newton, 1976). Pre-treatment of cells with cycloheximide, a chemical that prevents protein synthesis, does not prevent the transcription of IE genes. Furthermore, cells pretreated in this way also accumulate abnormally large amounts of IE messenger RNAs (mRNAs), indicating a possible negative feedback mechanism by proteins synthesised subsequently in a normal infection. Delayed early (DE) genes are those that are not transcribed until IE proteins have been produced. The

maximum rate of transcription of DE genes is at 5-7 hours. Late genes are either absolutely dependent on DNA replication (true late; TL) or are translated in small amounts before replication but increase in abundance after (early/late; EL). Late genes can further be distiguished from DE genes by their increasing rate of translation after 7 hours compared with the diminishing rate of production of DE proteins.

IMMEDIATE EARLY GENES

Five HSV-1 and 2 genes are transcribed in the absence of *de novo* protein synthesis on infection of a cell, and have therefore been identified as IE genes. The products of the genes are IE175K (ICP4), IE110K (ICP0), IE68K (ICP22), IE63K (ICP27) and IE12K (ICP47). This nomenclature is based on the apparent MW of the proteins on SDS-PAGE. Although the HSV 2 homologues of IE175K and IE110K have slightly different apparent MWs, this is ignored in order to show relationships. The alternative ICP nomenclature of Roizman is given in parentheses; the HSV 2 proteins have different designations in this system. The sequence data of IE175K and IE110K predict a much lower MW than that observed by SDS-PAGE. The discrepancy may be partially accounted for by the hydrophilic nature of many of the residues and the high proline content of the proteins. All but IE12K are phosporylated with multiple forms on 2 dimensional electrophoresis reflecting differently charged species (Ackermann *et al.*, 1984).

Numerous *ts* mutants of HSV that fail to express IE175K at non-permissive temperatures have been described (Preston, 1979). DE proteins are not synthesised, and by shifting to non-permissive

temperatures late in infection, IE175K was also shown to activate the transcription of late proteins (Watson & Clements, 1980). More evidence for the function of IE175K came from transfection experiments where the isolated IE175K gene is expressed in mammalian cell lines. IE175K was shown to be able to activate transciption of non-HSV genes, such as those of adenovirus (Feldman *et al.*, 1982). Furthermore, IE175K was shown to activate DE and late HSV genes in cell free systems (Pizer *et al.*, 1986), and did so by a demonstrable affinity for specific DNA sequences in the promoter regions of such genes (Faber & Wilcox, 1986). The protein was also shown to have an inhibitory effect on the transcription of all of the IE genes, including its own. The specificity of the action of this IE protein was unexpected, as it had been thought to have general activation properties, possibly mediated through cellular proteins.

IE110K has a similar composition to IE175K and would be expected to be able to bind to DNA to influence the rate of transcription. Increased rates of transcription of DE proteins mediated by IE110K have been demonstrated in transfection experiments (O'Hare & Hayward, 1985). Although it had not proved possible to obtain conditional lethal mutants of this gene, it was possible to artificially truncate the gene. IE110K deficient mutants grew normally in tissue culture, although there was evidence that at low moi's, the infectivity of the virus was compromised (Stow & Stow, 1986). A *ts* mutant of IE63K was shown to transcribe DE proteins normally but to be deficient in late gene transcription (Sacks *et al.*, 1985). No roles have been assigned to the other two IE proteins. It is notable that VZV, another neurotropic virus, has been shown to have counterparts to IE175K, IE68K and IE63K, but not IE110K or IE12K. By contrast, there are no homologies between any of the IE proteins of HSV or VZV with those of

CMV or EBV.

DELAYED EARLY GENES

The major role of DE genes in the replicative cycle of HSV is thought to be to mediate rapid synthesis of viral DNA. Although cells may contain the necessary enzymes for DNA replication, enhancement or reduplication of this function by virally encoded proteins is essential if the virus is to be able to productively infect cells that are no longer capable of division, or are in a resting phase of mitosis. Neurotropic viruses such as HSV spend a large part of their life cycle in such cells (neurones). This makes it difficult to determine the roles and requirements of DE and other regulatory proteins, since the *in vitro* models require actively replicating cells. Indeed many of the cell lines used are transformed and may not be susceptible to many of the regulatory controls of cellular replication.

The best characterised DE proteins are thymidine kinase (TK), DNA polymerase, DNA'ase and ribonucleotide reductase. TK is an important DE protein that is exploited by most currently used antiviral agents. Acycloguanosine (Acyclovir; ACG) has a specific antiviral effect due to the broader substrate specificity of viral TK, relative to its cellular counterpart. Viral TK phosphorylates ACG to ACG-monophosphate, while cellular enzymes process it further to di- and tri- phosphate forms. The latter nucleotide is a substrate for the virally encoded DNA polymerase leading to its incorporation into replicating viral DNA, causing premature chain termination. Cellular TK does not phosporylate ACG, nor do cellular polymerases recognise

the triphosphate form so the drug has a very low toxicity for uninfected cells. Since the widespread use of ACG and its predecessor, iododeoxyuridine, much attention has focussed on the possible mechanisms by which HSV may become resistant. HSV culture in increasing, initially sub-lethal doses of thymidine analogues *in vitro* commonly leads to the evolution of resistant strains deficient in TK production (Dubbs & Kit, 1965). While these mutants grow *in vitro* , they are non-pathogenic in experimental animals for the reasons discussed above. Less commonly, resistance may be acquired by changes in the specificities of virally DNA polymerase for ACG, rather than ablation of function (Coen & Schaffer, 1980). Should these mutants retain normal replicative abilities, then it is possible that they may also be fully pathogenic in experimental animals and in man. ACG resistant HSV has recently been isolated from a bone marrow transplant patient on long term ACG therapy (I. Smith, personal communication). The virally encoded DNA polymerase was unable to metabolise ACG-TP, yet the isolate retained some, but not total, pathogenicity in experimental animals.

The major DNA binding protein (MDBP) is another DE protein. The name derives from its observed properties *in vitro* , but its precise role is unclear. It is essential for DNA replication (Lee & Knipe, 1983) and evidence suggests that it operates in conjunction with DNA polymerase (Chiou *et al.*, 1985). A 136K early protein (ICP6) is a component of ribonucleotide reductase, the other being a 38 KDa DE protein (Frame *et al.*, 1985). The regulation of transcription of the former protein differs from the other DE proteins in that it is not dependent on IE175K and is transcribed along with the IE proteins. However, its transcription is not prevented by DE proteins, and the translation product rapidly accumulates in large amounts in the

cytoplasm and the nucleus during the DE phase of replication (Middleton *et al.*, 1982). By contrast, the 38K component has a solely nuclear distribution and is present in much smaller amounts. The functional significance of the apparent overproduction of the 136K protein is not known. Apart from DNA replication, DE gene products have been shown to prevent further transcription of IE proteins, and to influence the rate of transcription of late proteins.

LATE GENES

As mentioned previously, there are two classes of late gene, those that are transcribed from both parental and progeny viral DNA (EL) and those that can only be transcribed from progeny DNA (TL). There appears to be little functional significance in the distinction; for example, the major capsid protein, gD and gB are EL proteins, while gC is a TL protein. DE genes can be distinguished from both classes of late gene by observing the effect of DNA replication inhibitors on the abundances of transcripts of the three classes of gene. While the level of DE transcripts is not affected, EL transcripts are markedly reduced and TL are absent. The translation products of the late genes are primarily structural proteins, although regulatory functions may be attributed to some.

Specific antibody to gH-1 appears to prevent egress of mature virions if administered after infection of the cell (Gompels & Minson, 1986). Several lines of evidence point to role of components of the capsid in activation of transcription of IE genes, and in shutting off host cell protein synthesis. However, it is essential to point out the results of several investigators who found that naked single or double

stranded DNA of the HSV genome was capable of productive infection on transfection of a cell (Graham *et al.*, 1973; Sheldrick *et al.*, 1973). This discounts an "essential role" for capsid proteins in the initiation of infection. The conditions in which the experiments were performed were highly unnatural since very large moi's were required for infection. The duration of the replicative cycle was longer than that of cells infected with wild type virus. Thus, capsid components may be necessary for infectivity *in vivo* .

Shut-off of cellular protein synthesis appears to be mediated at the level of ribosomal RNA synthesis rather than suppression of synthesis of non-ribosomal mRNA. The very early swelling and disintegration of the nucleolus may be an effect of this shut-off. Fenwick & Walker (1978) mapped a gene product mediating shut-off to an area 0.52 to 0.59 map units by means of intertypic recombinants. Although this region encodes many proteins, ICP10 was thought to be the most likely effector of this function. Trans-activation of IE genes was mapped to a 65K phosphorylated protein, found in relatively large amounts in the tegument (Campbell *et al.*, 1984). The activity of the protein may be demonstrated even though the capsid is known not to enter the nucleus. The protein has no demonstrable affinity for DNA; this consideration and the low amounts of input protein on infection makes it likely that the protein operates through a cellular intermediary.

In summary, the biology of HSV is complex. Structurally, the virus is comprised of a large number of proteins, with diverse properties and antigenicity. The need for such a wide range of HSV-encoded glycoproteins is an enigma; conservation of the nucleotide sequence of genes encoding structural proteins between herpesviruses is evidence that they play diverse and subtle roles in the infectious process.

Understanding the method by which HSV takes over a cell and replicates is hampered by the large range of regulatory proteins produced by HSV and a fundamental ignorance of the normal metabolic processes within a cell. Assigning roles to individual proteins is complicated by the biochemical differences between the normal target cells of HSV *in vivo* , and the transformed, rapidly replicating cells often used *in vitro* .

CHAPTER 2

This chapter discusses the phenomenon of latency, and presents data on the role of the various arms of the immune system in protection from primary and recrudescent infection with HSV. In particular, attention is paid to the immune responses to individual proteins, and the implications for the proposed HSV vaccine.

PATHOLOGY

Primary and recrudescent infection with HSV cause an intradermal infection with accumulation of serous fluid containing infectious virus in vesicles. Typically, vesicles are lined with giant multinucleated cells with ballooning of the cytoplasm. There is an inflammatory response to HSV infection, with macrophages, neutrophils and killer (K) cells identifiable in pathological sections. It is long established that HSV becomes latent in the cell body of sensory neurones innervating the area of the primary infection. In animals, primary infection is prolonged and extended by reactivation of virus. Simmons & Nash, (1984) describe an animal model of primary and recrudescent infection in the mouse. Intradermally inoculated virus replicates in the skin reaching a peak by two days, accompanied by inflammatory processes. Virus is recoverable from the sensory ganglion by 2 days, reaching a peak titre by 4 days. Virus, reactivated from the ganglion becomes detectable in skin adjacent to the site of the original inoculation at 4 days and inflammatory changes develop subsequently. In this model, primary infection has two distinct components, the first being local infection, subsequently followed by

reinfection of the site by virus from the sensory ganglion. Immunochemical staining of epithelial sheets from the eyes of mice inoculated nasally showed that virus appeared in the eye at 3 days, and provided evidence for zosteriform spread of HSV to the eye (Shimeld *et al.*, 1986). Bernstein & Starberry (1986) describe the zosteriform spread of HSV-2 on vaginal inoculation of guinea pigs, as a model of primary and recrudescent genital infection.

LATENCY

The events leading to the establishment of latency in the neurone are unclear. HSV DNA can be readily detected in neurones of mice by southern blotting, at a copy number of 0.15 to 0.015 copies/cell (Rock & Fraser, 1983). The investigators failed to detect the terminal sequences of HSV, suggesting that the DNA was either circularised or in a highly concatamerised form. Puga *et al.*, (1984) presented evidence that there might be integration of virus sequences into the cellular DNA. The biological significance of these observations is not clear since it was not established that the DNA detected represented viral genomes with potential to reactivate, or whether it was an artefact resulting from the high doses of virus used to infect the experimental mice. In fact, Fraser *et al.*, (1981) found normal DNA sequences with no evidence of circularisation or integration in human brain tissue.

Efstathiou *et al.*, (1986) described experiments with both mouse models and human specimens. Linear DNA was found in the ganglia of mice during the acute stage of infection, but later, only endless, possibly circularised forms, were found. Follow-up of mice showed that

the endless form remained detectable for at least 4 months. Their analysis of cadaver trigeminal ganglia showed that endless forms of HSV DNA are also to be found in humans. Most recently, Mellerick and Fraser (1987) confirmed that HSV DNA was not integrated, and provided some evidence that it was circularised rather than concatamerised. These findings suggest that HSV could reactivate using the circularised form as a template for a rolling circle mechanism of replication.

A recent paper (Stevens *et al.*, 1987) descibes the discovery of an RNA species with an exclusively nuclear distribution in neurones containing latent HSV. The sequence of the RNA is complementary (ie negative sense) to the gene encoding IE110K, and may prevent IE110K mRNA transcription and hence expression of the regulatory protein. It is known that mutants deficient in IE110K production grow poorly *in vitro* , and it is possible that suppression of this protein *in vivo* could block HSV replication.

The immune system has also been shown to play a role in maintainance of latency. It is clear that immunosupression, either systemic or local (many of the stimuli that provoke recrudescence, such as pyrexia and ultra-violet light, appear to affect local cell-mediated immunity) leads to an increased frequency and severity of recrudescence. A clue as to how the immune system can prevent recrudescence comes from the observation that HSV may sometimes be isolated from patients who either do not suffer from recrudescences, or more commonly, from patients between recudescences. Regular sufferers of recrudescence often report experiencing prodromal symptoms such as tingling and hyperaesthesia but without subsequently developing any cold sores. Reactivation thus seems to have a variable outcome. Hill & Blyth

(1976) suggested a plausible hypothesis to explain the prevention of recrudescence by the immune system. They postulated that HSV reactivates frequently in all infected people, but brisk and effective immunity at the site of virus shedding normally prevents lesions forming. Whereas the majority of individuals are always able to contain reactivated virus, a minority occasionally fail to do so and suffer from periodic recrudescences. This hypothesis also accounts for the observation that many individuals maintain lifelong high titres of HSV antibody yet never have recrudescences. Repeated reactivation and exposure to the immune system but without recrudescence in such people would account for this observation. It is not known what proportion of people without recrudescences have regular reactivation of HSV.

HOST RESPONSES TO HSV INFECTION

The morbidity associated with HSV infection in man has been a major factor in current attempts to understand how the body responds to infection with HSV. Such knowledge is essential if effective preventive measures against HSV infection are to be developed. Much research has been concentrated on evaluating which arms of the immune system are responsible for protection from HSV. More detailed work that measures immune responses to individual viral components, such as individual glycoproteins, has been carried out, with the aim of developing an effective subunit vaccine to prevent primary infection.

NON-SPECIFIC HOST DEFENCES TO HSV

An intact skin barrier is a major factor in the prevention of infection. This is illustrated by the increased severity of disease caused by HSV in patients with eczema and burns. Natural killer (NK) cells kill target cells in the absence of any known antigenic stimulation and in the absence of antibody to the target cell. The method by which NK cells recognise their target is unknown. There is no apparent major histocompatibility complex (MHC) restriction, although they are more effective against target cells of the same species. Killing activity is enhanced by interferon. Bishop *et al.*, (1983; 1986) have presented evidence that there is specific recognition of gB and gC by NK cells of mice with no prior exposure to HSV.

Lopez (1981) reviewed a series of experiments that studied the role of inherited factors in resistance to HSV-1 in mice. There was a dominant trait in mice that protected them from the intraperitoneal, but not intracerebral, route of inoculation. Inheritance of this trait paralleled the inheritance of resistance to bone marrow allografts. This latter function is thought to be mediated by NK cells, so the evidence supports the idea that NK cells are important in resistance to HSV-1 infection. Whether NK function is an important factor in the human response to HSV infection remains to be established.

Macrophages are involved in many arms of the immune response, but they also have intrinsic anti-bacterial activity mediated by lectin-like compounds. It would seem unlikely that there would be any natural antiviral activity in these cells or in neutrophils that operate in a similar way. However, procedures that impair macrophage function

increase the susceptibility of mice to HSV infection inoculated intraperitoneally (Zisman *et al.*, 1970). Apart from using an inappropiate route, these studies are also subject to the objection that the proceedures may alter interferon levels (see below) and so increase the NK cell activity. Genetic differences in macrophage activity affected the resistance of mice to infection with HSV-2. The Chediak-Higashi syndrome is characterised by defective killing by macrophages, neutrophils and other phagocytic cells. Patients affected by the disease are susceptible to bacterial infection, but do not suffer from an increased prevalence or severity of infection by viruses, including HSV. All three interferons are important mediators of non-specific immunity to viruses, although the mechanisms by which they act are not fully understood and is probably multifactorial. α- & β- interferon are antiviral, while γ-interferon, a T-cell lymphokine, can modify the activity of various cells of the immune system. The role of interferons in controlling infection by HSV is unclear. It would appear unlikely that complement could directly act on virus in the absence of specific immunity. gC of HSV-1 binds to C3b but its role in pathogenesis is unclear, nor is it known if this binding is a primary function of the protein (see chapter 1)

SPECIFIC IMMUNITY TO HSV

Evidence for the importance of specific immunity comes both from observation of the effects of immunodeficiency states, both acquired and hereditary, and from experiments with the effector cells, both *in vitro* , and *in vivo* in animals. Interpretation of evidence from both sources is not without problems, in that apparently simple defects may have wide effects on other aspects of the immune system.

Investigating the function of the effector cells eg cytotoxic T cells, killer cells etc. *in vitro* has its own problems. Delayed-type hypersensitivity (DTH) cannot be studied readily *in vitro* and so there is very limited information on human DTH responses. Cytotoxic T-cells (CTCs) can be tested *in vitro* , but the antigen must be presented in association with self class I MHC antigens. This restricts experiments either to syngeneic mice or to human cytotoxic T-cells where it is possible to culture and experimentally infect target cells from the same patient. Antibody-dependent cell-mediated cytotoxicity (ADCC) can be readily studied *in vitro* since it is mediated by K cells that are not MHC restricted. All *in vitro* experiments suffer from the problem that only one aspect of the immune response can be studied at a time. Interaction and cooperation between cells does not occur and findings may not apply directly to the *in vivo* situation. *In vivo* experiments with HSV can only be performed readily in animal models. The main problems with these experiments is the artificiality of the situation.

IMMUNE RESPONSES TO ACUTE INFECTION

Many studies have been reported in which HSV is inoculated peripherally (either footpad or ear pinna) into a mouse and the immune response measured (Hill *et al.*, 1975; Nash *et al.*, 1980). Inflammation begins on the first day and becomes maximal within 4-5 days (Simmons & Nash, 1984). Neutralising antibody becomes detectable after 8 days, with maximal levels between 12-14 days post inoculation (Knoblich *et al.*, 1983). DTH can be observed within 4 days by injection of virus in the opposite ear pinna; the response becomes maximal after 8 days and persists indefinitely (Nash *et al.*, 1980). T

cells taken from the lymph node draining the site of inoculation show proliferation on exposure to antigen in mice *in vitro* . This occurs from the third day onwards. The proliferation response of helper T cells becomes maximal several weeks after inoculation and persists indefinitely. However, cytotoxic T cells are only found for about ten days after experimental infection of mice.

Although many arms of the immune response operate during primary infection, it is difficult to determine which of the responses are important in controlling its severity and spread. It is possible to study the role of humoral antibody relatively independently of T cell immunity. Mice treated with anti-IgM from birth lose the ability to mount an antibody response; all 5 classes of immunoglobulin are absent while their cellular immunity appears to be unimpaired (Gordon, 1979). Mice treated in this way showed equal susceptibility to HSV infection as the control group, with similar rates of clearance of virus from the site of inoculation (Kapoor *et al.*, 1982a). There was however, wider spread of virus in the central nervous system, with latency demonstrable in ipsilateral, adjacent and contralateral ganglia. This evidence is supported by findings of a normal course of HSV infection in human subjects with congenital B cell deficiencies. It also concords with findings with other human viruses. For example, reduced humoral immunity leads to an increased prevalence of paralysis in patients infected with poliovirus, presumably because of a failure to limit spread of the infection. Limiting the spread of HSV infection may be mediated by antibody alone, antibody and complement or antibody and K cells. It is not clear at present which of the three possibilities is effective *in vivo* .

In experimental animals, T cell mediated immunity appears essential for survival of HSV infection. Athymic mice rapidly perish from disseminated infection on challenge with HSV (Nagafuchi *et al.*, 1979; Metcalf *et al.*, 1979), and can be protected by transfer of syngeneic T cells taken from normal mice 7 days after viral challenge (Kapoor *et al.*, 1982b). Patients with diminished cell mediated immunity also appear to be at risk from a much more severe primary HSV infection than normal subjects.

Many methods can be used to evaluate the roles of the different components of cell mediated immunity. Transfer of various populations of syngeneic T cell from immunocompetent mice to athymic mice is one possibility. The donor and recipient mice could have the same class I and II markers, or they could differ in one or the other. Cell-mediated cytotoxicity (CMC) is restricted by class I and DTH by class II. Such studies have given conflicting results on the relative importance of CMC and DTH (Nash *et al.*, 1981b; Nash & Gell, 1983; Larson *et al.*, 1983). Further work on T cell responses will need more careful separation of T cells with different functions. On balance, it would appear that DTH makes a relatively greater contibution to immunity from HSV than CMC, although this remains disputed. The target specificity of T cells is also important; transfection experiments now allow responses to individual proteins, or protein fragments, to be studied. This is obviously important in view of the known functional differences between the various glycoproteins, and the variation in the susceptibility of HSV to neutralisation by antisera of differing target specificities.

Several studies of the antibody response to naturally occuring HSV infection in man have been described. Neutralising antibodies are detectable within a month of primary infection and readily cross-neutralise the type-heterologous virus. Gilman *et al.*, (1980; 1981) describe the results of using a radioimmunoprecipitation (RIP) assay to investigate the target specificity of human convalescent sera. The results showed that patients generally had antibody to a large number of virally-specified proteins but that patients differed in the range of apparent antibody specificities. Eberle *et al.*, (1983) used the western blotting or immunoblotting (IB) method to identify the targets of antibody in convalescent sera, and found reactivity with HSV glycoproteins and several capsid components. The results of Teglbjaerg *et al.*, (1986), who used both methods, are difficult to interpret, and reveal a major problem of the identification of bands produced by both assays. Convalescent sera reacted with the major capsid protein (MCP), ICP6, major DNA binding protein (MDBP), gB and ICP 20 and 25, in the RIP assay. However, many more bands were produced, but they were not identified. In the IB assay, several bands appeared, including MCP, ICPs 6 & 7, MDBP, gB, ICPs 25 & 36 and gD, and, again, over half were not identified.

Eberle *et al.*, (1984; 1985) and Bernstein *et al.*, (1984) used the IB method to study antibody response in primary infection. They found that the initial antibody response was not to a glycoprotein, but rather to the tegument protein, p40 (see chapter 1). This was a surprising result and conflicted with work by Zweerink & Corey, (1981), Ashley & Corey, (1984) and Ashley *et al.*, (1985) who showed, using the RIP method, that the initial immune response was confined to gB, gC-1 and the MCP of HSV-2, although their results differ in detail. Ashley & Corey (1984) reported also that, in some patients,

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the appearance of antibody to gD was delayed, and that this correlated with a decreased interval to the first recrudescence. Ashley *et al.*, (1985) reported that Acyclovir treatment reduced the humoral antibody response to primary genital HSV infection, and associated this finding with the clinical observation that patients on such treatment tended to have earlier and more severe recrudescences than those in the untreated group.

IMMUNOLOGICAL RESPONSES AND THE ESTABLISHMENT OF LATENCY

As described previously, the establishment of latency involves the migration of virus up the afferent axon to the nerve cell body in the sensory ganglion. In this latent state, the immune system is obviously incapable of eradicating the virus. There have been many studies to determine which arms of the immune system are responsible for preventing recrudescence. Recrudescences occur in the presence of high titres of neutralising antibody; indeed, patients with frequent recrudescence show, on average, higher antibody titres than patients with no recrudescences. This data discounts a role of humoral immunity in the maintenance of latency. Torseth *et al.*, (1987) demonstrated that peripheral lymphocytes of patients with previous exposure to HSV showed a lymphoproliferative response to synthetic gD, gB and gC. The γ-interferon produced by the T cells was quantitated; the level produced on stimulation with gD correlated with the frequency of recrudescence. Whether this is a cause or effect remains to be established. Recrudescence may be influenced by any aspect of cell-mediated immunity eg. cytotoxic T-cells, antibody dependent cell-mediated cytotoxicity, by DTH, or by other mechanisms.

In mice, many immunological functions can be demonstrated following the resolution of the primary infection. Mice retain a lifelong lymphoproliferative response to HSV antigen, indicating helper T-cell recognition. DTH is also permanent, but this function cannot be transferred to uninfected, syngeneic mice by transfer of spleen cells, as it could in earlier stages of the infection. Transfer of DTH is thought to be prevented by suppressor cells that appear after the primary response to primary infection. Suppressor cells of both B cell and T cell origin can be demonstrated in the mouse (Nash & Gell, 1983; Schreier *et al.*, 1983a). Suppressor T cells appear to comprise at least two distinct populations with different surface markers. A notable feature of T suppressor cells is that, while lymphoproliferative responses are observed in response to type heterologous virus, suppressor cell activity is type specific (Nash *et al.*, 1981a)

CTC activity wanes in both mice and humans after primary infection, and classic MHC-restricted cytotoxicity cannot be demonstrated in specimens from convalescent patients. A correlation between lymphokine production and recrudescence has been observed in both guinea pigs and humans. Guinea pigs vary in their susceptibility to recrudescence; the lymphocytes of those with frequent episodes show reduced lymphokine production on challenge with HSV antigen in a lymphoproliferative assay (Dounenberg *et al.*, 1980). A similar reduction can be observed with human T cells from patients with frequent recrudescence compared with controls (Shillitoe *et al.*, 1977; O'Reilly *et al.*, 1977). However, the production of a lymphokine, macrophage migration inhibition factor, was normal in patients between recrudescence; a reduction could be observed during recrudescence (Sheridan *et al.*, 1982). Another measure of immune function in humans, the T4 (helper)

to T8 (cytotoxic/supressor) ratio, is also lowered during recrudescence. At present it is not possible to establish whether reduced lymphokine production or reduced T4/T8 ratio are the cause or effect of recrudescence in humans.

VACCINE DEVELOPMENT

One of the aims of studies of the immune response to herpes simplex virus is to obtain information relevant to development of a vaccine for HSV. Although recrudescence occurs despite demonstrable cell mediated and humoral immunity, a vaccine administered before first exposure to HSV might prevent primary infection, or may prevent the establishment of latency. Preliminary observations indicate that a vaccine may be effective. Firstly, restriction enzyme analysis to differentiate strains of HSV has shown that patients are generally infected with only one strain, with the implication that the first infection prevents subsequent infection with other type-homologous strains. Another general observation concerns the epidemiology of HSV-2 infection. The reasons for the evolution of two serotypes of HSV are uncertain. One explanation for the divergence is based on the observation that recrudescences of genital HSV-1 infection are much less common than HSV-2. HSV-2 therefore appears better adapted to that area, and, in evolutionary terms, is more likely to be transmitted. But another explanation for the difference is that HSV-2 has diverged antigenically in order to avoid pre-existing immunity to HSV-1 acquired before the onset of sexual activity. The latter hypothesis is supported by evidence that genital infection with HSV-1 is rare in patients with previous oro-labial HSV-1 infection; HSV-1 genital infection is normally primary. If the latter explanation is valid,

then it would be clear evidence that pre-exposure to HSV can prevent or modify the outcome of subsequent HSV infection.

The safety of vaccines is paramount, particularly for infections such as HSV, that are often asymptomatic or trivial. UV irradiated HSV DNA has been shown to have transforming activity *in vitro* and *in vivo* , and any vaccine should be guaranteed to be free of any HSV genetic material. For these reasons, much attention has focussed on the possibility of a vaccine made by recombinant DNA technology. The advantages of such a product would be purity, ease of preparation and safety. However, it is necessary to establish if immunity induced by individual HSV proteins is sufficient for protection, and which proteins should be chosen.

To date, most work has concentrated on examination of immunity to individual HSV glycoproteins, and the protective effect subunit vaccination has on experimental animals. Potential efficacy of immunisation can be examined in several ways. 1) measuring neutralising antibody and cell mediated responses to individual glycoproteins; 2) determining the protective effects of passively administered antibody; 3) determining the protective effect of individual or multiple glycoprotein antigens in both limiting or preventing primary infection with challenge doses of virus, and preventing the establishment of latency.

Immunopurified and recombinant gD has been shown to protect mice from lethal challenge by HSV-1 and -2 (Long *et al.*, 1984; Eisenberg *et al.*, 1985a). Roberts *et al*, (1985) have extended this conclusion to gB and gC, again by using immunopurified glycoproteins. Glorioso *et al.*, (1984) compared the efficacy of gB, gC and whole virus immunogens

in protection from challenge by the intraperitoneal amd intracerebral routes. They found that all three protected mice from viral challenge by the i/p route, but only the whole virus vaccine protected mice from i/c challenge. Paoletti *et al.*, (1984a) made vaccinia recombinants containing an inserted gene for gD. Such constructs were then evaluated as possible vaccines. The same investigators (Paoletti *et al.*, 1984b) found 100% and 80% survival rates in mice challenged after receiving the vaccine, compared with 45% and 15% in the untreated groups. Similar results have been obtained by other investigators (Cremer *et al.*, 1985; Martin *et al.*, 1987). Dix & Mills, (1985) immunised mice with gD-1, gD-2, gB-1 and gB-2 and measured protection from challenge with HSV-1 and -2. All four immunogens completely protected mice from a footpad HSV-1 challenge that killed 70% of the control group. However, a dose of HSV-2 that killed 90% of the control group also killed 60 and 90% of the mice receiving the gB-1 and gB-2 vaccines, and 30 and 20% of mice receiving gD-1 and gD-2. The investigators then dissected out the sacral ganglia, and found extensive infection of the CNS with contralateral and ipsilateral infection in the control and gB-1 and -2 recipients, while in those receiving gD, infection was confined to the ipsilateral ganglia. These findings indicate that a vaccine could prevent the establishment of latency.

Antibody elicited by an immunisation could operate either by neutralisation of virus, by ADCC of infected cells by activated K cells, or by complement-mediated lysis. Passively administered antibody has been shown to be protective in mice (Dix *et al.*, 1981; Balachandran *et al.*, 1982 and others). Monoclonal antibodies have been extensively investigated for neutralising activity *in vitro* , and it has been demonstrated by several investigators that

neutralisation of HSV can be achieved by monoclonals against every glycoprotein so far investigated, even though not all of the proteins are essential for virus growth *in vitro* . Similarly immunisation with individual purified glycoproteins induces the production of neutralising polyclonal sera (Norrild, 1985). Monoclonal and monospecific antisera to gD been shown to have the highest neutralising titres. Neutralisation may not be the mode by which antibody limits primary infection in a vaccinee, since ADCC operates at much lower antibody concentrations. Furthermore, antibody participating in K cell or complement mediated cytolysis need not be neutralising; only recognition of infected cell membrane components is necessary to activate the pathways leading to destruction of infected cells. Kumel *et al.*, (1985) showed that there was no correlation between *in vitro* neutralisation by monoclonals and their *in vivo* protective effect when passively administered into mice. These experiments directly point to a role for ADCC by K cells, since the mice were congenitally deficient in complement activity. These findings disagree with those of Simmons & Nash, (1984), who found that only neutralising antibodies protected mice from experimental challenge.

Cell-mediated immunity induced by vaccination is likely to be more important in conferring protection, in view of its demonstrably greater role in limiting natural infection. Chan *et al.*, (1985) showed that passive transfer of T cells from gB immunised mice to syngeneic naive mice conferred protection from lethal challenge. Schreier *et al.*, (1983b) showed delayed type hypersensitivity reactions in mice protectively immunised with either gD and gC. As might be expected from the antigenic sites of gD and gC, protection and DTH was demonstrable against heterologous type virus following gD,

but not gC, vaccination. Naylor *et al.*, (1982) showed that the route of immunisation affected the type of immunity produced. Intraperitoneal immunisation, in many respects equivalent to intravenous, elicited an antibody response but no detectable DTH, while animals immunised subcutaneously showed DTH but no antibody response. Martin *et al.*, (1987) showed that a gD-vaccinia construct induced protection from lethal challenge, T cell lymphoproliferative responses and interleukin-2 production. However, no class I restricted cytotoxic T cells reactive with HSV were produced even on re-immunisation with whole HSV virions. The failure to induce CMC was not MHC related, and did not appear to be the result of an inappropiate route of inoculation since CMC to vaccinia was readily detectable. Low levels of CMC to HSV were mediated by T cells bearing class II antigens, although the significance of this is not clear.

In summary, the available information on subunit vaccines shows that a protective effect may be elicited in experimental animals, and that DTH rather than CMC is important in this protecive effect. Results from trials of such vaccines in humans should shortly be forthcoming. The data about the protective effect of antibody tends to indicate that it plays a minor role in the pathogenesis of HSV infection, possibly related to the late appearance of specific antibody relative to the rate at which HSV infects neurones. However, this does not mean that it is unimportant as an effector of protection by vaccination, and, in particular, it may localise the infection by rapid complement and K cell mediated killing of infected cells and neutralisation of released virus.

In man, Chen *et al.*, (1984) describe the efficacy of the "Skinner vaccine" in protection of the sexual partners of patients with genital HSV-2 infection. This work has been criticised from an ethical and safety point of the view, and inadequate selection and monitoring of the patients in the study make interpretation of the results difficult. The main problem is that no attempt was made to establish if the partners had had prior exposure to HSV-2 by serological assays. In patient selection and claims of efficacy, the investigators did not consider the fact that up to 50% of genital HSV infection may be asymptomatic. These criticisms emphasise the need for careful and thorough trials to establish the efficiacy of any candidate vaccine.

MEASUREMENT OF TYPE-SPECIFIC ANTIBODY TO HSV

The knowledge recently gained concerning the nature of, and serological response to, HSV glycoproteins has been used in the development of an assay that can detect and differentiate specific antibodies to HSV-1 and -2. The motivation is epidemiological and clinical, in that it often desirable to differentiate past exposure to HSV-1 from HSV-2, and from infection with both. In a clinical setting, there are instances where the results of such an assay would have a bearing on the clinical management of the patient, such as genital HSV infection during pregnancy, and in the diagnosis of HSV infection where isolations have proved unsuccessful.

Plummer (1973) reviewed very early attempts to develop a type-specific assay for HSV. These methods without exception used whole virus, and usually were found to be inadequate at differentiating sera with antibodies to both HSV-1 and -2 from monospecific sera. The

application of gel based methods led to the identification of proteins that appeared to elicit type-specific antibody. Vestergaard *et al.*, (1979) and Jeansson *et al.*, (1979) described the separation of apparently type-specific antigens by non-immunological purification methods. Dreesman *et al.*, (1979) and Eberle & Courtney (1981) used RIP analysis of HSV proteins to identify gC-1 and gC-2 (later found to be gG-2) as type-specific antigens. The proteins were purified and used in a type-specific assay. Proteins binding to *helix pomatia* lectin (gC-1, gG-2) were used in type specific assays (Suchanova *et al.*, 1984; Svennerholm *et al.*, 1984). Monoclonal antibodies have also been used to extract gG-2 from a cell lysate prior to use in a type-specific assay (Lee *et al.*, 1985).

gC-1 has since been shown to be related to gF (renamed gC-2) and cross-reactivity demonstrated in one of the two antigenic areas of the protein (Zweig *et al.*, 1984); it can no longer be condidered as a candidate type-specific antigen. More recently, the lack of homology between gG-2 and other HSV proteins has become apparent, and antibody to this protein would be expected to be type-specific. However, a cross-reactive oligopeptide serum reactive both with gG-2 and gG-1 has been described (McGeoch *et al.*, 1987; see chapter 1). Whether this would lead to cross-reactivity of antibody elicited by natural infection is unclear, since the antiserum was raised to an oligopeptide corresponding to a sequence of amino acids near the C terminus, within the viral envelope.

CHAPTER 3

The AIDS epidemic has reached alarming proportions, and it is unclear what will be the eventual outcome in terms of human mortality and morbidity. Rarely has so much effort been concentrated in fundamental and applied research to find a cure. It is now clearly established that AIDS is the terminal stage of infection with a retrovirus, human immunodeficiency virus (HIV). Preceding clinical disease, there is a prolonged carrier stage when patients are positive for HIV-specific antibody and may be infectious but symptom-free. This stage may last for several years before the onset of AIDS; prospective follow-up should establish whether HIV infection inevitably progresses, or whether some individuals are able to keep the disease in check indefinitely. AIDS, when it does occur, is uniformly fatal and there have been no reported cases of spontaneous remission of symptoms. HIV has been isolated from blood, semen, saliva and other secretions of an infected individual, but, while it has been established that HIV is commonly transmitted through sexual intercourse, there is currently little evidence for aerosol spread or infection through intact skin. Evidence that direct inoculation is required for infection comes from the strong association of HIV infection with intravenous drug abusers and haemophiliacs.

This introduction consists of a description of the syndrome (particularly of illness caused by the virus itself, with relatively less attention given to the range of disease secondary to immunosuppression). Further sections will describe the biology of HIV and the process of infection of a cell. Chapter 4 describes the laboratory markers of infection and describes the current research into the immune responses to HIV infection. Finally, a selection of

the numerous theories of pathogenesis will be reviewed.

DISCOVERY OF HIV

The AIDS syndrome was recognised several years before the discovery of the virus. Although the primary feature of the syndrome was recognised to be that of immunosuppression, the distribution of cases suggested an infectious aetiology, and a virus transmitted in similar ways to Hepatitis B was suspected as far back as 1982. Particularly suspicious was the exponential rise in numbers in certain well-defined risk groups, initially promiscuous male homosexuals in the San Franscisco area, but later haemophiliacs, drug abusers and heterosexual partners of any of these. This ruled out the earlier theory that certain features of the lifestyle of the first group was responsible for the observed immunosuppression. Human cytomegalovirus (CMV), hepatitis B (HBV; possibly in association with delta agent) and human T-lymphotropic virus type 1 (HTLV-1) were all considered as candidates for the causative agent of AIDS. However all three viruses were excluded as the primary cause on the basis of cases, particularly children, in whom evidence for infection with these viruses was absent. Barre-Sinnoussi *et al.*, (1983) found reverse transcriptase (RT) activity, suggestive of a retrovirus, in cultures of T lymphocytes from a patient with persistent generalised lymphadenopathy (PGL), a condition found in patients in the recognised risk groups for AIDS. The virus showed a tropism for replicating T-lymphocytes, formed syncytia with subsequent cell lysis, could be seen budding from the membranes of infected cells by EM, and showed weak serological cross-reactivity with HTLV-I by immunofluorescence. On this basis, Barre-Sinnoussi and colleagues classifed the isolate as a retrovirus,

and called it lymphadenopathy associated virus (LAV). Two further detections were subsequently made from patients with AIDS (Vilmer *et al.*, 1984). Proof that the three viruses isolated were the cause of AIDS was problematic. Gallo *et al.*, (1984) found viral particles and RT activity in 48 out of 70 T-cell cultures of patients with PGL or AIDS. Continuous propagation of one isolate was achieved and allowed large amounts of purified viral antigens to be prepared. Antibody reactivity with the prepared antigens was found in essentially all of the patients with AIDS and PGL and also in a proportion of well patients in the risk groups. Antibody reactivity was not found in patients in low-risk groups. Finally, retrospective testing of stored serum collections worldwide showed that seroconversion in AIDS-risk populations first began to occur about 3 years before the first AIDS cases were reported (Brun-Vezinet *et al.*, 1984; Chiengsong-Popov *et al.*, 1984; Safai *et al.*, 1984; Sarngadharan *et al.*, 1984).

CLINICAL FEATURES OF HIV INFECTION

The current Centres for Disease Control (CDC) classification of the clinical features of AIDS comprises:

Group I ACUTE INFECTION

Group II ASYMPTOMATIC INFECTION

Group III PERSISTENT GENERALISED LYMPHADENOPATHY

(cont'd)

Group IV OTHER DISEASE:

A) Constitutional disease. Fever more than one month, more than 10% weight loss, or diarrhoea for more than one month.

B) Neurologic disease. Dementia, myelopathy, or peripheral neuropathy.

C) Secondary infections.

i) *Pneumocystis carinii*, cryptosporidiosis, toxoplasmosis, strongyloidiasis, isosporosis, candiadiasis (of oesophagus or lungs), cryptococcosis, histoplasmosis, infection with *Mycobacterium avium or kansasii*, CMV infection, chronic or disseminated HSV infection, progressive multifocal leukencephalopathy.

ii) Oral hairy leukoplakia, VZV reactivation of more than one segment, Salmonella bacteremia, nocardiosis, tuberculosis, or oral candidiasis.

D) Secondary cancers. Kaposi's sarcoma, non-Hodkin's lymphoma, primary lymphoma of the brain.

E) Other conditions in HIV infection.

The four principle groups are based on chronology, and patients can only be reclassified downwards, even with remission of symptoms.

Patients may, however, fall into several subcategories of group IV. The classification was designed to remove uncertainty about the distinction between AIDS-related complex and AIDS, and it separates illness caused by the virus itself (groups I, II, III, IVa and IVb) from illness secondary to immunosuppression (IVc and IVd). There remain some problems. For example, while weight loss may be attributed to a hypercatabolic state and a direct infection of the intestinal mucosa by HIV, opportunisic infection with isospora and cryptosporidia undoubtedly account for a proportion of the symptoms observed. Weight loss could therefore be a feature of both groups IVa and IVc.

Following exposure, there is an interval of several weeks or months during which the patient remains well and seronegative. In a few cases (group I), there may be an infectious mononucleolosis-like illness, with fever, rash, malaise and generalised lymphadenopathy for several weeks. The signs are non-specific and it is unlikely that HIV infection could be reliably diagnosed on these findings alone. Information about the seroconversion events has either come from retrospective examination of patient records where the time of seroconversion was known, or from prospective studies of risk groups known to be at risk for AIDS.

After seroconversion, patients may remain symptom-free for several months to several years (group II). Increasingly, careful clinical scrutiny of patients after seroconversion reveals minor degrees of immunosuppression in almost all patients. For example, patients are more likely to suffer from shingles, a well known marker of mild deficiency in cell-mediated immunity. Laboratory investigations also may reveal characteristic changes also seen in the full AIDS syndrome, such as depressed T-helper cell number and raised immunoglobulin

levels. Leukopoenia (particularly neutropoenia), anaemia and thrombocytopoenia have all been described in asymptomatic patients. In particular, overt thrombocytopoenia, along with other hypersensitivity reactions, is frequently found on administration of trimethoprim and related antibiotics.

A proportion of patients develop PGL (group III). PGL is defined as enlargement of lymph nodes at two or more sites (other than inguinal), for more than three months with no other identifiable cause. Although originally considered as a preliminary step before the development of AIDS, a proportion of cases are considered to be incidental, in whom PGL is not prodromal to the full AIDS syndrome (Janossy *et al.*, 1985). PGL may in fact regress either spontaneously or on development of the full syndrome, while patients may have PGL for several years with no sign of advancing disease. Patients with PGL may have a variety of constitutional symptoms, but remain classified in group III rather than IVa on the basis of severity.

Constitutional disease (Group IVa) comprises fatigue, night sweats, and malaise of varying grades of severity. Patients appear chronically ill and often cachectic. Prolonged fevers and involuntary weight loss of more than 10% are found in severe cases. In Africa, the term "slim" is used to describe this condition. Patients may also have chronic diarrhoea, often with malabsorption. Hypersensitivity reactions, retinal infarcts (cotton wool spots), thrombocytopoenia and hypergammaglobulinaemia and raised levels of α-interferon have been described. In some respects, the features resemble the clinical features of systemic lupus erythematosis, an autoimmune disease. This suggests an immune complex aetiology for certain aspects of HIV pathogenesis.

Neurological disease (Group IVb) probably comprises at least two distinct syndromes, that can be distinguished both clinically and histologically. Progressive encephalopathy is a rapid form of the disease with an underlying inflammatory component. Patients initially show episodes of confusion or forgetfulness which evolves within weeks or months into a marked psychomotor depression, with apathy, withdrawal, mutism and profound dementia. Ultimately the patient becomes bedridden, incontinent and unresponsive. A slower course may be observed in some patients, in whom degeneration of the brain tissue is the prominent pathological finding. In such patients, subtle personality changes may develop over several years, often with profound depression or inappropiate, uncontrollable moods. Patients become increasingly withdrawn, and eventually enter a vegetative state similar to the other form of encephalopathy. Involvement of the peripheral nerves may lead to focal neurological abnormalities.

Secondary infection (Group IVc) by opportunistic organisms is one of the hallmarks of the full AIDS syndrome, and infection by the 12 organisms listed in part (i) constitutes the CDC definition of AIDS. However, infections with the pathogens listed in part (ii) are also indicative of profound immunosuppression (see Pinching, (1985) for a review of the clinical features of secondary infections in AIDS patients). The pattern of infections is similar, in many ways, to that observed in patients with congenital or acquired cellular immune deficiency. Characteristically, organisms that do not normally cause disease in the normal host (*P. carinii, M. avium,*), and those that cause mild and self-limiting disease (HSV, *Toxoplasma gondii,*) are pathogenic in AIDS patients, with severe, disseminated and persistent disease and poor response to conventional antimicrobial therapy. The spectrum of opportunistic infection in AIDS patients differs in detail

from that seen in other patients with other deficiencies in cellular immunity; infection with *Legionella pneumophila*, and *Listeria monocytogenes*, are characteristically rare in AIDS patients.

Secondary cancers (Group IVd) that comprise the CDC definition of AIDS are Kaposi's sarcoma, non-Hodgkin's lymphoma and primary central nervous system lymphoma. There is some evidence for the involvement of CMV and EBV in the aetiology of Kaposi's sarcoma and non-Hodgkin's lymphoma. (Safai *et al.*, 1984) The incidence of these tumours resembles that in other patients with secondary immunodeficiency. In the latter situation, discontinuation of iatrogenic immunosuppression is associated with regression of the tumour. This supports the idea that secondary cancers are primarily opportunist in AIDS patients.

Undoubtedly, new syndromes will be characterised in HIV-infected patients as the epidemic progesses. In the absence of an animal model for HIV-related disease, clinical features and associated pathology remain a primary source of information about the virus. Analogies do exist, however, between AIDS and visna infection in sheep, and such comparisons have led to a greater appreciation of the direct effects of HIV infection compared with those secondary to immunosuppression.

BIOLOGY OF HIV

Retroviruses are very diverse in host range, structure and pathogenicity, but share a common method of replication. The genomic material is in the form of positive sense RNA; on infection of a cell, it is transcribed to a double stranded DNA copy which migrates to the nucleus to initiate virus replication. The "reverse" transcription is mediated by a virally-encoded protein found within the capsid of the virion. The so-called reverse transcriptase is essential for the infectivity of the virus and is characteristic of the retrovirus family. At one time it was thought that this enzyme was uniquely present in retroviruses, but more recent work has established that it is found in cauliflower mosaic virus (CaMV), and, in a related form, in hepatitis B virus. Hull & Covey, (1986) discuss the relationships between these latter two viruses and retroviruses, and come to the surprising conclusion that they share sequence and functional features with each other and with transposable elements of drosophila and yeast. This data, if corroborated, would significantly broaden the definition of a virus.

Retroviruses can be divided into three main groups. The best characterised are the oncogenic retroviruses that cause cancers in their host species. There are at least two oncogenic retroviruses known to affect man (human T-lymphotropic virus (HTLV) types I and II) that cause certain types of T-cell leukaemia. Feline leukaemia virus, Rous sarcoma virus and Moloney murine leukaemia virus are just some of the many animal oncogenic retroviruses that have been investigated. The name oncogenic retrovirus should not be taken to imply that they all cause cancer by the same mechanism; at least five distinct ways have been described, ranging from direct transmission of oncogenes to

the cell they infect, to trans-activation and cis-activation of normally suppressed cellular genes. There is no evidence that HIV is oncogenic, and sequence data indicates that it is only distantly related to this family of retroviruses.

The spumavisuses are the second group of retroviruses. These have been little studied due to problems with culture, and because they appear to be non-pathogenic. They have been isolated from several mammalian species including man. There is a suspicion that a spumavirus is associated with the very rare de Quervain subacute thyroiditis (Werner & Gelderblom, 1979).

There are grounds for classifying HIV as a lentivirus, the third group of retroviruses, characterised by slow, progressive diseases found in several mammalian species. Visna virus (VV; sheep), progressive pneumonia virus (PPV; sheep and goats), caprine arthritis encephalitis virus (CAEV; goat) and equine infectious anaemia virus (EIAV; horse) are well characterised members of this group. A recently discovered relative of HIV, HIV-2 has been isolated in Africa from patients with AIDS, but who were seronegative for HIV by conventional antibody testing (Clavel *et al.*, 1986).

Kanki *et al.*, (1986) also reported a novel "human" retrovirus from Africa at the same time as the French group, but it is widely suspected that the virus they called HTLV-IV was merely a contaminant from primate isolation experiments. A primate retrovirus (simian T cell lymphotropic virus type III or simian immunodeficiency virus; STLV-III or SIV) causes an AIDS-lke disease in macaques, shows close sequence homology with HIV-2, but to be more distantly related to HIV-1 (Franchini *et al.*, 1987; Chakrabarti *et al.*, 1987). The SIV

sequence was found to be essentially identical to HTLV-IV, in contrast to the variability commonly observed between different isolates of the same retrovirus. There is now a consensus of opinion that rejects the claim of a human origin for HTLV-IV.

Figure 2 illustrates the HIV genome (after Rabson & Martin, 1985). Notable is its much smaller size than the HSV genome (only 9735 base pairs instead of 150 000), and the relatively fewer open reading frames (7 or 8 instead of around 50). In contrast to HSV, there is complete sequence data on several isolates of HIV, and of several other retroviruses. A major surprise was the discovery of extra open reading frames as compared with the oncogenic retroviruses. The latter generally only have three, *gag* , *pol* and *env* (although HTLV-1 and -II also have a transactivating protein pX). Subsequent sequencing of VV revealed a similar arrangement to HIV, with three extra genes encoding presumably regulatory proteins (Sonigo *et al.*, 1985).

PROCESS OF INFECTION

Infection of a cell by HIV is followed by release of positive sense genomic RNA in the cytoplasm. Reverse transcriptase (RT), present in the core of the virus, mediates the immediate synthesis of a complementary strand of DNA. RNA is removed from the DNA copy by endo H activity present in the RT protein. On completion, RT continues to produce a double stranded DNA from the newly synthesised negative sense DNA, and the linear duplex is then transported to the nucleus to initiate transcription of viral RNAs. The extent of viral DNA replication *in vivo* is not known, but since it is mediated by host cell DNA polymerase, it probably varies between different cell types. ie. depending on whether the cell is mitotically active or not.

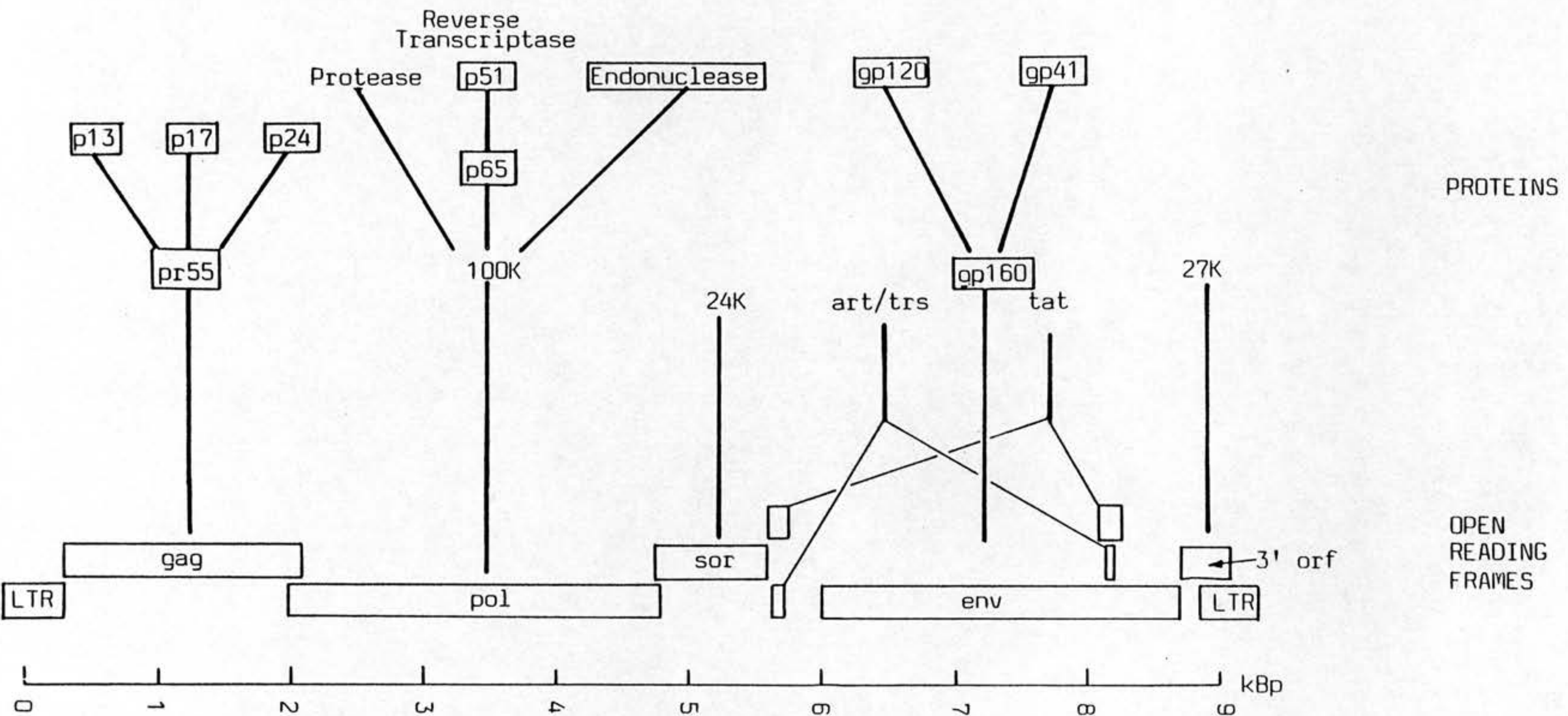


FIGURE 2: HIV genome and location of genes (based on Weiss, 1987). Virion components in boxes.

There are two origins for DNA replication, one at the 5' LTR and one at the the downstream end of the *pol* gene. Progeny genomes have a characteristic structure of a complete negative strand and a positive strand with a nick, corresponding to the junction between strands from the two initiation sites. The 5' end of the RNA has strong promoter sites for transcription by cellular RNA polymerase II.

Lytic infection of a cell occurs with the synthesis of structural proteins encoded by the *gag* , *pol* and *env* genes. There are two distinct mRNAs produced from the nuclear DNA for this step. Firstly there are full length transcripts from which the gag and pol proteins are translated. Initially a polyprotein spanning both the *gag* and *pol* genes is produced. This occurs despite a stop codon and a frame shift at the end of the *gag* gene. The stop codon is weak in that approximately one time in ten translation proceeds to the end of the transcript. This ensures that gag proteins are more abundant than pol proteins, as would be required for capsid assembly. The method by which a frame shift can be read through successfully has been described for Moloney murine leukaemia virus (Yoshinaka *et al.*, 1985) and may apply to HIV. A protease encoded by the 5' end of the *pol* gene divides the gag polyprotein into functional units during assembly of the virion. Env proteins are translated from a short, separate mRNA. An infected cell also produces genomic RNA for incorporation into the assembling virions during the latter stages of the replicative cycle.

REGULATION OF GENE EXPRESSION

The *sor* , *tat* , *art/trs* and *3'orf* genes are thought to encode regulatory proteins. Transfection of cells with full length DNA copies of HIV with deletions of each of the proteins has been used to investigate their roles in *in vitro* virus replication. Deletion mutants lacking *3'orf* grow *in vitro* (Fisher *et al.*, 1986b), while those lacking *sor* can replicate, but more slowly (Sodroski *et al.*, 1986b). More recently, sor has been shown to be essential for infectivity. Strebel *et al.*, (1987) showed that replication in culture of *sor* deficient mutants was only possible because of direct cell to cell spread, rather than through infection by extracellular virus in the supernate. Released virions with *sor* deletions were shown to be relatively non-infectious. Fisher *et al.*, (1987) showed that all stages in the replication of *sor* deficient mutants were normal, and postulated that the effect of sor was "post-translational". Although this might be a maturation or packaging function, she also considered that sor might actually be present in the virion, albeit in small amounts, and have a function in the early stages of infection. With the notable economy in coding sequences of HIV (and viruses in general), it is likely that 3'orf also plays an essential role in natural infection. Furthermore, patients infected with HIV develop antibodies to the 3'orf protein, indicating that it is expressed in natural infection (Arya & Gallo, 1986).

Mutants deficient in *tat* and tat/trs do not replicate *in vitro* or induce cytopathic effects (Dayton *et al.*, 1986; Fisher *et al.*, 1986a; Sodroski *et al.*, 1986a). Both gene products have been shown to have trans-activating functions, although their sites of action are not entirely clear. There is serological evidence for *tat* and

art/trs gene translation (Feinberg *et al.*, 1986; Knight *et al.*, 1987). It is unfortunate that different methods of investigation of the roles of *tat* have yielded conflicting results. Based on previous work by Sodroski *et al.*, (1985) on transactivation of HTLV-1 gene expression by pX, Cullen (1986) attached the 5' LTR promoter sequence to either chloramphenicol or interleukin-2 genes and found increased levels of mRNA production in the presence of tat. This is evidence for control at a transcriptional level, although it has been pointed out that the increased level of transcripts may be due to a stabilising effect of tat on these mRNAs).

On the other hand, deletion mutants of HIV lacking *tat* gene expression were shown to have normal levels of viral mRNAs, but failed to produce viral proteins (Rosen *et al.*, 1986; Feinberg *et al.*, 1986). This is evidence for regulation by tat at a translational level. The problem is not resolved at present. Disparate roles for art/trs have also been described. Sodroski *et al.*, (1986a) found normal levels of mRNAs encoding gag and env proteins, but reduced rates of synthesis (ie a similar effect to that found in *tat* deletion mutants). On the other hand, Feinberg *et al.*, (1986) found depressed levels of gag/pol and env mRNAs and disproportionately high levels of multiply spliced 2000 base pair mRNAs encoding non-structural proteins. Again, different investigators have found regulation at different levels of gene expression. Knight *et al.*, (1987) describe experiments that indicate a role for art/trs in trans-activation of the env transcript. Unlike cellular mRNAs, the promoter sequence of the env mRNA appears to have an intrinsic translational block, possibly mediated by loop formation from palindromic sequences, and that intervention from the 19 KDa protein is required for translation.

These experiments were generally performed in cells that supported a lytic cycle of infection, without the establishment of latency. It is therefore very difficult to speculate on what the role of the regulatory proteins might be in a real infection. HIV is known to infect a wide range of cell types, including T-lymphocytes, macrophages and astrocytes in the brain. It is speculated that, during the latent stage, the non-structural proteins mediate a transcriptional or translational block that is released on reactivation of the virus. HIV manages to continue to replicate in man in the presence of specific humoral and cell-mediated immunity. Cells bearing viral antigens would be expected to be rapidly destroyed by the immune system. An attractive theory for HIV reactivation supposes that the process is divided into two distinct phases. Firstly, the relatively slow process of mRNA and genomic RNA synthesis is carried out in the absence of translation of viral proteins. During the next stage, viral proteins are rapidly synthesised from the accumulated mRNAs, and the virions assembled before destruction of the cell.

To achieve this, HIV would require co-ordinated control at several levels of viral gene expression, necessitating a simultaneous switch to genomic and structural protein RNA synthesis followed by a co-ordinated derepression of translation. This model is consistent with the known ability of HIV to continue replication in man despite demonstrable specific immune responses to the virus (see below). It may also explain why only a small proportion of circulating T-cells in infected patients appear to contain viral proteins.

HIV STRUCTURAL PROTEINS

The sequence and arrangement of genes encoding the structural proteins resembles that of other retroviruses, and there are functional homologies between the proteins they encode. Figure 2 shows the translation and subsequent cleavage of HIV encoded proteins. The gag and pol proteins are relatively well conserved from an evolutionary point of view, but the env proteins are diverse. In particular, the env proteins of lentiviruses appear to have specific features to evade host immune responses (see below).

REVERSE TRANSCRIPTASE

The *pol* gene products include a 33 KDa endonuclease, a protease for viral protein cleavage and a 65 KDa glycosylated protein, subsequently cleaved to a 51 KDa form. Both forms have been shown to have reverse transcriptase activity, and both are found in the purified virion along with endonuclease. The functional significance of the two forms is unknown. Reverse transcriptase is the target for current anti-HIV drugs, of which 3'-azido-3'-thymidine (AZT) is the best known example, and which operates by mimicking thymidine and disrupting reverse transcription. Cellular thymidine kinase converts AZT to the monophosphate form, and thymydylate kinase and TDP kinase convert it to the triphosphate form. This is incorporated into DNA by HIV-specified RT but not by cellular DNA polymerase, and herein lies the basis of the drugs specificity. It does not have the dual recognition safety of ACG (that requires both virally encoded TK and DNA polymerase for activity), and is significantly cytotoxic. Larder *et al.*, (1987) investigated the substrate binding areas of the enzyme

by means of site-specific mutagenesis in attempts to design new drugs with greater specificity for HIV. Preliminary clinical trials of the efficacy of AZT indicate that only temporary remission of disease is achievable (Yarchoan *et al.*, 1986), and there may be worsening of the disease on cessation of treatment. AZT is inactive against the virus in its the latent form. A more effective drug would be one that inhibited reactivation of virus, rather than one that only operated during the initial stages of infection.

CAPSID PROTEINS

The capsid comprises predominantly p24, p17 and p13, the three cleavage products of the precursor *gag* gene product. The arrangement of the capsid proteins is unknown, and is likely to be complex as variable amounts of uncleaved precursor proteins are also found in the capsid. p13 has been shown to have RNA binding activity. None of the gag proteins are glycosylated. On natural infection, high levels of non-neutralising antibody to p24 are induced, with more variable reactivity with p17 and p13. The epitopes of p24 and p17 have been investigated by monoclonal antibodies. Ferns *et al.*, (1987) found reactivity of monoclonals with p24 and p17 to be mutually exclusive as would be expected. p17 monoclonals inhibited each other from binding indicating that they recognised a single antigenic site on the protein. p24 monoclonals did not normally cross-inhibit each other suggesting reactivity with several different antigenic determinants on the protein. However certain p24 monclonals were cross-inhibited by those reactive with p17, suggesting an antigenic area comprising both proteins. The data are, however, preliminary and would require corroboration. In particular, some monoclonals apparently failed to

inhibit their own binding in this experimental set-up. An apparent variability in the capsid proteins between isolates was indicated by the finding that recent African isolates of HIV were not recognised by several of the p24 monoclonals. Variation in the p24 protein was described by Higgins *et al.*, (1986). who showed that isolates from patients in the San Franscisco area showed differing reactivities with monoclonals, allowing them to be subtyped into those resembling the original HTLV-III islate of Gallo, and those resembling the Californian AIDS-related virus (ARV) strain.

ENVELOPE PROTEINS

The *env* gene encodes a 160 KDa membrane precursor protein. Cleavage by cellular proteases produces a C-terminal 41 KDa portion containing the transmembrane anchor region, and an external 120 KDa portion. Disulphide linkages established before cleavage hold the two fragments together, although shedding of gp120 can be demonstrated. gp41 contains approximately 350 amino acids, of which 150 are within the envelope. The reason for the large internal domain is not known. A sequence of 23 hydrophobic amino acids is thought to span the envelope. The external portion of the protein contains 4 sites for N-linked glycosylation.

gp120 is 480 amino acids long and contains several cysteine residues that determine the conformation of the protein, and mediate attachment to gp41. The protein has a total of 27 potential N-linked glycosylation sites. There is also evidence for O-linked glycosylation, so the fully processed protein would be considerably modified antigenically. Chang *et al.*, (1985) showed that env antibody

reacted predominantly with the C-terminal portion of gp120 and the adjacent attached gp41 region, both regions with relatively little glycosylation.

Comparison of isolates from different geographical areas shows greater variability in the env region that any of the other structural protein genes. Furthermore, within the gp120 region, certain areas are more variable than others. Certain regions encoding hydrophilic loops are found to be highly variable. Many of the potential glycosylation sites are found in such areas. Comparison of African and French isolates showed that half of the sites of N-linked glycosylation were not conserved (Alizon *et al.*, 1986), and this would be expected to affect profoundly the antigenicity of the proteins. The three hypervariable regions identified were bounded by conserved cysteines suggesting a common folding pattern. The requirement for a functional T4 receptor (see below) may account for the relative sequence conservation of 3 other regions of the protein.

VIRUS ATTACHMENT

Unlike HSV, the nature of both the viral and cellular receptors has been established. There is overwhelming evidence that a T4 phenotype is necessary for infection by HIV. Firstly, monoclonal antibodies reactive with the T4 epitope have been shown to block the attachment of HIV (Dalgleish *et al.*, 1984; Klatzmann *et al.*, 1984). Secondly, MacDougal *et al.*, (1986) described the formation of gp120-T4 complexes *in vitro* . Thirdly, several T- and B-cell and fibroblast human cell lines were rendered susceptible to HIV infection by prior transfection of the cells with the complete DNA sequence for the T4

receptor (Maddon *et al.*, 1986). It was thought that the ability of HIV to infect cells other than T-helper cells contradicted the role of T4 in adsorption. However, not only macrophages, but also microglial cells of the brain and endothelial cells of the intestine have subsequently been shown to show the T4 phenotype.

It was surmised that gp120 was responsible for attachment of HIV to its target, on analogy with other retroviruses, and by the observation that gp120, on its own, could cause syncytium formation in cells of the T4 phenotype (Lifson *et al.*, 1986). HIV was shown to bind to mouse cells transfected with the T4 gene but there was failure to internalise the virus. This suggests a secondary interaction, possibly between gp41 and another cellular protein (Maddon *et al.*, 1986).

According to one hypothesis, the regions within the gp120 protein that interact with T4 are likely to be highly conserved due to functional constraints. However, if there is immunological pressure for HIV to mutate in order to evade neutralisation, then only variation in the T4 binding site could be expected to be effective. This second hypothesis would predict neutralisation sites to be located in the variable regions. Sequence data indicates that the residues between 37-130, 211-289 and 488-530 are relatively invariant between French and African isolates (Alizon *et al.*, 1986). Ho *et al.*, (1987) raised antisera to synthetic oligopeptides corresponding to various regions of gp120. Ten of the 22 antisera reacted with native gp120, and of these, only four were neutralising. They corrresponded to residues 280-293, 458-484, 616-632 and 728-751. Overall, it seems that the neutralisation sites are not located in the conserved areas, and the data supports the second hypothesis. This data is preliminary and further work is required.

In summary, HIV causes a diverse and prolonged disease in humans. The regulation of HIV replication suggests methods by which the virus could maintain a state of latency. The heavy glycosylation and variability of the viral envelope may indicate that immunological pressures have shaped the evolution of HIV. The role that the T4 receptor plays in virus attachment accounts for the observed tropisms of HIV for various organs in the body.

CHAPTER 4

PATHOGENESIS OF HIV INFECTION

The processes by which HIV causes disease in humans have not been determined, despite the great advances made in understanding the virology of infection. This section will give an account of the principal laboratory markers of HIV infection, particularly those of immunosuppression. The cell-mediated and humoral immune responses to infection will be discussed. Finally, the numerous theories concerning the pathogenesis will be reviewed.

MARKERS OF INFECTION

The AIDS syndrome was described and investigated thoroughly before the discovery of HIV. Such studies could not investigate asymptomatic carriers and there is still a relative lack of information about this group of patients. In AIDS patients, it is difficult to distinguish the primary effects of infection with HIV from abnormalities arising from secondary infection, cancers, or simply prolonged illness. There was a tendency to attribute the entire range of AIDS symptomatology to immunosuppression. The discovery of HIV led to the identification by serology of a large number of infected but asymptomatic carriers. This has led to an intensive area of research to investigate the markers, pathogenesis and causes of progression to AIDS. This is a particularly interesting area, since the time course of progression is so variable between patients; furthermore, it is not known whether progression to

AIDS is inevitable or if some patients can keep the disease in check indefinitely. In terms of drug treatment, it would appear easier to prevent progression than to prevent primary infection.

EXPOSURE & SEROCONVERSION (GROUP I)

It is difficult to study the events around seroconversion because of the non-specificity of the clinical signs and the lack of a readily available animal model. Most information has come from retrospective serological testing, case studies and the experimental inoculation of chimpanzees. The specific markers of initial infection are viraemia, followed by the appearance of HIV-specific antibody.

There is some evidence that exposure does not necessarily lead to infection. This was noted in a study by Burger *et al.*, (1984) who described the absence of seroconversion in the wife of a haemophiliac infected with HIV. IgM was detected in the serum of the wife during exposure, but it disappeared on cessation of sexual contact. This observation is similar to that reported by Fultz *et al.*, (1986) who also detected a transient IgM response in a chimpanzee sharing a cage with another experimentally infected with HIV. It is not known whether infection is more readily transmitted by infected cells or by free virus. Obviously, free virus is infectious since factor VIII regularly infects haemophiliacs. Foreign cells would also be destroyed rapidly by the recipient, so there would always be an extracellular phase during the infectious process. Plasma from patients with HTLV-I and -II is not infectious; it has been shown that infection requires the inoculation of cells from an infected patient (Okochi *et al.*, 1984).

The interval between exposure and seroconversion is not known with certainty, but appears to be prolonged in certain documented cases. Much of the evidence is anecdotal, and has recently been reviewed by Pinching (1987). There is a general consensus that clinical illness associated with primary HIV infection precedes the appearance of HIV-specific IgG. Cooper *et al.*, (1985) describe infection in twelve male homosexuals. The times between exposure and illness and seroconversion could not be accurately established in this study, but were generally considered to be relatively short. In one patient, it was estimated that 6 days elapsed between exposure and illness. The time to seroconversion was in the range 19 to 56 days in three others. Features of the clinical illness described included fever, myalgia, malaise, lymphadenopathy and sore throat. A maculopapular rash affecting the trunk was found in half of the patients. Studies of T-cells showed transient elevation of T8 (cytotoxic/suppressor) cell numbers with relatively unchanged numbers of T4 (helper) cells. These T-cell findings are typical of many acute viral illnesses and do not reflect an abnormal immunological response.

In a well documented needlestick incident (Editorial, 1985), blood from a patient infected with HIV was inoculated accidently into the hand of a nurse. The nurse developed an infectious-mononucleosis -like illness by the thirteenth day; by 47 days she seroconverted for antibody. Tucker *et al.*, (1985) describe the illness associated with primary HIV infection in a haemophiliac transfused with contaminated factor VIII during a synovectomy operation. Transfusion was given over several weeks so the time of exposure could not be exactly determined. Features of the acute illness could be related to the time of seroconversion. Rash and splenomegaly preceded the seroconversion by about a week, while lymphadenopathy was first recorded one week after

the development of antibody. This is unlike the illness described by other investigators. Furthermore, a transient fall in T4 numbers was also detected, while T8 numbers were erratic. The authors found a transient depression in the T4/T8 ratio. However intercurrent bacterial infection during the operation and the known abnormalities in haemophiliac immune function may have accounted for these results.

Gaines *et al.*, (1987) describe symptomatic infection in a cohort of 20 male homosexuals in a prospective study and give accurate information on the time to seroconversion. The time from exposure to illness was determined in each patient and found to lie in the range of 11 to 28 days, while HIV-specific antibody was detected 2 to 7 weeks after the initial illness. Prominent symptoms reported included fever, sore throat, lymphadenopathy and rash. One patient had a transient meningoencephalitis. Laboratory evidence of thrombocytopoenia was found in twelve patients, while 15 showed varying degrees of leucopoenia. T4 and T8 numbers were not recorded. Denning *et al.*, (1987) and Carne *et al.*, (1985) describe acute myelopathy and encephalopathy in association with primary infection. Accepting the reservations about the applicability of animal models, Alter *et al.*, (1984) report an interval of about six weeks between inoculation and seroconversion in captive chimpanzees.

Variation in the specificities for individual HIV proteins has been reported during seroconversion. Using the immunoblotting technique, antibody to p24, a core protein, is normally the first specificity to be detected after seroconversion (Lange *et al.*, 1986a; Groopman *et al.*, 1986; Schuepbach *et al.*, 1985). The consensus between these papers is that p24 reactivity is shortly followed by antibody to pr55, the uncleaved gag precursor protein. Antibody to gp41 appears at a

much later stage, while anti-gp120 may not be detected at all in many patients. All patients eventually develop antibody to pol gene products, RT and endonuclease. Investigators using the RIP technique have reported different findings; the first signs of seroconversion are marked by the appearance of antibody to gp160, followed by gp120 and p24 (Gaines *et al.*, 1987). Results from their RIP assay showed that two patients developed antibody to gp160, at a time when they were negative for antibody in the conventional IB assay. p24 antibody reactivity first appeared several days later in these two patients in the latter assay. Two groups have shown that the RIP method is more sensitive for antibody to env proteins than the IB assay (Ulstrup *et al.*, 1986; Barin *et al.*, 1985) in convalescent sera, and this was advanced as the explanation for the discrepancy in the results of the seroconversion studies.

Allain *et al.*, (1986) used a new ELISA technique to study antibody responses to a recombinant *env* gene product (an unglycosylated gp41 protein, including a small part of gp120) and a similarly prepared synthetic p24 antigen. The method of preparation ensured that there was no contamination with other HIV proteins. Sera from 40 haemophiliacs taken around the time of seroconversion were tested retrospectively for antibody to the "env" and "core" proteins. The timing of samples made the analysis of the results difficult, but anti-"env" antibody was detected before anti-"core" in 21 patients and these results resemble those of the RIP assay.

Using an ELISA for HIV antigen, Allain *et al.*, (1986) reported the detection of free virus in the sera of 14 haemophiliacs before seroconversion. The antigenaemia was also shown to co-exist with anti-"env" antibody but became undetectable on development of antibody

to p24. The hypothesis advanced to explain these findings was that there was relative abundance of capsid proteins over envelope proteins and the former were produced in such large amounts on primary infection that it could overwhelm the initially restricted amounts of antibody to p24. During resolution of the viraemia and the detection of anti-"core", it would be predicted that immune complexes would be found. The clinical evidence of rash and myalgia, two immune complex phenomena, during primary illness would support this hypothesis.

The duration of the antigenaemia could not be accurately established due to the spacing of samples. A statistical estimate could be made on the basis that the prevalence of antigenaemia was 50% in a group where the frequency of sampling was on average 3.5 months, while in the group who were less frequently tested (5 months), the prevalence was reduced to 25%. There is, therefore, an association between sample spacing and detection of antigenaemia. Statistical analysis of this data was not attempted. Goudsmit *et al.*, (1986) used a similar antigen assay in a study of the seroconversions of male homosexuals. They reported a 31% incidence in antigenaemia where the average spacing of samples was 3 months. Data from the two groups will be discussed more fully later, since they are directly comparable to work carried out in this thesis. Other investigators have confirmed the existence of an antigenaemia before seroconversion; Wall *et al.*, (1987) report simultaneous antigenaemia and primary illness in two patients, followed by seroconversions 6-8 weeks later. There is currently little evidence for prolonged antigenaemia in the absence of seroconversion.

Parry & Mortimer (1986) described the development of an assay to detect HIV-specific IgM, and its application to the study of seroconversions. In contrast to many viral infections, IgM is not a prominent feature of the early immune response, and may not be detected at all in some patients. Where it is detected, it is generally present at low concentrations. Bedarida *et al.*, (1986) found IgM in sera of patients preceding seroconversion using an IB assay with labelled anti-human IgM. IgM was detected in three of the five experimentally inoculated chimpanzees by direct binding ELISA (Fultz *et al.*, 1986). No IgM was detected in the remaining two, despite daily testing. In the others, IgM was detectable 1-2 weeks before the appearance of HIV-specific IgG. The problems of measuring IgM in the presence of IgG, and the possible effects IgM would have on RIP and competition ELISAs are discussed more fully later.

ASYMPTOMATIC INFECTION (GROUP II)

As mentioned previously, patients classified as group II may show minor degrees of immunosuppression or constitutional disease. This is frequently reflected in a fall in absolute numbers of T4 cells, or, more commonly, a rise in T8 cell numbers (Horsburgh *et al.*, 1986; Melbye *et al.*, 1986). A sudden fall in T4 numbers has been shown to be a poor prognostic sign (Biggar *et al.*, 1985), correlating with progression to ARC or AIDS. Weber *et al.*, (1986) reported, however, that the variation of T4 numbers in promiscuous male homosexuals tended to vary greatly for other reasons and they could not find a correlation between T4 numbers and progression. Asymptomatic patients show a reduced proliferative response to mitogens, such as phytohaemagglutinin (PHA), and specific antigen in an *in vitro* assay

of helper T cell function. Hypergammaglobulinaemia, a feature reported in AIDS patients, is also found to a lesser extent in asymptomatic patients (Nicholson *et al.*, 1985; Jaffe *et al.*, 1985)

Antibody to HIV is present in patients during the asymtomatic period. Antibody has been detected to all of the structural and non-structural proteins of HIV. The presence of antibody is a reliable indicator of past exposure, and is the method of choice in screening blood for infectivity. Much data has been presented on the relative sensitivities of currently used tests for HIV antibody. In general terms, there is no significant difference between the results of the different tests, whether they be direct-binding, competitive or capture ELISA's, indirect immunofluorescence or haemagglutination assays. The discrepancies arise from testing sera collected around seroconversion, where some of the less sensitive tests fail to detect the very low levels of antibody, often with restricted target specificity, found in sera from patients at this time. This is discussed later.

Antibody levels to HIV have been reported to be reduced in patients with prodromal AIDS (Biggar *et al.*, 1985). Although there is reported to be no change in titre to gp41 (Weber *et al.*, 1987), there is accumulating evidence that there is a characteristic reduction in antibody to gag proteins in patients progressing to AIDS. Schuepbach *et al.*, (1985) first noted a reduction in p24 reactivity in an IB assay in patients who progressed to AIDS, but also reported greater reactivity with gp41 in such patients compared with patients in the early stages of the disease. Essentially similar findings were reported by Lange *et al.*, (1986b) in a larger study of male homosexuals. Whether the decline in p24 antibody levels was the result

of immunosorption of antibody by an increased rate of virus replication and release, or whether it was due to clonal exhaustion of the B cells producing HIV antibody was not resolved. Lange *et al.*, (1986c) described the testing for p24 antibody of sequential samples from a cohort of children infected with a single plasma donor. They found an association between p24 antibody and lack of disease progression (but not the converse); similar findings with adult patients were made in two independent cohorts of infected patients from London (Weber *et al.*, 1987).

Groopman *et al.*, (1986) contested these data and showed that by extending the incubation times in the IB assay, p24 reactivity could be detected in all patients in their cohort, whether symptomatic or not. They did not, however, titrate sera for p24 antibody; if they had, it is likely that they would have found higher titres in the asymptomatic group and would have arrived at essentially the same conclusion as the other investigators. They also advanced the hypothesis that lack of reactivity with gp41 observed in some patients in their study group indicated the existence of variant forms of gp41 encoded by the virus infecting those patients IB assay. This is unlikely.

Lange *et al.*, (1986a) used IB, RIP assays for HIV antibody and an ELISA for HIV serum antigen detection in a follow-up study of a cohort of male homosexuals. They found a strong correlation between disappearance of p24 antibody and subsequent antigenaemia. This is analogous, but opposite, to the course of events during seroconversion. The detection of antigen late in infection was surprising in view of the similarities to HIV seroconversion with that of HBV. In the latter case, infection is

accompanied by an antigenaemia which resolves on development of anti-surface antigen (anti-HBs). Reappearance of surface antigen does not occur in patients with anti-HBs. Goudsmit *et al.*, (1987a) also detected HIV antigenaemia in their cohort, in association with disease progression, although significantly, there were patients who were persistently antigenaemic, negative for p24 antibody but who remained well. Antigen has also been detected in the cerebrospinal fluid of patients with HIV infection (Goudsmit *et al.*, 1986). The risk of progression differs in different groups of patients. In particular, children have been reported to have a high rate of antigenaemia, low p24 reactivity, while remaining symptom-free (Lange *et al.*, 1986c). A similar picture may be seen in chimpanzees, where there is prolonged antigenaemia, low levels of p24 antibody (Goudsmit *et al.*, 1987b), and no progression to overt AIDS.

The relationship between infectivity and antigen level has not been established, although there is much evidence that antigen-negative blood may be infectious. By analogy with HBV, it is possible that the antigenaemia, where there is apparently excessive production of core proteins over those of the envelope, may indicate defective virus production, and not increased infectivity. Virus may be isolated from patients at all stages of infection, including from those who are antigen negative and with high anti-p24 levels. Most investigators report that it is more difficult to isolate from patients in the terminal stages of AIDS, where there may be high serum antigen levels. This is most likely to be a function of the poor proliferative response of T cells during the isolation procedure.

Neutralising antibody may be detected in the convalescent stage of HIV infection, but only from a proportion of patients and at generally low

levels (Folkes *et al.*, 1985; Weiss *et al.*, 1985; Robert-Guroff *et al.*, 1985). Weber *et al.*, (1987) investigated the neutralising ability of sera from patients infected with HIV and related the data to the rate of progression to AIDS. There was a slight trend towards increasing neuralisation titre with time in the patients, but no independent correlation with disease progression. The low neutralisation titres to HIV generally observed, compared with those found in HTLV-I infection, probably accounts for the increased transmissability of HIV in cell-free inoculation. HTLV-I typically induces neutralisation titres 300 fold higher than HIV; this may explain why HTLV-I infection can only be acquired by cell inoculation.

PERSISTENT GENERALISED LYMPHADENOPATHY (GROUP III)

This is a poorly defined group and probably encompasses several distinct pathologies. Janossy *et al.*, (1985) present evidence that there are two distinct histological changes found in lymph nodes, each with a distinct prognosis. Details of the histology are complex, but are reviewed by Racz *et al.*, (1986). In summary, there may be an explosive follicular hypertrophy in patients who Janossy regarded as having a good prognosis, while degenerative changes were seen in those with prodromal AIDS (Fernandez *et al.*, 1983). Further studies are necessary to confirm this correlation, and to investigate the relationship with the the two distinct changes in T cell subset numbers (ie. rise in T8 numbers or fall in T4) observed in patients in groups II and III.

ARC and AIDS (GROUP IV)

The laboratory markers of advanced disease vary between patients, and there is always the possiblilty that secondary infections and cancers might obscure the underlying pathological changes. The most striking feature of AIDS is the depletion in T4 cell numbers; T8 numbers may be elevated or relatively normal. There is some evidence that T4 cells of different function might be differentially affected. The numbers of T4 cells involved in the lymphoproliferative response to antigenic stimuli may be relatively unaffected, with a relatively greater reduction of T4 cells effecting the DTH response (Nicholson *et al.*, 1984).

T4 cells show impairment of function in addition to reduced numbers. There is a reduced proliferative response to pokeweed mitogen and failure to provide help for immunoglobulin production in an *in vitro* assay of T cell function (Lane *et al.*, 1983). Reduced lymphoproliferative responses have also been reported for CMV and HSV antigens (Hersch *et al.*, 1985). The proliferative response to HIV antigen was investigated by Wahren *et al.*, (1987) in asymptomatic and unwell patients (PGL, ARC and AIDS). Patients with advanced disease showed poor responses to HIV compared with aymptomatic patients. There was a selective reduction in the proliferation to whole HIV virions; interestingly the response to individual virion components (p24, gp41 and gp120) was greater than that to the whole virus; overall, p24 elicited the strongest proliferative response. HIV proteins did not inhibit the proliferative response to HSV or CMV, ruling out a non-specific immunosuppressive effect of the virus. Interleukin-2 increased the response to antigens and mitogens, and the reduced sensitivity of helper cells for interleukin-2 has been advanced as an

explanation for their functional abnormalities (Gluckmann *et al.*, 1985).

DTH responses are reported to be reduced in AIDS patients on skin challenge; such patients also show a failure to produce granulomas. Monocytes reduced in number and there is a defect in the maturation process to macrophages (Hersch *et al.*, 1985). Macrophage function was found to be defective in AIDS patients; the recent discovery of HIV infection in cells of the monocyte line may account for this, although the effect described may be due to a failure of T cell-macrophage cooperation. γ-interferon can restore the ability of macrophages from AIDS patients to phagocytose candida (Murray *et al.*, 1984; Estevier *et al.*, 1986).

Several studies have investigated cytotoxic T-cell responses to HIV antigens. Plata *et al.*, (1987) found a high proportion of alveloar macrophages from AIDS patients to be infected with HIV. They were killed by autologous cytotoxic T cells in three of four patients tested; killing was shown to be class I restricted. Cross-reactivity between the effector and target cells was demonstrable between patients of the same HLA type. This indicates that HIV strain variation was not a significant factor in the effectiveness of the cytotoxic response. The authors suggest that a component of the lung pathology observed in AIDS (interstitial pneumonitis) may be due to infiltration of cytotoxic T cells. Walker *et al.*, (1987) used a vaccinia recombinant with inserted *env* or *gag/pol* genes to infect B cells. T cells from the patients were then incubated with the infected cells in a chromium release assay. Specific cell lysis of cells expressing gag/pol proteins was found in three of the eight patients, while all patients killed env-expressing cells. Neither

paper gives data on the relation between CTL activity and progression. Only this would show whether there was a defect in HIV-specific cytotoxicity concomitant with other defects in immunity. CTL reactivity with other antigens was shown to reduced in AIDS patients (Sharma & Gupta, 1985).

B cells are present in normal numbers in AIDS patients although they show abnormal function. There is a polyclonal hypergammaglobulinaemia, with IgG levels typically being raised 50% above their normal value. Titres of antibody to previously encountered antigen are similarly raised. More striking is the failure of AIDS patients to develop an antibody response to neoantigens, or to show a rise in titre to recall antigens (Lane *et al.*, 1983). Immune complexes are frequently detected; this may indicate immune dysfunction (Euler *et al.*, 1985), but it may also reflect the HIV antigenaemia that often accompanies advanced disease. Morrow *et al.*, (1986) analysed polyethylene glycol precipitates of sera from AIDS patients and detected HIV core proteins in the immune complexes by immunoblotting. Lange *et al.*, (1987) detected HIV antigen in the immune complexes of in a wide range of HIV-infected patients. They used a modified ELISA after disassociation of the complexes, and were able to find antigen in patients who were negative in the conventional ELISA test for antigen.

In summary, the main feature of AIDS is a reduction and loss of function of T4 cells. At present it is not known whether the B cell and macrophage defects are secondary to this, or reflect a different pathological mechanism. The current evidence shows, increasingly, that HIV infects a wide range of cells in the body, and macrophages are considered by some to be the main target of HIV, and the pathogenesis of HIV infection may involve more than simple T4 cell destruction.

THEORIES OF PATHOGENESIS

Many theories have been advanced to explain various aspects of the pathogenesis of HIV infection. The simple theory originally held was that HIV was an infection of T4 helper cells, and that the disease was caused by a progressive destruction of these cells with a concomitant loss of immune function. Several lines of evidence challenge this version of events.

Firstly, sequence data showed HIV to be related more closely to visna and other lentiviruses than to HTLV-I and -II. Lentiviruses are known to cause a range of diseases independently of immunosuppression, and to be able to infect a wide range of different cell types, in particular monocytes. HIV has been shown to infect a high percentage of lung macrophages (Plata *et al.*, 1987) and cells from the brain (Gartner at al., 1986). There is also evidence that macrophages mediate the copious diarrhoea via inflammatory responses in the bowel, and cause the weight loss observed in patients with HIV by release of cachexin (Torti *et al.*, 1985).

Secondly, HIV appears to be resistant to neutralisation as compared with HTLV-I and -II, with demonstrable consequences in transmission and spread in the body. HIV causes disseminated disease in the face of an immune response whereas HTLV-I remains confined to the T cells that were originally infected. Thirdly, widespread infection of T4 helper cells in HIV-infected patients, even those with advanced disease, could not be readily demonstrated when hybridisation probes were developed and used to test for virus-specific sequences. Harper *et al.*, (1986) found only 1 T4 cell in 20 000-100 000 to contain specific sequences of either RNA or DNA. Fourthly, while it is appreciated that

HIV induces syncytium formation in T cells *in vitro* as part of the infectious process, such syncytia have been rarely observed in lymphocytes from infected patients; the only evidence for a cytopathic effect *in vivo* is the description of giant cells in the brain of infected individuals (Vaseux *et al.*, 1986).

This last section of the introduction will review current theories of HIV infection. Many are contradictory, while others could be considered to describe different aspects of the disease.

RESISTANCE TO THE IMMUNE SYSTEM

It is necessary to explain how HIV infection progresses in the face of an immune response. The neutralisation titres of patients with HIV are generally around 1/10 while those induced by HTLV-I infection are 1/3000-4000 (Weiss *et al.*, 1985). There is circumstantial evidence that the high degree of glycosylation of gp120 of HIV is responsible; HIV has 19 potential sites for N-linked glycosylation, while HTLV-1 has only two. The extra glycosylation may modulate the immunogenicity of the protein by shielding the amino acid chain from recognition. The existence of a strongly immunogenic carbohydrate epitope on gC-1 of HSV (see chapter 1) indicates that this may not be the entire explanation.

Equine infectious anaemia virus (EIAV) has been shown to undergo rapid point mutation in the envelope region of the genome in order to evade neutralisation (Montelaro *et al.*, 1984). Infection is characterised by episodic reactivations of virus. Each replicative cycle produces virus that is resistant to neutralisation by antibody collected during

previous episodes (Salinovitch *et al.*, 1986). This is clear evidence for rapid evolution providing a method by which virus may evade the immune system. Visna virus also shows rapid evolution of the env protein on experimental infection of sheep, but the inoculum strain persists alongside the new variants (Lutley *et al.*, 1983). This is evidence that immunologically driven variation is not a feature of infection with this virus.

The *env* region of HIV does show relatively high rates of variability in sequential isolates from the same patient (Hahn *et al.*, 1986), but less than between isolates taken from different patients, which is, in turn, less than that between isolates from different geographical areas (Alizon *et al.*, 1986; Starcich *et al.*, 1986; Hahn *et al.*, 1986). The details of gp120 variability have been discussed in chapter 3. The sequence data comparing sequential isolates from the same patient require caution in their interpretation. Bolton *et al.*, (1987) showed that the isolation procedure may in some circumstances peripheral blood lymphocytes (PBLs) imultaneously in the same patient. More specifically, they showed that PBLs in a sample from an infected patient contained two antigenically distinct strains. While both could be demonstrated on primary isolation, transfer to a permanent cell line led to the disappearance of one of the strains. Hahn *et al.*, (1986) used such a cell line in their isolation procedure, and therefore may have selected a variant form for the eventual sequencing study. The possibility remains, that, like visna, the original strain may have persisted in the patient alongside the variant they investigated.

Another explanation for the poor neutralising response to gp120 is that it resembles the MHC class 2 antigen and is therefore tolerated

by the immune system. On the basis that both gp120 and the MHC 2 antigen interact with the T4 receptor and that both are glycosylated, it is possible they might have a similar conformation, and cross-react serologically. Circumstantial evidence to support this theory again comes from the observation that HTLV-I, to which there is a strong neutralising response on infection, does not bind to a cell via a specific receptor. Sattenhau *et al.*, (1986) have presented evidence that there is limited sequence homology between the gp120 and MHC class II protein. Against this hypothesis is, firstly, the observation of high neutralising titres to viruses such as polio, EBV and many others that bind to specific cellular receptors, and, secondly, the generally low neutralising titres in laboratory animals experimentally immunised with HIV. Since the MHC antigens differ between species, there ought to be no immunological tolerance to the binding site of HIV. One way to prove or disprove the hypothesis would be to simply measure the reactivity of anti-gp120 antisera raised in animals with human MHC class 2 antigens.

Another theory for the poor immunogenicity of HIV envelope proteins comes from the observation of shedding of gp120 after release from the cell (Gelderblom *et al.*, 1985). The authors ascribed this to a failure of the gp41 and gp120 to form disulphide bridges as the precursor gp160 is cleaved on budding. Another possible explanation comes from the observation of a nonsense stop codon in some clones of HIV-2 and SIV *env* genes, present in the transmembrane anchor region of the protein (Franchini *et al.*, 1987; Chakrabarti *et al.*, 1987). If this were expressed, then the protein would be secreted rather than incorporated into the viral envelope. The stop codon is not found in HIV-1 *env* sequences, but this may be because the isolates have changed during the isolation procedure; some clones of HIV-2 also do

not have this codon.

gp120, shed in large amounts, could enhance the infectivity of the virus in a number of ways. It could temporarily absorb neutralising antibodies and allow the virus to survive the extracellular phase for long enough to infect other cells. Secreted gp120 might be able to mediate immunosuppression by binding to T helper cells and thus prevent a normal immune response. T4 bearing cells with complexed gp120 on the surface would be the target for cytotoxic T cells, ADCC by K cells or complement lysis (the latter two mediated by non-neutralising antibody). The wide distribution of the T4 marker on other cell types might explain the extensive inflammatory pathology seen in patients. gp120 mediated destruction of T4 positive cells resembles graft versus host disease that can be induced experimentally in mice. Shearer & Moser, (1986) describe the immunological abnormalities produced when mouse T cells are transferred to another mouse that is genetically identical apart from the MHC class 2 locus, and discuss the similarities with the immune dysfunction in AIDS patients.

An immune response may require the interaction of an antigen presenting cell (of macrophage origin) with an HIV-specific helper T cell. If a large proportion of the macrophages were infected by HIV, then such interactions may lead to a rapid depletion of HIV-specific cells and a selective defect in immune response to HIV. Klatzmann & Gluckmann (1986) advance this hypothesis as a possible explanation for the loss of p24 antibody reactivity with time in patients infected with HIV (see above). They explain the persistence of antibody to gp41 and gp120 by postulating that variation in the *env* gene may cause the original specific T cells to fail to recognise the modified form

during antigen presentation. There are several problems with this theory. Firstly, exposure to other viruses in HIV-infected patients should lead to selective loss of immunity to them by the same mechanism; this is not observed (HSV, EBV, CMV). Secondly, p24 antibody returns on AZT treatment, at the same time as the antigenaemia disappears. This suggests that p24 antibody disappears in advanced disease due to immunosorption by antigen rather than by a specific defect in immunity.

Another method of evading the immune response involves the phenomenom of latency discussed in chapter 3. This is the evidence for specific adaptations in HIV and other lentiviruses to enable them to cause silent infections of cells. At a later stage, the virus can replicate very quickly after removal of the block on translation of accumulated mRNAs; thus virus assembly and release may take place before destruction of the cell. Regulation of gene expression undoubtedly mediates latency of HIV, and cells infected in this way would not be susceptible to immunological challenge. In the case of visna virus, the targets of infection are known to be monocytes (Peluso *et al.*, 1985). Such cells are latently infected and their mobility in the circulation and the tissues allows rapid and wide dissemination of infection. Reactivation may then occur in several sites including the CNS where immunological responses are generally poor. Since the discovery of monocyte infection by HIV in humans, an analogy may be drawn with visna to provide an explanation for immunological evasion in the human disease (Haase, 1986). In particular, the theory explains the finding of large numbers of infected macrophages in the lungs of patients with AIDS (Plata *et al.*, 1987), and the subsequent inflammatory response by cytotoxic T cells. Spread of latently infected macrophages also accounts for the observed dissemination of

infection in the brain and the subsequent encephalopathy, and to the gut and subsequent enteropathy.

Endogenous retroviruses may contribute to the immunological evasion of HIV. One theory postulates that the *env* sequence resembles that of endogenous retroviruses, and that expression of the latter during ontogeny makes the immune system tolerant for this particular protein. Evidence for expression of endogenous retroviral products is discussed in Simpson, (1986), who proposes that many of the minor non H-2 histocompatability antigens in mice are retroviral products. However, none of the hybridisation experiments have found reactivity of *env* gene probes of HIV with human chromosomal material, indicating an absence of sequence homology between HIV and endogenous retroviruses.

A related theory proposes that HIV may recombine with sequences of such viruses integrated into the host cell DNA. HIV recombinants would therefore bear a proportion of tolerated antigens. Such viruses would, however, be immunogenic on transmision to another human. This theory predicts, therefore, that infection would be followed by rapid recombination events, and isolates taken subsequently will differ from the inoculum strain. Isolates from different patients would be predicted to differ more than sequential isolates from the same patients, since different individuals, unless related, inherit different endogenous retroviruses. To an extent, available sequence data does support this proposal (Hahn *et al.*, 1986), although there are alternative explanations for the findings (see above).

THEORIES OF IMMUNOSUPPRESSION

Although destruction of T4 cells plays a part in the immunosuppressive effect of HIV, particularly in the late stages of infection, several investigators have proposed alternative mechanisms to account for the reduced T cell help and other immune dysfunctions.

Infection of macrophages by VV has been shown to induce the production of a factor resembling interferon (Kennedy *et al.*, 1985). The relationship between it and ordinary interferon is not known, but it is thought to resemble the abnormal interferon induced in SLE in humans. It is suggestive that many of the symptoms of SLE resemble those of HIV infection. The factor produced during VV infection of sheep leads to abnormal macrophage function, and Denman, (1986) proposes that this contributes to the immunopathology, particularly the inflammatory changes in the lungs and brains in the absence of active viral infection in the majority of cells. The pathological changes consequent to abnormal interferon production resemble those seen in autoimmune disease; in particular there is a striking similarity to the arthropathy associated with SLE and other autoimmune disorders.

Ruddle (1986) proposed that lymphotoxin production mediates the lysis of infected T4 helper cells on viral replication. In particular, she proposes that the *tat* gene product has a direct effect on expression of the lymphotoxin gene. Lymphotoxin has been shown to be expressed during normal helper cell proliferative responses, although in high concentrations it is cytopathic. A recent paper (Stricker *et al.*, 1987) reports the discovery of antibody reactivity in AIDS patients to a 18 KDa protein expressed on the surface of PHA stimulated T cells,

but not on resting cells. The identity of this differentiation antigen is not known. Sera from patients who were uninfected or who were in the asymptomatic stage of HIV infection were reported not to react to this protein, while reactivity was found in all patients with ARC and AIDS. This antibody reactivity may be a useful marker for progression, but the authors also contend that this autoreactive antibody may contribute to helper cell destruction and mediate the immunosuppression observed in AIDS patients. Interestingly, lymphotoxin has the same MW as this protein, and they may be one and the same, particularly as they are both only expressed on differentiation. More recent work that looked for the presence of lymphotoxin in the supernate of HIV infected cells produced negative results (Ratner *et al.*, 1987). This, and the growing evidence of macrophage, rather then T-lymphocyte, infection in the pathogenesis of HIV makes the involvement of lymphotoxin unlikely.

Ziegler and Stites (1986) propose that immunosuppression arises from the possible cross-reactivity of gp120 and the MHC class 2 antigens. They discount the possibility that this would merely lead to tolerance of the T4 binding area of gp120, and they propose that HIV infection would lead to an autoimmune response to the class 2 antigens. In time, an anti-idiotypic response might then develop to the auto-antibodies; it would therefore be reactive with the T4 receptor. In view of the wide distribution of the T4 phenotype on many cells other than T helper cells, the improbabilty of the immune system mounting an immune response to the widely distibuted MHC antigen, and the general lack of evidence for naturally occurring anti-idiotypic responses, taken together make this theory extremely unlikely. The theory would also predict grave consequences for vaccination in humans with subunit vaccines comprised of gp120, since this too might lead to an

autoimmune response. Zagury, in correspondence with the journal, Nature, described the administration of a vaccine containing gp120 to himself and twelve African patients. Despite subsequent seroconversion, no ill effects have so far been reported. This is evidence against the above theory.

A glycoprotein of feline leukaemia virus (p15E) has been shown to have an immunosuppressive effect, preventing responses to a wide range of antigens and mitogens (Pahwa *et al.*, 1985). Sandstrom *et al.*, (1986) proposed that gp41 of HIV also had a non-specific immunsuppressive effect on the basis of the observation of reduced proliferative responses to mitogens in the presence of inactivated HIV virions. Wahren *et al.*, (1987) tested the immunosuppressive effect of individual viral components, gp120, gp41 and p24 and whole virion on the proliferative response to CMV or PHA. There was no difference in the response to the stimuli whether the viral proteins were present or not and they concluded that a direct immunosuppressive effect by HIV was unlikely.

SUMMARY

It seems likely that there is a primary involvement of macrophages in the pathogenesis of HIV infection, and that many of the features of HIV infection may be accounted for by macrophage dissemination in the infected patient and inflammatory changes at the sites of reactivation. HIV differs from other lentiviruses in the degree of immunosuppression caused. No theory at present explains the wide range of immunological abnormalities found in AIDS patients.

CHAPTER 5

PRELIMINARY WORK

The author had previously tested a wide range of sera for antibodies to HSV by immunoblotting (IB) and immunofluorescent (IF) techniques before embarking on the PhD thesis. Convalescent sera from patients with prior exposure to HSV showed marked reactivity in the IF assay, and reacted with several bands in the IB assay. However, sera from patients with primary infections with both HSV-1 and -2 showed only faint membrane fluorescence by IF and restricted reactivity with 4 bands of similar molecular weight in the IB assay. The identity of these bands (35-42 KDa) was not known; it seemed unlikely that they were glycoproteins in view of their small size (the smallest HSV glycoprotein is gD, MW 65 KDa). Reactivity of sera with only one or two of the 4 bands was rarely observed, suggesting that they were related antigenically. Substituting anti-human IgG with anti-human IgM in the IB assay showed that the target of IgM in these sera was the same as IgG.

Attempts were made to identify the target of this early antibody. The sera from patients with primary infection that showed restricted reactivity were tested by a live-cell IF assay. The purpose of this assay was to measure antibody reactivity with antigen expressed on the surface of the HSV infected cell; live cells were used to ensure the integrity of the cell membrane and prevent reactivity with HSV proteins present in the nucleus or cytoplasm. An assumption about this experiment was that only glycoproteins would be expressed on the cell surface. This was tested by comparing the reactivity of several

glycoprotein and non-glycoprotein monoclonals by the conventional IF assay using acetone fixed cells, and by a live cell assay. The results showed that the glycoprotein monoclonals reacted in both assays, while the p40 monoclonal and another against the major capsid protein (both non-glycoproteins) reacted only in the fixed cell assay. This showed that p40 was not on the surface of infected cells. In fact, p40 and the major capsid protein were only found in the nucleus in the fixed cell assay.

The reactivity of sera from patients with primary infection showing restricted reactivity in the IB assay and in the fixed and live cell IF assays was compared. In each case, reactivity with both live and fixed cells was found, and constituted preliminary evidence that the acute sera contained a hitherto undetected additional specificity for a glycoprotein not detected in the IB assay. At around this time, Ashley & Corey (1984) published results using a radioimmunoprecipitation (RIP) assay that showed that sera from patients with primary HSV-2 reacted predominantly with gB, a finding in agreement with the results of the IF assay.

AIMS

The aim of the first part of the work described here was to compare the sensitivity of different tests for antibody of differing specificity, and by testing the same sera by a number of tests, to establish which method detected antibody first during seroconversion. This work, while of interest in the study of humoral immune responses to HSV infection, was also of major use when setting up similar assays to detect antibody to HIV. In the latter case, the widespread use of screening for antibody to HIV as an indicator of past exposure to the virus and infectivity of the patient made the development of a test of high specificity and sensitivity a priority. High levels of antibody are elicited by infection with HIV and since they are maintained during all stages of the disease, it is relatively easy in the majority of patients to determine past exposure and hence infectivity. However, very sensitive tests are required for general screening in order to detect the very low levels of antibody during seroconversion.

By analogy with other viruses, notably HBV, it was hypothesised that patients would be most infectious during primary infection, at a time when antibody levels might be very low. This consideration was of particular interest to the Blood Transfusion Service (BTS), since they were considering which test to adopt in screening of donors. That HIV can be transmitted by blood and blood products was readily appreciated at the time, on the basis of case reports of patients transfused with infected blood, and, tragically, by the early discovery of AIDS and ARC in a high proportion of American and, later, European haemophiliacs using factor VIII concentrate. One of the aims of the thesis was to compare the sensitivity of different commercially available tests for antibody, and to develop other tests, notably IB,

using experience gained from work with HSV.

This work was aided considerably by the availability of a wide range of stored sera from differing patient groups. Of particular value in the seroconversion studies were sera from a cohort of haemophiliac patients under the care of Dr Ludlam. That they were infected with HIV was established in 1984 using the forerunner of the Wellcozyme enzyme immunoassay developed by Dr R. Tedder at the Middlesex Hospital, London. Discovery of past infection in these patients was a surprise since many had been maintained solely on locally produced factor VIII. Using patient records, it was possible to establish which batch of factor VIII led to infection of the haemophiliacs, and to calculate the time of exposure and relate this to the time of seroconversion. In total, 18 patients were shown to have been infected by the implicated batch (Ludlam *et al.*, 1985), and the stored serum samples were ideal for studying early antibody responses to infection, particularly in view of the fact that the time and dose of exposure was also known.

It was possible to study follow-up samples from the same cohort, and to compare serological reactivity during the asymptomatic and symtomatic stages of infection. The patients were relatively intensively followed up, and regular serum samples were taken and stored. This contrasts with other patient groups, particularly intravenous drug abusers, from whom it was rarely possible to obtain more than one sample. Another advantage of the haemophiliac cohort was that all of the patients were probably infected with the same strain of virus; differences in clinical outcome therefore must reflect differences in individual susceptibility to HIV infection. This laid the basis of research relating HLA haplotype to disease progression carried out by Dr M. Steel at the Western General Hospital. By the

same token, the follow-up serological studies were undertaken in the absence of reinfection by other strains of HIV, thus considerably simplifying the interpretation of results, and adding weight to the conclusions.

MATERIALS

HERPES SIMPLEX VIRUS

CELLS. Vero cells (Flow) were grown in Eagle's minimum essential medium (EMEM; Gibco) supplemented with 5% new born calf serum (NBCS; Gibco); 2mM L-glutamine; 10% tryptose phosphate broth; 300 IU/ml benzyl penicillin (Glaxo); 0.3 mg/ml streptomycin sulphate and 120 mg/ml sodium bicarbonate (growth medium; GM).

VIRUS. Strain 1657 (Peutherer, 1970) was the reference HSV-1 strain used in most experiments. It was typed by immunofluorescence and microneutralisation. The type was subsequently confirmed by restriction enzyme analysis. MS (Plummer, 1964) was the reference type 2 strain used in most experimemts. For comparison of different HSV-1 strains, 6 genital isolates (2490, 2746, 5028, 6517, 88 & 14739) and two sequential isolates of HSV-1 from a member of staff with recurrent labial HSV-1 infection (6785 & 14778) were available. All of these had been typed as above.

CONTROL SERA. Rabbit pre-bleed, anti-HSV-1 and anti-HSV-2 sera were available (Peutherer, 1970). Large volumes of HSV-1 antibody positive serum (JFP) from a member of staff with culture proven recurrent HSV-1 infection were available. Human anti-HSV-2 serum was not available in such large amounts, and sera from more than one patient with culture proven genital infection were used on different occasions (S27629 was normally used). Negative sera included S122 and S123, which were taken from 2 children aged 5 and 8 with no evidence of past exposure to HSV.

MONOCLONAL ANTIBODIES. Table 1 (Columns 1 to 4) lists the numbers and specificities of several monoclonal antibodies (supplied as ascites fluid) from Dr A. Cross, MRC Virology Unit, Institute of Virology, Glasgow, and from Dr A.C. Minson, University of Cambridge.

HUMAN IMMUNODEFICIENCY VIRUS

CELLS. Lymphoblastoid cells (CCRF-CEM; Flow) were grown in RPMI (Northumbria) supplemented with 10% foetal calf serum (Gibco); 20mM L-glutamine, 300 IU/ml benzyl-penicillin (Gibco); 0.3 mg/ml streptomycin sulphate and 120 mg/ml sodium bicarbonate.

VIRUS. CEM cells persistently infected with strain HIV-RF (CEM-RF), isolated from a patient with AIDS in the Caribbean (Popovic *et al.*, 1984), was used as antigen in an indirect immunofluorescence assay for antibody to HIV.

ANTIGEN. Gradient purified HIV-1 (strain IIIb) was purchased from Dupont and used as antigen in an IB assay for HIV-specific antibody.

CONTROL SERA. Serum from an HIV-infected intravenous drug abuser (J2) was available in large amounts and used in most experiments as a positive control. Negative serum (JFP) was available in large amounts from a member of the laboratory staff.

LAB NUMBER	CLONE NUMBER	SOURCE[2]	TARGET[1]	CROSS REACTIVITY[3]	REACTIVITY IN ELISA	INHIBIT- -ABILITY[4]
1	1892	G	gD	Y	++	+++
2	2462	G	gC-1	N	++	+
3	2975/22	G	gB	Y	+	+++
4	3104/23	G	gE-1	N	-	ND
5	5010	G	p40	Y (IB only)	+	-
6	AP2	C	MCP	Y	-	ND
7	1079	G	MCP	?	-	ND
8	LP10	C	gG-1	N	+	+/-
9	AP1	C	gG-2	N	+++	+/-
10	1917	G	gB	Y	++	++
11	SP62	G	p40	N	+++	-
12	3114	G	gE-1	N	+++	Non-specific
13	p110	G	gD-2	Y/N	++	++
14	LP14	C	gD	Y	ND	ND
Others:	2001	G	gD-1	N	++	++
	2245	G	gD-1	Y/N	+	++
	p105	G	gD-2	Y/N	+	+++

1 Targets as characterised by producer of the monoclonal
2 G : Kindly supplied by Dr A. Cross, MRC Virology Unit, Glasgow
C : " " " Dr A, Minson, Dept. Pathology, Cambridge
3 Y=cross-reactive; N=type-specific; Y/N=partially cross-reactive
4 Inhibitability with standard control sera in MABIA; measured on a scale of - : uninhitable to +++ : completely inhibited by positive sera; ND=not done

TABLE 1

Numbering, reactivity, inhibitability and source of the monoclonals used in the MABIA.

METHODS

HERPES SIMPLEX VIRUS

VIRUS CULTURE. Stock vero cells were split 1:3 twice weekly in GM. When not in use, vero cells were harvested, resuspended in GM containing 10% dimethylsulphoxide, and frozen slowly, eventually being transferred to liquid nitrogen for permanent storage. For routine passaging of HSV-1 and -2, confluent monolayers of vero cells were infected with virus at a multiplicity of infection (moi) of 0.1 in a small volume for one hour at 37C. The inoculum was then removed and replaced with GM and incubated at 37C until a complete cytopathic effect (cpe) was observed (generally 2-3 days). Cells were then harvested into supernatant GM with glass beads, and the volume reduced to 10ml by low speed centrifugation (1000 x g, 5 min.). Cells were lysed in an MSE ultrasonicator at full power for 20 seconds and the infectivity determined by plaque titration.

PLAQUE TITRATION. Serial tenfold dilutions of virus ultrasonicate were made in GM. 50 ul of virus dilution, 50 ul of vero cell suspension (at 0.75 x 10 cells/ml) and 50 ul GM were pipetted into wells of a microtitre plate (Sterilin, tissue culture grade; generally 8 or 12 duplicates at each virus dilution). The plate was then covered and incubated for 3 days at 37C in a humidified incubator containing 5% carbon dioxide. Plaques were counted at each dilution and the virus concentration, expressed in plaque forming units (pfu)/ml calculated. Titrated virus was stored in aliquots at -70C for up to 3 months.

FIXED CELL IMMUNOFLUORESCENCE. Vero cells were infected at a moi of 10 with HSV-1, -2 or mock infected, harvested at 18 hours with glass beads and washed twice with phosphate buffered saline (PBS) using low speed centrifugation as above. The cell concentration was adjusted to 0.5 x 10^6 cells/ml in PBS and 50 µl drops applied to PTFE-coated slides. After drying, the slides were fixed in acetone for 5 min. at room temperature. Slides prepared in this way were either used immediately or stored for up to 3 months at -20C. Sera to be tested were diluted either 1/50 (human and rabbit), or 1/200 (mouse monoclonal) in PBS supplemented with 5% NBCS (PBSS) and 20 µl applied to slides bearing fixed HSV-1, -2 or control antigens. The slides were placed in a moist box and incubated at 37C for 45 min. Slides were washed in PBS and fluorescein isothiocyanate (FITC) conjugated anti-human IgG (Scottish Antibody Production Unit; SAPU) at 1/50, anti-rabbit IgG (SAPU) at 1/50 or anti-mouse IgG (ICN Biomedicals) at 1/100 in PBSS added to each well. The second incubation was also 45 min. at 37C in a moist box. After washing, slides were mounted and observed with incident ultraviolet light using a Leitz Lux SM microscope.

ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA) FOR HSV ANTIBODY. Confluent monolayers of vero cells were infected at an moi of 10 with HSV-1, -2 at 24 hours [illegible]. Cells were harvested with glass beads, and washed twice with PBS. Cells were resuspended at a cell concentration of 10^7 cells/ml in 0.1M glycine, pH 10.0, 0.1M NaCl (coating buffer) and ultrasonicated for 2 min. at full strength in an MSE ultrasonicator. The cell lysate was centrifuged at 10 000 x g for 30 min. at 4C to remove cellular debris. The protein concentration of the supernate was determined by absorbance measurements at 260 and 280 nm in an ultraviolet spectrometer. ELISA plates (Nunc immunoplate 1) were

normally coated at a protein concentration of 10 μg/ml diluted in coating buffer, and left overnight at 4C. The plates were either used immediately or stored for up to a month at -70C. Normally, alternate columns of wells of the ELISA plates were coated with HSV (either HSV-1 or -2) and control antigen. Test serum was diluted in PBS containing 0.05% tween-20 (PBST), 1% bovine serum albumin (PBSAT), and 5% normal rabbit serum (PBSATR), and 200 μl added to each well. The first incubation was for 60 min. at 37C followed by washing 5 times in PBST using a hand washer (wash volume 5 x 300 μl). The second incubation used 150 μl/well of either horseradish peroxidase (HRP) conjugated anti- human IgG (SAPU) or anti-mouse IgG (ICN biomedicals) diluted 1/500 and 1/2000 respectively in PBSATR, with an incubation time of 60 min. at 37C. After washing, 150 μl of substrate (1.125 g Na_2HPO_4 ; 0.28 g citric acid, pH 6.0; 10 mg o-phenylene diamine (OPD); 10 ul H_2O_2 ; 50 ml distilled water, dH_2O) was added to each well and incubated at room temperature for 30 min. 50 μl of 4M H_2SO_4 was added to each well to stop the reaction. The plates were read immediately at 490 nm in an automatic plate reader (Dynatech MR 580).

MONOCLONAL ANTIBODY BINDING INHIBITION ASSAY (MABIA). Antigen coated ELISA plates prepared as previously were used as the solid phase in this assay. 100 μl of a 1/100 dilution of human or rabbit serum in PBSTA was added to a well, followed by 100 μl of monoclonal antibody diluted appropiately in PBSTA. The plate was incubated for 1 hour at 37C, washed as above and 150 μl/well of HRP anti-mouse IgG at 1/2000 in PBSAT supplemented with 10% normal human serum (HSV antibody negative) added and incubated as above. Substrate development and reading the plate were as above.

POLYACRYLAMIDE GEL ELECTROPHORESIS. Confluent monolayers of vero cells were infected at a moi of 10 with HSV -1, -2 or mock infected. Cells were harvested at 24 hours, washed twice in PBS and resuspended in sample buffer (1% sodium dodecyl sulphate, SDS, BDH; 0.0625M Tris-HCl, pH 6.8; 1% 2-mercaptoethanol; 10% glycerol; bromophenol blue marker dye) at a cell concentation of 5 x 10^6 cells/ml and heated to 100C for 3 min. Solubilised HSV-1, -2 or mock infected cell extracts were electrophoresed on 10% polyacylamide gels using the discontinuous buffer system of Laemmli, (1970). Samples were electrophoresed at 60V using a high pH electrode buffer (0.025M Tris; 0.192M glycine; pH 8.3; 0.1M SDS) through the stacking gel (0.125M Tris-HCL, pH 6.8; 4.44% acrylamide, BDH; 0.12% NN-methylene-bis-acrylamide, bis; 0.1% SDS polymerised by ammonium persulphate in the presence of NNN'N tetramethylethylene diamine). After stacking, proteins passed into the separating gel (0.375M Tris-HCl, pH 8.8; 10% acrylamide; 0.27% bis; 0.1% SDS; polymerised as above) and electrophoresed at 150V until the dye front reached 10 cm from the top of the separating gel. Gels were stained conventionally (Coomassie blue) or blotted onto nitrocellulose as described below. Purified proteins (trypsin inhibitor, 20 100 Da; lactate dehydrogenase, 36 500 Da; glutamate dehydrogenase, 55 400 Da; phosphorylase B, 97 400 Da; Boehringer) were used for molecular weight determination.

PROTEIN BLOTTING. The method was based on that described by Towbin *et al.*, (1979). Resolved proteins were blotted electrophoretically on to nitrocellulose (Schleicher & Schuell) at 15V overnight at 4C in transfer buffer (0.025M Tris; 0.192 glycine; pH 8.3; 20% methanol).

IMMUNOSTAINING. The nitrocellulose sheet, after transfer of proteins, was blocked in 3% gelatin in tris buffered saline (TBS; 20mM Tris-HCl, pH 7.5; 0.5M NaCl) for 30 min. at room temperature. After washing in TBS, sheets were either used directly or sealed in plastic bags and stored at -70C indefinitely. For analysis of the target specificities of various human and rabbit antisera, and that of monoclonal antibodies, a dilution of serum (generally 1/20; 1/100 for monoclonals) in TBS supplemented with 1% gelatin (TBSG) was made and incubated with strips of nitrocellulose bearing the viral antigens. This first incubation was for 2 hours at room temperature with constant agitation on an MSE orbital shaker. Washing comprised an initial rinse in dH_2O, followed by two ten minute washes in TBS plus 0.05% tween-20 (TBST). The second incubation used HRP conjugated anti-human IgG (SAPU; 1/500); anti-rabbit IgG (Sigma; 1/200) or anti-mouse IgG (ICN; 1/500) diluted in TBSG for 2 hours at room temperature with agitation as above. After washing as above, the nitrocellulose strips were incubated in substrate (100 ml TBS, 60 µl H_2O_2; 20 ml methanol, 60 mg 4-chloro-1-napthol; mixed immediately before use) for 15 minutes at room temperature. The strips were washed in water, dried and stored in the dark. Despite this latter precaution, there was inevitably fading of bands, and yellowing of the nitrocellulose over years of storage.

NON-DENATURING IB. Confluent monolayers of vero cells were infected at an moi of 10 with HSV-1, -2 or mock infected. Cells were harvested at 24 hours with glass beads, washed twice in PBS and finally resuspended in lysis buffer (PBS; 0.2 mM phenylmethyl sulphonyl fluoride, PMSF; 0.1 mg/ml DNAse II type IV, Sigma; 0.1 mg/ml phosphodiesterase I type IV, Sigma) supplemented with detergents as described in the results. Cells were incubated in lysis buffer for 15 min. on ice, and then

centrifuged at 100 000 x g for one hour to remove insoluble material. Supernate was either used immediately, or stored at -20C. The cell lysate was diluted in sample buffer and electrophoresed using the gel system as described above but with SDS in the sample buffer, electrode buffer and gel solutions replaced with other detergents as described in the results. Electrophoresis was performed at 4C instead of room temperature to avoid degradation of proteins. In some experiments, the acrylamide concentration was reduced to 7% (bis reduced to 0.18%) in the separating gel. Blotting, blocking and immunostaining were as described above. Purified proteins (α-lactalbumin, 14 200 Da; carbonic anhydrase, 29 000 Da; ovalbumin, 45 000 Da; BSA, 66 000 Da & 132 000 Da; urease, 240 000 Da; Sigma) were electrophoresed to investigate the relation between rate of migration and protein size.

RADIOLABELLING. Confluent monolayers of vero cells were infected at a moi of 10 with HSV-1, -2 or mock infected. After absorption for one hour, the inoculum was replaced with a small volume of methionine-free EMEM (supplemented with 0.5% NBCS; 20 mM glutamine; 300 IU/ml benzyl-penicillin; 0.3 mg/ml streptomycin sulphate and 120 mg/ml sodium bicarbonate), and the cells incubated for 4 hours at 37C. [^{35}S] methionine (Amersham; 800 mCi/mmol) was added to each bottle to produce a final radioactive concentration of 50 μCi/ml and incubated for a further 14 hours. Cells were harvested with glass beads, washed twice in PBS and resuspended at a cell concentration of 10^7 cells/ml in lysis buffer supplemented with 1% sodium deoxycholate (DOC), 1% triton X-100 (TX100). After incubation on ice for 15 min., cell lysates were centrifuged at 100 000 x g for 60 min. to remove insoluble material, and stored in aliquots at -20C until required.

RADIOIMMUNOPRECIPITATION ASSAY (RIPA). 10 µl of human or rabbit serum or mouse monoclonal antibody, 25 µl of a 50/50 suspension of sepharose-staphylococcal protein A (Pharmacia) in PBS and 200 µl of PBST were added to a 0.5 ml conical bottomed plastic tube. The tube was incubated at room temperature for 60 min. with agitation to keep the sepharose in suspension. Unbound serum components were removed by two cycles of washing with PBST followed by low speed centrifugation (500 x g, 1 min.). 50 µl of radiolabelled antigen prepared as above, and 200 µl of PBS containing 1% DOC, 1% TX100 were added to the tube and incubated for a further 60 min. After washing, 100 µl SDS sample buffer was added to the sepaharose and the tube boiled for 3 min. 5 µl (1/20 of the precipitated sample) was removed and dried onto glassfibre discs for liquid scintillation counting. The remainder of the immune precipitate was analysed by SDS-PAGE as described above. Bands were visualised by autoradiography for 2 weeks at -70C with Kodak X-Omat P film.

HUMAN IMMUNODEFICIENCY VIRUS

VIRUS CULTURE. Stock CEM cells were passaged twice weekly splitting cells 1 to 3 or 4 depending on cell density. CEM persistently infected with HIV-RF were maintained similarly. Cells were grown in a humidified incubator, in 5% carbon dioxide. Cells were stored in liquid nitrogen as above when not in use.

IMMUNOFLUORESCENCE. CEM and CEM-RF cells were washed twice in PBS and resuspended at a concentration of approximately 10^6 cells/ml. The cell supension was applied to slides and fixed as for HSV described above.

Subsequent testing of human sera was similar to the method used in the fixed cell IF assay for HSV antibody.

ELISAs FOR ANTIBODY TO HIV. Enzyme immunoassays (EIAs) for HIV-specific antibody from Wellcome, Pasteur, Abbott (env/core confirmatory assay) and Dupont were used according to the manufacturers instructions. Quantification of antibody levels in the latter two assays was performed as described in the results.

IMMUNOBLOTTING. 50 µg of purified HIV-IIIb (Dupont) viral lysate was mixed with 1.5 ml sample buffer, and electrophoresed using the SDS-PAGE system used for HSV. Modifications included increasing the acrylamide concentration to 12% (bis to 0.32%); blocking in PBS plus 2% BSA; human serum diluted 1/50 in PBS plus 0.2% tween-20; anti-human IgG conjugate diluted 1/200 in PBS plus 0.2% tween-20 and finally the substrate changed to 0.05% diaminobenzidine, 0.05% H_2O_2, in PBS, pH 7.6. For some experiments, a commercially available IB antibody detection system (Biorad) was used.

SERUM ANTIGEN DETECTION. Two capture ELISA kits (Abbott and Dupont) were used to test human sera for HIV p24 antigen according to the manufacturers instructions.

DEVELOPMENT OF METHODS

ELISA FOR HSV ANTIBODY

The concentration of antigen required to coat ELISA plates was not known. Non-specific binding of antibody to cellular proteins was more likely with high coating concentrations, so titrations of coating HSV-1, -2 and control antigens were performed. Figure 3 shows the variation with HSV-1 or 2 antigen dilution (on an inverted scale) with binding of several monoclonals. Non-specific reactivity of monoclonals with control antigen was very low (OD less than 0.03). The graph demonstrates maximum binding of each monoclonal antibody at antigen concentrations of 11 µg/ml and above; a concentration of 10µg/ml was therefore used in all subsequent experiments. The uniformity of the antigen dilution effect with each monoclonal was thought to result from the limited protein binding capacity of the ELISA plate.

Figure 4 shows titrations of human HSV-1 amd HSV-2 antisera against type-homologous antigens in the ELISA. A straight line relationship can be seen between antibody concentration (on a linear scale) and OD in the range 0-0.7, with HSV-2 antisera showing a intercept with the y-axis at zero. HSV-1 sera however showed an intercept at around 0.04, signifying the binding of conjugate to HSV-1 in the absence of test serum (at an infinite dilution). This occurred despite addition of normal rabbit serum to the conjugate to block HSV-1 Fc binding activity. An allowance for this binding was made in subsequent experiments. The linear dilution response of sera allowed quantitation of antibody levels in test sera by a simple ratio of the OD with that

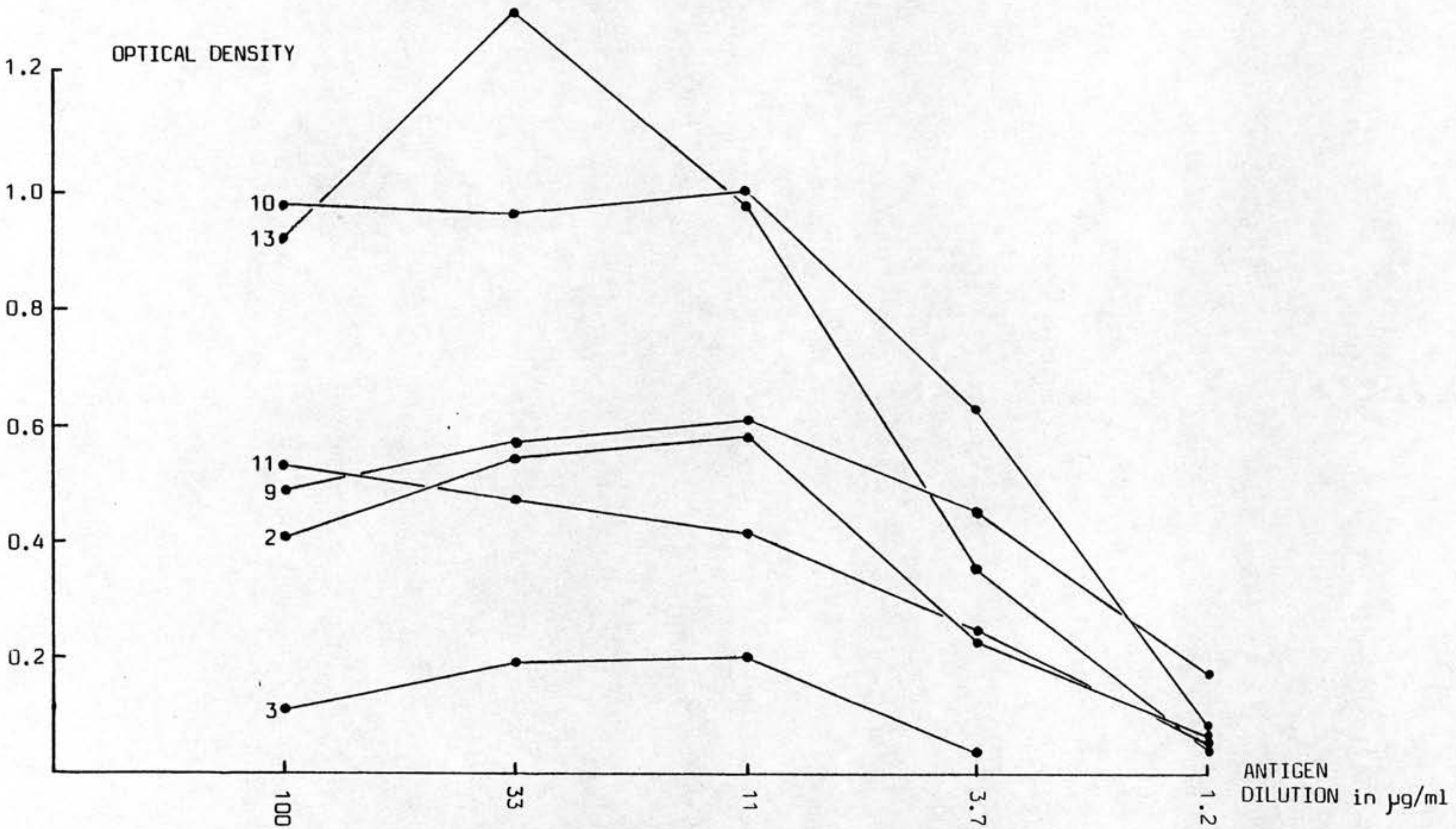


FIGURE 3. Reactivity of monoclonal antibodies in ELISA for HSV antibody plotted against coating antigen dilution. See Table 1 for identification of monoclonals; OD at 290 nm of HSV-1 or -2 minus control antigen (net OD).

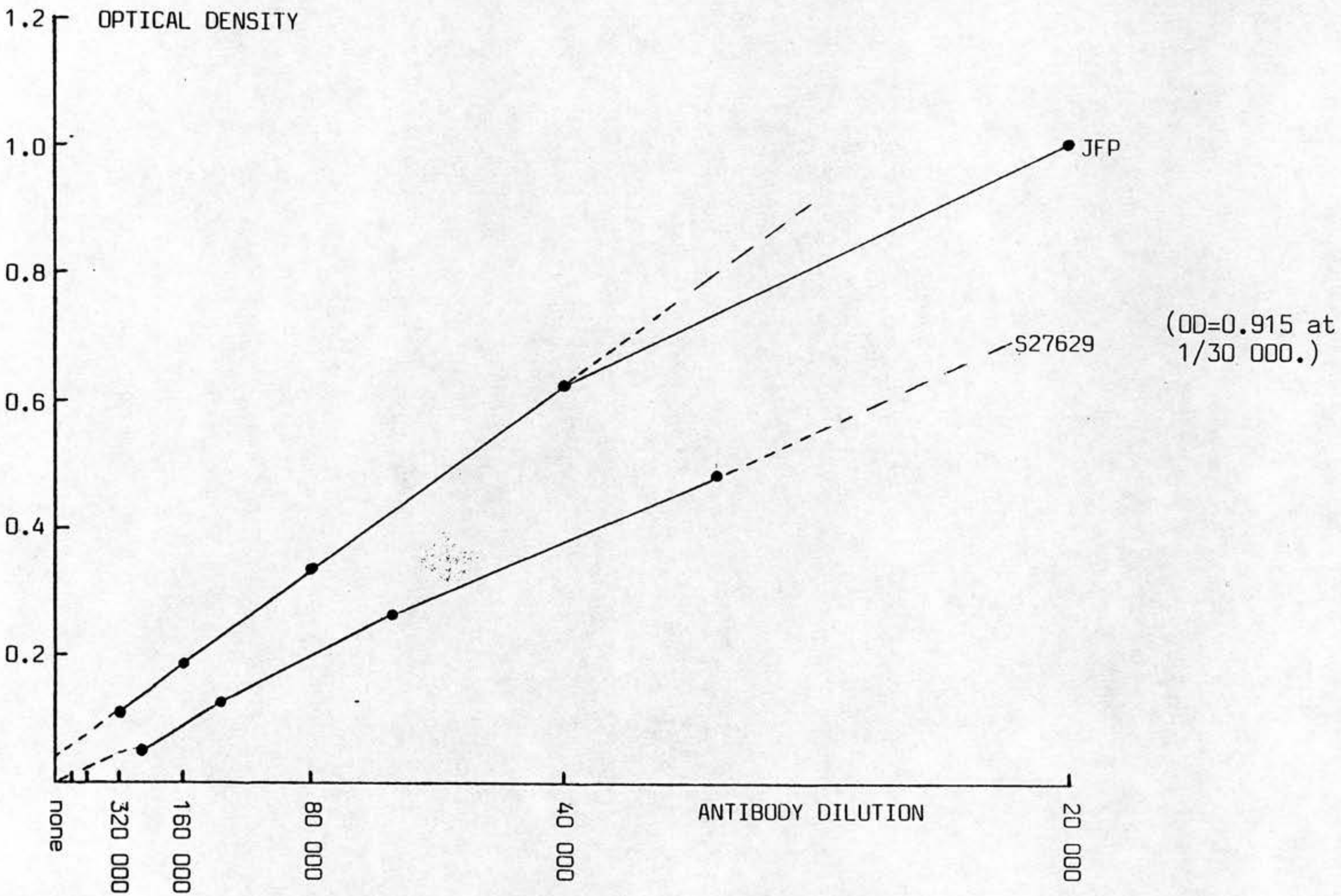


FIGURE 4: Titration of HSV-1 (JFP) and HSV-2 (S27629) human antisera against type-homologous antigen in ELISA for antibody to HSV. Net OD on y axis; linear serum dilution scale on x axis.

of a positive control, taking into account any difference in assay dilution between the test and control sera. Antibody levels varied widely so, in practice, test sera were tested at two dilutions, generally 1/1000 and 1/10 000 to ensure that at least one OD fell in the linear range of the graph. Testing at two dilutions confirmed that almost all sera with appropiate OD values showed linear dilution responses, confirming the validity of the test. For routine screening of sera, multiple replicates of JFP with HSV-1 and S27629 with HSV-2 were used as positive controls in each assay run. Antibody levels in test sera were expressed in units, with the positive controls arbitrarily assigned 1 unit (U; 1000 mU).

MABIA

To maximise the sensitivity of this assay for blocking activity in test sera, each monoclonal was first titrated against HSV antigen. Low dilutions of monoclonal saturated the available binding sites (plateau region); as the dilution was increased a point was reached where there was a rapid drop in OD (linear region), where the amount of monoclonal took over as the limiting factor. Figure 5 shows titrations of the monoclonals used in the blocking assay. An inverted dilution scale was used in order to to encompass the wide range in titres observed; replotting the data as in figure 4 confirmed that there was a linear increase in OD with concentration of each monoclonal, but only over a small range in those with low saturation OD readings. Using figure 5, the concentration of monoclonal producing an OD midway between zero and the saturation level was calculated and used subsequently in the blocking assay.

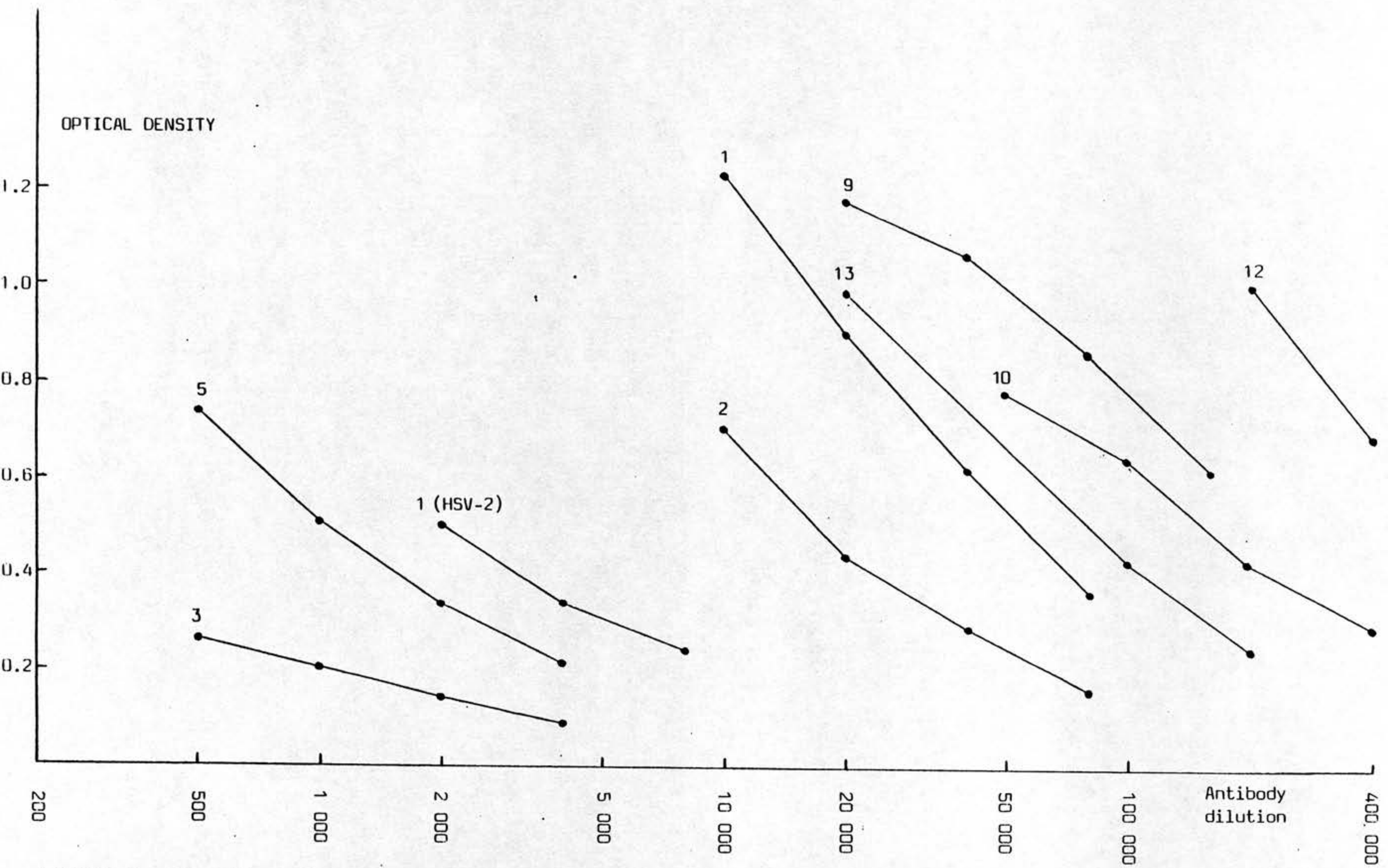


FIGURE 5. Titration of monoclonal antibodies log inverse scale against HSV antigen in ELISA for HSV antibody
Monoclonals titrated against HSV-1 antigen, except nos. 9 and 13 (HSV-2); net ODs recorded.

One problem encountered with the MABIA in its initial stages of development was the inappropriate reactivity of anti-mouse IgG with human IgG. The first experiments involved incubating a positive and a negative human serum with monoclonal no.1 (gD-specific), washing, and then measuring the amount of monoclonal binding by tracing with the anti-mouse label. The ODs achieved by the negative and positive sera were similar. To investigate this, a second experiment was performed in which the monoclonal was omitted. In this case, the OD with the negative serum was roughly zero, while the OD with the positive serum was about 0.6. This was caused by cross-reaction of conjugate with human serum and completely overshadowed the inhibitory effect of the positive serum in the first experiment. Inhibition in the first experiment had actually occurred; the OD with the negative serum being caused by the monoclonal binding. Although one could have attempted to remove cross-species reactivity by prior immunosorption on a human IgG coated column, it was simpler to simply block the conjugate during the second incubation by diluting it in PBSAT containing 1% negative human serum. This proved to be completely effective providing that care was taken in ensuring that the human serum was HSV-antibody negative and free from interfering substances such as rheumatoid factor. In practice, an HSV negative serum from a 5 year old boy (S122) happened to be readily available in large amounts and was used for blocking in all subsequent MABIAs.

To test the specificity of the assay system, HSV-1 antiserum (JFP) was tested at various dilutions against suitable dilutions of two monoclonals, nos. 1 and 9. Monoclonal 1 reacts with native gD-1 and-2 (type-1 & 2) and might reasonably be supposed to be inhibitable. No. 9 reacts with both native and denatured gG-2, and in view of the complete lack of antigenic cross-reactivity of gG-2 antibody with

HSV-1 proteins, might reasonably be presumed to be uninhibitable by an HSV-1 antiserum. Figure 6 shows the results of titrating JFP serum on a linear scale in a blocking assay with the two monoclonals. The graph shows concentration dependent blocking of no.1 by JFP, but no significant blocking of no.9 at any serum dilution.

Subsequently, sera were tested for blocking activity at a dilution of 1/100. The calculation to measure inhibition should take into account the reactivity of monoclonal and/or serum with control antigen. Therefore all experiments were performed with both HSV and control proteins, and the latter subtracted from the former to give a net OD. The next factor to be taken into consideration was variablility between batches in the net OD achieved by the monoclonals. Minor differences in incubation temperature or duration, or substrate activity affected this, and so each batch contained several replicates of monoclonal alone. The last consideration was the contribution made by test serum on the OD achieved on incubation with the monoclonal (additive effect), distinct from the inhibition (subtractive effect). The additive effect arose from slight residual cross-reaction of the anti-mouse conjugate with human antibody, despite blocking. Since this would vary with the titre of HSV-specific antibody, it had to be measured for each test serum.

The calculation used to measure inhibition was as follows:

$$\text{RESIDUAL BINDING} = \frac{(\text{Net OD:Test serum + monoclonal}) - (\text{Net OD:Test serum})}{\text{Net OD:monoclonal}}$$

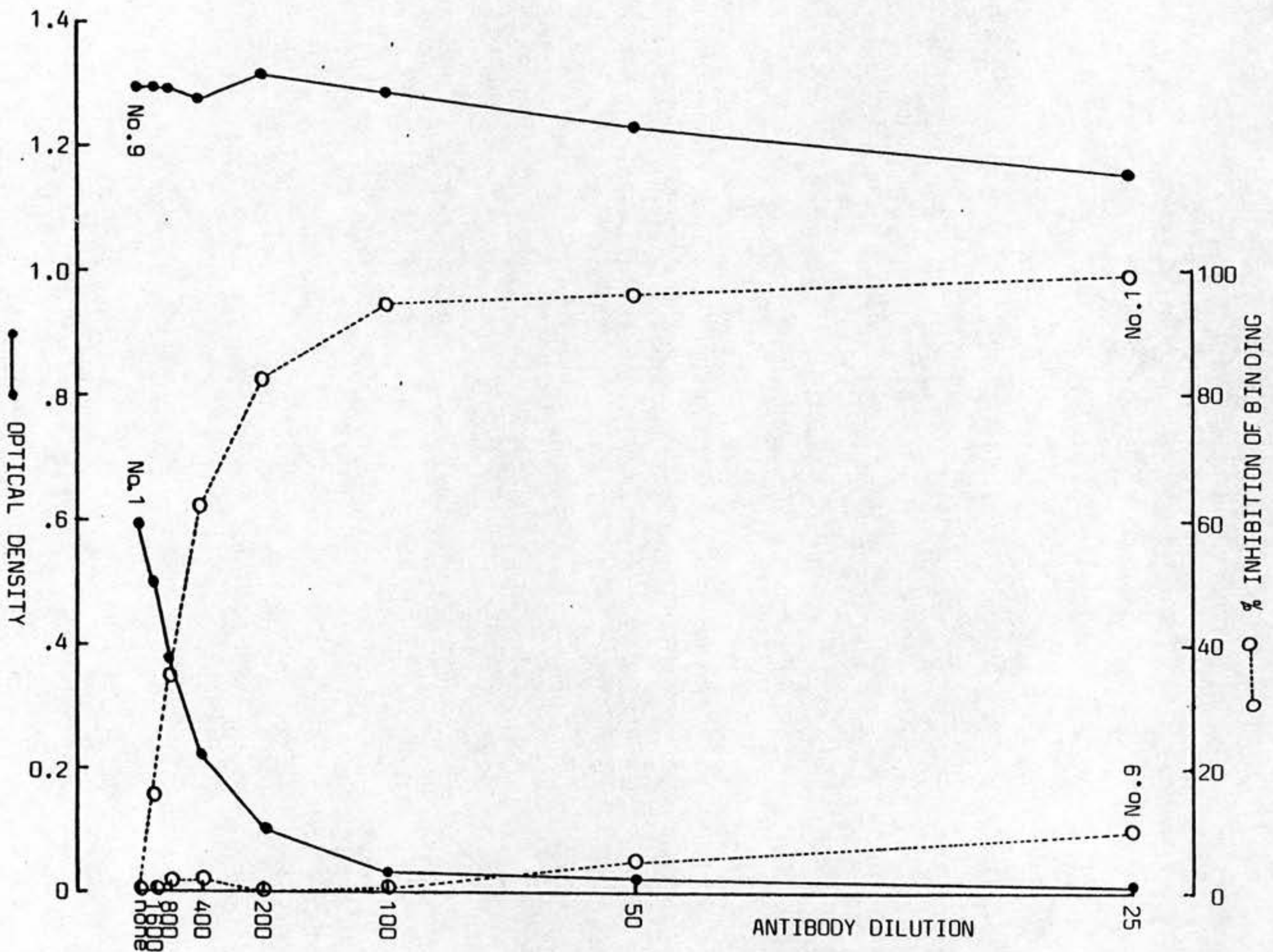


FIGURE 6. Titration of HSV-1 positive serum (JFP) in MABIA against two monoclonals, no.1 (gD) and no.9 (gG-2). •—• Net OD; o----o inhibition of binding.

Table 2 shows the results of another experiment investigating the specificity of the MABIA. The calculation of the residual binding value is shown for each serum and monoclonal combination. There was little or no inhibition by either pre-bleed rabbit sera of any of the monoclonals. The rabbit anti-HSV-1 serum and JFP showed almost complete blocking of no.1, partial blocking of no.2 and no blocking of no.9. Although the rabbit antiserum blocked no.5 partially, JFP (and several other human sera, not shown) did not. The rabbit and human (SB13166) anti-HSV-2 sera showed inhibition of nos. 1 and 9, but not of no.5. The human serum did not block the type-specific monoclonal, no.2; however, the type 2-specific rabbit antiserum produced an inhibition value with this monoclonal below those of the negative sera, indicating a possible weak inhibitory effect.

To investigate the relation between cross-reactivity of monoclonals and cross-inhibition, as observed above, an experiment with four gD monoclonals reported to be type-specific was performed (Table 3). Two HSV-1 and 2 HSV-2 specific monoclonals were tested with type-homologous and type-heterologous antigen to measure cross-reactivity; cross inhibition was measured by blocking the monoclonals with prebleed, anti-HSV-1 and anti-HSV-2 rabbit sera. Monoclonal 2001 did not react detectably with HSV-2, yet was measurably blocked by the type 2 serum (pre-bleed 76%; anti-HSV-1 19%; anti-HSV-2 47%). 2245 did cross-react, but with approximately 1/5th of the OD. It was cross-inhibited almost completely by the type 2 antiserum. p110 cross-reacted extensively with HSV-1 (1/4 of the OD) and was almost completely cross-inhibited by the type 1 serum. p105 reacted poorly in the ELISA, and the inhibition figures are based on very low OD readings and are less accurate than those of the other monoclonals. It shows, too, extensive cross-reaction, and even greater

SERUM	No.5 (p40)			No.2 (gC-1)			No.1 (gD)			No.9 (gG-2)		
	A[1]	B[2]	C[3]	A	B	C	A	B	C	A	B	C
NONE	.503			.687			.447			.378		
NEG1[4]	.597	.562	117%	.754	.710	103%	.555	.520	116%	.487	.473	125%
POS1[4]	.332	.297	59%	.322	.287	42%	.048	.013	3%	.350	.336	89%
NEG2[4]	.596	.584	116%	.695	.683	99%	.598	.586	131%	.491	.474	125%
POS2[4]	.464	.452	90%	.513	.501	73%	.083	.071	16%	.207	.190	50%
JFP	.457	.440	87%	.345	.328	48%	.043	.026	6%	.420	.411	109%
SB13166	.469	.456	91%	.691	.679	99%	.223	.210	47%	.321	.296	78%

1 This column gives the net OD reading of a 1/100 dilution of serum or none and a suitable dilution of monoclonal.
2 The OD after subtraction of the net OD of test serum without monoclonal.
3 The ratio of the OD in column 2 to the net OD of monoclonal without serum, expressed as a percentage.
4 NEG1, POS1, NEG2 & POS2: pre-bleed and HSV-1 immunised rabbit sera; pre-bleed and HSV-2 immunised sera

TABLE 2

Reactivity of control sera in MABIA with monoclonals 5, 2, 1 & 9.

MONOCLONAL	TARGET	DILUTION	REACTIVITY WITH: hom.[1]	het.[1]	INHIBITION BY: neg.[2]	hom.[2]	het.[2]
2001	gD-1	1/20000	1.003	0.002	83%	27%	64%
2245	gD-1	1/2000	0.573	0.109	97%	16%	57%
p110	gD-2	1/20000	0.568	0.147	84%	13%	24%
p105	gD-2	1/2000	0.265	0.050	62%	1%	12%

1 Reactivity with type-homologous and type-heterologous antigen, expressed as net OD.

2 Inhibition by pre-bleed (neg.), rabbit antiserum immunised with type-homologous (hom.) or type-heterologous (het) antigen

TABLE 3

Investigation of the cross-reactivity and cross-inhibitability of four type-specific monoclonals to gD-1 and gD-2.

cross-inhibition. This small study shows that sera cross-inhibit to greater extent than the monoclonals cross-react. It is evidence that inhibition is not necessarily epitope specific; rather it may occur by binding to neighbouring epitopes that do not share the type-specificity of the target epitope of the monoclonal (steric hindrance).

To increase the sensitivity of the MABIA for gG-2 and other antibody specificities, blocking of no.9 and no. 13 (gD-2) by rabbit HSV-2 antiserum was investigated at several coating antigen dilutions. By reducing the target for monoclonals, greater inhibition of binding might be observed in sera with low levels of blocking antibody. However, the blocking was similar at antigen concentrations in the range 10 ug/ml to 0.12 ug/ml (data not shown). Similarly, within this range of antigen concentration, HSV-1 antiserum (JFP) showed no change in the inhibition of binding of monoclonals 1, 2, 3 and 10.

Numerous preliminary experiments were performed to find a suitable panel of monoclonals with which to screen test sera for blocking activity. Table 1 summarises the findings that led to selection of monoclonals 1, 2, 3, 10, 9 and 13 for subsequent screening. Monoclonal 4 was unreactive in the ELISA; no.5 was slightly reactive, but was of low titre, and not inhibited by any human serum. No.6 and 7 were both unreactive. No 8 (gG-1) was weakly reactive in the ELISA with a maximum OD of .220 at dilutions of 1/20000 and less. With such low ODs, it was difficult to obtain accurate and reproducible measurements of inhibition of the control sera. Within these limits, it appeared that the monoclonal was no more than 50% blocked by HSV-1 positive sera, and was unaffected by negative and type 2 sera. No.12 was the only monoclonal so far tested that appeared to be blocked by both

negative and positive sera with no correlation between antibody titre and extent of inhibition. The reason for this non-specificity is not known, but could be explained by postulating that the monoclonal is directed to an epitope within the Fc binding region of gE. Incubation with either positive or negative sera would lead to binding to the site, non-specifically blocking the monoclonal.

Table 4 summarises the protocol adopted for the screening of test sera. Since multiple replicates of positive and negative controls were included in each batch, it was possible to calculate the reproducibility of the assay, and the range of error in calculating the results. These are also shown in this table. Monoclonal no.11 (p40) was found to be uninhibitable by the control sera. It was included in the screening of test sera to act as a control. In any inhibition assay, there is the possibility of non-immunological blocking by interfering substances in certain sera, particularly those with bacterial contamination. All sera tested in the MABIA were also tested against no.11, but none caused a reduction in the binding of no.11 (data not shown). This confirms the immunological specificity of the inhibition values obtained with the other monoclonals on screening.

IMMUNOFLUORESCENCE ASSAY

Attempts were made to reduce non-specifity caused by Fc binding by gE in this assay. Gallo, (1986) describes the use of low concentration of acetic acid as a pretreatment for slides to be used in a fixed cell IF assay for antibody to HSV. To test this, a comparison of slides pretreated with 5% acetic acid and untreated slides was made (table

Monoclonal	Dilution	Replicate + control values[1]	Average	Error
For HSV 1 antibody detection :				
1	1/50 000	5% 6% 4% 4% 0% 1%	3.3%	∓2.1%
2	1/20 000	43% 47% 41% 42% 45% 43%	43.5%	∓2.0%
3	1/2000	2% 13% 0% 6% 0% 2%	3.8%	∓4.6%
10	1/100 000	9% 14% 8% 4% 4% 3%	7.0%	∓3.8%
11	1/400 000	81% ND 87% 92% 87% 94%	88.2%	∓4.5%
For HSV 2 antibody detection :				
1	1/50 000	13% 15% 11%	13.0%	∓1.6%
3	1/2000	0% 0% 0%	0 %	-
10	1/100 000	45% 30% 31%	35.3%	∓6.8%
9	1/200 000	43% 50% 39%	44.0%	∓4.5%
13	1/100 000	0% 6% 2%	2.7%	∓2.5%

Antigen : 10 µg/ml protein; pH 10 coating buffer; 18 hours; 4C
1st Incubation : 200 µl; 75 min; 37C; PBSAT diluent
Wash : 6 times; PBST
2nd Incubation : 175 µl; 75 min; 37C; PBSAT + 5% - human serum
Wash : 6 times; PBST
Substrate : OPD; citrate buffer, pH 6.0; 30 min; room temp.

1 Replicate control values from all of the assay runs of test sera. Positive controls: JFP (HSV-1); S27629 (HSV-2)

TABLE 4

Protocol adopted for screening sera for monoclonal blocking activity

5). The results show that membrane fluorescence of HSV-1 and 2 infected cells with negative serum was abolished by the acid treatment, while not reducing the binding of positive serum. Diluting test serum and fluorescein conjugate in PBS + 2% NBCS also reduced non-specific binding (data not shown).

IMMUNOBLOTTING

Preliminary work established the amount of antigen necessary for the assay; the blotting conditions to ensure complete transfer of resolved proteins to nitrocellulose; the composition of buffers to avoid background staining and the optimum concentration of conjugate. Results from this work led to the adoption of the method described in the methods section. Control sera were tested against both HSV and control antigen in the IB assay (figure 7; table 6). No sera reacted significantly with control antigen, and the results have not been illustrated. Pre-bleed rabbit sera did not react with any HSV proteins in the assay (lanes 1, 10); negative human sera were also unreactive (lanes 3-5, 12-14). Rabbit anti-HSV-1 and anti-HSV-2 sera and JFP were reactive in the assay, producing several bands, reflecting broad polypeptide specificity (lanes 2, 6 & 11).

Proteins were identified by the reaction of monoclonal antibodies of known specificity in the assay. However, several were unreactive due to denaturation of the target epitopes by the antigen solubilisation proceedure. Nos. 14, 2, 5 and 9 did produce bands, and these are shown in figure 7. The target of no.14 is gD and this appears as a band of approximate MW 57 KDa (lane 7). gD of HSV-2 produced a band of similar molecular weight. The target of no.2 is gC-1 and produced bands of

		Control	HSV-1	HSV-2
Untreated slide	S123 (negative) at 1/10	none	++	+
	1/100	none	+	+
	JFP (anti-HSV-1) 1/10	none	++++	+++
	1/100	none	+++	++
Treated slide	S123 at 1/10	none	none	none
	1/100	none	none	none
	JFP at 1/10	none	++++	+++
	1/100	none	+++	++

TABLE 5

Comparison of acetic acid treated and untreated slides for specificity and sensitivity for HSV-1 antibody. Slides were read double blind; membrane fluorescence graded from none to ++++.

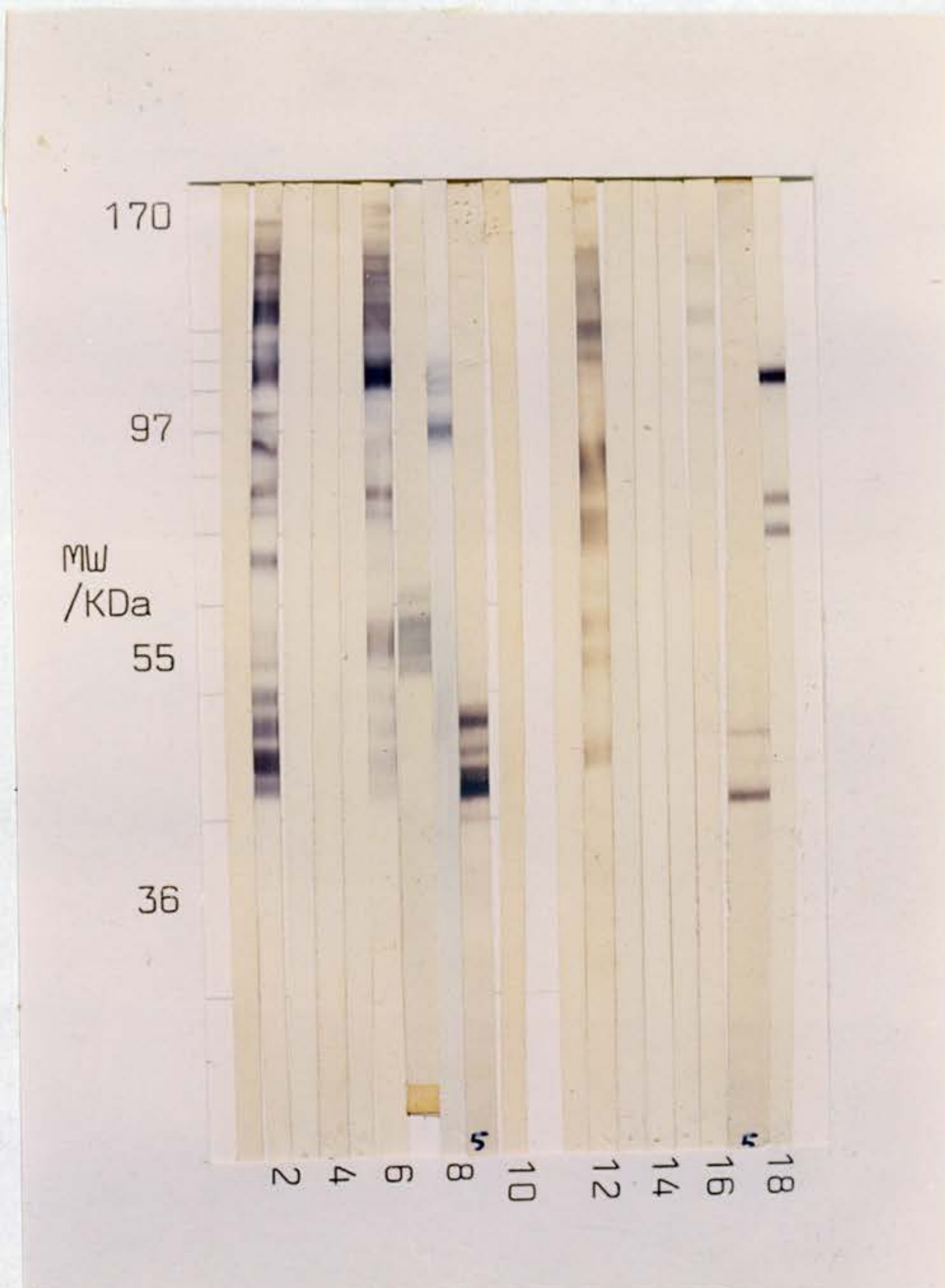


FIGURE 7. Reactivity of control sera in the IB assay.

HSV-1. Lanes 1 & 2: pre-bleed and HSV-1 immunised rabbit antisera; lane 3, 4, 5: three negative human sera; lane 6: human anti-HSV-1 positive serum (JFP); lanes 7, 8, 9: monoclonals no. 14 (gD), no. 2 (gC-1) and no. 5 (p40); lane 10: negative mouse serum.

HSV-2. Lanes 11 & 12: pre-bleed and HSV-2 immunised rabbit antisera; lanes 13, 14, 15: three negative human sera; lane 16: human anti-HSV-1 postive serum: lanes 17 & 18: monoclonal antibodies no. 5 (p40) and no. 9 (gG-2).

SERUM	IB bands[2]	cpm[1]	RIP bands[2]
Pre-bleed 1	NONE	22	NONE
anti-HSV-1	150-140K 130K 108-104K gC 78K 75K 67K p40	21870	gC gB 80K gD/gG
Pre-bleed 2	NONE	134	NONE
anti-HSV-2	150K 130K 120K 110K 73K 69K gD p40	13770	MCP 130K 120K gB 95K 80K 72K gD 38K
S20264	NONE	151	NONE
S20326	NONE	108	NONE
S20495	NONE	218	NONE
JFP	150-140K 130K 108-104K 78K 75K gD/gG p40	13112	gC gB 80K gD/gG p40

1 cpm refers to the radioactivity of 1/10th of the immune complex as measured by liquid scintillation counting

2 name of the proteins given, or MW in KDa if not definitely identified

TABLE 6

Reaction of the control sera with type homologous virus in the IB and RIP assays

102, 100 and 90 KDa (lane 8) with HSV-1, and none with HSV-2. The reaction of nos. 5 (p40) and 9 (gG-2) are shown in lanes 9, 16 & 17.

Two procedures were performed to investigate variability in antigenicity between different isolates of HSV. The first method involved producing antigen from six isolates from patients with primary genital HSV-1 infection The antigen was electrophoresed, blotted and reacted with sera taken from each of the six patients. The aim was to investigate whether patients reacted more strongly with their own isolate than with those of other patients. The results (figure 8) show that there was considerable variation between the bands produced by each of the sera, and some difference between the intensities of the bands between isolates. There was little consistent evidence for a stronger reaction with their own (homologous) isolate than with those of other patients (heterologous). A similar experiment compared the reactivity of JFP with two homologous isolates and 1657, the standard HSV-1 strain. Again there was little difference in reactivity of JFP with the three viruses. On the basis of the results, it was felt reasonable to use 1657 as the standard strain of HSV-1 for all subsequent experiments.

The other proceedure to examine variation in antigenicity of the virus used for IB assays involved passaging 1657 and MS at high and low moi's. Passaging at high moi leads to an increasing proportion of defective viruses and may lead to altered protein synthesis and a different pattern of bands. Very early samples of 1657 and MS were obtained from Dr I.W. Smith and passaged 4 times at an moi of 10 (high) or at 0.001 (low). The former cultures showed complete cpe at 24 hours; those of the latter typically showed individual plaques at 3 days which coalesced to give a complete cpe at 5 days. After

FIGURE 8. Reactivity of sera from six patients with previous primary genital HSV-1 infection with homologous (from same patient) and heterologous (from other patients) isolates. Each panel comprises antigen prepared from one of the 6 isolates; strips within the panel reacted with each of the six sera from the patients. Homologous reactions arrowed.

passaging, antigen was produced from each of the four cultures and used in an IB assay. No difference in the number, apparent MW or antigenicity of the bands was detected between the high and low moi cultures (data not shown).

RADIOIMMUNOPRECIPITATION ASSAY

Control sera were tested by RIPA, and compared with the results of IB (table 6). The table gives both the counts of the immune precipitate and the bands detected subsequently by SDS-PAGE. Antibody negative sera produced low counts and no bands on analysis of the precipitate. Positive sera produced elevated counts and several bands by autoradiography. Several protein identifications were made by monoclonals (figure 9; lanes 7-10 & 13). gD-1 (lane 7) and gD-2 (not shown) both appear as broad bands of approximate MW of 66 KDa on reaction with no.1. The bands are distorted by large amounts of co-migrating IgG. There is also a weaker band of MW 121 KDa in lane 7, probably the result of slight co-precipitation of other HSV proteins. Richman *et al.*, (1986), showed that gG-1 appeared in their RIP assay as a band of similar apparent MW, and appearance as gD-1. Bands of this size produced by test sera cannot be positively identified as either so will be referred to as gD/gG.

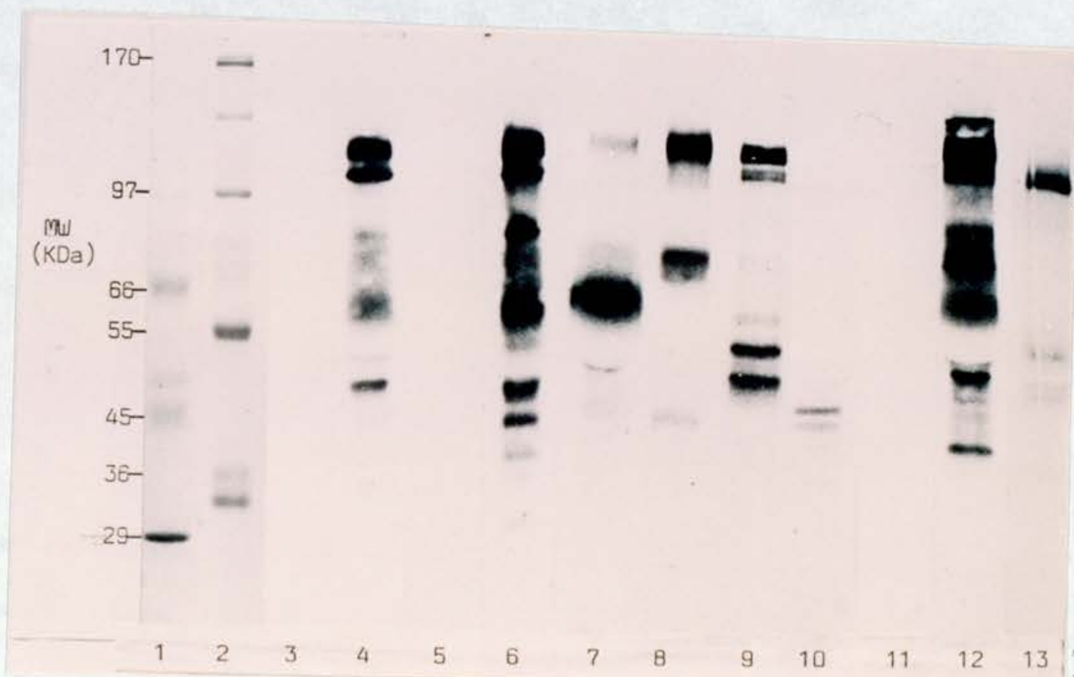
gC-1 (no.2, lane 8) appears as a prominent band MW 121, with weaker bands at 75K and 45K. gB-1 (no.3; lane 9) has two prominent bands of size 118K and 112K, with two lower molecular weight bands of 56K and 50K. These probably represent cleavage products. gB-2 (lane 13) has a major band of 110K and similar lower MW bands. No.4 (gE-1) was unreactive in the assay. No.5 (p40) produced weak bands of MW 47.5, 45

FIGURE 9. Reactivity of control sera in the RIP assay.

Molecular weight markers (coomassie blue stain). Lane 1: carbonic anhydrase (29 KDa); ovalbumin (45 KDa); bovine serum albumin (66 KDa). Lane 2: lactate dehydrogenase (36.5 KDa); glutamate dehydrogenase (55.4 KDa); phosphorylase (97.4 KDa); γ-macroglobulin (170 KDa).

HSV-1. Lanes 3 & 4: pre-bleed and HSV-1 immunised rabbit antisera; lanes 5 & 6: negative and positive human sera (S122 & JFP); lanes 7, 8, 9, 10: monoclonal antibodies no. 1 (gD); no. 2 (gC-1); no. 3 (gB) and no. 5 (p40).

HSV-2. Lanes 11 & 12: pre-bleed and HSV-2 immunised rabbit antisera; lane 13 monoclonal antibody no. 3 (gB).



and 42.5 KDa with HSV-1 antigen (lane 10), and no bands with HSV-2. No.6 produced faint bands of 158 KDa with both HSV-1 and 2 antigen (not shown).

NON-DENATURING IMMUNOBLOTTING

Several detergents were investigated as alternatives to SDS for immunoblotting. One paper describes the use of triton X-100 for electrophoresis (Dewald *et al.*, 1975), and this was the system tried. The discontinuous buffer system described by Davis (1964), and subsequently adopted by Laemmli (1970) for disassociated proteins, was used with the addition of 1% Triton X-100 to all buffers, including that used for solubilisation of the antigen. On electrophoresing the sample, it was observed that migration was very slow; after 6 hours at 180 V, the dye front had moved only 3 cm. The gel was divided; half was stained for protein, and the other half was blotted onto nitrocellulose and reacted with an HSV-1 antiserum. Both procedures showed that there had been no protein separation; instead there was clumping at the top of the gel with only very faint bands in the separating gel. Similar results were obtained when the triton concentration in the electrode, stacking and separating gels was reduced to 0.1% or omitted entirely; the only difference was that the marker dye migrated at its normal rate. These findings suggested that the micelles formed by triton were too large for satisfactory electrophoresis. Only water soluble, hydrophilic proteins that do not interact with the detergent would be separated in this gel system.

The quoted weight of triton micelles is 120 000 Da (Helenius, 1976), and a detergent with smaller micelles was sought. Sodium deoxycholate

was chosen (micelles 12 000 Da) and the experimemt as described above was repeated substituting this detergent. Although sodium deoxycholate appeared soluble at pH 6.8 (pH of stacking gel), the detergent precipitated during electrophoresis, both in the stacking gel and in the lower electrode buffer (cathode) reservoir. Protein staining was impossible since the entire gel became opaque in the staining solutions; blotting revealed again that the HSV antigens were clumped at the top of the separating gel.

3-[(3-cholamidopropyl)dimethyl-ammonio]-1-propane sulphonate (CHAPS) is a cholic acid derivative of small micellar size and this was the next candidate. Serum, sheep red blood cells, and HSV-infected cells were solubilised in a 1% solution of the detergent and electrophoresed in the presence of the detergent. Haemoglobin remained red and the protein separation could be observed during electrophoresis. There was effective stacking of the haemoglobin and presumably of the other proteins, and a normal rate of migration of the marker dye. A protein stain of the gel showed effective separation of all of the serum components, and several of the proteins in sheep red blood cells. However, in the HSV-infected cell lysate, there was again clumping of material at the top of the gel; although much protein had entered the separating gel, it was smeared with only faint bands. An immunostain of the gel confirmed that the antigenic material was largely clumped at the top with some smearing down the gel. A subsequent experiment with molecular weight markers showed clear resolution of the proteins, although there was no relation between distance migrated and molecular weight. These latter experiments showed that the only proteins resolvable in this system were water soluble, and the detergent was having little effect on the separation of membrane proteins.

The same experiments were repeated with sodium taurodeoxycholate (TDCA; cells solublised in 1% TDCA; 1% in separating and stacking gels; 0.3% in electrode buffers). This is also a cholic acid derivative of small micellar size, but differs from CHAPS in being negatively charged in the pH range for electrophoresis (CHAPS is zwitterionic, with no net charge). It was thought that a negative charge might improve resolution of detergent solubilised proteins (cf SDS). It also remains in solution at low pH (at least pH 4) so it should not precipitate during electrophoresis. Experiments with this detergent bore out these expectations. Firstly, there was normal stacking of haemoglobin and other soluble proteins, and there was no precipitation of detergent. A protein stain of the HSV-infected cell lysate revealed an absence of clumping at the top of the separating gel, and good resolution of numerous proteins (Figure 10; lane 1). An immunostain of the cell lysate confirmed that all of the HSV proteins had entered the gel to form a major band at Rf 0.15 (lane 4), and several minor bands above and below. A subsequent experiment with molecular weight markers showed no relation between migration and molecular weight (data not shown). It was deduced that the detergent solubilised and assisted in the separation of membrane proteins, but did not interact with water soluble proteins. In this latter respect, it differs from SDS.

Immunoblotting with TDCA was used as decribed above to test the control sera. Figure 10, lanes 5-8 shows the development of reactivity with the protein of Rf value 0.15 in the two HSV immunised rabbits. Negative human sera were unreactive in the assay. Monoclonals were used to identify proteins. No.3 (gB; lanes 2 & 3) reacts with the prominent band (0.15 HSV-1, 0.13 HSV-2). No.1 reacts very weakly with the band at Rf value 0.65 (data not shown).

FIGURE 10. Reactivity of control sera in the TDCA IB assay. Lane 1: HSV-1 infected cell lysate, coomassie blue stain; lanes 2 & 3: reactivity of monoclonal antibody no. 3 with strips prepared from HSV-1 and -2 antigen; lane 4: human positive serum (JFP) with HSV-1 antigen; lanes 5 & 6: pre-bleed and HSV-1 immunised rabbit antisera with HSV-1 antigen; lanes 7 & 8: pre-bleed and HSV-2 immunised rabbit antisera with HSV-2 antigen.

Monoclonals nos.2 (gC-1), 4 (gE), 5 (p40), 6 (MCP) and 9 (gG-2) failed to react with strips prepared in this assay. To investigate the reasons for this, HSV-1 infected cells were solubilised in TDCA, centrifuged, and then resolubilised in SDS and electrophoresed conventionally. After blotting, strips were reacted with JFP serum, and the three monoclonals previously shown to be reactive with denatured proteins, nos. 2, 5 and 9. Only two bands appeared on reaction with JFP; one had an apparent MW of 100 KDa, and the other was of MW 65 KDa. It is likely that these two proteins correspond to gB-1 and gD-1, the only proteins detectable in the original TDCA IB method. Furthermore, none of the three monoclonals (2, 5 & 9) reacted with the resolubilised material. It would appear that only gB and gD are efficiently solubilised in TDCA; the other proteins must have been lost during the high speed centrifugation of the cell lysate. While this might be expected for the two capsid proteins, p40 and MCP, it was surprising that the other glycoproteins were also apparently insoluble.

There was the unlikely possibility that monoclonals nos. 2 and 9 (gC-1 and gG-2) failed to react in the TDCA IB assay because the target epitopes were concealed when the protein was in its native configuration. To prove that this was not the case, strips prepared by TDCA IB were pretreated by boiling in conventional SDS-containing sample buffer, and then tested with the monoclonals. Reactivity was not restored, confirming the absence of the proteins after TDCA solubilisation.

To solubilise a greater proportion of HSV proteins, the TDCA IB assay was modified by the inclusion of 3M urea in all buffers, along with the TDCA. However, this caused smearing of proteins, and could not be

used. Another approach was to modify the sample buffer and run proteins on conventional SDS-containing gels. This was the approach used by Cohen *et al.*, (1987), in studies of gD. The first modification was to use ordinary sample buffer, with mercaptoethanol, but not to boil the cell lysate. Heating to 56C for 5 minutes gave identical results to boiling. Keeping the cell lysate at room temperature after addition of sample buffer led to clumping of a large proportion of antigen at the top of the gel, but with similar resolution of other proteins. Another method tried was to substitute TDCA with 0.1% SDS in the conventional solubisation proceedure, incubating on ice for 15 minutes with protease inhibitors and centifuging at 100 000 x g to remove insoluble material. Electrophoresis was performed at 4C with all electrophoresis buffers supplemented with 0.1% SDS. However, there was extensive smearing of proteins, clumping at the top of the separating gel, and little reactivity of blots with JFP serum.

HUMAN IMMUNODEFICIENCY VIRUS

IMMUNOBLOTTING

The conventional IB proceedure was used with HIV, except that the polyacrylamide concentration was increased to 12% (bis to 0.32%). Since purified virus was used, there was no background reactivity and it was possible to increase the sensitivity of the assay without non-specific bands appearing. HRP conjugated anti-human IgG was used at twice the concentration used for HSV. Diaminobenzidine was found to be a more sensitive substrate than 4-chloro-1-naphthol and was used for all experiments. Experiments with other substrates were also performed, with mixed results. Taketa *et al.*, (1986) report the use of nitro blue tetrazolium in the presence of NADH and phenol. The method was tried, but although it was more sensitive than 4-chloro-1 naphthol, it was inferior to diaminobenzidine. Supplementing diaminobenzidine with imidazole has been reported to enhance detection, but, when tried with HIV, merely increased background staining of the strips, with no intensification of the specific bands. The normal brown bands produced by diaminobenzidine can be changed to purple by the addition of 0.1% nickel chloride to the substrate; this significantly darkens the bands and can give a useful increase in sensitivity.

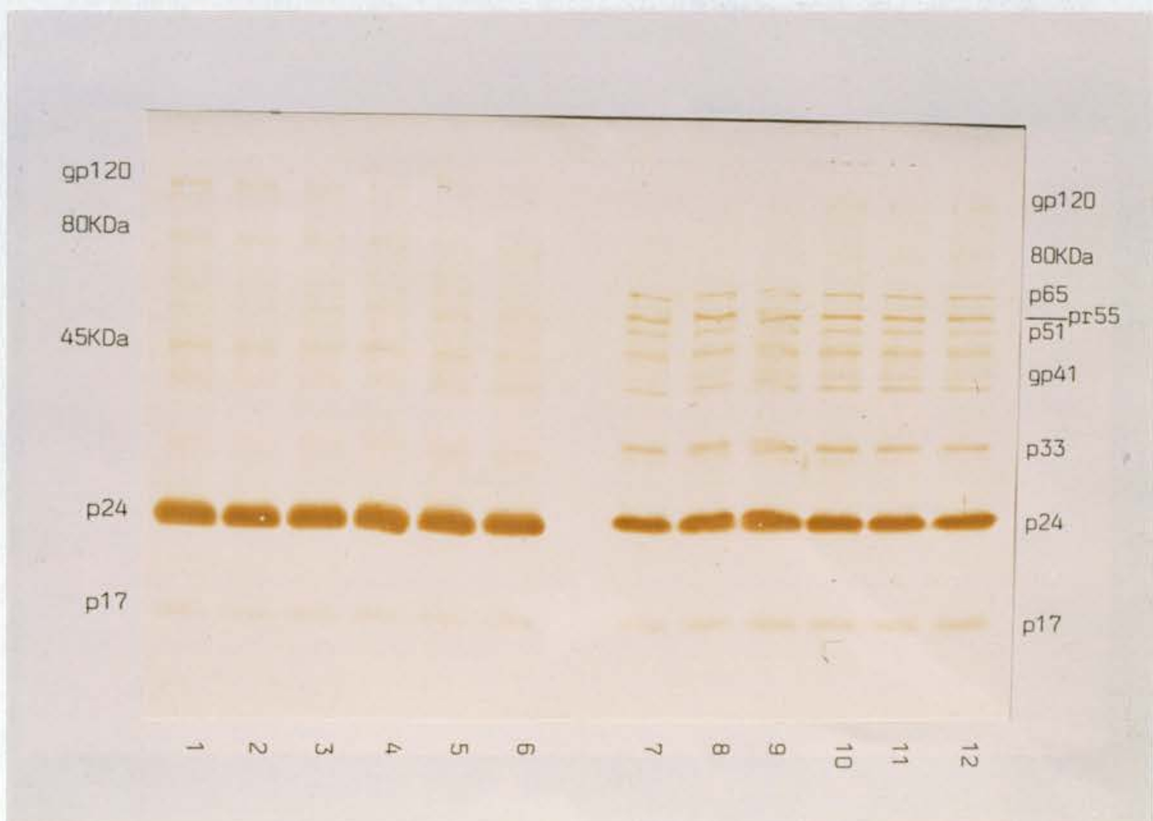
The purified virus was supplied by Dupont in a buffer containing triton. This, and its high price restricted developments of alternative solubilisation procedures. However, an investigation into the effect of heating the antigen prior to conventional SDS-PAGE was carried out. Antigen was added to sample buffer with 2 % 2-mercaptoethanol and to sample buffer without reducing agent. Heat

treatment included none (room temperature); 5 min at 37C; 30 min at 37C; 5 and 30 min at 56C, and finally 5 min at 100C (normal method). The samples were electrophoresed conventionally, blotted, and then probed with J2 (figure 11; see Results section for identification of bands). The blot showed poor resolution of proteins without reducing agent, and there were no extra high MW bands, indicating an absence of disulphide linked protein complexes. Comparison of the heat treatments showed a difference in the antigenicity of samples. Samples, both reduced and non-reduced, that were prepared at 37C, or unheated, showed equal resolution as the boiled samples, and showed an extra band corresponding possibly to gp120 or gp160.

ELISA FOR ANTIBODY TO HIV

A commercially available EIA for HIV antibody was used to quantitate levels of antibody. Figure 12 shows titration of J2 in the ELISA. The solid circlesshow replicate titrations of the positive serum plotted on an inverse scale. The readings were averaged and replotted on a linear scale to determine the relation between concentration and OD (hollow circles). It can be seen that there was a linear relationship in the range 0-0.8 OD units. If this were the case with other sera, it would be possible to quantitate antibody levels by simple comparison of the OD of the test serum with that of the positive control, taking into account the difference in dilution of test and control sera (ie. the same method as used to quantitiate HSV antibody levels). In practice, some sera did not show a linear dilution response; this is presumably due to there being predominant reactivity with a minor antigen on the ELISA plate. Sera not showing a linear dilution response would give different antibody levels when tested at different

FIGURE 11. Effect of solubilisation conditions on the antigenicity of HIV proteins in the IB assay. Lanes 1 to 6: non-reduced; lanes 7-12: reduced in 2% 2-mercaptoethanol. Lanes 1 & 12: Room temperature; lanes 2 & 11: 37C, 5 min.; lanes 3 & 10: 37C, 30 min.; lanes 4 & 9: 56C, 5 min.; lanes 5 & 8: 56C, 30 min.; lanes 6 & 7: 100C, 3 min.



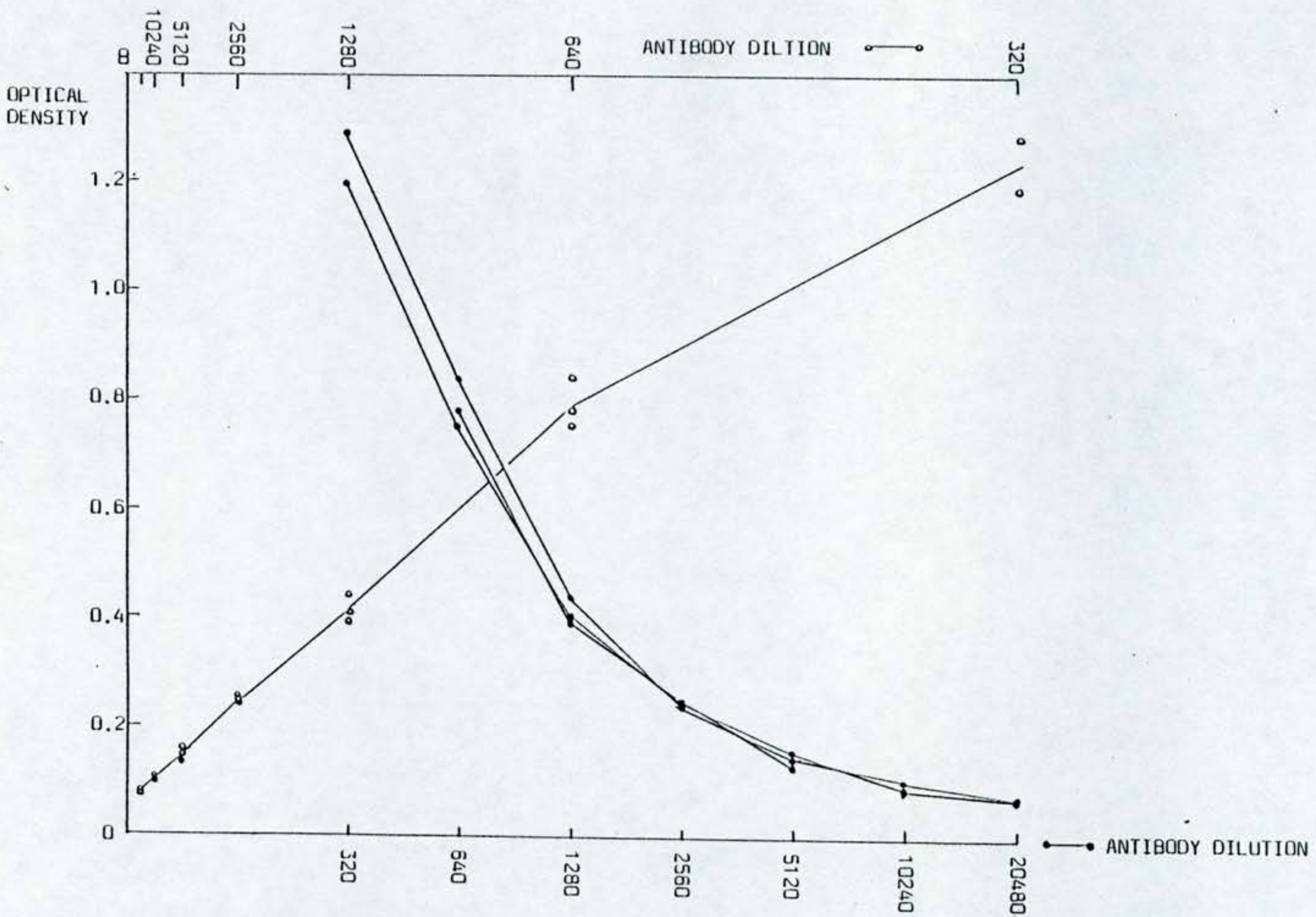


FIGURE 12: Titration of J2 in ELISA for antibody to HIV. y axis: OD at 290 nm; solid circles: replicate dilutions on inverse log dilution scale; hollow circles: averaged values on linear dilution scale.

dilutions. If this was the case, then the OD from the dilution that predicted the highest antibody level was used (generally at higher dilution). Test sera were generally tested at 1/1100 and 1/11000 in the ELISA, although some sera taken close to seroconversion had low levels of antibody and were tested at lower dilutions.

QUANTIFICATION OF "env" AND "core" ANTIBODY

In theory, it would have been possible to modify the Abbott confirmatory EIA for "env" and "core" antibody by dispensing with the anti-HIV label used for competition, and using the recombinant antigen coated beads in a direct binding assay. This would have allowed easier quantitation of antibody levels, but would have brought in the possibility of reduced assay specificity and would have prevented direct comparison of the results with those of other investigators. Figure 13 shows that there was not a straight line relation between log inverse serum dilution of J2 and OD in the conventional assay. Replotting serum dilution on linear and other scales also failed to show a simple relationship. To quantitate antibody levels in a test serum, the ODs at two serum dilutions were plotted on figure 13 and the distance horizontally between it and the standard curve measured. This distance, which could be either positive or negative, is proportional to the log of the difference in antibody level between the test serum and the positive control. The anti-log gives the ratio of antibody levels between the test serum and the positive control. Since J2 was arbitrarily assigned 1 unit in both the anti-"env" and anti-"core" assays, the ratio calculated for a test serum corresponded to its antibody level in units. Several sera were tested at multiple dilutions, and the antibody level calculated from each. Provided the

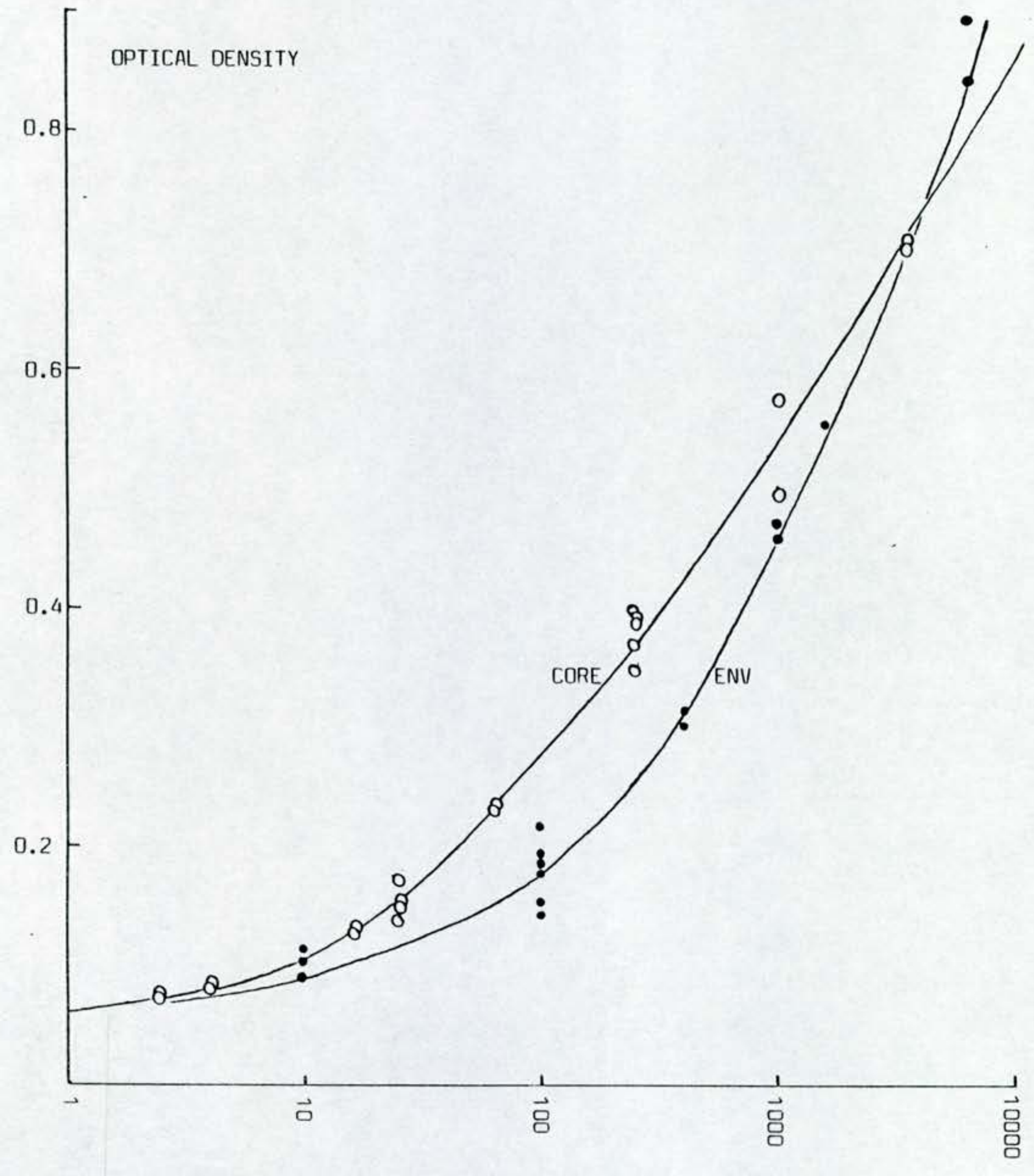


FIGURE 13. Titration of J2 in anti-"env" and anti-"core" competition assay. ● anti-"env"; o anti-"core".

OD fell within the range 0.15 to 0.7, the antibody levels calculated from the OD at each dilution were similar. For use in screening, sera were tested at 1/1000 and 1/10000 for "env" antibody, and at 1/2.5, 1/25 and/or 1/250 for "core" antibody.

RESULTS

HSV STUDY GROUPS

NEGATIVE SERA. Since one cannot rely on a patient's medical history for evidence of past HSV infection (because primary infection is often undiagnosed and recrudescences not universal), it was decided to use sera from children aged 3 to 15 that appeared to be antibody negative by conventional methodologies (complement fixation). The young age would make it less likely that there had been infection with HSV so long ago that antibody levels subsequently waned to a level below the sensitivity of the screening test. The results obtained from the use of these sera were supplemented with those from the pre-bleed rabbit sera when necessary.

HSV-1 POSITIVE SERA. A similar problem from unreported past exposure with HSV-2 precluded the extensive use of adult sera that were HSV-1 antibody positive. There would no guarantee, for example, that HSV-2 antibodies did not contribute to the reactivity of adult sera with HSV-1 antigen. The most satisfactory solution was again to use child sera since they are very unlikely to have been exposed to HSV-2. Any antibody detected was assumed to be HSV-1 specific. Sera from children in the age range 3 to 15 with complement fixing activity were used in several experiments. Other HSV-1 antibody positive sera were available from laboratory workers, and from HSV-1 immunised rabbits. For completeness, sera from adults with recurrent genital HSV-1 infection were also tested, although of course there is no definite information about past HSV-2 infection.

HSV-2 POSITIVE SERA. Although sera from adult patients with recrudescent HSV-2 infection were readily available, there was no information about prior infection with HSV-1. Sera from patients with primary HSV-2 infection were available, but since the patients were generally not followed up for more than 2 weeks, they were not typical of convalescent sera in that they would probably contain IgM and have a very limited range of IgG antibody specificities. Also, since they were taken from adults, they may have had low level HSV-1 antibody. The only sera guaranteed to be HSV-2 specific were immunised rabbit sera. Sera from patients with recrudescent HSV-2 infection were used, but the possible contribution of HSV-1 antibody to the results should be borne in mind.

REACTIVITY OF NEGATIVE SERA. These sera were tested against HSV-1 and HSV-2 antigen in the IB assay. The vast majority of sera were completely unreactive with any proteins in the assay. However, a small proportion did show weak reactivity. The following bands appeared in the IB assay with HSV-1 antigen: two bands of 40 and 90 KDa (sera nos. 4, 17, 18), 110 KDa (serum no. 20) and 65 KDa (no. 22). Two sera (nos. 9 & 11) reacted weakly with HSV-2 antigen in the IB assay, producing bands at 90 and 110 KDa respectively. The identity of any of these bands could not determined.

The same sera were tested by other methods (table 7). The ELISA results show that the vast majority of sera are unreactive with HSV-1 antigen (antibody levels of 5 mU and below were considered to be negative). The only three sera to react significantly with HSV-1 antigen were sera 22 (63 mU), 20 (29 mU) and 17 (30 mU). The MABIA showed no inhibition of any of the monoclonals by all but 3 of the sera, and possibly a fourth. These correspond to sera 22, 20 and 17,

SERUM	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
ELISA/mU	0	1	1	0	1	0	2	1	2	7	6	1	0	1	11	0	30	5	3	29	1	63	6
Inhibitions:																							
No.1	94	89	88	90	96	85	93	90	97	95	95	91	95	88	84	91	63	86	94	80	90	25	99
2	96	98	95	88	92	92	83	94	96	95	92	93	96	93	88	87	92	79	93	97	91	64	109
3	76	93	88	87	86	75	61	82	77	86	90	61	82	87	81	87	61	74	75	69	91	12	93
10	97	101	93	95	110	88	81	90	95	99	107	89	100	88	84	86	56	85	100	75	90	60	99
9	100	97	89	103	93	93	91	100	98	95	109	97		105	109	97	97	108	103	105	86	109	98
13	84	74	84	91	81	81	80	76	92	87	91	83		91	83	74	73	90	89	81	76	73	82
HSV IF	-	-		-		-	-					-					+	-		+		+	-
EBV	+	-	+	+	+	-	-	+	+	+	+	-	-	-	-	+	-	+	-	-	+	-	+
CMV	<8	<8	16	<8	<8	16		<8	8	16	<8	16	<8	<8	16	8	8	<8	<8	64		<8	<8
VZV	<8	16	<8	<8	<8	16		<8	<8	<8	16	16	<8	16	<8	<8	<8	32	16	<8		<8	<8

TABLE 7

Reactivity of the negative sera in ELISA for HSV antibody, MABIA against full panel of monoclonal antibodies, and IF assay with HSV-1 antigen. EBV antibodies determined by indirect ELISA with acetone fixed Raji cells, CMV and VZV antibodies estimated by complement fixation.

also reactive by ELISA and IB, as can be seen in the table. Serum 18, IB positive, ELISA negative showed borderline inhibition of nos. 1, 2 and 3 although the significance of this is uncertain. The immunofluorescence antibody assay with HSV-1 and mock infected cells, using the modification to prevent Fc binding, was used to test these doubtful sera. The results were read double blind by the author and another laboratory worker experienced in HSV IFAs; the latter read the slides with no knowledge of the other results of the sera. The assay confirms the presence of antibody reactivity with sera 22, 20 and 17, and agree precisely with the ELISA results. Fluorescence was confined to the cytoplasmic membrane, suggesting reactivity with HSV envelope proteins.

Further testing was performed for antibody to other herpesviruses that may possibly cross-react in the assays. The results showed no interference of the other herpesvirus antibodies with any of the tests; the majority of sera were unreactive despite variable levels of antibody to each of the other viruses. However, it is possible that the high titres of antibody to CMV (1/64) in serum 20 and to VZV (1/32) in serum 18 may have been the cause of the apparent reactivity of these sera with HSV. The numbers of sera involved are too small to be definite one way or the other.

The negative sera, apart from the three sera of uncertain status, were plotted on a scatter diagram to show the average, range and standard deviation of inhibition of the various monoclonals used in the MABIA. The diagram (fig. 14) shows the relatively tight clustering of inhibition figures around the 90-100% range. The average figures of the negative sera varied between the different monoclonals, with no.3 and 13 (gB and gD-2) clustering around 80-82% in contrast to the

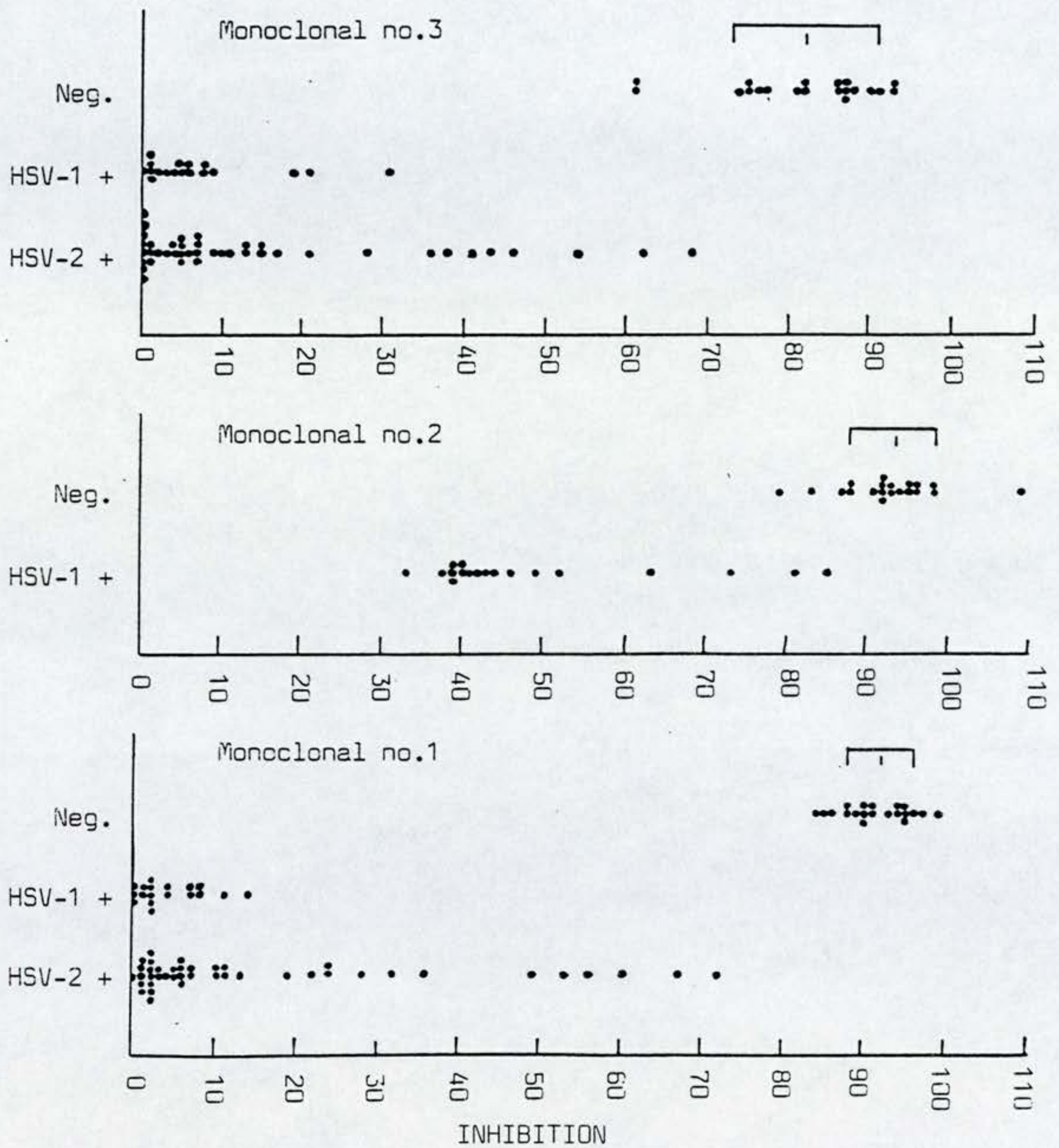


FIGURE 14: Scatter diagram representing the results of testing HSV antibody negative sera, HSV-1 antibody positive sera, and sera from patients with HSV-2 infection. Mean and 1 standard deviation from mean of negative sera values indicated above plotted values. Diagram continued overleaf.

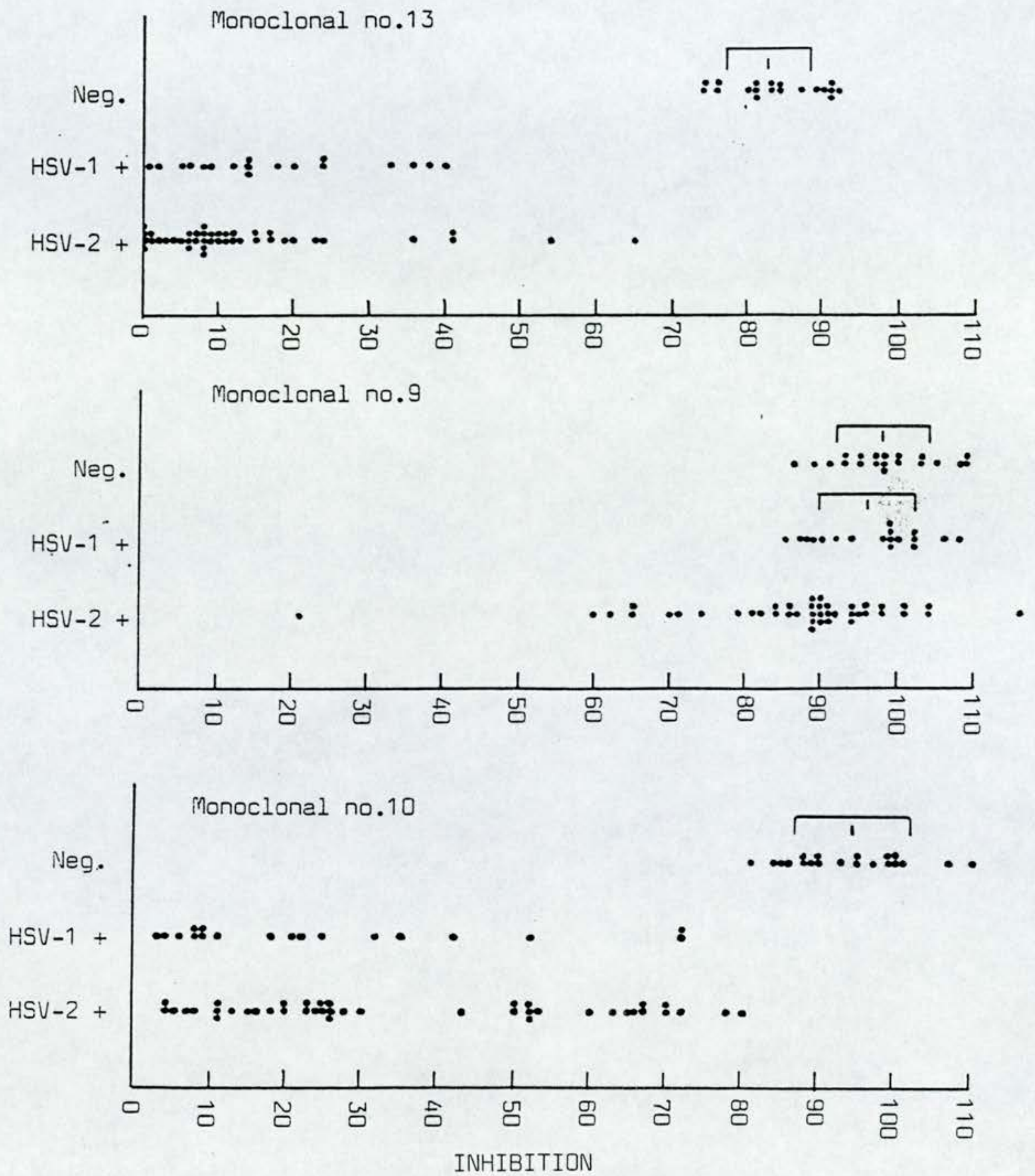


FIGURE 14 (cont'd): See preceding page for legend.

others which were clustered from 90 to 95%. No.3 shows significantly greater variability than the others, possibly a reflection of the errors associated with the low OD values that this monoclonal achieved. In fact, the standard deviation of the values of these negative sera is only slightly larger than batch to batch variation revealed by the multiple testing of the replicate positive controls (table 4).

TARGETS OF ANTIBODY IN CONVALESCENT SERA.

HSV-1 SERA. Figure 15 and table 8 show the results of testing the child and adult sera by IB, ELISA and MABIA. The IB assay shows very broad reactivities with several HSV-1 specified proteins (there was no significant reactivity of these sera, or any others in this study with mock antigen in the IB assay; data not shown). There was variation in the strength of reaction (intensity of bands) and breadth of antibody (number of bands visible) between the sera. One problem with analysing these IB results is the number of bands and the smearing in some places on the strip. It was not really possible to distinguish reactivity with the major glycoproteins gB, gC-1 and gE by this method, although p40 and gD/gG are more clearly resolved. All sera are positive for antibody to p40, while 18/21 are positive for gD/gG. There is prominent reactivity with a protein of MW 108K-104K in 15 sera, weaker reactivity in two others and absent reactivity in the remaining four. The antibody levels, as determined by ELISA are in the range 300 to 1670 mU, with sera distributed evenly throughout the range.

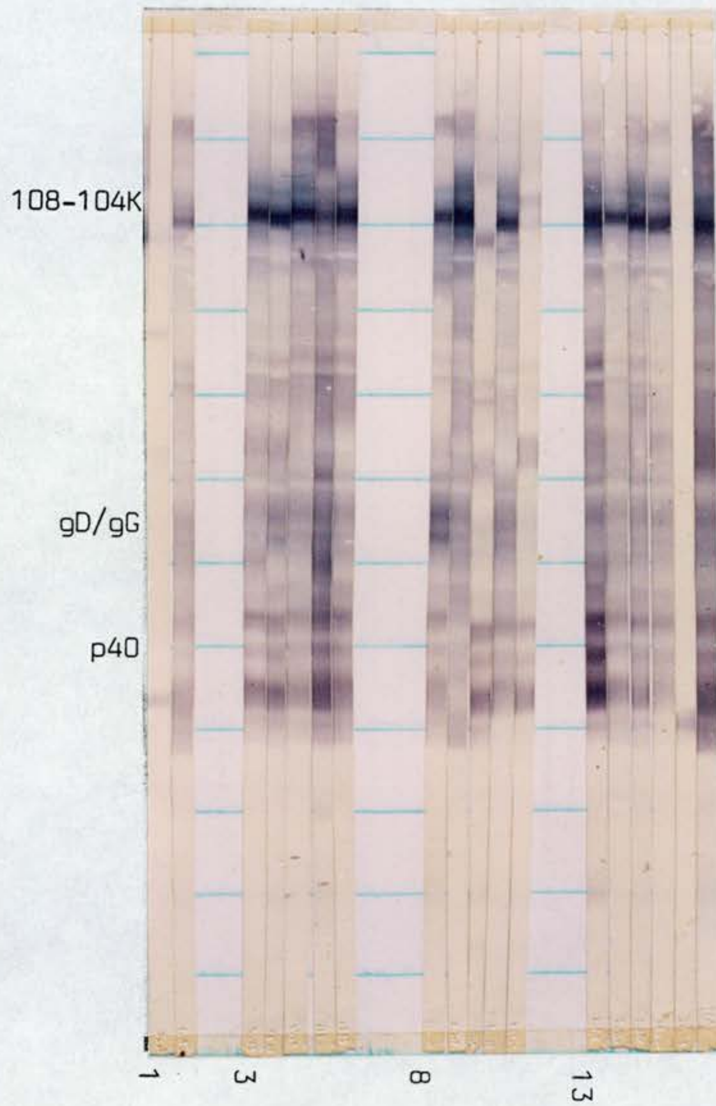


FIGURE 15. Reactivity of HSV-1 antibody positive human sera in the IB assay with HSV-1 antigen.

Serum[1]	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
ELISA/U	.32	1.0	1.1	1.7	1.6		.99	.55	1.3	1.0		.70	.98	.25	.52	.38	.30	1.1
Inhibitions:																		
No.1	4	4	1	2	2	0	2	2	0	11	14	7	8	8	2	7	0	2
2	46	42	39	40	41	39	49	44	39	85	81	51	43	73	33	63	40	38
3	21	6	4	5	0	1	2	1	1	9	6	3	8	31	19	8	5	1
10	9	4	21	3	8	6	9	25	42	22	72	8	11	52	32	72	35	18
9	100	108	102	89	94	99	98	87	85	99	90	88	92	102	99	106	102	99
13	40	24	18	1	2	8	14	14	5	6	33	14	38	36	20	24	12	9

1 Serum number corresponds to lane number in figure 15.

TABLE 8

Reactivity of the HSV-1 positive sera in ELISA for HSV antibody, and MABIA against the full panel of monoclonal antibodies.

The MABIA gave numerical data on antibody levels to individual proteins (table 8; figure 14). The HSV-1 positive sera without exception showed significant levels of blocking activity to monoclonals nos. 1 (gD), 3 (gB) and 10 (gB), and the vast majority showed blocking activity with no. 2 (gC-1). While the blocking activity of nos. 1, 3 and 10 was concentrated in the range 0-10%, the blocking of no.2 was around 40% and never less than 33%. The former monoclonals were therefore completely blockable; the partial blocking observed with no.2 presumably involves an epitope not exposed during the normal course of infection. A possible clue to this observation comes from the behaviour of the monoclonals in the IB assay. Only no.2 reacted with the denatured form of the target, and is therefore directed towards a linear epitope. It is possible that it is only partially exposed on the surface of the glycoprotein. The conformational epitopes of the other monoclonals are presumably part of the major antigenic areas of the proteins.

The sera from the children, assumed to not contain HSV-2 antibody, were also tested against the HSV-2 monoclonals in the MABIA. The results with no.9 confirm the results with the control sera, in that there was no detectable blocking activity, with the average and range of the inhibition values very similar to that of the negative controls. This emphasised the specificity of the assay. The results with no.13 show another feature of the assay; with this monoclonal, there was a range of blocking activity from 1 to 65%, concentrating around 12%. This is despite the apparent type-specificity of the monoclonal. Experiments with the control sera also showed more extensive cross-inhibition than cross-reactivity of the monoclonals.

There was no obvious difference between males and females in antibody levels, IB patterns or inhibition of monoclonals. There appeared to be no difference betweeen the adult and the child sera.

HSV-2 SERA. Figure 16 and table 9 show the results of testing the sera from patients with HSV-2 infection. The analysis is complicated by the possible presence of HSV-1 antibody in a large proportion of the sera, particularly when comparing the results of the inhibition experiments with those of the ELISA and IB assays. The IB assay again showed a broad reactivity with numerous HSV-2 specified proteins. There were differences between sera in the patterns of reactivity, in that some sera had antibody to one or two proteins, while others reacted with a wide range, particularly in the range 110 to 70K. All sera appeared reactive with p40 in this assay. There appeared to be reactivity with gG-2 in the IB assay, on comparison of the bands produced with many of the sera and those with monoclonal no.9. Antibody to gD was not readily detectable, in contrast to the IB results with HSV-1 sera.

The ELISA antibody levels were in the range 40 to 1480 mU, and correlated poorly with the observed intensity of the bands in the IB assay. The MABIA with cross-reacting and HSV-2-specific monoclonals revealed a wide range of inhibition values, greater than that produced by the HSV-1 sera, and not correlating with the IB or ELISA results. Analysis of the MABIA results on the scatter diagram (figure 14) showed several interesting features. Inhibition of no.9 was varied, but with an average figure lower than that of the negative or HSV-1 sera. The partial inhibition achieved by the HSV-2 sera is even less complete than the inhibition of no.2 with HSV-1 sera. As mentioned previously, no.9 was also reactive in the IB assay, showing the target epitope to be linear, and possibly not part of the antigenic sites of

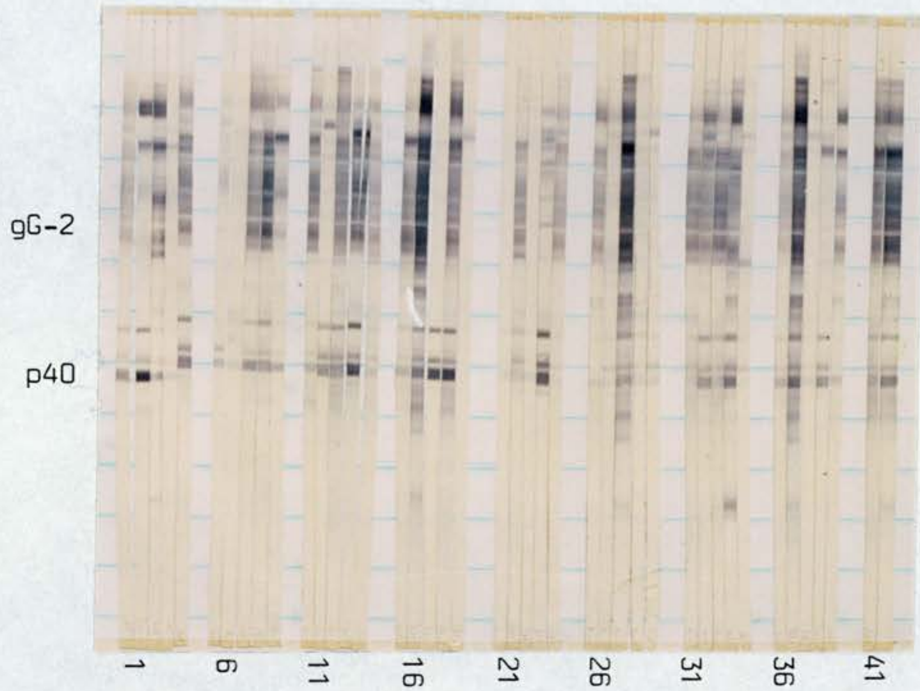


FIGURE 16. Reactivity of sera from patients with culture proven genital HSV-2 infection in the IB assay, with HSV-2 antigen.

Lane	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
ELISA/U	.53	.68	.38	.73	.67	.79	.25	.81	.77	.96	.26	.52	1.1	1.5	.79	.63	1.5	.54	.69	.21	.25	.42	.07	.54	1.1
Inhibitions:																									
No.1	1	67	72	4	19	6	6	10	11	36	60	7	1	49	3	28	22	13	1	11	10	1	5	3	2
3	7	54	62	2	15	7	6	13	9	43	68	13	3	38	0	4	21	0	0	15	36	0	7	5	5
10	16	66	67	43	25	11	63	24	13	70	70	11	4	72	15	53	52	8	30	26	26	18	67	20	4
9	91	98	21	94	89	101	116	90	87	90	89	104	74	65	81	89	62	104	79	84	94	91	98	90	86
13	0	65	54	8	12	8	17	1	5	15	41	9	2	23	0	15	1	8	6	19	20	10	12	7	6

TABLE 9a

Lane	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42
ELISA/U	.22	.04	.77	.41	.76	.60	.44	.46	.49	.69	.22	.96	.32	.88	.30	.76	1.1
Inhibitions:																	
No.1	53	1	34	2	2	3	7	6	24	2	6	32	2	2	56	5	0
3	11	1	0	1	5	10	5	7	46	0	28	17	4	1	41	0	0
10	65	20	60	11	25	52	28	50	80	23	52	50	26	7	78	23	5
9	84	89	82	96	94	70	89	60	92	91	96	65	95	101	90	86	71
13	36	7	6	10	0	4	24	3	11	8	11	8	13	9	41	17	0

TABLE 9b

Reactivity of sera from patients with culture proven genital HSV-2 infection in ELISA for HSV antibody, and by MABIA against type-common and HSV-2 specific monoclonal antibodies.

gG-2. Inhibition of no.13 was almost total with most sera, with an average less than that of the HSV-1 sera, as might be expected. Inhibition of the cross-reacting monoclonals, nos. 1, 3 and 10 was similar to that of HSV-1, but there a second, smaller clustering of sera showing partial inhibition. This was most pronounced with no.10, but can also be discerned with the other two. Checking with the table showed that the three clusters comprised roughly the same sera. These results could be interpreted as making a partial distinction between sera with HSV-1 and HSV-2 antibodies (complete inhibition of nos. 1, 3 and 10), and those with HSV-2 infection only (intermediate inhibition values).It would require a type-specific assay to show unequivocally whether this was indeed the case.

SEROCONVERSION STUDIES

STUDY GROUP. Patients presenting to the GUM clinic with symptoms and signs of genital HSV infection were investigated by viral isolation and serology. Blood samples were collected routinely at the first visit and at a subsequent visit approximately two weeks later and tested by complement fixation for HSV-specific antibody. Patients with a positive isolation and a standing titre were considered to have had a recrudescence, while those showing a rise in titre were considered to have had a primary infection if the first serum was negative. First and second sera from patients with primary infection of both types were used in the study. The low sensitivity of the CF test required a further check with a more sensitive assay for antibodies in the first sera. This was to ensure that there was no pre-existing low levels antibody present, either from previous exposure or because the patients had presented relatively late in the course of the primary

illness. The test used was IB, and patients with first sera reactive by IB, however weakly, were excluded from the study. As will be seen later, this was not the most sensitive test to use. Several patients, although negative by IB, were positive by other assays for HSV-specific antibody.

FIRST SERA. Table 10 gives the full results of testing the first sera from all 20 patients in the study. Sera were tested for antibody to other herpesviruses, and the results recorded in columns 4-6. As with the child sera, such antibody did not appear to interfere with testing for HSV-specific antibody. Column 7 records the duration of symptoms of genital HSV infection before attending the GUM clinic and collection of the first serum sample. This appeared to affect the likelihood of detecting antibody in the first serum. Column 8 gives the IB result; as mentioned previously all first sera were unreactive in this assay. Columns 9 and 10 record the indirect ELISA and IF assay results; the IF slides were read double blind as for the negative sera above. Columns 11 gives the TDCA IB results, and 12 and 13 record the counts and bands in the RIP assay. Finally, columns 14 to 19 record the results of the MABIA with the full panel of monoclonals.

Sera were tested for HSV-specific IgM in an indirect ELISA. These results were not satisfactory, since during development of the test it was noted that negative sera reacted detectably with HSV antigen, both HSV-1 and -2. Blocking with control antigen was effective at eliminating much of the reactivity with the control wells, but there remained apparently specific binding to the viral antigens, by both negative control sera and the anti-human IgM conjugate. However, the positive controls gave high OD readings distinct from the negative sera. Testing the first sera revealed a range of specific reactivities

PATIENT	SEX	HSV	CMV[1]	EBV[1]	VZV[1]	SYMPTOMS[2]	IB[3]	ELISA /mU[4]	IF	TDCA IB bands	RIP cpm	RIP bands	MABIA Inhibition of:[5] 1	2	3	10	9	13
1	F	1	-	+	-	7	-	14	++	gB	743	gB(wk)	92	97	81	82	95	70
2	F	1	-	+	-	3	-	3	-	-	40	-	99	95	93	95	89	67
3	F	1	-	+	-	NK	-	0	-	-	115	-	99	99	95	96	96	97
4	F	1	+	+	-	NK	-	7	+/-	gB(wk)	217	-	89	87	56	82	89	73
5	F	1	-	+	-	4	-	15	+/-	gB	827	gB	88	74	78	71	93	81
6	M	1	-	-	+	5	-	4	+/-	-			87	80	48	90	95	72
7	M	1	+	+	+	2	-	11	+	gB(wk)			84	89	73	92	102	73
8	M	1	+	+	+	3	-	3	+	gB(wk)			71	89	46	79	91	68
9	M	1	+	+	-	6	-	0	-	-			93	109	103	110	93	89
10	F	2	+	+	-	7	-	1	+/-	gB	438	130K gB	93	99	62	95	89	74
11	F	2	+	+	-	6	-	0	-	gB	108	-	83	105	115	98	84	76
12	F	2	-	+	-	4	-	6	++	gB	0	130K gB	81	99	98	99	93	75
13	F	2	+	+	-	3	-	17	+	gB	93	130K gB	89	97	94	105	100	77
14	F	2	+	-	-	5	-	0	-	-	14	130K gB	89	91	91	92	93	65
15	M	2	-	+	+	6	-	3	++	gB(wk)			104	99	81	87	91	82
16	M	2	-	-	-	2	-	1	+/-	-			95	104	83	102	69	78
17	M	2	-	+	+	7	-	5	-	-			89	92	98	93	92	78
18	M	2	-	+	+	2	-	1	-	-			95	86	88	100	74	77
19	M	2	-	+	+	NK	-	4	+	gB(wk)			86	93	101	92	101	87
20	M	2	+	-	-	4	-	3	+	-			90	95	81	102	93	79

TABLE 10

Reactivity of first sera in several assays for HSV-specific antibody.

1 Antibody to other herpesviruses determined as in table 9.
2 Duration of symptoms, in days, before collection of first serum.
3 All sera negative for antibody in the IB assay.
4 Antibody levels in units. Sera with more than 10 mU considered to be positive; 2-10 mU borderline, below 2 negative.
5 Sera showing inhibitions more than one standard deviation below the mean of the negative sera underlined.

with HSV antigen. Of the patients with HSV-1 infection, nos. 6 and 7 showed some IgM reactivity distinctly different from that of the negative controls. Patients 14, 19 and 20 showed greater than background serological IgM reactivity with HSV-2 antigen.

IgG antibody was unequivocally present in some of the first sera, albeit at a very low level. The detailed comparison gives data on the sensitivity and specificity of each test for antibody to HSV. Sera that were clearly positive for IgG are indicated, and were from patients 1, 4, 5, 7, 8, 10, 12, 13, 15 & 19, based on the consensus of results from columns 8 to 13. Inhibition of monoclonal antibody binding in the MABIA is not evidence for specific IgG, since apparent reactivity may be mediated by IgM.

Patient 1 showed reactivity in the ELISA, by IF, by TDCA IB and in the RIP assay. Patient 4 was reactive by TDCA IB and RIP; the results of the ELISA and IF assays are equivocal. Patients 5 was strongly reactive in the RIP assay, by TDCA IB and by ELISA. Patient 7 was reactive in the IF, ELISA and TDCA IB assays; there are unfortunately no RIP results for this patient. Patient 8 is similar although reactivity in the ELISA is borderline. Patient 10 is reactive by RIP and TDCA IB, but equivocal by IF and negative by ELISA. Patient 12 and 13 are positive for IgG by RIP, TDCA IB, IF and ELISA. Patients 15 and 19 are positive by TDCA IB and IF although the ELISA results are equivocal. There were some discrepancies in the results of these patients; patient 10 would appear to have a false negative ELISA result. Similarly, the counts in the immune precipitate of patient 12 are zero, despite showing gB and 130 K reactivity by autoradiography.

Sera from patients 2, 3, 6, 9, 14, 16, 17 and 18 were clearly negative in the assays for IgG, although patient 14 showed some reactivity in the RIP assay. The counts recovered in the immune precipitate from this latter serum were low, despite apparently showing reactivity with 130 KDa and gB proteins on SDS-PAGE.

This leaves two sera of uncertain status from patients 11, and 20. Patient 11 is negative by ELISA, RIP, and IF, but reacts in TDCA IB assay. Patient 20 is negative by TDCA IB, borderline by ELISA, but reactive in the IF assay. There is little evidence to show that the IF, RIP or TDCA IB assays are systematically more sensitive for IgG antibody than each other. This can be seen the results of patients 11 and 20, where the tests coming up positive differ between the two patients. The ELISA gives the most discrepant results, showing equivocal and negative results in several sera clearly positive by the other tests.

Of particular note is the detection of gB antibody reactivity by the RIP and TDCA IB methods in sera that were negative by conventional IB. The identity of the 130 KDa band produced with HSV-2 sera in the RIP assay is uncertain. The differences between the results of the TDCA IB and SDS IB assays indicate that it was the solubilisation of antigen that was responsible for the reduced sensitivity of the conventional IB assay; this was the only methodological difference between the two assays.

The results of the MABIA are not directly comparable to those of the other tests, since positive results may have arisen from reactivity with HSV of other immunoglobulin classes, notably IgM. Sera from patients 4, 5, 6, 7 and 8 with HSV-1 infection showed inhibition

figures of monoclonal antibodies nos. 1 and 3 significantly below the range of the negative sera. Comparing with the IgG results, patient 1 was HSV-IgG positive, but negative in the MABIA. Of the MABIA positive sera, sera from patients 4, 5, 7 and 8 were also positive for HSV-1 specific IgG. Sera from patients 6 and 7 showed reactivity in the IgM assay (with due respect to the preliminary nature of this data); this may acccount for the reactivity of the IgG-negative serum from patient 6 in the MABIA.

Turning to HSV-2 infection, inhibition of no. 13 was the most frequently observed (sera from patients 10, 11, 12 & 14). This compares with the results of the assays for IgG that showed 10, 12, 13, 15 and 19 to be reactive, 11 and 20 to be borderline and 14, 16, 17 and 18 to be negative. IgM reactivity was found in sera from patients 14, 19 and 20. Therefore, overall, there was poor correlation between the MABIA results and those for specific immunoglobulins in the sera from patients with HSV-2 infection.

SECOND SERA. Table 11 shows the results of applying the same battery of tests to the second sera. The second column gives information on the number of days elapsed between collection of the first and second sera. The remaining columns give the serological results in the same format as the previous table.

All sera showed reactivity in the IB assay, although in two patients, 19 and 20, the bands produced were very weak. Seventeen out of the twenty sera reacted with p40; this was the predominant antigen in both HSV-1 and -2 infection both in prevalence and in intensity of staining. All sera were positive by ELISA with a range of antibody levels to type-homologous virus from 20 to 860 mU. Similarly, all

PATIENT	DAY[1]	IB bands	ELISA /mU	TDCA IB bands	RIP cpm	RIP bands	MABIA Inhibition of: 1	2	3	10	9	13
1	9	gD/gG p40	270	gB	3159	gB	44	76	48	58	92	59
2	12	112K p40	130	gB	3104	gB	50	91	45	80	92	59
3	11	112,85,70K gD/gG,p40	190	gB	2791	gB	52	93	59	58	103	64
4	13	112K p40	380	gB	3644	gB	45	80	50	33	96	64
5	15	112K p40	860	gB,gD	7939	gB,gC	28	47	0	0	86	64
6	12	80K p40	170	gB			53	84	42	47	99	67
7	19	112K p40	140	gB			48	87	57	73	97	69
8	14	112K p40	190	gB			56	99	55	69	102	66
9	15	112K p40	260	gB			61	105	52	42	100	70
10	10	p40	160	gB	2386	MCP,gB 130,95,38K	67	96	68	73	86	56
11	10	150,130,107 82-66K,gD,p40	360	gB	2181	MCP,gB 130,95,32K	60	98	38	48	77	56
12	8	107K p40	190	gB	1789	MCP,gB 130,95K	67	88	67	80	85	57
13	18	107K p40	ND[2]	gB 0.63	3832	MCP,gB 130,95K	ND					
14	8	103K(?gG) p40	110	gB	769	MCP,gB 130,95K	80	94	71	84	92	68
15	8	gG,p40	66	gB(wk)			86	91	58	60	90	71
16	14	gG	108	gB			79	106	71	49	89	59
17	11	80K,gG p40	190	gB			76	102	62	34	81	67
18	14	80K,gG	55	gB(wk)			94	93	65	73	80	67
19	14	60K	20	gB(wk)			95	90	84	65	84	67
20	13	103K	55	gB(wk)			104	95	75	66	91	68

TABLE 11

Reactivity of second sera in several assays for HSV specific-antibody.

Footnotes as for table 10.

1 Number of days elapsed between first and second sera.

2 ND: not done.

second sera were reactive with gB in the TDCA IB assay, although two sera showed further specificities for other bands; in the case of patient 5, the band had been identified as gD by monoclonal no.1. Reactivity with gB was exceptionally weak in some sera, as indicated, and this correlates with the ELISA antibody level. Finally, the RIP results show elevated counts with the sera tested, and universal reactivity with gB on SDS-PAGE analysis. Sera from patients with type 2 infection also reacted with MCP, a 130 K protein, a 95 K protein and some lower MW species. Patient 5 also showed reactivity with gC, but not to gD as found in the TDCA IB assay.

Figures 17, 18 and 19 show examples of the reactivity of the first and second sera in the SDS-IB, RIP and TDCA IB assays. They are from patients 2, 5, 9 and 7, and show the absence of reactivity of the first sera in the IB assay, despite gB antibodies in the other two assays. Figure 17 shows the prominent reactivity of the second sera with p40 in the IB assay, while figure 18 and 19 show the reactivity with gB in the same sera.

Figure 20 is a diagram to show the inhibition results more clearly. The negative sera mean and 1 standard deviation ranges are shown above a set of arrows that represent the change in inhibition figure that occurs between the first and second sera. The arrows are in the same order as the table, with the HSV-1 sera above those from HSV-2, and the females above the male patients. With monoclonals nos. 1, 3 and 10, there are very large decreases in inhibition figures, signifying increases in antibody levels to the target proteins. As is found in the experiments with the convalescent sera, sera from patients with type 1 infection inhibit monoclonal no.1 more effectively than those with type 2, while there is little difference in inhibition of nos. 3

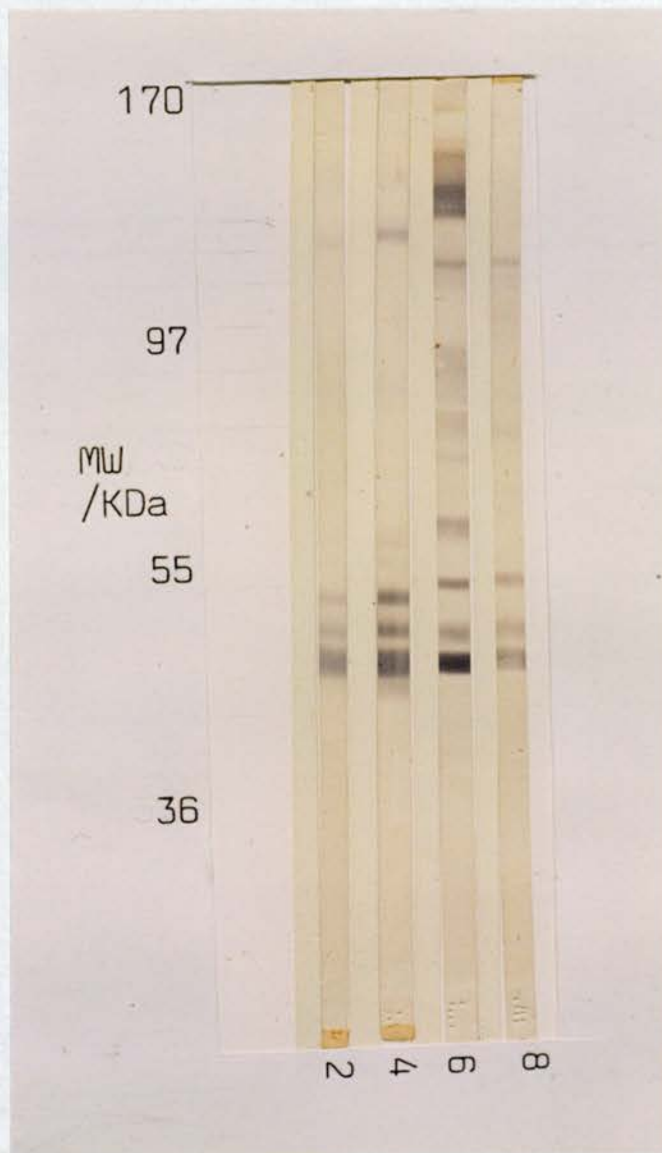


FIGURE 17. Reactivity of first and second sera from patients with primary HSV infection in the IB assay with type-homologous antigen. Lanes 1 & 2, first and second sera from patient 2; lanes 3 & 4, patient 5; lanes 5 & 6, patient 9; lanes 7 & 8, patient 7.

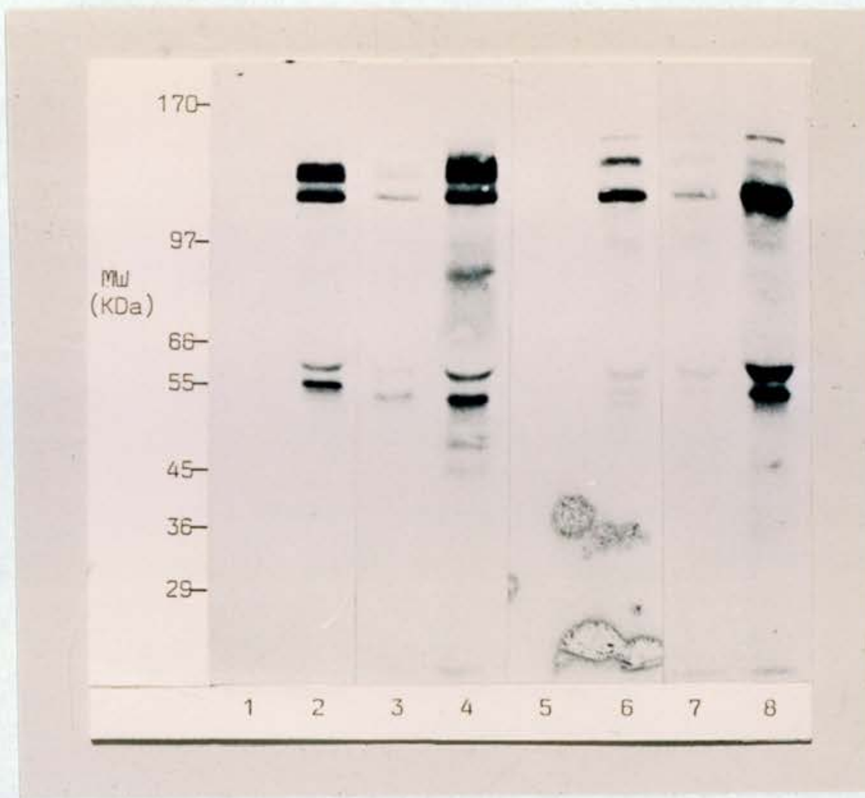


FIGURE 18. Reactivity of first and second sera from patients with primary HSV infection in the RIP assay with type-homologous antigen. Lanes as in figure 17.

FIGURE 19. Reactivity of first and second sera from patients with primary HSV infection in the TDCA IB assay with type-homologous antigen. Lanes as in figure 17.

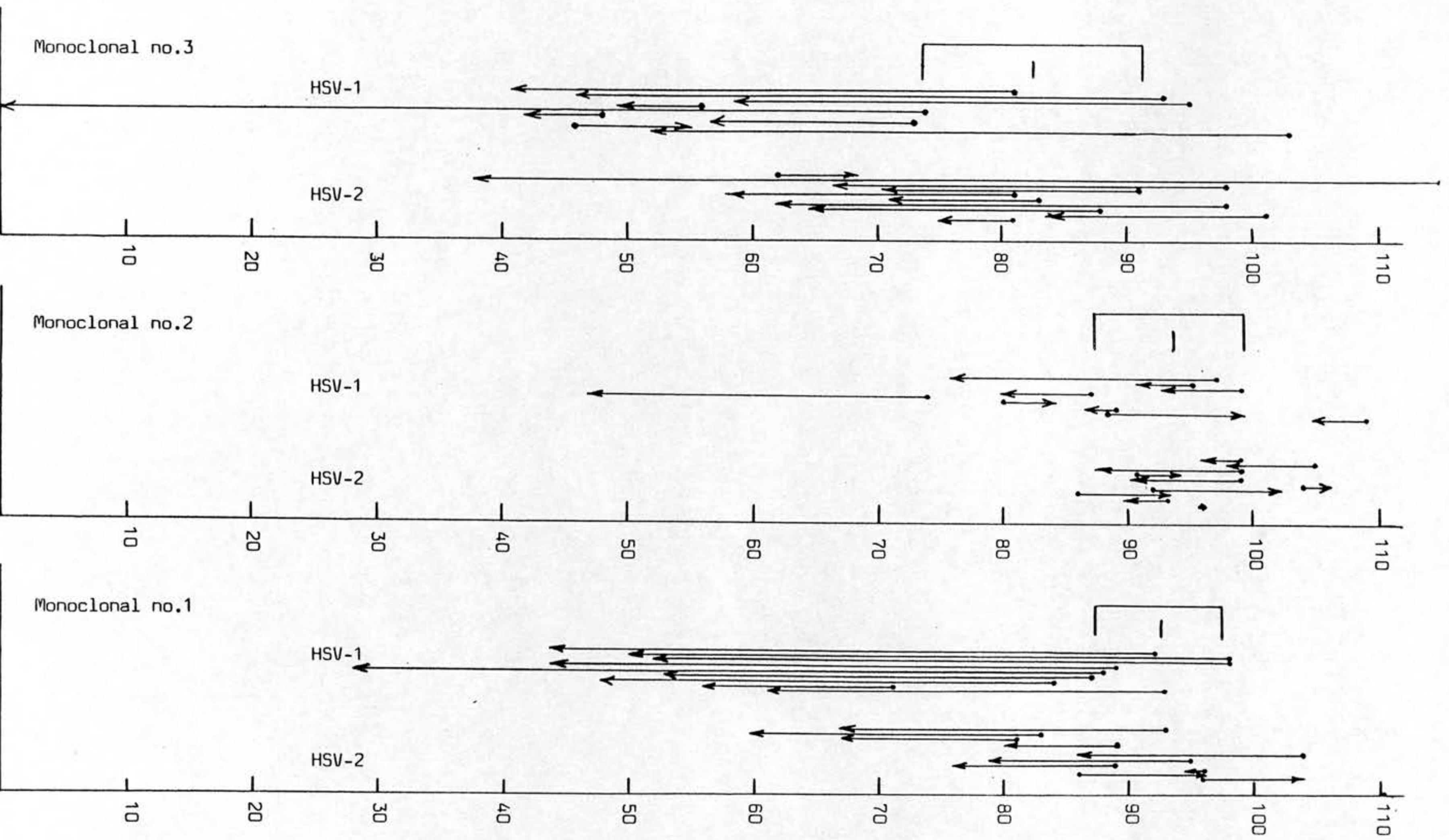


FIGURE 20: Diagram to illustrate change in reactivity between first and second sera of patients with primary HSV infection in MABIA. Inhibition values on x axis. Mean and range of negative sera illustrated above plotted values for comparative purposes. Figure continued overleaf.

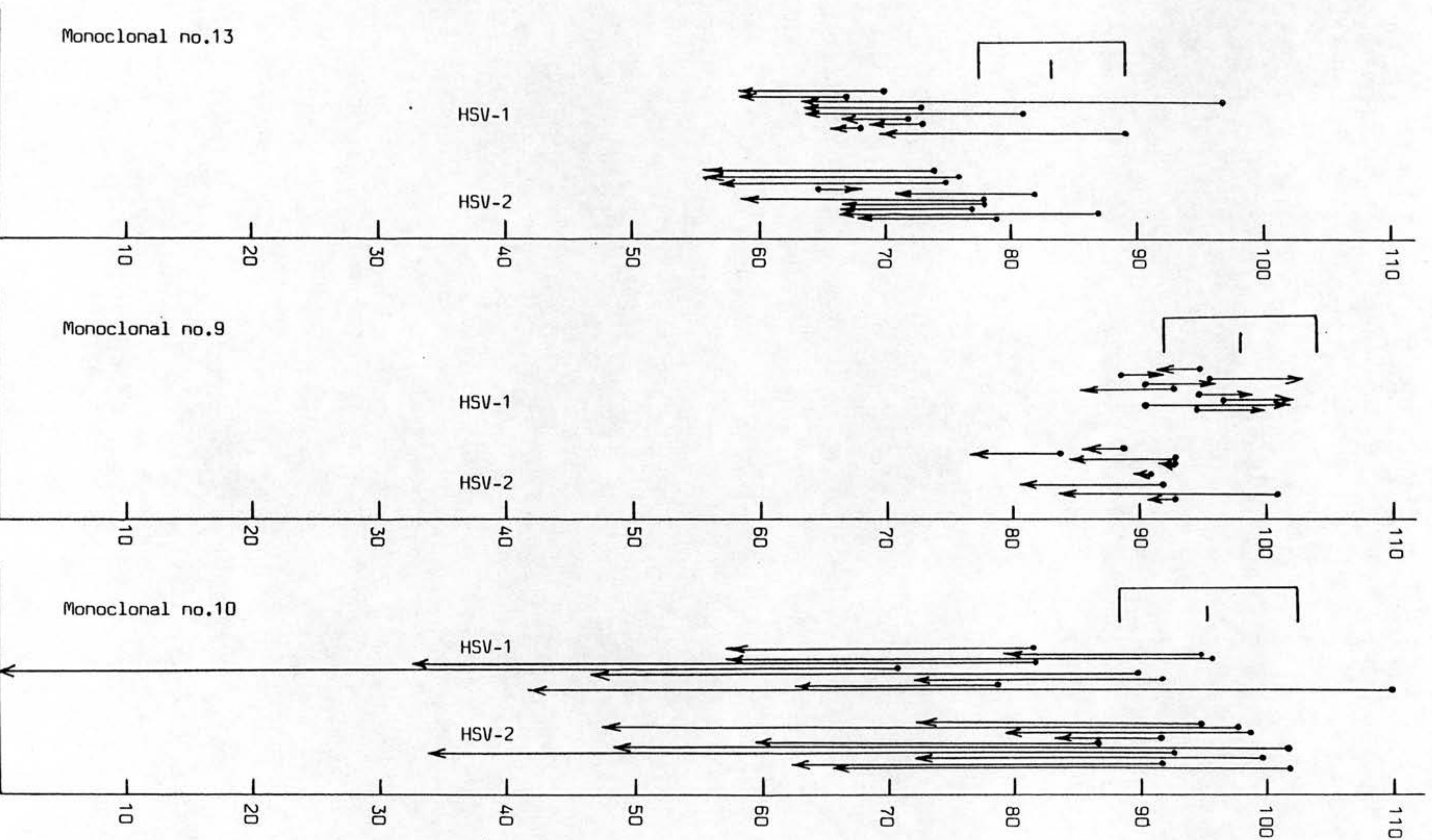


FIGURE 20 (cont'd): See preceding page for legend.

and 10 between HSV-1 and -2 antisera. Although several HSV-2 sera failed to develop reactivity with the target of monoclonal no.1, the results with no.13 confirm the presence of gD antibody in the majority; testing sera from patients with HSV-1 infection showed, in turn, less inhibition of this monoclonal than sera from patients with HSV-2.

Patients who did not develop antibody capable of inhibiting a given monclonal antibody normally show very short arrows that point in both directions within the negative range of the monoclonal. This appears to be the case with HSV-2 sera with monoclonal no.2. However, sera from some patients with HSV-1 infection show definite decreases in the inhibition figure, signifying the appearance of gC-1 specific antibody. The reverse applies to monoclonal no.9 (gG-2), where there is no clear trend with the HSV-1 sera, and the arrows lie largely within the negative range. By contrast, there is a leftward trend with the arrows from the HSV-2 sera, with several second sera clearly being positive for antibody to gG-2.

Overall, there was good correlation between the tests as to which second sera were strongly reactive, and which ones were weak. Typically, sera that produced high numerical results and low inhibition figures also appeared to be more broadly reactive in the immunoblotting and RIP methods. However, there were marked differences in the sensitivity of the tests for antibody of differing target specificities. The insensitivity of coventional IB for glycoprotein antibody is demonstrated, firstly, by its failure to detect such antibody in any of the first sera, and by the prominence of the reaction with p40, a tegument protein in most of the second sera. The TDCA IB and RIP methods showed sensitivity for glycoprotein antibody, but did not

detect antibody to p40 or, in the case of HSV-1 sera, to any capsid proteins. However, both methods failed to detect gG antibody in any of the HSV-2 sera, despite the evidence for such antibody by conventional IB and, to a limited extent, by the MABIA. Taken together, the tests demonstrate antibody reactivity with a range of glycoproteins, and to at least one capsid protein, in the second sera, and clear evidence for gB, and possibly gD, antibody at low levels in some of the first sera.

Unfortunately, there are no RIP results for most of the male sera. However, the ELISA antibody levels show an average male level of 127 mU and and average female level of 300 mU. The variability in antibody levels results in their ranges overlapping, so the figures are not significant statistically, although they do point towards a sex-related difference in antibody response.

HUMAN IMMUNODEFICIENCY VIRUS

STUDY GROUPS

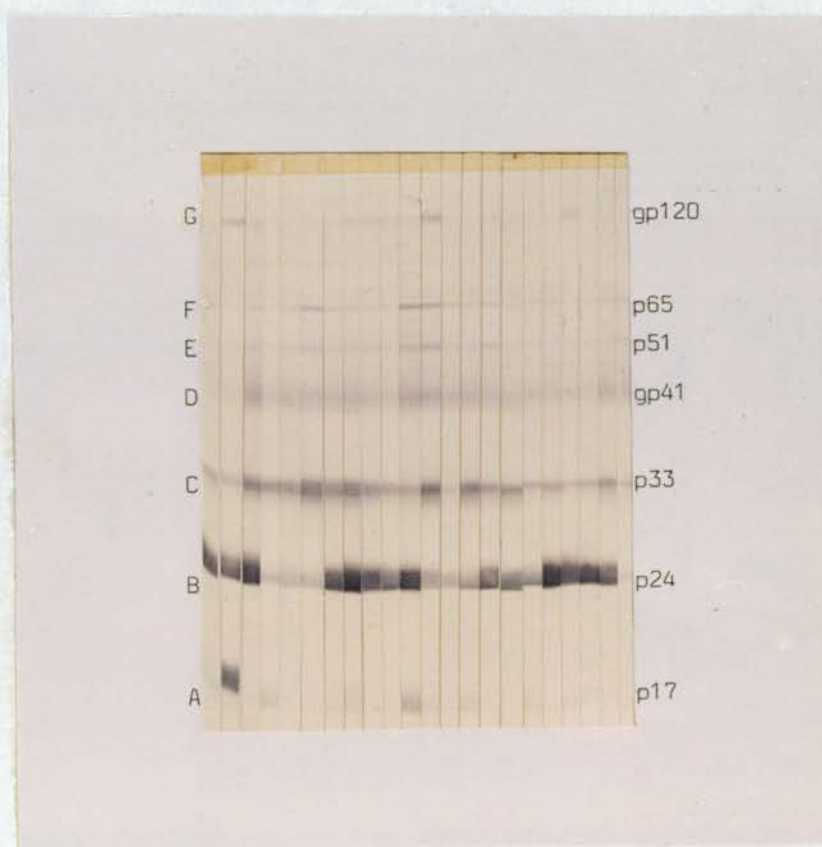
The immunoblotting method for the detection of HIV antibody was used to confirm the presence of antibody in sera referred to the reference laboratory. Sera were sent from various hospitals and the Blood Transfusion Service that had been found to be positive by conventional screening assays. The IB method was also used to investigate sera that showed borderline or discrepant reactivities in the screening tests. The results both clarified the diagnosis of patients, and was useful in the investigation of the different types and causes of non-specific reactions, and comparison of the sensitivity and specificity of the

different commercially available tests. Two years ago, sera were screened by the Abbott direct binding assay, and confirmed with the Wellcome test. However the Abbott test produced so many false positive reactions with stored or heat inactivated sera that its use was discontinued. Only the results comparing the IB assay with the Wellcome and Pasteur assays are presented. One problem with this work stems from the impossibility of knowing for certain whether the patient had been exposed to HIV in the past. In particular, many sera were from blood donors, in whom risk factors could not be assessed. The majority of the positive sera confirmed by IB were from IVDAs, although several positive male homosexual and haemophiliac sera were also referred.

Figure 21 shows the result of testing a number of HIV-antibody positive sera by the IB assay. The sera show a typical range of reactivities with several HIV-encoded proteins. The identification of proteins was made on the basis of molecular weight, and comparison with the results of other investigators. Table 12 shows the identification of bands, their apparent MW, and, in the case of prominent bands, the prevalence of reactivity to them in a panel of 66 HIV antibody-positive sera. Samples were considered to be positive if they reacted with at least two bands in the IB assay, provided the bands indicated reaction with proteins from different genes.

Sera referred to our laboratory could be divided into two groups for the purposes of this investigation. The unreactive group comprised sera that produced no bands by IB, while the reactive group was made up of sera that broad reactivity in the IB assay, reactive with at least three bands. Sera showing restricted reactivity with only one or two bands were investigated separately in the next section.

FIGURE 21. Reactivity of 22 HIV antibody positive sera in the IB assay with purified HIV as antigen. Accepted nomenclature of bands on right; coded nomenclature for use in tables on left.



NAME	CODE[1]	ORIGIN[2]	APPARENT MW[3] /KDa	PREVALENCE n=66
p12		*gag*	-	-
p17	A	*gag*	19	34 (52%)
p24	B	*gag*	25	66 (100%)
p33	C	*pol*	31	63 (95%)
pr40		*gag*	40	-
gp41	D	*env*	41	55 (83%)
pr45		*gag* ?	45	-
p51	E	*pol*	55	55 (83%)
pr55		*gag*	60	-
p65	F	*pol*	65	64 (97%)
gp120	G	*env*	120	20 (30%)

1 code adopted for scoring IB results for routine laboratory use
2 gene identified as coding for the protein
3 molecular weight on IB, note some differences with quoted molecular weights

TABLE 12

Apparent molecular weight and prevalence of antibody reactivity with HIV proteins in the IB assay

The results of testing sera unreactive in the IB assay by the Wellcome EIA (competition assay) and in the Pasteur assay (direct binding) are shown in figure 22. Several sera were only tested by the Wellcome assay. These results are presented in a separate graph to the left of the figure; however, the axes are aligned so the results may still be compared. The results from the Wellcome assay are expressed as the ratio of the ODs of the cut-off value (calculated from control sera provided in the kit) to the test result. Therefore, a ratio of 1.0 or greater indicates a positive test result; all sera with ratios below 1.0 are to be considered negative. The average reading of these unreactive sera was 0.653 with a standard deviation of 0.227. However, the distribution is skewed, with a greater than normal numbers of sera with high ratios. The ratios in the Pasteur assay were calculated by dividing the test value with the cut-off value (determined from control sera). Ratios of unreactive sera in this test tended to cluster around zero (average 0.69, range ∓ 1.50). While the Pasteur assay gave no positive reactions, 8 sera were above the cut-off in the Wellcome assay, and a total of 21 sera were above 1 standard deviation from the negative mean. The ratios of these sera in the Pasteur assay suggested that these sera were actually negative. However, these sera were repeatably more reactive than average in the Wellcome test, and the evidence from the BTS suggests that donors with sera that produce high ratios continue to do so at subsequent donations, often over some years.

Serum A and a follow-up serum, marked B, from the same individual were referred by the North London BTS, as being positive in their screening test for antibody to HIV. On testing, they showed repeatable reactivity in the Wellcome test, but could not be confirmed as positive by either Pasteur or IB, and were investigated more

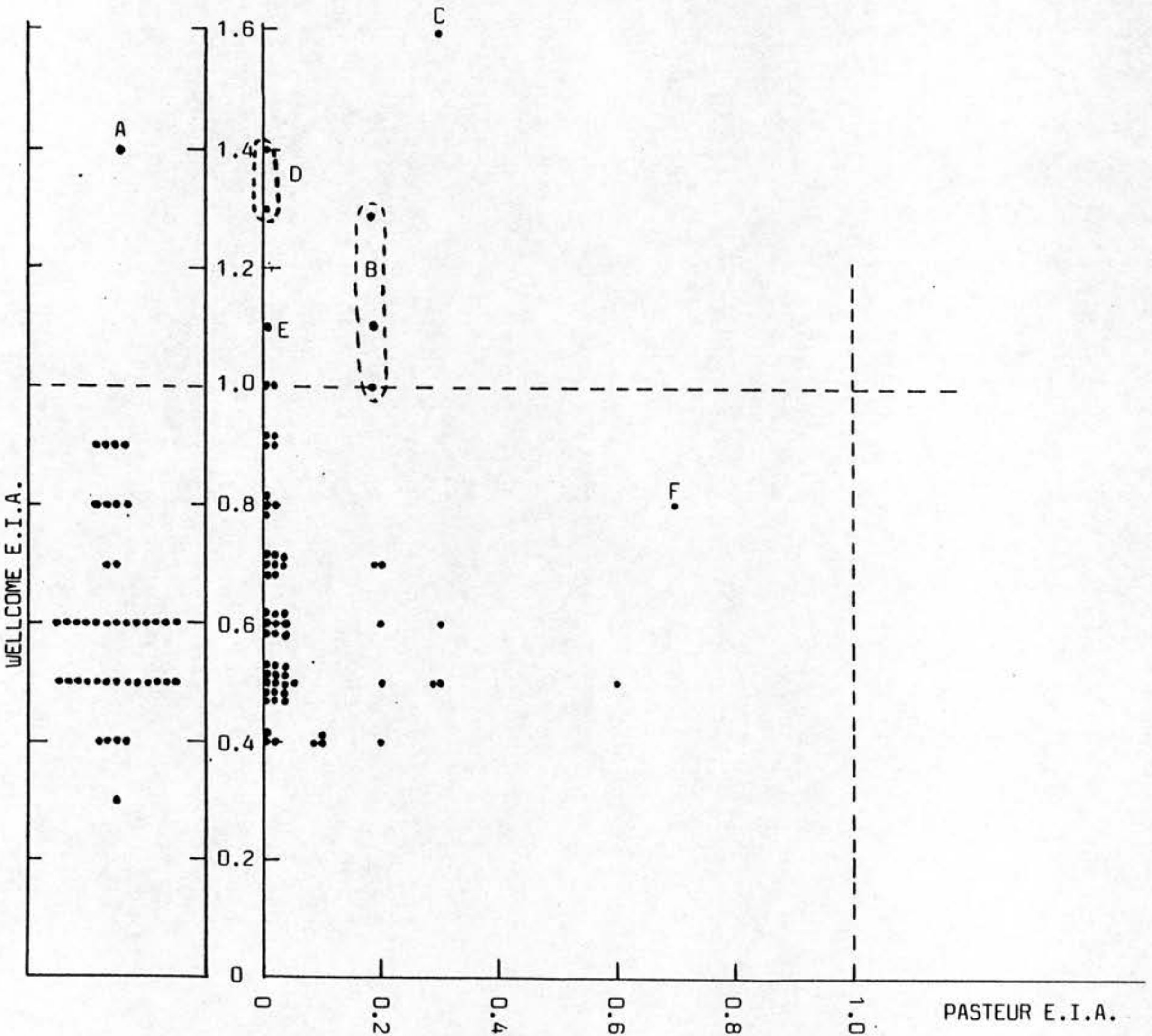


FIGURE 22: Reactivity of IB negative sera in Wellcome and Pasteur EIAs. Results expressed as test to cutoff OD ratio (Pasteur) or cutoff to test (Wellcome); results of sera not tested by Pasteur EIA plotted on left. See text for an explanation of points A-F.

extensively by several other assays. Both sera reacted non-specifically with uninfected T-lymphocytes to a far greater extent than normal sera in the IF assay, with no evidence of increased fluorescence with CEM cells infected with the RF strain of HIV. Serum A was negative for antibody in the Dupont direct binding assay and both sera were negative for "env" and "core" antibodies in the highly specific Abbott confirmation assay. The extra tests confirmed that the serum was negative for HIV antibody, although if the non-specificity was caused by antibody to cellular components, as suggested by the IF assay, then it is not clear how such antibodies could interfere with the Wellcome assay.

Sera marked C and F were referred by Dr R. Tedder, Middlesex Hospital, London. They were taken from low risk patients and were known to cause non-specific reactivity in a wide range of tests. Serum C showed slight or no reactivity in the Pasteur EIA, but was relatively reactive in the Wellcome assay. Serum F showed some reactivity in both tests, although was negative by IB. Samples marked D and E were referred from Dundee BTS; they showed weak reactivity in the Wellcome assay, but could not be confirmed by the other two tests. No sera have yet been found that give a false postive result in the Pasteur assay, although this may be a reflection of screening policy. In both this laboratory, and in the BTS, sera are generally screened by Wellcome, and reactive samples confirmed by Pasteur. If the Pasteur assay was used for primary screening, then non-confirmable reactive samples may be found in this assay.

The results from testing the reactive sera are shown in figure 23 (note the change in scale from figure 22). All but two sera were positive in both the Wellcome and Pasteur assays. There was a weak

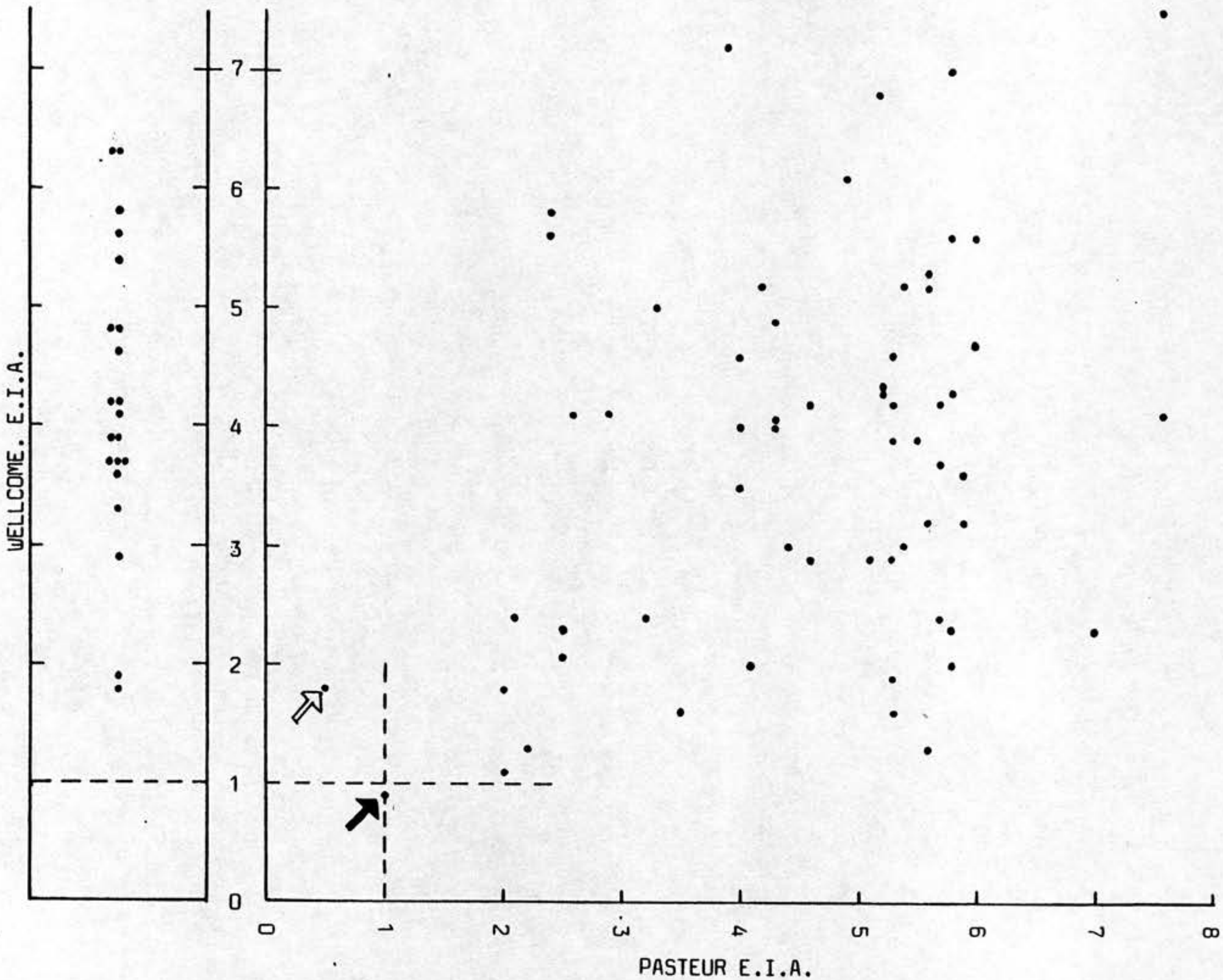


FIGURE 23: Reactivity of IB positive sera in Wellcome and Pasteur EIAs. Results expressed as in Figure 22 (note change of scale). See text for explanation of results indicated by arrow.

correlation between the ratios produced by the two tests. The serum marked with the solid arrow was from a patient who was the recent sexual contact of an HIV carrier. The serum was antigen positive on preliminary screening suggesting that the patient was very close to seroconversion for antibody. The reactivity of the serum in the IB assay was weak, with antibody restricted to p24, pr55, p51 and p65. The serum was on the cut-off value of the Dupont direct binding assay. Although this serum was positive, the ratio was indistinguishable from many of the negative sera. In the seroconversion studies to be described, borderline ratios with the Wellcome test are often associated with seroconversion, where antibody levels may be very low. The serum labelled with the hollow arrow was repeatably negative in the Pasteur assay, but positive by Wellcome and reactive with several bands in the IB assay. The reason for the discrepancy has not been found, but may reflect a sensitivity difference between the tests.

The last group of sera that arose from routine screening of referred sera comprised those that produced abnormally restricted patterns in the IB assay, and were investigated further by a number of methods. Due to differing availability of various tests for HIV antibody at different times, and the small volume of some of the samples, the analysis of some sera has not been comprehensive. Several sera have shown reactivity with p17 and no other HIV protein. One serum in an evaluation panel from Colindale showed this pattern, but there was insufficient to investigate it further. It had been reported as being of borderline reactivity in the original Abbott EIA. However, the same pattern was found in two unrelated blood donors from Fife, who had been identified as borderline in a Dupont ELISA screen. The samples from these two patients were negative by Pasteur (0.2, 0.0); negative by Wellcome (0.5, 0.5); and negative for both anti-"core" and

anti-"env" in the Abbott confirmation assay. Since they were blood donors, it was difficult to determine risk factors, but they were aged 55 and 60 making HIV infection unlikely. Neither had travelled to Africa. Samples from previous donations from both patients were available as far back as 1983. These were tested by IB and also found to have the same pattern. This discounts a seroconversion as a cause for the restricted reactivity. Furthermore, the prevalence of HIV infection in Fife in 1983 was so low as to discount HIV as a cause of this antibody reactivity. Sera from both patients were negative for HTLV-I antibody.

Seroconversion accounted for several very restricted patterns observed in referred samples. Sera from a haemophiliac (p24 only, Wellcome 1.0, anti-"env" positive, anti-"core" positive, p24 antigen positive), a male homosexual (p24, pr55, Wellcome 1.0, Pasteur 1.6, anti-"env" positive, anti-"core" positive), and two sera from a blood recipient taken three days apart (both p17, p24, Wellcome 0.5, 0.5, Pasteur 0.5, 0.5, both anti-"env" borderline, both anti-"core" negative, both antigen positive) were picked up on routine screening. Follow-up sera from all three patients taken after 1 week, 2 months and 1 week, were clearly positive by all tests. Further sera from the haemophiliac patient and the blood recipient were investigated in more detail (see next section).

The most unusual serum came from a middle-aged man who had worked in Kampala, Uganda. A blood donation from this patient was outside the normal range on the Wellcome screen but below the cut-off value. The serum reacted very weakly with p25 and pr55 in the IB assay, but was negative by Pasteur and Dupont direct binding assays. It was repeatably weakly positive for anti-"env" antibody in the Abbott

confirmation assay, but very strongly reactive for anti-"core" (stronger than any other serum so far tested, including those positive for HIV antibody). The serum was negative for HTLV-I antibody, but obviously requires testing for HIV-2 (not available at the time of writing).

SEROCONVERSION STUDIES

Using the stored serum collection in the Hepatitis Reference Laboratory, it proved possible to trace back known antibody positive patients and to identify the time of seroconversion. In some patients, notably haemophiliacs, several sera were collected at close intervals around seroconversion, and therefore would be suitable source material for critical comparison of the various tests. Sequences of sera from 8 patients were suitable for the comparison of the sensitivity and specificity of the IB assay, the Wellcome EIA, the Dupont EIA, the Abbott confirmatory EIA and the immunofluorescence assay described in the methods. Table 13 presents the data obtained from these patients. Column 4 records the number of days elapsed between collection of the last antibody negative serum of and collection of subsequent sera. As can be seen, this inevitably was very variable between patients, making the analysis of the results more difficult.

Column 5 records the IB results. None of the negative sera produced any bands, while the first positive sera commonly showed reactivity confined entirely to p24 (band B) and pr55 in many patients. Subsequent sera showed a broadening of antibody reactivity to proteins encoded by the *pol* and *env* genes. The table does not record the increased intensity of the bands with time after seroconversion. To

PATIENT	DIAGNOSIS	DATE	DAYS[1]	EXPERIMENTAL RESULTS							
				IB ASSAY bands[2]	WELLCOME ratio[3]	ABBOTT ratio[4]	IFA[5]	DUPONT /mU	ANTI-"env" /mU	ANTI-"core" /mU	ANTIGEN /pg/ml
1	Blood donor	13/12/83	0	none	0.4	0.8	-	-	ND	ND	ND
		10/04/84	119	AB DE	0.8/0.6	1.0	+/-	+	..	..	..
		10/07/84	210	BCDEF	2.0	2.2	+	+	..	..	..
		09/10/84	301	BCDEF	2.6	3.2	+	+	..	..	..
2	Blood recipient	10/01/84	0	none	0.6	2.6	-	0.3	0	0	
		06/03/84	56	AB	0.6	0.7	-	0.6	1.5	0	
		09/03/84	58	AB	0.6	0.7	-	1.3	1.0	0	
		26/03/84	76	AB	1.1	3.4	+		42.2	56.2	
		20/05/84	131	AB D F	1.9	6.3	+				
3	Haemo-philiac	15/10/84	0	none	0.5	0.5	-	-	0	0	
		27/11/84	43	B	1.2	0.8	+/-	+	32	2.4	
		09/01/85	86	AB F	1.2	1.6	-	+	100	17.8	
		16/01/85	93	AB DEF	2.4	2.2	-	+			
4	Haemo-philiac	09/04/84	0	none	0.4	0.6	-	0	0	0	3600
		29/05/84	50	AB D	1.4	1.0	+	21	89	3	0
		05/09/84	149	ABCDEFG	1.9	4.8		370			
		31/10/84	205	ABCDEFG	2.4	5.7					
5	Haemo-philiac	01/02/84	0	none	0.5	0.9	-	0	0	0	
		29/05/84	118	AB	1.2	0.7	+	6	38	1.7	0
		06/07/84	156	ABCDEF	2.2	1.8	+	18			
		05/10/84	247	BCDEF	3.1	4.6		108		75	
6	Haemo-philiac	16/04/84	0	none	0.6	1.0	-	0	0	0	0
		21/07/84	95	ABCDEF	1.5	2.5	+	17	53	2.7	0
		23/08/84	129	ABCDEF	1.7	3.6		63			0
		21/09/84	158		1.1	3.8		80		4.7	0
7	Haemo-philiac	17/04/84	0	none	0.5		-	0	0	0	30
		23/04/84	6	B	1.0			8	27	2.7	50
		08/05/84	21	B DEF	1.7		+	19		4.0	0
		14/05/84	27	BCDEF				26		6.7	0
		29/05/84	42	BCDEF				32		7.1	
8	Haemo-philiac	27/03/84	0	none	0.5	1.9	-	0	0	0	
		29/05/84	63	AB DEF	0.8	2.3	+	24	21	3.2	0
		31/07/84	126	ABCDEF	1.0	3.1	+	48			
		05/09/84	162		1.2	3.3	+	52			

1 Days elapsed since the last antibody negative serum.
2 IB bands identified alphabetically (see table 12); A - p17; B - p24; C - p33; D - gp41; E - p51 F - p65; G - gp120
3 cut-off to test ratio
4 test to cut-off ratio
5 results results read double blind; +/- weak or indeterminate

TABLE 13

Reactivity of sera from patients infected with HIV close to the point of seroconversion in several assays for HIV-specific antibody.

show this, figure 24 (a-e) shows several examples of seroconversions. The seroconversion from patient 7 (figure 24e) is particularly useful since the samples happen to very closely spaced. The restricted reactivity of the first sera would cause problems with interpretation if they were found on routine screening. However, with the knowledge that follow-up sera from the patients were subsequently clearly positive by several assay methods, reactivity with p24 alone in the IB assay appeared to be significant. This is despite the fact that the sera would be classified as negative by conventional criteria (reactivity with at least two bands encoded by different genes).

The next column shows the cut-off to test ratios of the sera in the Wellcome assay. The ratios of the negative sera were in the range 0.4 to 0.6. The first positive sera were clearly reactive in 4 of the 8 patients, borderline in one, and unreactive in the remaining three. The ratios of the sera from patients 1 and 8 were marginally elevated from those of the negative sera, but the second and third sera from patient 2 were indistinguishable from the negative sera. The two sera were tested by the Edinburgh BTS, who, with much larger batches and numerous controls, routinely apply statistical methods to detect weak reactions in the Wellcome assay that would not be found in a diagnostic laboratory. Both sera were completely unreactive and indistinguishable from a large panel of negative blood donors, while the weak reactivity of second serum from patient 1 was detected by the Edinburgh BTS.

The Abbott results are recorded in the next column. The result from the first serum of patient 8 indicates the non-specificity of this test. The serum produced a ratio of 1.8, but subsequent investigation showed that the patient had not been exposed to HIV by that date (see

(a)

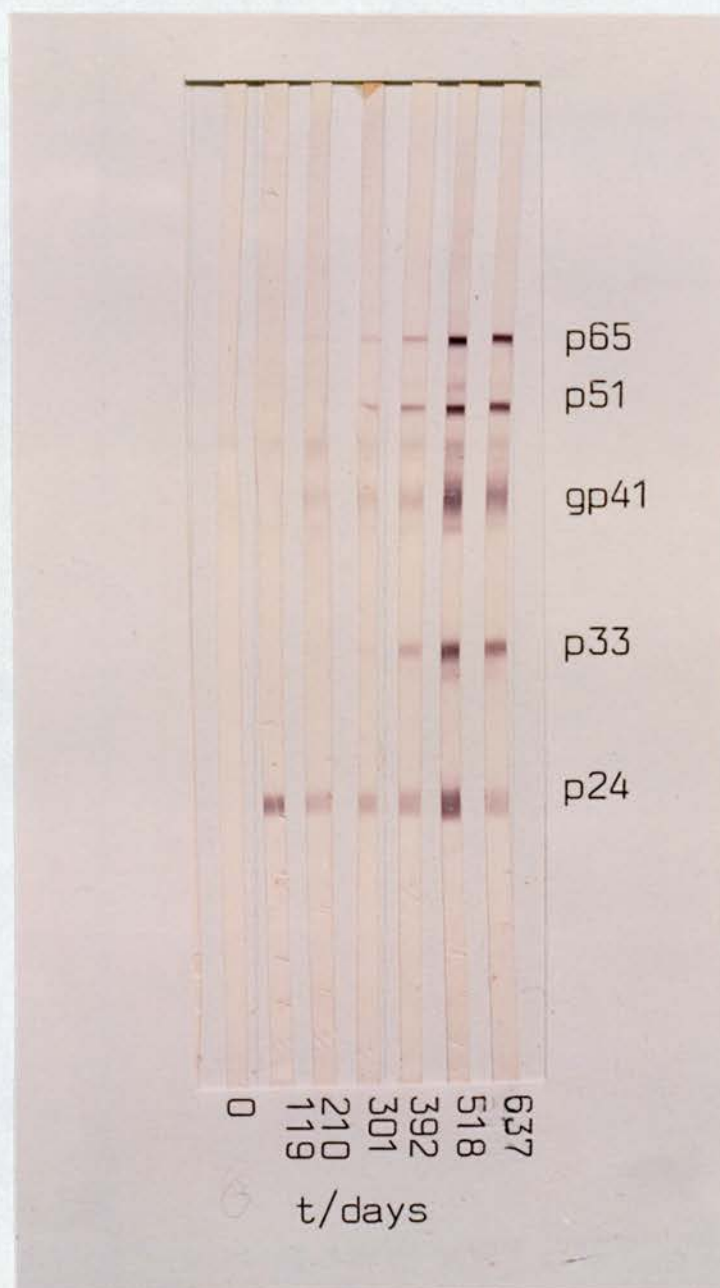


FIGURE 24. Reactivity of sera from patients seroconverting for antibody to HIV in the IB assay. Time, in days, from date of collection of last antibody negative serum indicated under strips. Panel a: patient 1

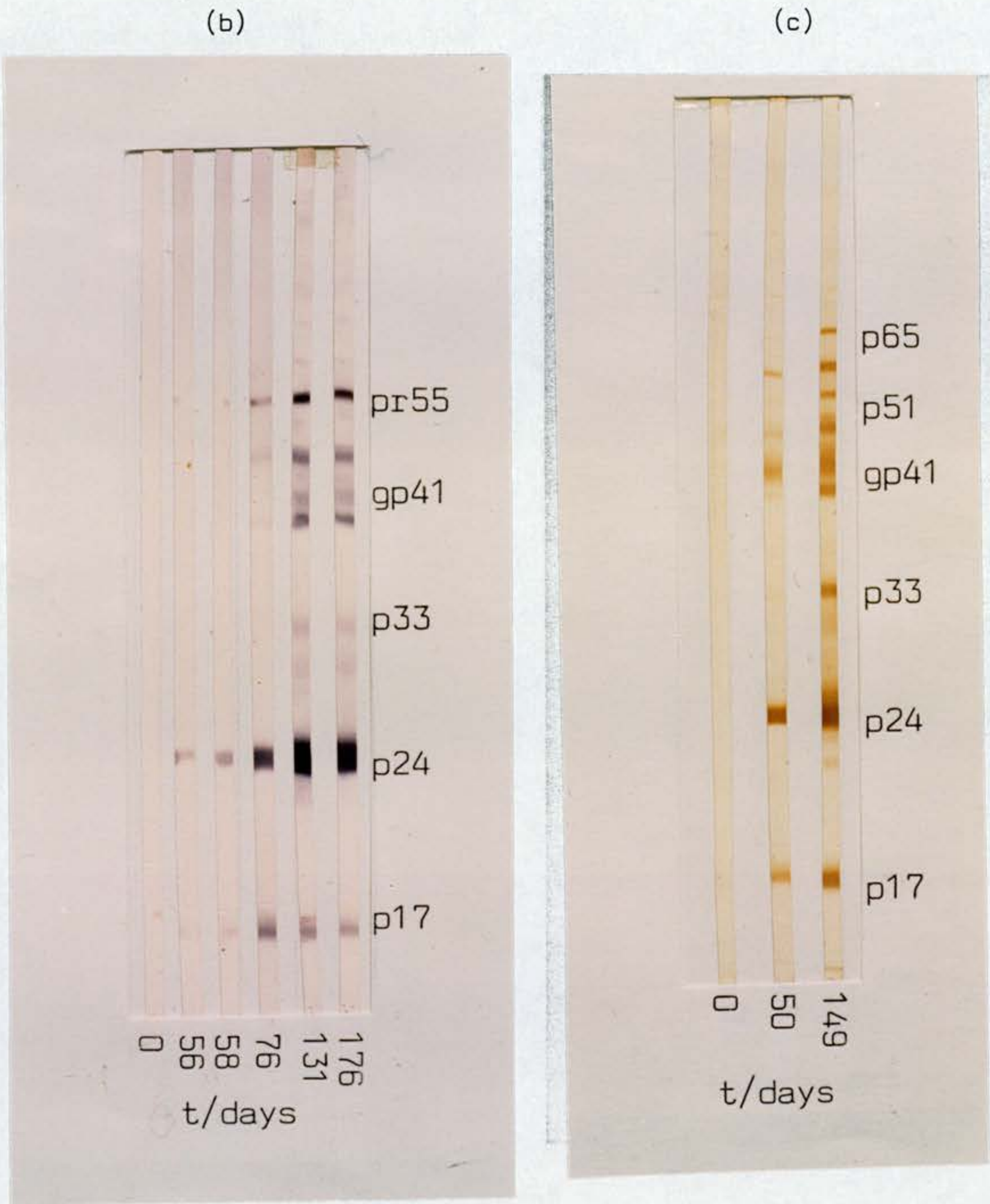


FIGURE 24 (cont'd). See above for legend. Panel b: patient 2; panel c: patient 4.

(d)

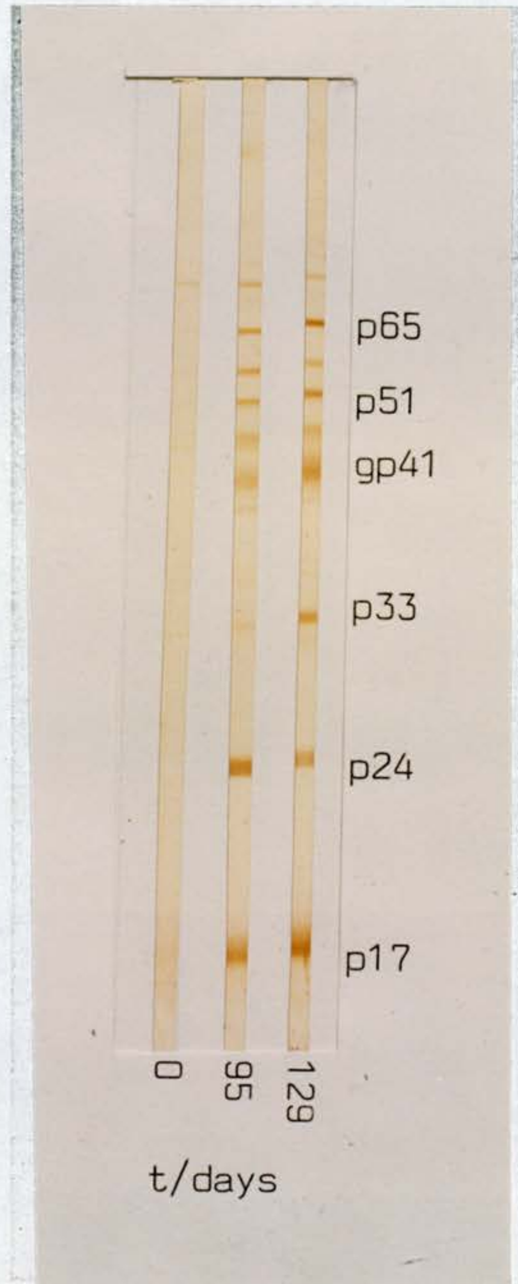
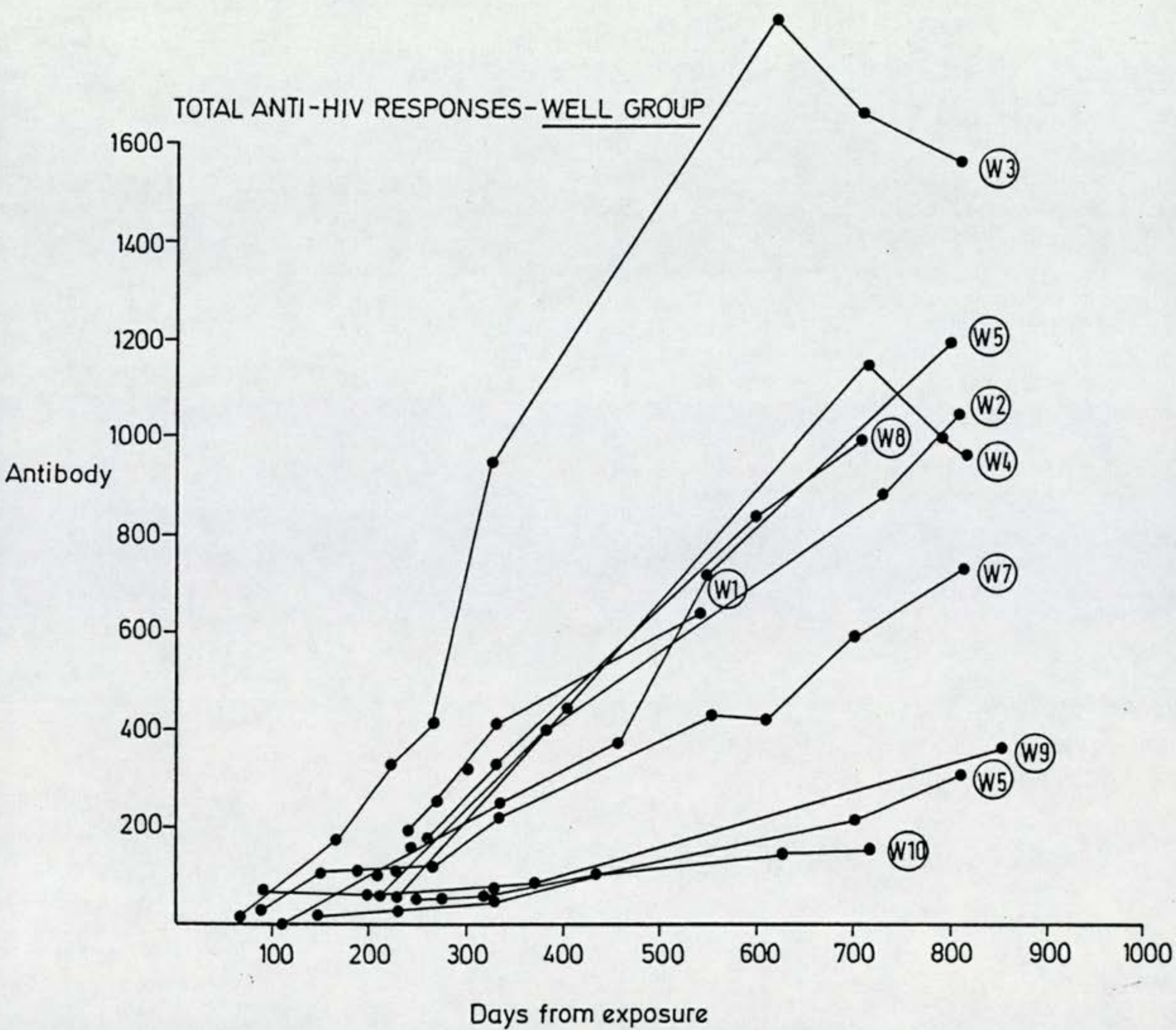


FIGURE 24 (cont'd). See above for legend. Panel d: patient 6.


TOTAL ANTI-HIV RESPONSES-WELL GROUP
1600
1400
1200
1000
800
600
400
200
Antibody
100
200
300
400
500
600
700
800
900
1000
Days from exposure
W3
W5
W2
W8
W4
W1
W7
W9
W5
W10

(e)

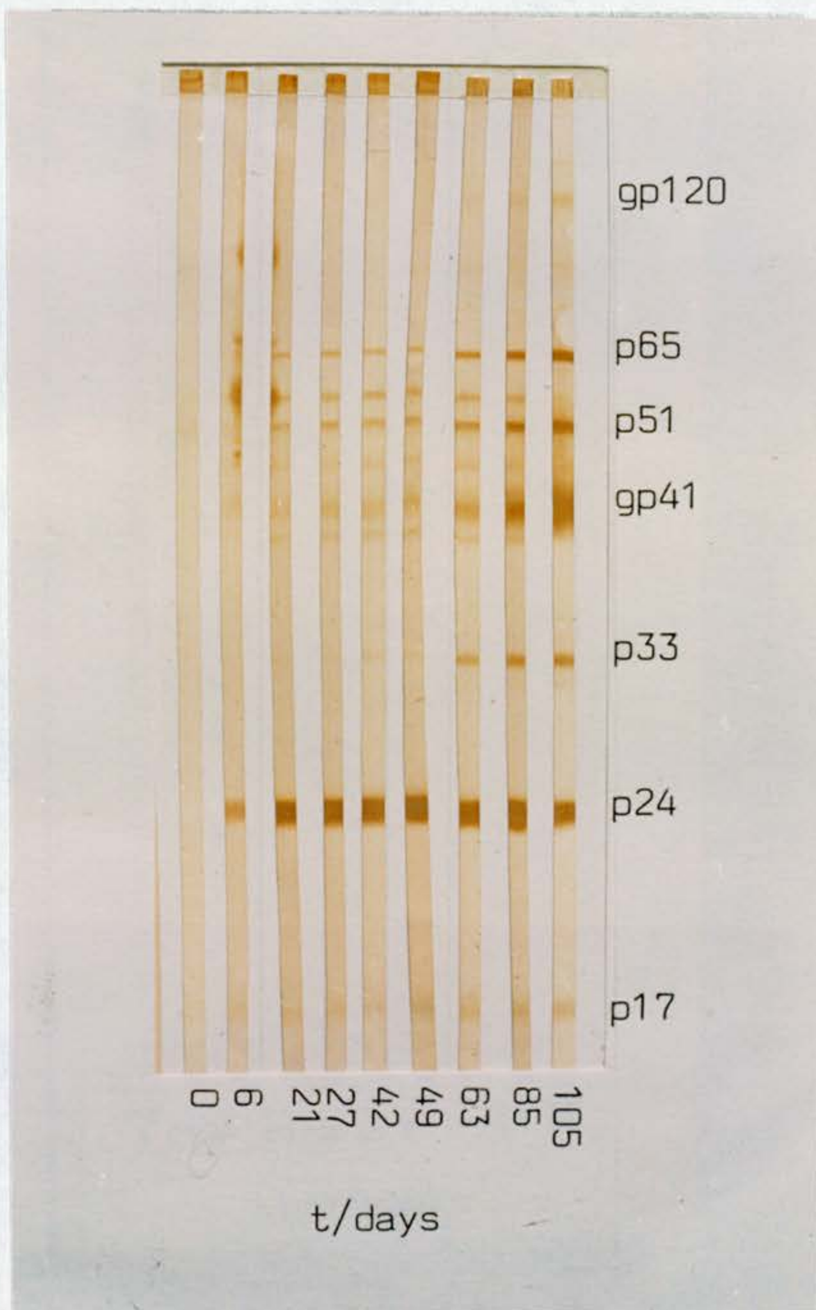


FIGURE 24 (cont'd). See above for legend. Panel e: patient 7.

next section). Since this serum and 2 other negative sera were reactive in the Abbott assay, there was little point in comparing its sensitivity with the other tests. Sera taken several months after the seroconversion did, however, show strong reactivity in the Abbott test, clearly distinct from the range of reactivities found with negative sera.

The IF assay results, read double blind, showed good agreement with the IB results, with no false positive reactions. However, the second and third sera from patients 2 were IF negative, despite clear evidence for the presence of low levels of HIV antibody. The second sera from patients 1 and 3 were graded as +/- and were distinguishable from negative sera by this test. However the weak reaction would lead to problems of interpretation if the test were used with unknown sera.

The results of Dupont assay were quantified as described in the methods. There was good agreement with the IB assay, and the test detected weak antibody reactivity in the second and third sera from patient 2. However, if the criteria for positivity recommended by the kit had been followed, any sera with less than 2 mU of antibody would be classified as negative. The second sera from all the other patients were, however, clearly positive. Steadily rising levels of antibody was found in all of the patients after seroconversion.

The Abbott Confirmatory EIA was designed to be a highly specific and sensitive test for HIV antibody. The detection of anti-"env" proved to be as sensitive and specific as the IB assay. The cut-off value calculated from the controls supplied with the kit would produce a level of around 0.3 mU, so the levels detected in the second and third sera of patient 2 were significant. The anti-"core" component of the

assay failed to detect antibody in these two sera, although positive results were obtained with the second sera from all other patients. Finally p24 antigen was detected in the first (antibody negative) sera of patients 4 and 7, in the second serum of the latter patient, and in the second and third sera of patient 2. The association of antigenaemia with seroconversion is investigated in the next section.

Although there was good agreement between the IB and anti-"env" assays, it is noteworthy that the IB assay detected antibody reactivity confined to p24, a core protein, in most of the first antibody positive sera, while the same sera all had detectable anti-"env", specifically gp41, antibody in the Abbott assay. As with the various assays for HSV antibody there appeared to be a difference between the tests in sensitivity for antibody of differing specificities. This study demonstrates the lack of sensitivity of some of the screening ELISA's for HIV antibody and gives information about the significance of restricted patterns of reactivity in the IB assay.

THE HAEMOPHILIAC COHORT

The circumstances by which some haemophiliacs in Edinburgh became infected by HIV differ markedly from the experience elsewhere, and constitute a unique group with which to study the course of infection with this virus. As described in the introduction, the source of infection of eighteen haemophiliacs was a single batch of factor VIII concentrate. The prevalence of HIV infection in Edinburgh in 1983 was so low that it is probable that the batch was contaminated by a single blood donation, and as such constitutes a single source of infection. Apart from the opportunity to study viral evolution in this cohort, it

constitutes a relatively homogenous group in which the duration and dose of infectious material administered to each patient was known. Furthermore, this batch was the only source of infection of all but one of the cohort.

Although the 18 patients in the study were infected at approximately the same time with the same material, they differ in their current clinical condition. Since one of the aims of studying this cohort was to relate serological results to progression to AIDS and related illnesses, the patients were designated as either well (W) or unwell (U) depending on clinical assessments made by Dr Ludlam and Dr Cuthbert over the first three years following exposure. 10 patients (W1-10) have remained asymptomatic; table 14 gives details of the remaining 8 patients who developed symptoms before March 1987 (U1-8). Two of the patients classified as well developed splenomegaly; although this may be the result of HIV infection, it may occur in haemophiliacs for other reasons. There was no other evidence of disease progression in these two patients and so they have been classified in the well-group. The unwell group (8 patients) comprises those with AIDS, ARC and persistent generalised lymphadenopathy (PGL). One patient (W2) emigrated after receiving the contaminated batch but before seroconversion. It has not proved possible to study his sera, and he has been omitted from the study. Two patients (U1 and U8) were lost to follow-up after the first year of infection, but samples were available in 1987. Patient U2 received commercial factor VIII after seroconversion, and possible re-infection with another strain of HIV needs to be borne in mind when considering the serological results form this patient.

Number	Time of appearance of symptoms after exposure
U1	Group II at 12 months, group III at 17 months
U2	Group I illness at seroconversion, group IVa at 9 months, IVc at 31 months.
U3	IVa at 28 months
U4	IVa at 29 months
U5	III at 11 months
U6	IVa diagnosed at 31 months, onset probably 25-26 months.
U7	Group II at 6 months, III diagnosed at 12 months
U8	IVa at 22 months

TABLE 14

Clinical status of patients in the unwell group, based on CDC classification, assessed March, 1987; 3 years after exposure.

SEROCONVERSION FOR ANTIBODY. Sera were tested by IB and and by the anti-"env" component of the Abbott confirmatory assay for the earliest signs of seroconversion. The two methods were in complete agreement over the timing of the appearance of antibody in each patient and allowed the last negative and first positive sera from each patient to be identified. These sera were then tested further by other assays, including the Dupont ELISA for p24 antigen. The full results are presented in table 15.

The second and third columns record the time and dose of exposure to the implicated batch. This data was obtained from Dr R. Cuthbert of the Haematology Department, but has been repeated here since it relates directly to detection of antibody and antigen. In 6 patients, the last HIV antibody negative serum was collected before the first exposure to the implicated batch. While these sera have been tested for antigen and found to be negative, the data are not included in the table since they are not directly relevant to the study described here. The antigen results of the first sera from the remaining 11 patients are shown in column 4. A range of antigen levels from 15 to 3600 pg/ml was found in five of the 11 sera.

The time between exposure and collection of the first antibody positive serum in each patient is shown in the next column. These sera were tested in the Dupont EIA, for anti-"core" and for antigen. All sera were reactive in the Dupont EIA, although the serum from patient U1 would have been scored as negative if the kit recommendations had been followed. Three sera were negative for anti-"core", as measured by the Abbott confirmatory assay, although two were reactive with gag proteins in the IB assay. In patients W5 and W1, anti-"core" antibody appeared in subsequent sera, collected 126 and 13 days days later

PATIENT	EXPOSURE[1]	DOSE[2]	LAST NEGATIVE[3]	ANTIGEN pg/ml	FIRST POSITIVE[3]	ANTIGEN pg/ml	IB bands[4]	DUPONT /mU	ANTI-"env" /mU	ANTI-"core" /mU
W1	09/03/84	20	N/A[5]	-	77 days	0	B EF		60	1.6
W3	01/03/84	81	N/A	-	68 days	0	B	14	40	2.1
W4	08/03/84	10	109 days	0	230 days	0	B D	49	178	56
W5	27/03/84	20	N/A	-	210 days	0	BCDEF	85	224	0
W6	02/04/84	60	98 days	20	148 days	0	AB EF	14	28	4.5
W7	02/03/84	60	25 days	0	88 days	0	AB	24	21	3.2
W8	19/03/84	81	106 days	600	150 days	0	B		71	1.4
W9	14/03/84	23	N/A	-	91 days	0	ABCDEF	62	56	126
W10	13/03/84	20	77 days	0	254 days	0	EF	46	274	0
U1	09/04/84	9	157 days	15	197 days	50	B	1	3	0
U2	10/03/84	54	30 days	30	36 days	50	B	8	27	2.7
U3	25/03/84	10	22 days	0	117 days	0	AB	17	53	2.7
U4	05/03/84	50	N/A	-	85 days	0	B		27	1.7
U5	08/03/84	30	21 days	0	77 days	0	B EF	26	178	10
U6	22/03/84	43	14 days	0	198 days	0	B EF	50	112	11
U7	07/03/84	20	N/A	-	83 days	0	B	6	38	1.7
U8	05/03/84	109	35 days	3600	85 days	0	B	21	89	3.0

1 Date of first exposure to implicated batch of factor VIII
2 Dose of infected material in number of bottles administered
3 number of days elapsed since first day of exposure
4 bands coded as for table 12
5 no sample taken between exposure and seroconversion

TABLE 15

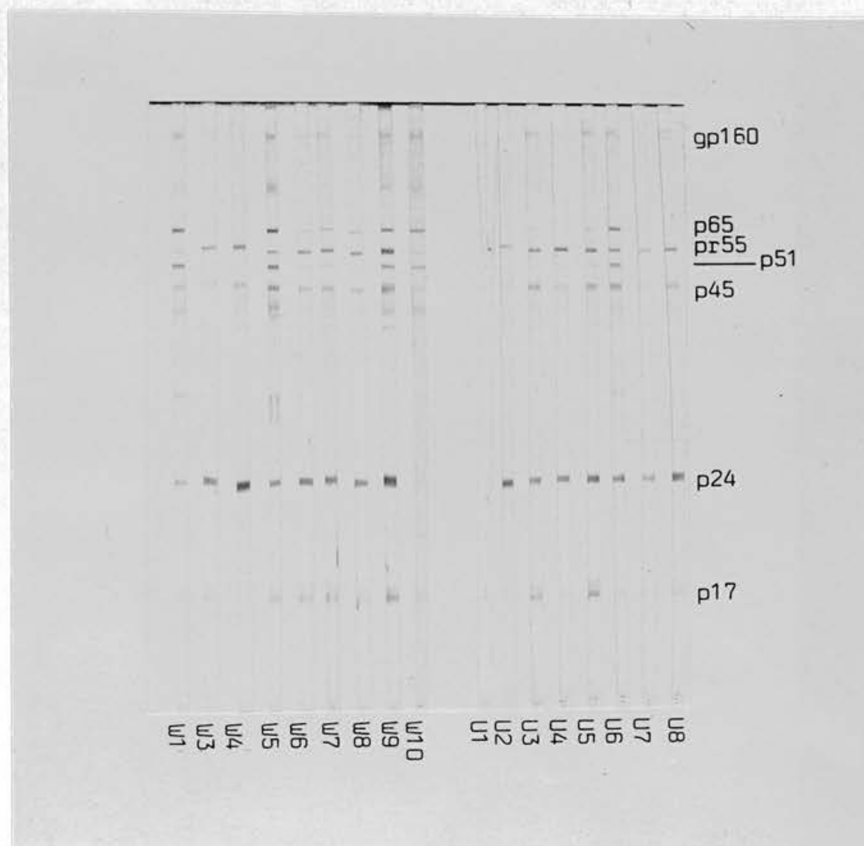
Detection of antigen and antibody in sera taken immediately before and after seroconversion for antibody.

respectively. However, all subsequent sera from patient W10 remained negative for anti-"core" and unreactive with gag proteins in the IB assay.

The results of the first positive sera in the IB assay are shown in figure 25. All but one sera reacted with p24, and the precursor gag protein, pr55. Reactivity with gp41 and *pol* gene product was less frequent, and generally the intensity of the bands was weaker than those of gag proteins. There was some correlation between breadth and intensity of antibody reactivity in the IB assay with anti-"env" antibody and Dupont antibody levels. Patient U1 showed extremely weak reactivity in the anti-"env" assay (3 mU) and only one faint p24 band in the IB assay. Furthermore, it would have been classified as negative in the Dupont assay if the calculated cut-off value had been used. The opposite extreme is represented by the first positive serum from W9, who shows broad reactivity in the IB assay, and high levels of antibody in each of the three ELISA's. All first positive sera were tested for antigen. Two of the 17 sera were positive (U1, U2). Subseqent sera from both patients were antigen negative (13 and 15 days later). The first positive serum from patient U2 was simultaneously anti-"core" and p24 antigen positive.

The relationship between time, duration and dose of exposure with time to seroconversion and detection of antigenaemia is illustrated in figure 26. In some patients, samples were available prior to the last antibody negative sample, and were tested for antigen in order to fix a definite time limit on the duration of the antigenaemia. Sera from patient W8 were antigen and antibody negative; antigen postive antibody negative; and antigen negative, antibody negative in successive samples; similar patterns may be seen in patients U1 and

FIGURE 25. Reactivity of the first positive sera of patients in the haemophiliac cohort in the IB assay.



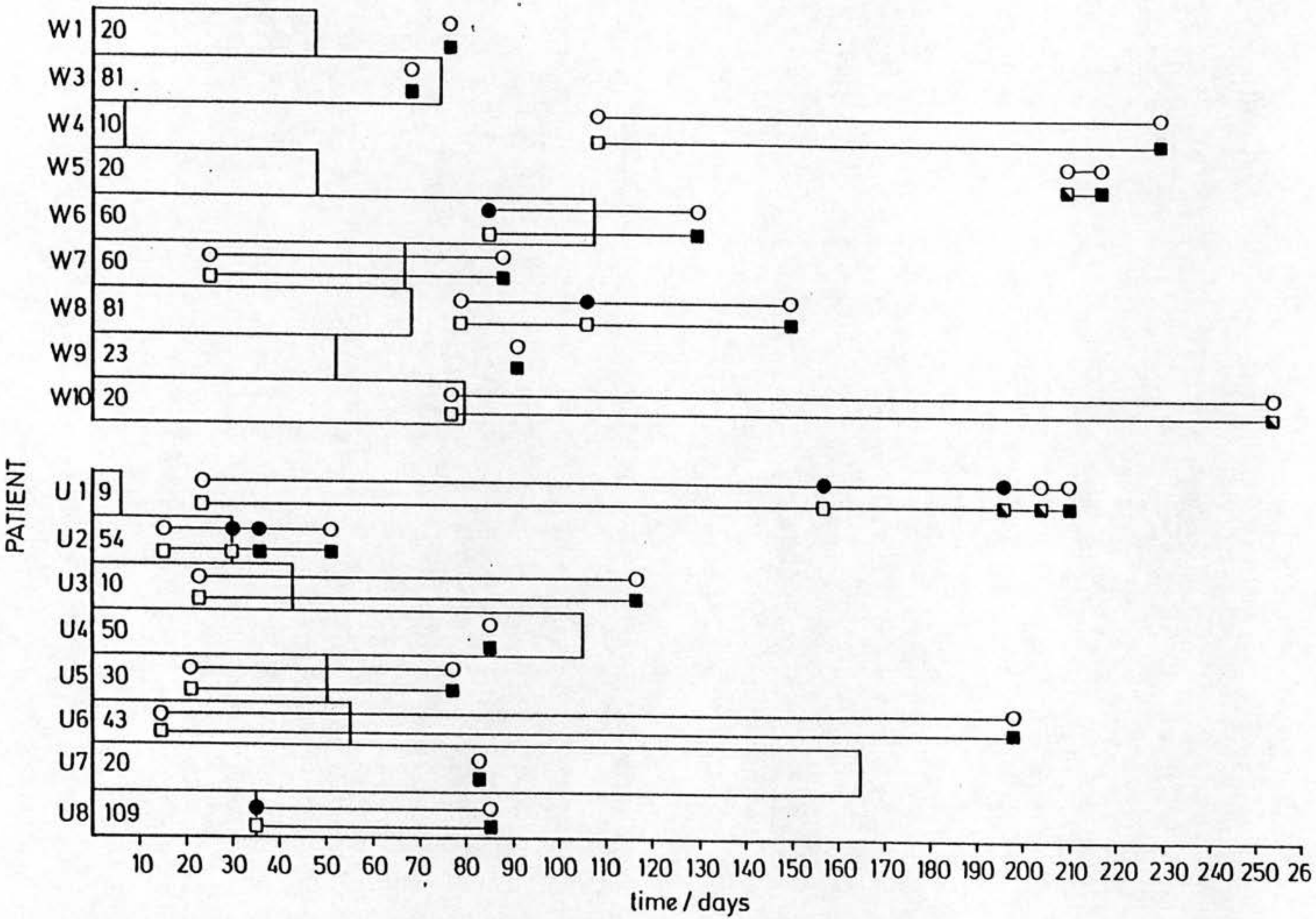


FIGURE 26: Diagram of the seroconversion events in the haemophiliac cohort. Boxes indicate duration of exposure to implicated batch of factor VIII; figures within boxes: number of bottles transfused; ○ Antigen negative; ● antigen positive; □ antibody negative; ■ antibody positive; ◪ antibody positive, anti-"core" negative.

U2. The duration of antigenaemia appears to be short; in patient U2, the duration had an upper limit of 37 days.

Despite the difficulties caused by the irregular spacing of samples from different patients, it was possible to see variation in the time to seroconversion after exposure. At least 157 days elapsed before the appearance of antibody in patient U1, while antibody appeared in patient U2 between 30 and 36 days. Comparison of the time to seroconversion and dose administered reveals a weak association, in that patients W4 and U1 (10 and 9 bottles respectively) seroconverted at least 108 and 157 days after exposure. This data is currently being analysed statistically by Dr R. Cuthbert.

FOLLOW-UP PERIOD

Sequential sera from the patients in the cohort were available in the 3 years following exposure. Table 16, 17 and 18 and figures 27, 28, 29, 30 and 31 show the results of testing sera from the patients in mid-1985, mid-1986 and mid 1987 by a number of tests. While the first positive, mid-1985, and mid-1986 sera from each patient were tested with the same kit batch for anti-"env" and anti-"core" antibody levels, the mid-1987 samples from each were tested using a different batch and did not give comparable results. Seasonal differences in incubation temperature may have accounted for some of the differences. However, the main cause of the problem probably results fom minor alterations in the antigen coating and conjugate between different batches of the Abbott Confirmatory EIA.

PATIENT	time[1]	ANTIGEN pg/ml	IB bands[2]	DUPONT /U	ANTI-"env" /U	ANTI-"core" /mU
W1	445 days	20	A CDEF		1.2	0
W3	323 days	0	ABCDEF	0.95	1.5	2100
W4	403 days		ABCDEF	0.44[3]	0.75	710
W5	455 days	0	ABCDEF	0.37	2.4	7
W6	312 days	0	ABCDEF	0.05		71
W7	551 days	0	ABCDEF	0.43	0.89	560
W8	332 days	20	ABCDEF	0.33	1.0	0.5
W9	369 days	0	ABCDEF	0.08		28
W10	435 days	0	DEF	0.10	1.5	0
U1	385 days	0	ABCDEF	0.52	1.1	75
U2	416 days	0	ABCDEF	0.69	4.0	1
U3	330 days	30/0[4]	A CDEF	0.21	0.67	0
U4	416 days	0	ABCDEF	0.80	1.9	1
U5	509 days		ABCDEF	0.55	0.4	270
U6	433 days	0	BCDEF	0.42	1.4	4
U7	381 days	0	BCDEF	0.17	2.5	15
U8	373 days	0	ABCDEF	0.42	2.7	71

1 number of days elapsed since first day of exposure to HIV
2 bands coded as for table 12;

3 result of sample taken at 329 days after exposure.
4 reactivity in Dupont ELISA but not confirmable by neutralisation.

TABLE 16

Antibody levels and detection of antibody in sera from the haemophiliac cohort in mid-1985.

PATIENT[1]	time	ANTIGEN pg/ml	IB bands	DUPONT /U	ANTI-"env" /U	ANTI-"core" /mU
W1	599 days	18	ABCDEF	0.70	1.6	0
W3	819 days	0	ABCDEF	1.6	8.4	9000
W4	817 days	0	ABCDEF	0.97	5.0	8900
W5	798 days	0	ABCDEF	1.1	2.8	120
W6	792 days	0	ABCDEF	0.31	0.48	160
W7	825 days	0	ABCDEF		1.7	360
W8	844 days	200	ABCDEF	0.99	3.5	9
W9	853 days	0	ABCDEF	0.36	0.25	790
W10	717 days	0	DEF	0.15	2.2	0
U2	808 days	0	ABCDEF	1.1	8.9	0
U3	807 days	50/0	CDEF	0.61	1.6	0
U4	833 days	0	BCDEF	1.9	7.1	18
U5	873 days	0	ABCDEF	0.69	1.3	790
U6	887 days	0	ABCDEF	0.72	3.6	9
U7	916 days	0	BCDEF	0.22	3.2	2

Footnotes as in table 16

1 No mid-1986 samples obtained from U1 or U8.

TABLE 17

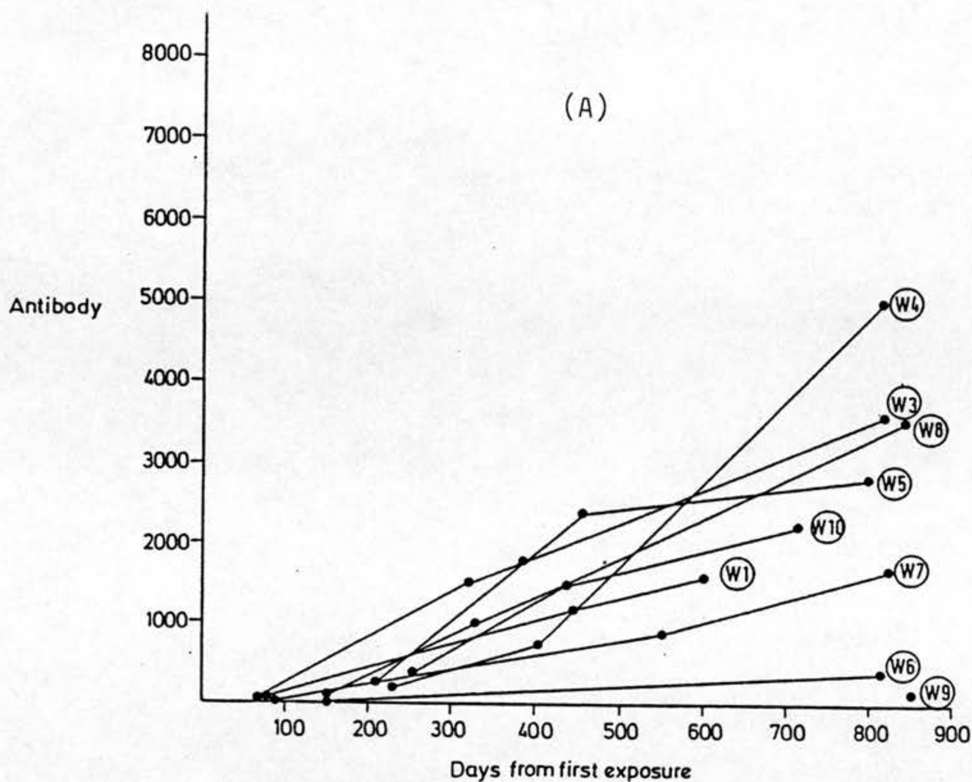
Antibody levels and detection of antibody in sera from the haemophiliac cohort in mid-1986.

PATIENT	time	ANTIGEN pg/ml	IB bands	DUPONT /U
W1	1054 days	60	ABCDEF	0.68
W3	1230 days	0	ABCDEF	0.80
W4	1212 days	0	ABCDEF	3.78
W5	1199 days	0	ABCDEF	2.10
W6	1134 days	0	ABCDEF	0.32
W7	1068 days	0	ABCDEF	0.57
W8	1150 days	250	ABCDEF	0.80
W9	1168 days	0	ABCDEF	2.05
W10	1211 days	60	DEF	0.58
U1	1141 days	0	BCDEF	0.61
U2	1059 days	30	CDEF	0.58
U3	1090 days	30/0	DEF	0.46
U4	1214 days	30/0	BCDEF	1.42
U5	1216 days	0	BCDEF	0.57
U6	1195 days	0	BCDEF	0.86
U7	1098 days	45	CDEF	0.76
U8	1153 days	45/0	BCDEF	0.30

Footnotes as in table 16

TABLE 18

Antibody levels and detection of antigen in sera from the haemophiliac cohort in mid-1987.



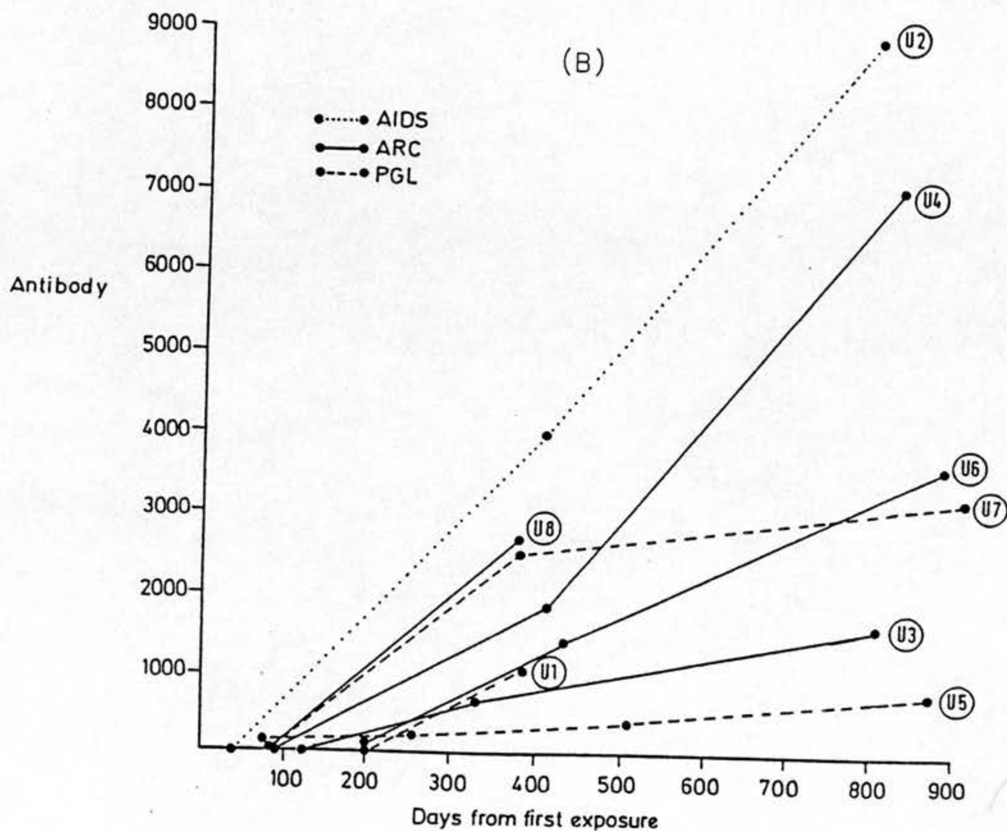


FIGURE 27: Reactivity of sera from the haemophiliac patients in ELISA for anti-"env" antibody. Levels in mU; (A) well group; (B) unwell group.

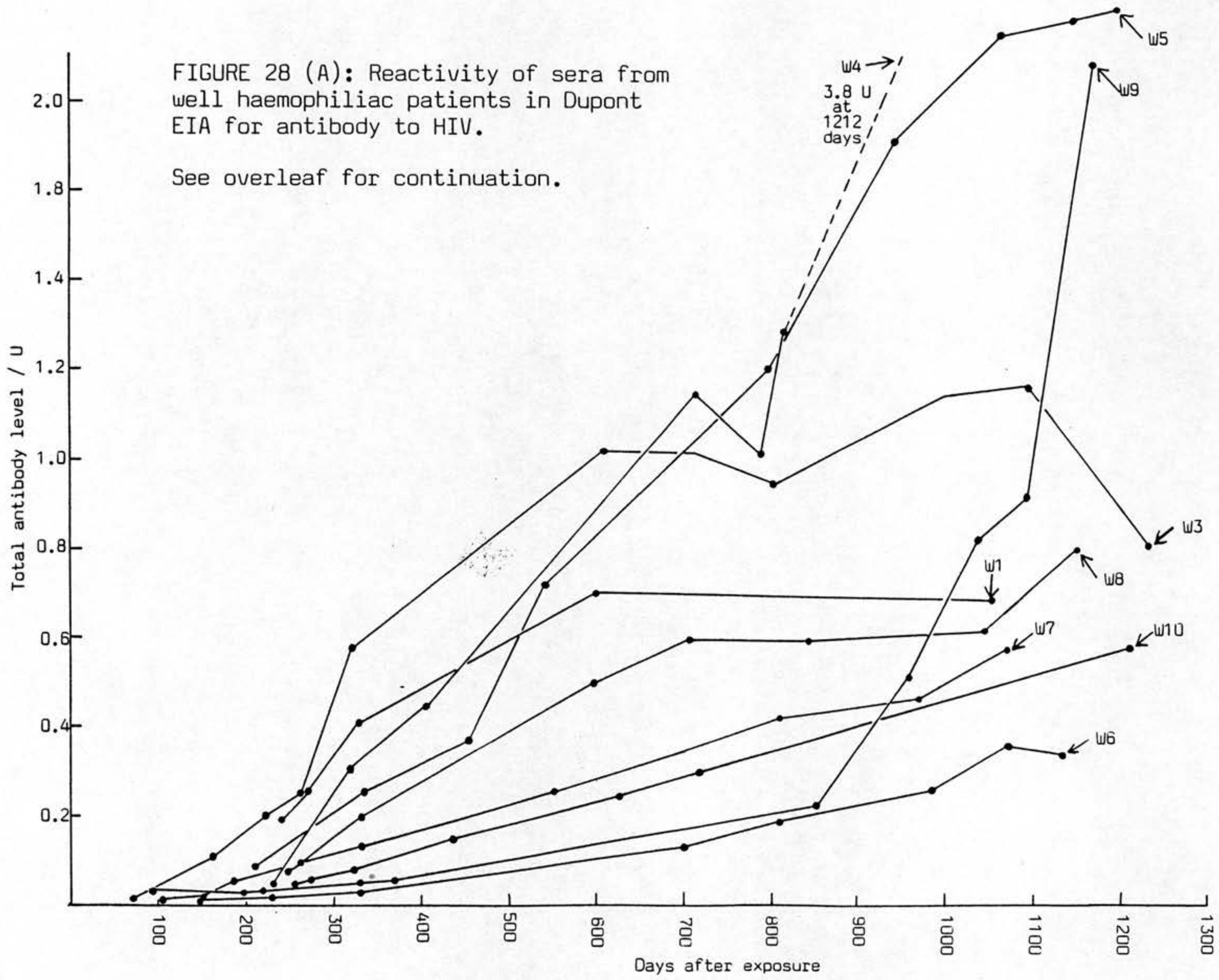


FIGURE 28 (A): Reactivity of sera from well haemophiliac patients in Dupont EIA for antibody to HIV.

See overleaf for continuation.

FIGURE 28(B): Reactivity of sera from unwell haemophilac patients in Dupont EIA for antibody to HIV.

See preceding page.

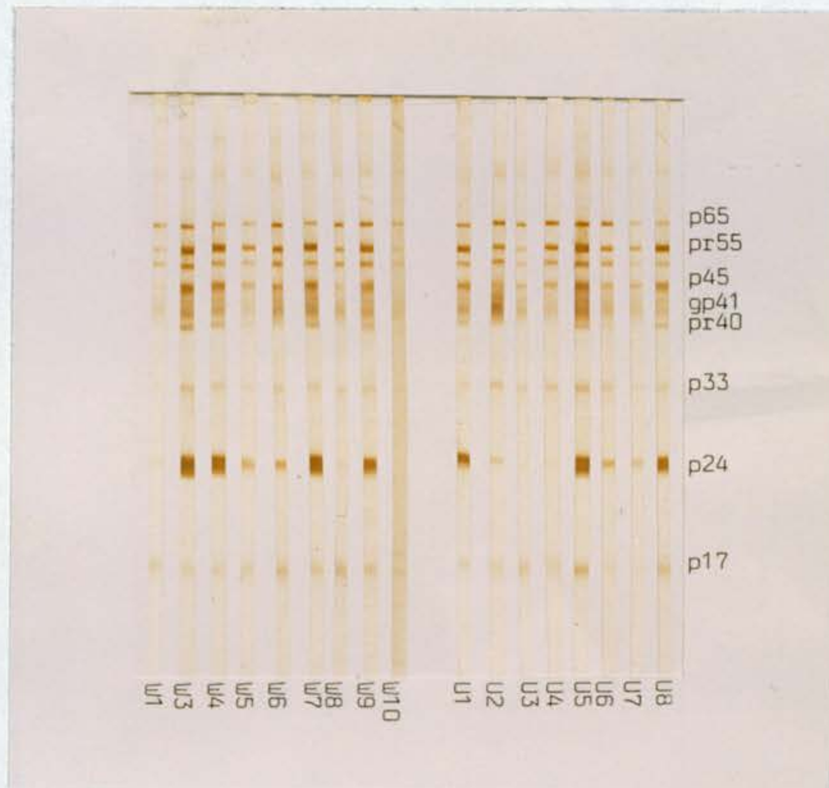
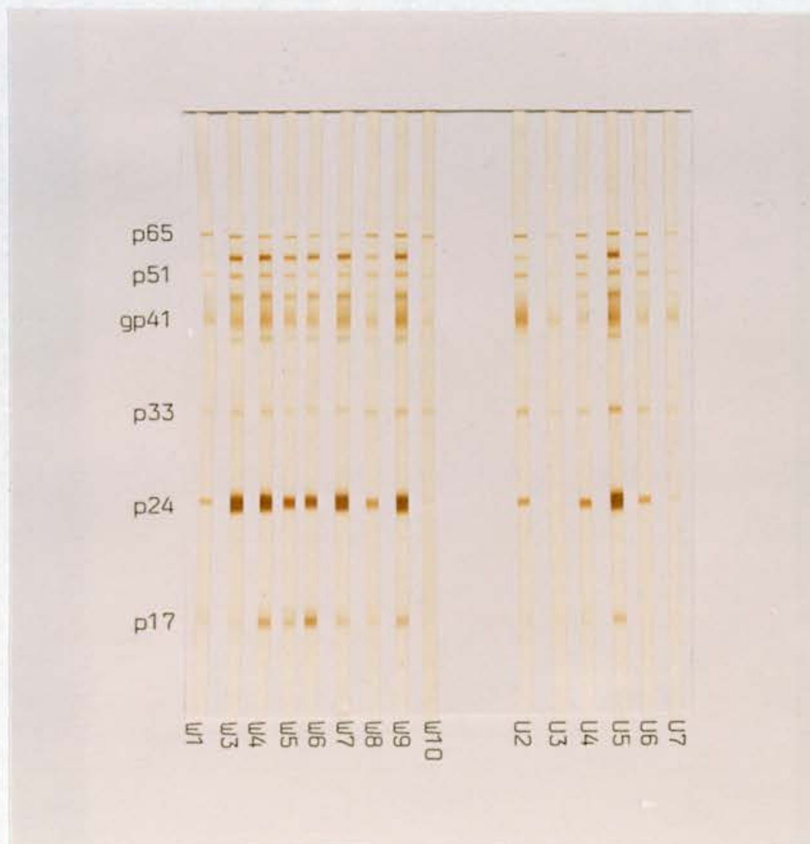


FIGURE 29. Reactivity of sera taken in mid-1985 from patients in the haemophiliac cohort in the IB assay.

FIGURE 30. Reactivity of sera taken in mid-1986 from patients in the haemophiliac cohort in the IB assay. No sera available from patients U1 or U8.



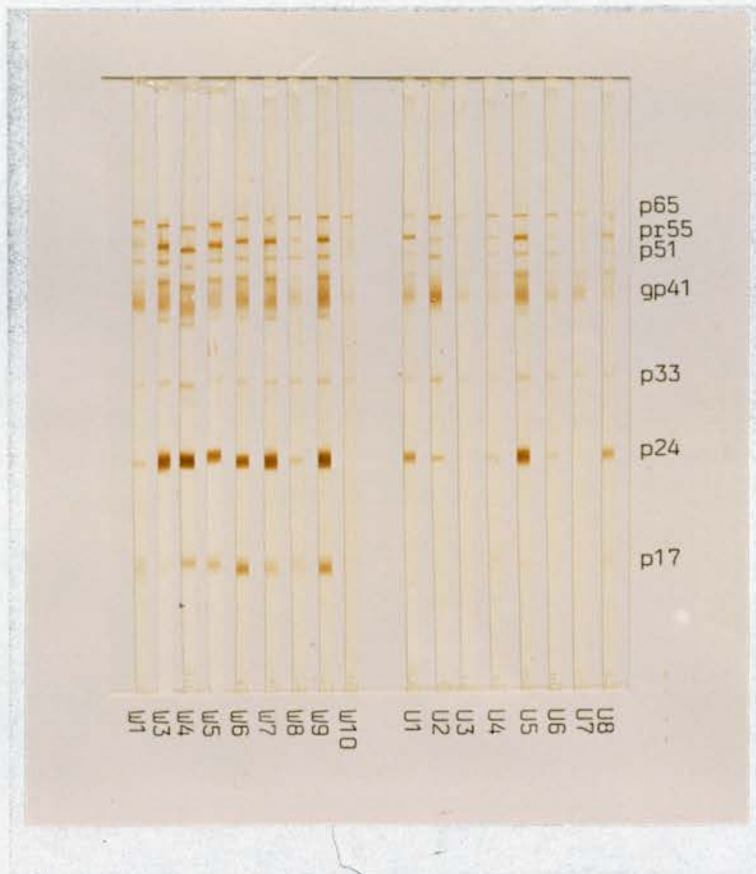


FIGURE 31. Reactivity of sera taken in mid-1987 from patients in the haemophiliac cohort in the IB assay.

The results with the anti-"env" component of the test are shown in in column 6 of tables 16 and 17 and graphically in figure 27. All patients showed steady rises in anti-"env" antibody levels with time after exposure. There were differences in the level of antibody finally achieved, although this did not correlate with clinical progression; there was no statistical difference in the average level of antibody between the well and unwell groups at any of the three sampling times.

Antibody levels in the Dupont assay were recorded in column 5; tables 16, 17 and 18, and graphically in figure 28. All patients showed a steady rise in levels of antibody over the first two years, The majority of patients, both well and unwell, continued to show small rises into the third year of the study. The relative uniformity of the serological response between the well and unwell groups is striking, in view of the differences in clinical condition between the patients in the first two years of infection.

However, differences between patients emerged thereafter. Patients W4, W5 and W9 showed marked increases in antibody level, achieving levels many times higher than the average for the well group. In contrast, patients U2, U3 and U8 showed prolonged falls in antibody level in the third year, possibly reflecting clinical progression and dysfunction of the immune system. The patient with AIDS (U2) showed the most drastic and sustained fall of the three patients. This fall began at about the time AIDS was diagnosed, and continued until death from secondary infections at 1100 days (the last sample was taken 7 days before). The falls observed in patients W3 and U7 are based on the result of testing single sera and would need to be corroborated by further sampling. However, it is notable that patient W3 developed

constitutional symptoms of sufficient severity to warrant inclusion in group IVa within the last three months, co-incident with the fall in antibody level.

Sera were screened for antigen, and positive samples confirmed by neutralisation. Two patients (W1 and W8) showed evidence of long-term antigenaemia (column 3), and were tested in greater detail. Antigen appeared in patient W1 at 329 days, and has remained until the present day, at the same low levels. Antigen first appeared in patient W8 at 245 days (30 ng/ml). Levels rose throughout 1985 and 1986 to a level of 200 ng/ml at 844 days. Two further patients (W10 and U7) had detectable antigen, confirmed by neutralisation, in the mid-1987 samples only. Patient U3 showed low level antigenaemia in four sera taken after seroconversion. Two were retested in an attempt to demonstrate neutralisation but were found to be unreactive in the presence of both positive and negative sera. However, the most recent sample (tested by S. Rebus) was found to be antigen positive, and confirmable (60 pg/ml). The mid-1987 sera from patients U4 and U8 were initially weakly reactive in the assay, but were not confirmable. It is is possible that these patients will become antigenaemic, by analogy with U3.

Figures 29, 30 and 31 and column 4, tables 16, 17 and 18 show the results of testing the sera with the IB assay. Comparison with the first positive sera (figure 25) with those from mid 1985 shows that a marked increased antibody reactivity to several HIV proteins developed over the first year on infection. The only exception was patient W10, who continued to show an unusually weak pattern of reactivity at all times after infection. That this patient was truly infected and not merely immunised is shown by the appearance of antigenaemia in 1987

and steady increases in antibody level in the Dupont and anti-"env" assays over the follow-up period.

However, reactivity with p24 and p17 was variable between patients, with many showing a loss of reactivity p24 and p17 over the first year. The anti-"core" assay (column 11, table 15; column 7, table 16) measures antibody reactivity to recombinant p24 in a competition ELISA, and provides numerical data on reactivity with core proteins. It can therefore be used to corroborate the findings of the IB assay. Patients W1, W8, U2, U3, U4, U6 and U7 all showed reduced reactivity in the IB assay, and falling levels of anti-"core" antibody in the specific ELISA over the first year of infection.

In contrast, the changes in antibody reactivity between sera taken in 1985 and those from 1986 and 1987 were relatively minor. Little change in reactivity to proteins encoded by the *env* and *pol* genes was noted in the majority of patients. However, patients U2, U3, U7 and U8 showed a general weakening of reactivity with these bands, and this correlates with the fall in total antibody level found on testing in the Dupont assay (see above). Reactivity to gag proteins also varied little in the last two years of follow-up. Patient U8 showed a fall in reactivity with p24 in the IB assay, and a fall in anti-"core" level, between 1985 and 1987. Unfortunately, there are no samples available for 1986, so it is not known when the loss of antibody occurred. This patient developed antigenaemia over the same period.

The seven patients showing falls in antibody reactivity to *gag* -encoded proteins in the first year were investigated more thoroughly. The results of testing sera taken at more frequent intervals are shown in table 19. Part A of the table shows

A

Patient Group[1]											
U2 IVc	Days after exposure	36	101	123	243	416	607	808	1048	1059
	Anti-"core"[2]	2.7	8.4	5.7	2.8	1.1	1.2	0	0	0
	[Antigen][3]	50	0	0	0	0	0	0	0	30
U3 IVa	Days	27	117	180	330	807	1090			
	Anti-"core"	0	2.7	4.7	0	0	0			
U7 III	Days	83	212	381	728	916	1098			
	Anti-"core"	1.7	75	15	15	0	0			
W1 II	Days	77	271	475	629	1054				
	Anti-"core"	1.6	0	0	0	0				
	[Antigen]	0	20	18		60				

B

Patient Group[1]										
U4 IVa	Days after exposure	85	107	210	231	416	540	694	833	1214
	Anti-"core"	1.7	1.9	0.5	0	1.1	2.6	4.8	18	2.4
W8 II	Days	106	150	245	332	598	844	1150		
	Anti-"core"	0	1.4	1.7	0.5	7.9	8.9	7.5		
	[Antigen]	600	0	35	20	80	200	250		
U6 IVa	Days	14	198	258	327	433	887	1195		
	Anti-"core"	0	11	4	11	4	9	1.3		

1 Using current CDC classification of HIV infection
2 Anti-"core" levels in units (postive control=1000 units)
3 Sera were p24 antigen negative unless indicated (in pg/ml)

TABLE 19

Anti-"core" and antigen levels in sera from selected patients in the haemophiliac cohort. (A) Patients showing sustained fall in anti-"core" levels over follow-up period; (B) patients showing fluctuating levels of anti-"core".

quantification of the initial increase followed by loss of anti-"core" antibody. There was good correlation between the observed intensity of the p24 band in the IB assay with the anti-"core" level. An example of the IB results is shown in figure 32. As can be seen, the p24 band weakened, but did not disappear, between days 123 and 1048 days; this is further evidence for the greater sensitivity of the IB assay for p24 antibody over the specific ELISA for anti-"core" antibody. Low level antigenaemia was detected and confirmed in the last sample available from the patient before death. Patients U3, U7 and W1 also show loss of anti-"core" antibody; the latter patient also became p24 antigen positive at the same time as p24 antibody was lost. The time after seroconversion that the loss of reactivity occurred varied between these four patients.

The three patients in table 19, part B also showed losses in anti-"core" reactivity, but subsequent events distinguished them from the patients those in part A. Sera from patient U4 showed a fall in anti-"core" level between days 107 and 210; antibody became undetectable by day 231. However, anti-"core" subsequently re-appeared and was still rising by day 833. Figure 33 shows the result of testing these sera in the IB assay, and confirms that a fall followed by a rise in antibody to core proteins had occurred. The mid-1987 sample (1214 days), however, showed a fall in the level of anti-"core" and reduced reactivity with p24 (figure 31). The patient remained antigen negative throughout the follow-up period.

A fall in anti-"core" followed by a rise was also seen in patient W8, and confirmed by the IB assay. Serum p24 antigen was detectable from day 245 onwards, the first appearance co-inciding with the initial fall in antibody levels. Antigenaemia persisted, however, despite the

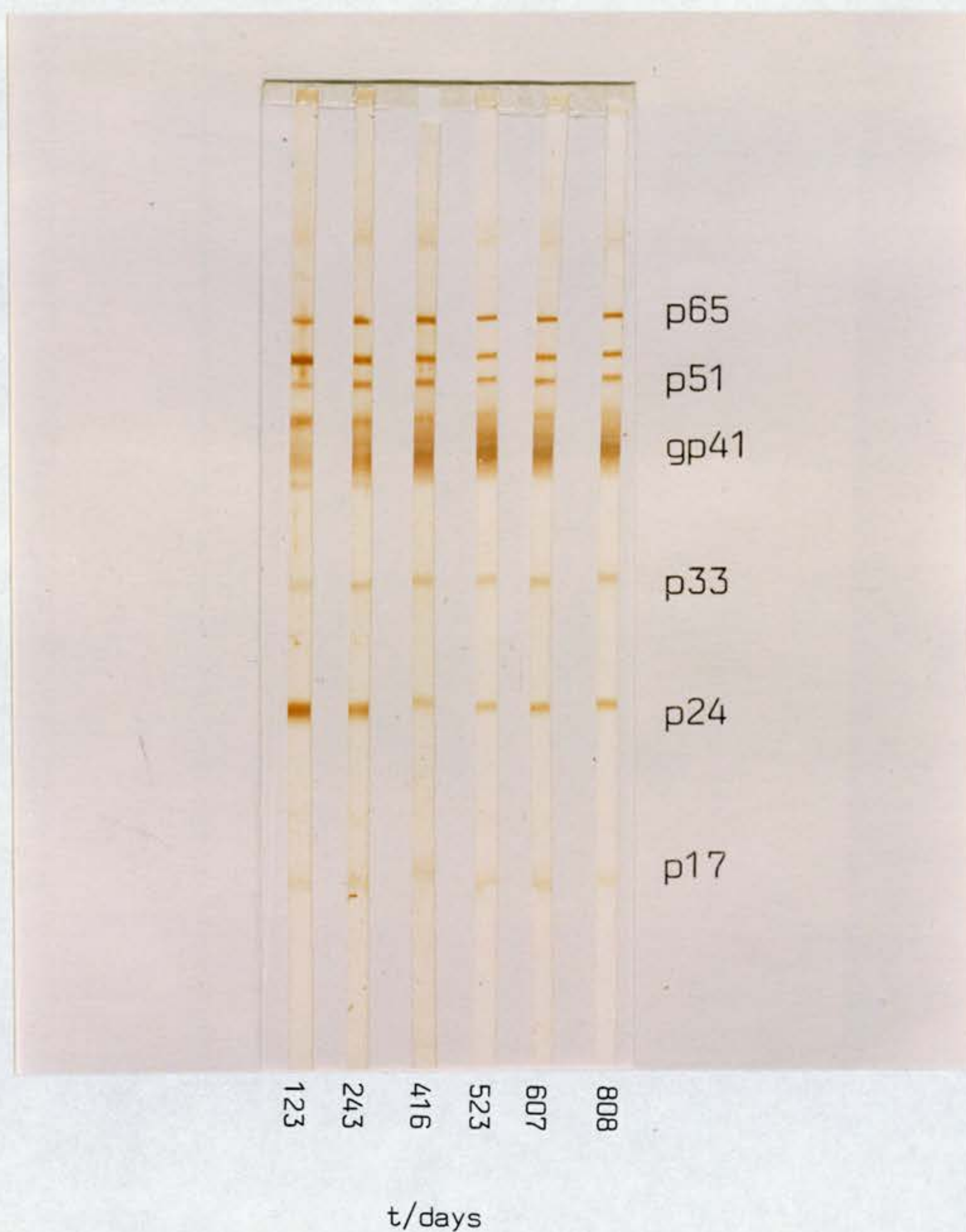


FIGURE 32. Reactivity of sequential sera from patient U2 in the IB assay. Days elapsed from first exposure to implicated batch of factor VIII indicated under the strips.

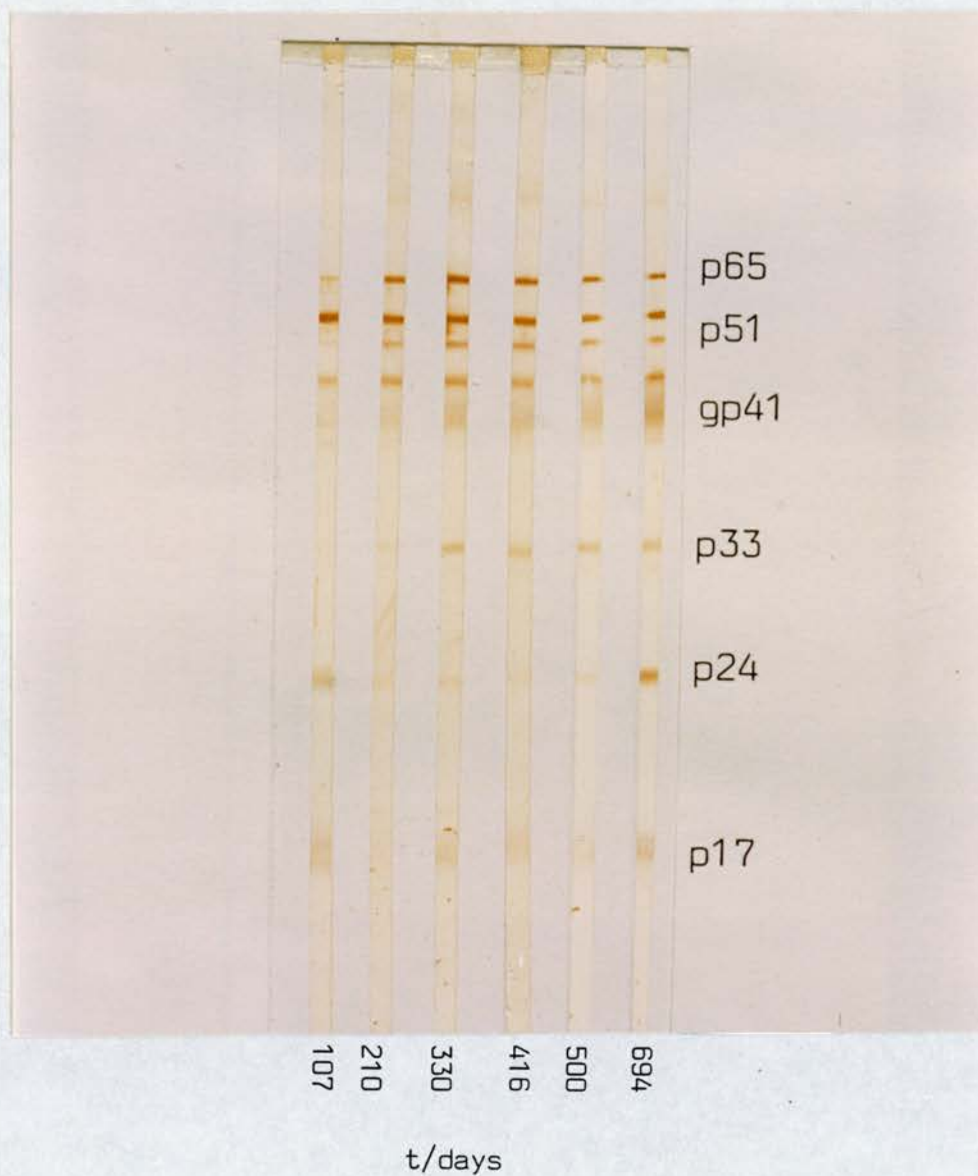


FIGURE 33. Reactivity of sequential sera from patient U4 in the IB assay. Days elapsed from first exposure to implicated batch of factor VIII indicated under the strips.

reappearance of anti-"core". Finally, patient U6 showed fluctuating levels of anti-"core", relatively constant low level reactivity with p24 in the IB assay, and remained antigen negative.

There was some overall correlation between anti-"core" levels and p24 reactivity with clinical progression in this cohort. Patients who showed steadily rising levels of anti-"core" over the two year follow-up could be grouped in the normal response group. Patients showing this pattern were W3, W4, W5, W6, W7, W8, W9, and patient U5 (PGL). Patients showing falls in anti-"core", fall followed by rise, fluctuating or absent anti-"core" levels could be put into the abnormal response group. Patients in this group were W1, W8, W10, U2, U3, U4, U6, U7 and U8. If PGL were not considered as evidence of progression to AIDS, then the figures would read 8 well and no unwell patients in the normal response group, and 3 well and 5 unwell (4 ARC, 1 AIDS) in the abnormal response group. It remains to be seen whether the abnormal immunological responses to core proteins in the latter three well patients is prodromal for progression.

DISCUSSION

ANTIBODY TO HERPES SIMPLEX VIRUS

It is well established that infection with HSV in man elicits a humoral immune response. Antibodies may be detected to a wide range of HSV-encoded polypeptides, of which a proportion are neutralising. The specificities of the IB, RIP and TDCA IB assay were determined by preliminary experiments with a number of control sera. Subsequently, the assays were used to investigate the antibody response to primary infection with HSV; IB was also used to investigate the targets of antibody in a number of human negative and HSV-1 antibody positive sera, as well as those from patients with recrudescent HSV-2 infection.

IMMUNOBLOTTING ASSAY

One of the problems with the IB assay was that of identfying the bands produced on reaction with antibody positive sera. There are two main reasons fór this. Firstly, many of the monoclonals used for identification of proteins failed to react in the assay. This was presumably due to denaturation of their target epitopes during the antigen solubilisation procedure prior to electrophoresis. This concords with the results of Braun *et al.*, (1983), who reported denaturation and loss of antigenicity of the targets of some, but not all, monoclonal antibodies in their IB assay. In the present study, only those reactive with gD, p40, gC-1 and gG-2 (nos. 14, 5, 2 and 9) reacted with denatured epitopes. Comparison of the bands produced by

these monoclonals with those produced by control or test sera allowed some identifications to be made. However, although similar bands to those produced by no.14 were found on testing human sera in the IB assay, caution was needed before identifying them as gD. Richman *et al.*, (1986) describe the comigration of gG-1 to the same area of the gel and it is possible that the bands found on reaction with sera from patients with HSV-1 infection reflected an antibody specificity to this latter protein.

In theory, further identifications of proteins could be made by comparing the IB results with those obtained from assaying monoclonal antibodies in the RIP assay. Although the methodologies of the two assays are different, both use SDS-PAGE to resolve individual proteins, which would be expected to migrate to the same areas of the gel. In practice, however, it was found that the appearance and apparent mobility of proteins in the two assays differed. This can be seen by comparing the bands produced by no.2 (gC-1) in both assays (lane 8, figure 7 with lane 9, figure 9) and nos. 1 and 14 (gD; lane 7, figure 7 with lane 8, figure 9). The reason for the discrepancy in the appearance of gC-1 in the two assays is unclear. However, it is possible that herpes proteins are subject to a degree of proteolytic cleavage in the second incubation of the RIP assay, despite the use of inhibitors (see below); this may modify their appearance on SDS-PAGE.

Some investigators have used monospecific rabbit antisera to individual HSV glycoproteins for identification of proteins, in order to circumvent the problem of monoclonals failing to react in the IB assay (Eberle *et al.*,, 1984; Bernstein *et al.*, 1986). Unfortunately, these were not available for the work described here.

The second problem with identification of proteins was caused by the large number of bands in the 80 to 130 KDa range produced by many convalescent sera in the IB assay. Even if the MWs of the target proteins are known, it was rarely possible to differentiate reactivity with them from that to the numerous other bands in the same area. It would be hazardous to distinguish reactivity to gB, gC-1, gG-2, gH-1 and MDBP on the basis of MW in these experiments, all of the proteins being so similar in weight. Furthermore, even though the monoclonal no.9 revealed the appearance of gG-2, and no.2 identified gC-1 in the IB assay, it was impossible to clearly identify reactivity to them in the control antisera (figure 7). Similarly, it was difficult to identify the targets of many of the HSV-1 antibody positive sera and those from patients with recrudescent HSV-2 infection (figures 15 and 16).

In summary, the identification of the target proteins of sera in the IB assay is complicated by the large number of comigrating bands. The similarity in MW of several proteins leads to the possibility of misidentification through comigration of bands. In this study, and in those of other investigators (Eberle *et al.*, 1983; Lehtinen *et al.*, 1985; Teglbjaerg *et al.*, 1986), the use of IB to investigate the target specificity of convalescent sera is limited by this consideration. Investigation of sera taken from patients with primary infection is more straightforward because of their restricted range of reactivity.

Eberle *et al.*, (1985) and Bernstein *et al.*, (1984); Eberle *et al.*, 1984) described the reactivity in the IB assay of sera taken from patients with primary HSV-2 and HSV-1 infections respectively. In the former case, patients were shown to develop antibody to p40 and the

MCP on seroconversion, and subsequently, to other proteins. In primary HSV-2 infection, antibody specificities for p40, gG-2 and, to a lesser extent, to gD and an 80K protein were detected in acute phase sera. The highest titres of antibody were to p40. The identity of p40 was shown by reactivity of a monoclonal antibody in the IB assay. A restricted pattern of reactivity to p40 was found in guinea pigs 14 days after experimental inoculation with HSV-2.

p40 reactivity was found in all of the patients with primary HSV-1, but only in 7 out of 11 of the patients with HSV-2 infection. Reactivity of monoclonal no.5 in the IB assay confirmed the identification (compare lane 9, figure 7 with lanes 2, 4, 6 & 8, figure 17). In most cases, it was the most prominent band produced, although additional specificities for other proteins were found in almost all sera (table 11). In contrast to the findings of Eberle *et al.*, 1985, no reactivity with MCP was found in any of the patients with primary HSV-1 infection. Reactivity with a 112 KDa protein, was, however, frequently found. Reactivity with gG-2, 107 KDa and 103 KDa was found in many of the HSV-2 sera.

RADIOIMMUNOPRECIPITATION ASSAY

More monoclonal antibodies were reactive in the RIP assay than in the IB assay, and allowed the identifications of gB, gD, gC-1, and p40 to be made. The migration of gD was clearly affected by large amounts of comigrating immunoglobulin carried over from the immune precipitate, leading to a diffuse band clearly different from that produced by no.14 in the IB assay. gC-1 appeared to have a MW of 121 KDa, with weaker bands at 75 and 45 KDa. Both gB-1 and gB-2 also showed lower

molecular weight forms. The multiple forms of gC-1 and gB in the RIP assay are probably the result of proteolyic cleavage during the second incubation of the assay. Attempts were made to prevent this by the addition of PMSF to the lysis buffer. This appeared to be insufficient to prevent the action of the released cellular proteases. Proteolytic cleavage of gB and gC is not found in the results of other investigators using the RIP assay, and this may be due to methodological differences between the various assays. For example, Ashley & Corey, (1984) incubated serum and antigen together on ice; in this case, the lower temperature probably prevented the proteolysis, perhaps at the expense of reducing the antibody/antigen binding reaction. p40 appears only faintly in the RIP assay, in contrast to the IB assay. This may be a reflection of the low solubility of HSV capsid and tegument proteins in non-denaturing detergents, and is probably the reason for the failure of monoclonal no.6 (MCP) to react in the RIP assay (see below).

Co-precipitation is one of the problems of the RIP assay. The separate bands produced on SDS-PAGE analysis of an immune precipitate do not necessarily indicate antibody reactivity to each protein. The detergents used for RIP assays are commonly non-denaturing, and non-dissociating. One way to minimise this problem is to ultracentrifuge the cell lysate before use in order to remove insoluble material. Preliminary experiments carried out during the development of the RIP assay illustrated that co-precipitation does occur, and does affect the results.

Comparison of the proteins in a HSV infected cell lysate by SDS-PAGE before and after ultracenrifugation showed the disappearance of a 155 KDa band, as well as many other in the 50-80 KDa range. EM analysis of

the pellet produced during ultracentrifugation of the cell lysate revealed relatively large amounts of intact and degraded nucleocapsids. Surprisingly, many nucleocapsids were associated with what appeared to be viral envelopes. Comparison of spun and unspun HSV-1 antigen in the RIP assay showed the 155 KDa protein to be present only in the immune precipitate of the unspun material. Furthermore, the total counts of the immune precipitate were markedly increased, as was the intensity of all of the bands, including those later identified as glycoproteins, on SDS-PAGE analysis. This data indicated that the nucleocapsid is poorly soluble in the commonly used detergents for RIP assay. There is also some evidence for the incomplete solubilisation of the viral envelope.

Steps were taken to remove insoluble material by ultracentifugation from the cell lysate before use in the RIP assay, and the reaction of the monoclonals in the RIP assay showed that there was independent precipitation of gC-1, gB and p40. However, the immune precipitate of no.1 appeared to contain relatively large amounts of a 65 KDa protein (gD) and a small amount of a 130 KDa protein of uncertain identity (figure 9, lane 7). The MCP was not found on testing the HSV-1 antibody positive control sera with spun antigen in the final version of the RIP assay (table 6; figure 9). However, RIP analysis of the HSV-2 antibody positive control sera, as well as those from patients with primary HSV-2 infection, revealed the presence of a a weak 155 KDa band corresponding to the MCP of HSV-2. It is not certain if this is due to incomplete removal of insoluble material, or that the proteins of HSV-2 nucleocapsids are more soluble than those of HSV-1.

Zweerink and Corey, (1982) did not ultracentrifuge the cell lysate before use in their RIP assay. Mann & Hilty (1982) spun the antigen at only 10 000 x g prior to incubation with serum. This is inadequate to pellet intact nucleocapsids remaining after solubilisation. The results presented by both research groups indicate apparent reactivity of all antibody sera tested with 148 and 152-159 KDa proteins respectively. Prominent reactivity with MCP was not found by other investigators (Gilman *et al.*, 1980; 1981; Ashley & Corey, 1984; Ashley *et al.*, 1985), all of whom spun the antigen at 100 000 x g before use. On this basis, the results of Zweerink & Corey (1982) and Mann & Hilty (1982) must be disregarded.

The three negative human and both pre-bleed rabbit sera were unreactive in the RIP assay. Non-specific reactivity in the RIP assay has been reported. Zweerink & Corey (1982) found apparent reactivity of negative sera with MCP and gp130. As mentioned before, the cell lysate was not ultracentrifuged and undoubtedly contained whole virions, and it is possible that the known stickiness of HSV particles contributed to this non-specific reaction. Baucke & Spear (1979) report the Fc binding activity of gE. In theory, this might be expected to cause non-specific reactions since binding of a radiolabelled HSV protein to IgG in the control or test serum might occur independently of immunological recognition. The result would explain the apparent reactivity of negative sera with gE. However, in the experiments described here, and in those of others investigators using the RIP method, no such reaction occurs. The RIP assay of Johnson & Feenstra (1987) appears to show non-specific reactivity with gB; this may be a reflection of the high specific activities used in the radiolabelling required to show the reactivity of g70.

Human and rabbit control antisera produced numerous bands in the RIP assay, of which gB-1, gC-1 and gD/gG-1 (HSV-1) and MCP, gB-2 and gD-2 (HSV-2) could be recognised. Gilman *et al.*, (1980; 1981) also found predominant reactivity in almost all convalescent sera with HSV-1 proteins (MW 133, 99 and 82 KDa) and HSV-2 proteins (131 and 101 KDa). Reactivity with other proteins was weaker and more variable. Eberle & Courtney, (1981) used a glucosamine label, and hence did not investigate reactivity to non-glycoproteins. Convalescent sera were found to react with gA (pgB), gB, gC and gD in this assay.

gB was the predominant target of antibody elicited by primary infection in the RIP assay described here. Not only did all of the second sera tested show reactivity with this protein, but also 6 of the first sera. In 4 of the 5 patients with HSV-1 infection, antibody reactivity to gB was the only target specificity detectable. The second serum of patient 5 showed reactivity with gC-1. Of all of the second sera, this showed the strongest reactivity in the ELISA. All of the second sera from patients with HSV-2 infection showed additional reactivity with MCP, and 130 and 95 KDa proteins; two of these showed reactivity with lower MW proteins. The identity of the 130 and 95 KDa proteins could not be ascertained. The 95 KDa protein was generally very faint, and may have been a precursor or degradation product of gB. The 130 KDa protein was, however, prominent, and the sharpness of the band makes it unlikely to be a glycoprotein. Ribonucleotide reductase and MDBP are present in relatively large amounts in the HSV infected cell and are both candidates for this protein. Ashley & Corey (1984) also detected reactivity to a 130 KDa protein, and identified it as a form of gB (the other being of MW 110 KDa). The reactivity of monoclonal no.3 in the RIP assay (figure 9, lane 13) shows that the observed MW of gB-2 is lower than 130 KDa in the SDS-PAGE system used

in the work described here.

There was no evidence of antibody reactivity with any of the other HSV glycoproteins in the RIP assay. Ashley & Corey, (1984) and Ashley *et al.*, (1985) found initial reactivity of sera from patients with primary HSV-1 and -2 infection to gB. In sera from patients with HSV-1 infection, reactivity with gC-1 and, to a lesser extent with MCP, was also found, while those from patients with HSV-2 infection showed strong reactivity with gB and an 88 KDa non-phosphorylated protein. Some reactivity was also seen with MCP. On follow-up, patients with HSV-1 infection developed antibodies to a 88 KDa protein, gE, gD and a 66 KDa protein. Sera from patients with HSV-2 infection showed reactivity with gC-2/gE-2 (not differentiated), gD and the 66 KDa protein.

NON-DENATURING IMMUNOBLOTTING

There is surprisingly little in the literature concerning the separation of membrane proteins by non-denaturing PAGE. Most of the methods published describe the separation of water soluble proteins on native gels, in the absence of any detergent. Since the proteins of HSV are either membrane inserted, or are part of a self-assembling nucleocapsid, mild dissociating conditions are required to bring the individual HSV polypeptides into solution. Developing a non-denaturing IB system involves the selection of a detergent capable of solubilising HSV proteins effectively yet retaining their antigenicity, and permitting electrophoresis on a size basis. Satisfying the first two considerations involves a compromise, since a powerful dissociating detergent inevitably denatures proteins (cf

SDS), while mild detergents may fail to solubilise a proportion of the virion components. In particular, the nucleocapsid seems to relatively insoluble in the Triton/deoxycholate detergent mixture used in the RIP assay (see above).

Dewald *et al.*, (1975) published a method using triton to solubilise membrane proteins prior to PAGE in a detergent-containing gel. This was the method originally tried in the work described here. However, the failure of this method with HSV proteins led to considerations of the properties of detergents and the selection of those showing optimal properties. Helenius & Simons, (1985) and Helenius *et al.*, 1979 reviewed the properties of a wide range of detergents and these descriptions were used to select TDCA as a candidate. The failure of PAGE in the presence of triton could be explained by its large micellar size; proteins solubilised in triton would have a minimum size of 120 000Da, and this probably explains the failure of the proteins to migrate. Subsequently, only detergents of smaller micellar size were considered. Experiments had shown that sodium deoxycholate (micellar size 12 000 Da) precipitated below pH 7.0 and therefore could not be used, while proteins solubilised and electrophoresed in CHAPS failed to migrate, possibly due to the fact that this zwitterionic detergent has no net charge. TDCA appeared to suffer from none of the shortcomings so far mentioned. Firstly, it is of small micellar size, similar to that of SDS and sodium deoxycholate, secondly it has a low pKa, and in pratice remained soluble at pH4. Finally, it had a net negative charge, which would be expected to assist the separation of proteins.

When used to replace SDS in the IB assay, these expectations were borne out to a certain extent. Membrane proteins solubilised in TDCA

could be separated on a gel, as indicated by the lack of aggregation of protein at the top of the separating gel on coomassie blue staining. Furthermore, gB was found to have an Rf value of 0.15, on reaction with the appropiate monoclonal antibody. Assaying the control antisera in the TDCA IB assay also indicated the existence of other faster migrating HSV-encoded proteins, of which one could be identified as gD-1. Electrophoresis of water soluble marker proteins by TDCA PAGE showed that there was no relation between molecular weight and distance migrated, unlike the behaviour of proteins, whether water soluble or not, on analysis by SDS PAGE. The observed relationship between migration and MW observed with SDS solubilised proteins may be accounted for by the equal binding of both hydrophilic and hydrophobic proteins to SDS. Since SDS contributes the majority of the negative charge of the solubilised protein, only the size of the protein rather than its charge/mass ratio influences its rate of migration on electrophoresis. The absence of any relationship between MW and rate of migration in TDCA PAGE indicates that TDCA failed to bind to water soluble proteins. Experiments that used TDCA PAGE to separate different membrane proteins of known MW would establish whether there was constant or variable binding of TDCA to water insoluble proteins.

The failure of the other monoclonal antibodies to react in the TDCA IB assay was investigated by SDS-PAGE analysis of the TDCA-solubilised material, and by treatment of TDCA IB strips by boiling in conventional SDS sample buffer, in the hope of revealing hidden epitopes. The former investigation revealed that p40, gC-1 or gG-2 were not detectable in the TDCA cell lysate by conventional SDS IB analysis using specific monoclonals reactive with denatured proteins. The second experiment showed that immunogenicity of p40, gC-1 or gG-2

was not restored by SDS treatment. Taken together, these results indicate that TDCA fails to solublise not only a tegument protein, but also two HSV glycoproteins. By implication, the absence of reactivity of no.4 and no.6 (gE and MCP) and the weak reactivity of no.1 (gD) in the TDCA IB assay may also be accounted for by poor solubility. It would appear that gB is more soluble than the other glycoproteins in this detergent. This is a surprising finding which may have consequences for other detergents and other assays.

Cohen *et al.*, (1986) reported the application of a non-denaturing IB assay to study the discontinuous (denaturation sensitive) and continuous (resistant) epitopes of gD with a panel of monoclonal antibodies. The method involved initial solubilisation of proteins in SDS at room temperature, followed by electrophoresis on a detergent free gel. The results showed that the native gel system retained the antigenicity of denaturation sensitive epitopes. Unfortunately, it was not made clear whether the method was capable of resolving other proteins; monoclonal antibodies of other specificities were not used, nor was a protein stain of the gel shown. However, Snowden *et al.*, (1985) and Snowden & Halliburton (1985) reported a similar method in the analysis of antibody to gB. They modified the conventional SDS PAGE method by omitting the boiling step prior to electrophoresis, and demonstrated that this retained the antigenicity of one or more epitopes of gB of HSV that cross-reacted with those found on equivalent proteins of other herpesviruses.

Modifications of the SDS-PAGE method were tried, and the results confirmed that gB could be resolved by omitting the boiling step. However, there was clumping of proteins at the top of the gel, and generally very poor resolution of proteins, not only with detergent

free gels, but also with gels supplemented with 0.1% SDS. These experiments were carried out at 4C; the reason for the failure to detect antibody to gD may be that there was more efficient solubilisation at the room temperature used by Cohen *et al.*, (1986) for electrophoresis. Further work should investigate whether room temperature SDS-PAGE efficiently solubilises proteins other than gB and gD, and retains their antigenicity.

In collaboration with Dr B. Cohen, Western General Hospital, Edinburgh, attempts were made to resolve HSV-encoded proteins by isoelectric focussing (IEF) in the presence of triton. As mentioned above, PAGE in the presence of triton failed possibly because of the detergent's large micellar size. However, IEF does not rely on a seiving effect by the gel for the resolution of proteins, rather, proteins migrate to their isoelectric point on a pH gradient established by applying an electric field to a solution of ampholines. Consequently, the gel may made from agarose which does not obstruct the movement of micelles of any size. The method can be used with native gels, or those containing non-ionic detergents. This ruled out the possiblity of using detergents other than triton for the solubilisation of HSV proteins.

Triton solubilised lysates of HSV-1, -2 and mock infected cells were resolved by IEF, followed by blotting to nitrocellulose and probing with control sera. The results were not presented formally in this thesis due to technical problems with the method. A single HSV-specific band was observed with both HSV-1 and -2 antigen on reaction with positive serum (JFP). The absence of other bands may have been the result of failure of the separation or immunoblotting stages. However, in the light of the results with TDCA. it is possible

that the poor solubility of several HSV proteins in triton accounted for the failure of IEF IB to detect more than one HSV-specific band.

In summary, the TDCA IB method allowed the separation of a proportion of HSV-encoded proteins. The reactivity of monoclonals nos. 1 and 3 in the TDCA IB showed that the method retained the antigenicity of both gB and gD otherwise lost in the conventional IB assay. The method was used to test the first and second sera of the patients with primary HSV infection. Reactivity with gB was found in all of the second sera. Only one showed additional specificities for gD. The surprising feature of the TDCA IB results was the detection of antibody reactivity to gB in some of the first sera, when conventional IB found them all to be antibody negative. A comparison of the sensitivities of the tests is to be found in the next section.

COMPARISON OF THE TESTS FOR HSV ANTIBODY

The most striking feature of the results of testing sera from patients with primary HSV infection was the detection of antibody reactivity in many of the first sera, all of which had been screened by IB and found to be negative. To a certain extent, the ELISA and IF results of the same sera confirmed the presence of antibody in some of the apparently positive first sera, although the results showed variability and some contradictions between tests. Undoubtedly, the low levels of specific antibody in the first sera contributed to the uncertainty.

The other main difference between the results of the IB assay with those of the RIP and TDCA assays was the identity of the target of antibody elicited by infection. It seems likely that the extensive

denaturation of HSV antigen prior to electrophoresis contributes to the insensitivity of the conventional IB assay for antibody of certain specificities. It would appear that the epitopes of p40 are relatively less sensitive to denturation than those of the HSV glycoproteins. The similarity of the TDCA IB results with those of the RIP assay confirm that it was the solubilisation of the antigen rather than any other methodological difference between the RIP and SDS IB assays that led to the discrepancy in the results.

The TDCA IB results are therefore useful in discounting a number of alternative explanations based on other differences between the IB and RIP tests. One possibility would have been that there was an IgG subclass difference in target specificity. The IB assay used an anti-human IgG second antibody to detect binding of test serum to the HSV antigens. However, most RIP assays, including this one, use staphylococcal protein A (SPA) in the immunoprecipitation. It is well known that SPA fails to bind to IgG subclass 3, yet it has been established that, in viral infection, specific antibodies of subclass IgG3 and IgG1 predominate. That the absence of IgG3 binding by SPA did not affect the results was shown by the TDCA IB results, which agreed with the RIP assay, yet used the same anti-human IgG conjugate as the IB assay. Ashley *et al.*, (1985) suggested that the discrepancy between her results and those of Eberle *et al.*, (1984) arose from differences in the affinity of antibody to p40, which was supposed to bind more efficiently to immobilised proteins (in the IB assay) than to those in solution (RIP), the converse being true for antibodies to gB. The TDCA IB assay showed that reactivity to p40 may not be detected because of reduced solubilty in the detergents used in RIP analysis. Furthermore, the reactivity of first sera and monoclonal no.3 with gB in the TDCA IB assay showed the antigenicity of gB to be

retained even when immobilised; loss only occurring after SDS treatment.

While denaturation accounts for the insensitivity of the IB test for glycoprotein-specific antibody elicited during primary infection, it is difficult to account for the observed absence of antibody to other glycoproteins on testing acute phase sera in the RIP and TDCA IB assays. Before concluding that the early humoral immune response is confined to one glycoprotein, certain experimental findings need to be accounted for.

As mentioned above, the MCP showed poor solubility in the detergents used in the RIP assay, and this accounted for its absent or weak reactivity in the RIP and TDCA IB assays. A similar argument could be made for p40, which showed weak reactivity with monoclonal no.5 (in contrast to the strong reactivity of this monoclonal in the IB assay), and possibly for some of the other capsid and tegument proteins. Analysis of TDCA IB solubilised material indicated that, not only was p40 undetectable, but also gC-1 and gG-2. The latter two proteins would be expected to be fully soluble in most detergents. The absence of these two proteins, and possibly of several of the other HSV glycoproteins, is one reason for the inability of the TDCA IB assay to detect antibody to proteins other than gB and gD in hyperimmune or convalescent antisera.

Similar constraints may apply to the RIP assay, since similar detergents are used, and the solublisation procedure is identical to that of the TDCA IB assay. An argument against this is the observed reactivity of monoclonal antibodies of numerous specificities in the RIP assay, both reported here and extensively elsewhere. However, the

extreme sensitivity of the RIP assay and the relative excess of antibody may mask differences in the ability of the RIP to detect antibody to different proteins. Insolubilty of glycoproteins may result from intermolecular associations of homologous or heterologous glycoproteins in the viral envelope, or perhaps between glycoproteins and proteins of the tegument mediating attachment of the envelope. Both gB (Snowden & Halliburton, 1985) and gD (Cohen *et al.*, 1986) have been observed to form dimers in the presence of SDS; this is evidence to show that the non-covalent intermolecular associations may survive the relatively powerful dissociating effect of this detergent. Stannard *et al.*, (1987) have observed a degree of organisation of glycoproteins in the viral envelope, with different HSV glycoproteins associated with distinct morphological elements on the virion surface. It is not impossible that these structures, or parts of them, may resist dissociation in non-ionic detergents, thereby preventing full solubilisation. Different glycoproteins would be expected to show greater or lesser solubility; some may be effectively dissociated and remain in solution during the ultracentifugation step of the solubilisation procedure, while others may be largely removed at this stage. Evidence to support this comes from EM analysis of the pellet obtained on centrifugation of HSV-1 infected cells in 1% triton and 1% sodium deoxycholate. Abundant nucleocapsids were found, both unenveloped and partially enveloped.

If it were established that there was differential solubility of HSV glycoproteins (the data presented here is preliminary and the discussion speculative), then the RIP results presented here, and those of previous investigators, would need to be re-interpreted to account for the possibly greater abundance of, and hence greater sensitivity for antibody to, proteins such as gB, compared with the

apparently less soluble gC-1 and gG-2 glycoproteins. The discussion above illustates some of the problems in comparing antibody levels to different proteins. The MABIA was developed as an alternative to these gel-based methods to provide further information on the target specificity of sera.

MONOCLONAL ANTIBODY BINDING INHIBITION ASSAY

Early experiments with the MABIA were designed to investigate the nature of the inhibition shown by antibody positive sera on the binding of HSV-specific monoclonals. The first experiment compared the blocking activity of a HSV-1 antibody positive serum (JFP)) on two monoclonal antibodies (one type-common, one HSV-2 specific) and showed that there was specific inhibition of the former, but no blocking of the type 2 monoclonal at any serum dilution. Further experiments with a range of positive and negative human sera, and pre-bleed and immunised rabbit antisera established that the test could demonstrate the specific inhibition of a range of monoclonals. No monclonals, other than no.12 (gE-1 specific), were affected by negative sera, while there was virtually complete inhibition of binding of monclonal no. 1 and partial inhibition of nos. 2, 5 and 9 by antibody positive sera. As mentioned in the results, no.12 may be a special case, in view of the known Fc binding activity of gE. The possible localisation of the site of binding of no.12 within this area would allow non-immunological blocking by IgG.

The target epitopes of the monoclonals were not characterised, but it was possible, indirectly, to obtain information on whether inhibition was epitope specific, or whether only reactivity within the same

antigenic area was necessary to mediate inhibition. Two HSV-1 specific and two HSV-2 specific monoclonals to gD were obtained and tested by ELISA. The results indicated varying amounts of cross-reactivity of the monclonals (table 3). The same monclonals were than tested in a MABIA with pre-bleed, HSV-1 and HSV-2 antibody positive rabbit antisera. The results of monoclonal no. 2001 show that, although it did not cross-react with type-heterologous antigen, it was measurably inhibited by the HSV-2 antiserum, albeit less efficiently than that of HSV-1. The other monclonals showed varying degrees of cross-reactivity, and still greater "cross-inhibition" by type-heterologous antisera. From this data, it could reasonably be inferred that precise blocking of the target epitope of the monoclonal by the antiserum was not necessary to mediate inhibition of monoclonal binding, although type homologous antisera did block more efficiently than sera of the opposite type.

Experiments that directly measure the inhibition of one monclonal by another have been reported and tend to confirm the above conclusion. Marlin *et al.*, (1985) investigated the cross-inhibition of 14 monoclonals to gC-1. By measuring the ability of each monclonal to block the binding of each of the others, they could be assigned to two groups, 1 and 2. Group 1 monoclonals effectively inhibited each other, but had no effect on those of group 2. The converse was true for those of group 2. Experiments with truncated proteins localised the antigenic site of group 1 monoclonals to the N-terminus half of the molecule, while those of group 2 reacted with all truncated forms indicating reactivity with the C-terminus half. The later findings of Sjoeblom *et al.*, (1987) indicate that one of the two antigenic areas of gC-1 may be composed of carbohydrate.

Several monoclonal antibodies were screened for reactivity in the ELISA and inhibitability by control antisera. The results revealed a range of inhibition values, two p40-specific monoclonals being resistant to inhibition by human sera. A possible explanation for this latter observation would be that the mouse was immunised with a preparation of HSV antigen bearing epitopes not normally exposed during the course of infection in man. Evidence to support this hypothesis comes from the partial inhibition of no.5 by the HSV-1 rabbit antiserum, but not by the human sera (table 2); the rabbit, too, may have received unnatural immunogens.

Experiments with the negative sera, those positive for HSV-1 antibody, and those from patients with HSV-2 infection provided much data on the specificity of the test. The negative sera without exception showed no significant inhibition of any of the monclonals used, with little variation in inhibition values. Since it was established that the patients with HSV-1 antibody had not been exposed to HSV-2, it was possible to compare the inhibition values of no.9 (gG-2) from these sera with the negative sera to further establish the specificity of the test. The results showed that there was no inhibition of no.9 by any of the sera positive for HSV-1 antibody, and the average and range of the values was similar to that of the negatives.

Binding of monoclonals nos. 1, 3 and 13 was completely inhibited by most HSV-1 and HSV-2 antisera, although a minority of sera of both types showed only partial inhibition. However, monoclonals no.2 and 9 were less readily blocked. No sera showed inhibition values below 32% with no.2, while a proportion of sera from patients with HSV-2 infection showed inhibition figures within the range of the negative sera. The susceptibility of monoclonal no.10 to blocking by HSV-1 and

-2 antibody sera was intermediate in extent between monoclonals nos. 1, 3 and 13 and no. 2 and 9.

The reason for the difference in inhibitability of the monclonals is not entirely clear. It is notable, however, that the monclonals found to react in the IB assay, and hence with linear epitopes, were poorly inhibited by most antibody positive sera. In contrast, the monoclonals unreactive in the IB assay were readily inhibited (nos. 1, 3 and 13). There does seem to be an association between inhibitability and the nature of the epitope. Conformational epitopes may be relatively large, often involving the interaction of more than one polypeptide chain. For example, Eisenberg *et al.*, (1985b) showed that the conformational epitopes designated I, III, IV and VI of gD-1 were found on distinct hydrophilic loops, yet monclonals to each of them showed a degree of cross-inhibition. This data, and the predic ted tertiary structure of the protein indicated that the four epitopes associated to form a relatively large antigenic area on the protein. Generalising from this information, it can be seen that inhibition of a monoclonal reactive with one of the conformational epitopes in a large antigenic area, such as described for gD-1, could be mediated by a wide range of human antibodies to gD. In this case, the size of the antigenic area would appear to the important factor mediating susceptibility of a monoclonal to blocking.

It is well established that epitopes retaining antigenicity in the IB assay are linear, and therefore depend on the amino acid sequence of the polypeptide rather than its conformation. Eisenberg *et al.*, (1985b) showed that, for gD-1, linear epitopes could be precisely localised and were not found in association with the hydrophilic antibody binding site of the protein. Indeed, epitope V is found on

the interior side of the membrane and may not be exposed to the immune system during natural infection. Unlike the conformational epitopes, the target for inhibition is relatively small since there are no neighbouring epitopes. This may be the explanation for the relatively ineffective blocking of monclonals nos. 2 and 9 in the MABIA. Human sera would require relatively large amounts of extremely specific antibody to effectively block monoclonals to linear epitopes, in contrast to the relatively low specificity required to block those to conformational epitopes.

There is little in the literature to compare with the results obtained in the MABIA. Ross *et al.*, 1985 describe a similar assay and investigated the blocking ability of four gB and gC monoclonals by several human sera. However, there were several problems with this work. The assay was insensitive, in that no change in inhibition values of any of the four monoclonals could be found in sera from patients with primary infection. The monoclonals were not titrated before use, and inhibition values were tested at a fixed monoclonal antibody concentration of 1/100 (human serum at 1/25). This excessive amount of monoclonal antibody was probably one reason for the insensitivity of the assay. The inhibition values of the negative sera were extremely variable, and overlapped considerably in range with those of convalescent HSV-1 and HSV-2 antisera.

DETECTION OF ANTIBODY BY MABIA DURING SEROCONVERSION

Figure 20 summarises the results of testing the first and second sera from patients with primary infection with HSV-1 and -2. All of the patients with HSV-1 infection showed the development of antibody to gD, since all showed a significant fall in inhibition value with monoclonal no. 1. Not all patients with HSV-2 infection showed significant reactivity with no.1, but did so with monoclonal no.13. There is clear evidence therefore that antibody to gD is elicited by infection with both HSV-1 and -2.

All patients developed antibody to gB, as shown by the significant inhibition of both monoclonals nos. 3 and 10 in all of the second sera and in proportion of the first. The relatively ineffective blocking of monoclonal no.2 by convalescent sera is reflected, perhaps, in the failure of the MABIA to detect the development of gC-1 antibody in a proportion of patients with HSV-1 infection. However, on comparison with the results of patients with HSV-2 infection, there does seem to be a trend towards lower inhibition values in the second sera. While the arrows representing the results of patients with HSV-2 point in both directions and are largely confined to the negative range, those of patients with HSV-1 infection tend to point leftwards, and to show inhibition values significantly lower than the negative range. The precise converse holds true for patients with HSV-2 infection. There in no evidence for gG-2 antibody in patients with HSV-1 infection, while a convincing case could be made for the presence of antibody to gG-2 for at least five of the patients with HSV-2 infection.

It is unfortunate that a wider range of monclonals was not available to study the evolution of antibody during primary infection. In

particular, it would have been desirable to have used monoclonals to gC and gG that were more readily inhibited. In all probability, the data obtained do not exhaust the possible repertoire of antibody specificities to be found in primary infection.

There are two problems when comparing the data on antibody specificity obtained by the MABIA with that from the IB and RIP assays. The first one has been mentioned; the observed inhibitions may have been mediated by IgM, while the other tests only detect IgG reactivity. Secondly, the inhibition figures obtained by different monoclonals cannot be used to compare the relative amounts of antibody to each protein. Firstly, monoclonals differ in their ease of inhibition, possibly as a result of the size of the antibody binding site associated with the epitope. Secondly, the extent of inhibition depends on the affinity constant of the monclonal relative to the range of values found in the polyclonal antiserum used for competition. A monoclonal with high affinity may bind to its target, despite the relative excess of antibody of similar specificity but lower affinity in the antiserum.

From a theoretical point of view, the MABIA can only detect the presence of antibody; it cannot indicate its absence. It is only by collecting statistical information on the reactivity of a large number of antibody positive sera that reasonable inferences can be made about the likelihood of an apparently negative serum in the MABIA actually containing HSV-specific antibody or not. Fortunately for the work described here, the results were largely positive. The author was initially reluctant to develop the MABIA, since it may simply have produced predominantly negative results that would defy interpretation. This may also be the explanation for the apparent lack

of reports in the literature concerning its use.

Although the MABIA results cannot be directly compared with those of the other methods, the ready detection of antibody of several target specificities does support the idea that not only the IB, but also the RIP and TDCA IB assays show different sensitivities for antibody to different proteins. Investigation of the solubilty of HSV proteins in non-ionic detergents indicated that gB may be over-represented (see above). This may well be the explanation for the apparent restricted reactivity of acute phase sera in the RIP assay found here and by other investigators. Although the antibody to gD, gC-1 and gG-2 found in the MABIA may have entirely of the IgM class, this seems unlikely in view of the ready detection of HSV-specific IgG to other proteins, such as gB and p40, at the same time.

SUMMARY

The comparison of numerous methods to study the target specificity of sera from patients exposed to HSV has revealed more about the limitations of the conventional tests than about the humoral antibody response to viral infection. The IB assay was shown to be of limited sensitivity as a result of denaturation of antigens. The apparently low solublity of proteins, including certain HSV-encoded glycoproteins, in the detergents used in the TDCA IB assay accounted for its failure to detect antibody to proteins other than to gB and gD-1. The similarity of the detergents used in the TDCA IB assay with those of the RIP assay led to the suspicion that the RIP may, too, be insensitive for antibody of numerous specificities.

The ready detection of antibody to gD, gC-1 and gG-2, as well as to gB by the MABIA in sera from patients with primary infection contrasts with the apparently restricted specificity for gB found in the same sera in the RIP assay. The results from the MABIA do not contradict the idea that antibody to all of the HSV proteins may be elicited on primary infection. Although direct comparison of MABIA results with those of the RIP assay may be faulted, it would appear that the observed restricted nature of the early antibody response found by the RIP assay in the work described here, and by other investigators, is an artefact of the methods used. The differential sensitivity of the RIP assay, in particular for gB, may not be noticed on assaying sera with relatively high titres, such as those from immunised animals, monoclonals and convalescent sera from humans. By contrast, testing for the extremely low levels of antibody found in patients close to seroconversion, subjects the RIP method to much closer scrutiny, from which it may be shown to be deficient.

FUTURE WORK

It would be difficult to devise alternative solubilisation conditions with the current range of available detergents to improve the performance of the RIP and IB assays. There is a compromise between effective solubilisation and preservation of antigenicity, as the discrepancy in the results of the IB and RIP assays shows. It may be possible to subject proteins dissociated in SDS or other powerful detergents to a renaturation procedure, as has been achieved for some insoluble enzymes. However, a procedure that has been shown to be effective at restoring the activity of a single enzyme would be unlikely to be able to restore the antigenicity of any more than a

small proportion of the numerous epitopes on the wide range of HSV-encoded proteins.

A possible method to quantify antibody response would involve the titration of test sera against a panel of individually purified HSV proteins in an indirect ELISA. One could ensure that equal amounts of target protein were available for reaction with the test serum (unlike the RIP assay) by adjustment of the protein content prior to coating on the solid phase. However, the problems of dissociating proteins in the original preparation would remain, and again there would be a compromise between yield of protein and retention of antigenicity. The method would also be elaborate and costly to use. It would also suffer from the continual problem of ensuring that the preparations of the individual proteins were pure. As an alternative, recombinant proteins could be used as antigen, although this would inevitably lead to problems in ensuring that the synthetic protein folded in the same conformation as the wild-type, and that all of the epitopes were present. Successful comparison of relative antibody levels to individual proteins elicited by HSV infection is likely to remain an elusive goal.

The MABIA has many features that would make it suitable for use in a type-specific assay for HSV antibody. The problems with the current attempts using purified type-specific antigens are as follows. Firstly, they rely on the solubilisation and subsequent immunological or non-immunological purification procedures to isolate the type-specific protein. Production of pure indivdual proteins is very difficult to accomplish, yet the specificity of the test is totally dependent on this being achieved. The isolation of proteins relies on the solubility of the proteins in detergents, and in practice is

confined to glycoproteins. Thirdly, since the whole protein is used as the antigen, no differentiation is made between antibody binding to the different antigenic sites. Although type-specific antibody binding sites are commonly found on HSV proteins, it is unusual to find proteins with no cross-reacting areas. Although antibody to gC-1 was at one time considered to be type-specific, it has since been shown that there is a second partially cross-reacting area on the protein. The exterior parts of gG-1 and gG-2 appear to be totally unrelated, and these two proteins may be candidates as type-specific antigens. However there is cross-reactivity of the internal part of the proteins. Furthermore, homology between gG-1 and gD-1 has recently been reported (see chapter 1). It is not clear whether these proteins would be suitable for use.

None of these disadvantages are found with the MABIA. Firstly, its specificity resides in the monoclonal, and no purification of antigen is required before use. Secondly, the inhibition of binding of monoclonals in confined to reactivity within the same antigenic area, rather than to the whole protein. Thus, a type-specific assay based on the carbohydrate area of gC-1 would be a possibility. Another advantage is that the assay may be used with insoluble proteins. This would allow the use of some of the proteins of the tegument or capsid, should any be shown to be type-specific.

Further work could be pursued to find and investigate more monoclonals with a view of developing a MABIA for type-specific antibody. No. 9 is unsuitable since it is only poorly inhibited, but another monoclonal to gG-2, preferably against a conformational epitope, would be ideal. For HSV-1 antibody detection, monoclonals against gG-1 or the carbohydrate epitope of gC-1 could be used. There is a relative lack

of monoclonals to gG-1, LP10 being of no use in the MABIA. No. 2 (gC-1) was only partially in hibitable and was not found to be suitable, although other gC-1 monoclonals may be. In view of their relatedness, monoclonal antibodies to other proteins of HSV would be less likely to be successful in a type-specific MABIA. Although many monoclonals appear to be type-specific, cross-inhibition might be expected if the monoclonal reacted within an antigenic area shared by its type-heterologous counterpart.

HUMAN IMMUNODEFICIENCY VIRUS

The limitations of the IB assay for antibody to HSV could also be expected to operate on testing sera for antibody to HIV. Unfortunately, the problems involved in HIV culture and the preparation of antigen confined studies of antibody to HIV to commercial sources of antigen. The results of a RIP assay using metabolically radiolabelled HIV and mock infected cell lysates as antigen were considered too preliminary for inclusion in the thesis, although it is hoped that this method will subsequently be improved and used to complete the seroconversion studies. Reference will be made in this discussion to the results of other investigators who have used the RIP assay more extensively.

TARGET OF ANTIBODY IN CONVALESCENT SERA

Table 12 records the prevalences of reactivity to the various bands in the IB assay of HIV-antibody positive sera. The sera were those referred to the laboratory for the confirmation of the presence of antibody, and were taken from patients in a wide variety of risk groups. Of all of the bands, only reactivity to p24 was observed in all of the sera tested, although there was also a high prevalence of antibody to p65, p51, gp41 and p33. Antibody reactivity with p17 was more variable, being found in approximately half of the patients tested. Antibody to gp120 was less readily detected in this assay.

The identification of bands was relatively straightforward, largely as a result of the relatively small numbers of HIV encoded proteins. The 55 KDa band was identified as pr55, the precursor polyprotein formed

on translation from the *gag* gene and cleavage from that of the *pol* gene. The cleavage of pr55 *in vivo* leads to the appearance of bands of MW 25 (p24) and 19 (p17) KDa in IB assay. There is a further fragment of 12 KDa, but this was too small to be resolved by this gel system. The proportion of protein in the uncleaved precursor form relative to the lower MW forms varied on the source of virus. Comparison of the bands on strips prepared from antigen obtained from the National Institute of Health, USA in figure 21 with those in subsequent figures, prepared from antigen purchased from Dupont shows that pr55 is present in only small amounts in the former preparation. It was a little surprising to find such relatively large amounts of a precursor protein in purified virions, and suggests that cleavage of the gag polyprotein may not be an essential stage in virus assembly. An alternative explanation may be that the cleaved gag proteins of the nucleocapsids remain aggregated even after boiling in SDS and mercaptoethanol. This seem unlikely.

There is also evidence that bands of MW 45 KDa and 40 KDa are also derived from the *gag* gene. This is on the basis that differences in antibody reactivity to p24 and pr55 correllates with the appearance of these two bands (see figures 24; 25; 29-33) on the strips. Again, there was variation between different sources of antigen in the detection of these bands. It is probable that these bands are intermediate products in the cleavage of the gag polyprotein, pr45 perhaps representing the combination of p24 with p17, while p40 represents the combination of p24 with p12. Henderson *et al.*, 1987 showed that virions of the closely related equine infectious anaemia virus contained several intermediate proteins in cleavage pathway of the gag polyprotein, and showed that alternative pathways were operative.

A monoclonal antibody to p24 of HIV, kindly provided by Dr B. Ferns, Middlesex Hospital, showed weak reactivity with a 28 KDa proteins as well as with the 25 KDa band (data not shown). Furthermore, a hyperimmune serum obtained by immunising a mouse with purified HIV also showed reactivity with this protein, as well as strong reactivity to the other *gag* encoded proteins (data not shown). This band has not been observed on reaction with human sera. Although the evidence suggests this 28 KDa protein is derived from *gag*, it does not seem to fit into any possible cleavage pathways. It therefore may be a degradation product of one of the larger gag proteins.

p65 and p51 are derived from the *pol* gene and represent the RT enzyme found in the nucleocapsid. The lower MW form is derived from p65 by cleavage of a 12 KDa RNAse fragment. p33 represents the endonuclease. Comparison of the IB results shows that there is correlation between antibody reactivity to the three proteins. There is no evidence for the 100 KDa percursor protein in the IB results. Several authors have mis-identified p65 and p51. Lange *et al.*, (1986a) imagined that p65 was a glycoprotein derived from the *env* gene, although work published subsequently from this group no longer contains this error.

Antibody to gp41 is readily detected in convalescent sera in the IB assay; however, antibody to gp120 and the precursor gp160 is not commonly detected. That this partially due to denaturation of the antigen can be seen from the results of varying the temperature of the sample buffer prior to electrophoresis (figure 11). gp120/gp160 of unheated HIV, both reduced and non-reduced, shows slightly more antigenicity on reaction with J2 than that heated to 56C or boiled. The bands, however, remain extremely faint and one needs to consider

whether this is the explanation for the failure of IB to detect antibody to the protein.

Gelderblom *et al.*, (1985) provided evidence that gp120 is shed from the envelope of the virus on budding from an infected cell. The functional significance of this has been discussed in chapter 4 of the introduction. It is possible that extensive shedding of gp120 occurs during the purification of virus and that little remains in the antigen used for the IB assay. No band corresponding to gp120 can be seen on a protein stain of the purified virus from Dupont after PAGE. However, no bands corresponding to gp41 can be seen either, yet immunological reactivity with this protein can be readily detected in the IB assay.

At first sight, the results of the RIP assay would appear to support the idea that gp120 is absent in the IB assay. The RIP studies published (Kitchen *et al.*, 1985; Barin *et al.*, 1985; Gaines *et al.*, 1987; Chiodi *et al.*, 1987 and many others) all show prominent reactivity of sera with gp120 and the precursor gp160. Since RIP assays generally use cell lysates as a source of antigen, it might be expected that relatively larger amounts of gp120 might be available for reaction, since much of the viral material is intracellular at the time of harvesting. On the other hand, it is established that solubilisation of antigen in the RIP assay is less denaturing, and the prominent appearance of gp120 may be the result of greater reactivity of antisera with non-denatured antigen. The RIP results contribute little to the settling of the problem.

Pan *et al.*, (1987) used HIV infected cell lysates as antigen in an IB assay for virus-specific antibody. Although this may lead to problems

of non-specific antibody binding to cellular proteins, there was some evidence of reactivity to several HIV proteins. In particular, and in contrast to studies using purified virus as antigen, promin ent reactivity was seen with a band identified as gp160. Weaker and more variable reactivity was seen with bands identified as gp120 and gp41. However, caution should be exercised in the interpretation of these results. The use of MW markers for the calculation of MW of the observed bands was not reported. No positive identifications of proteins were made by monoclonal antibodies of known specificity. Furthermore, test sera were not tested with uninfected cells as a control, in order to exclude the possibility that some of the bands produced were, in fact, cellular proteins. These are serious omissions in view of the different appearance of the blots compared to those of other investigators, particularly the apparent detection of HIV-specific antibody to unusual proteins such as those of 48 and 28 KDa. No explanation was given for the abnormal dilution response of positive sera on titration. While antibody to p24, gp41 and the 55 KDa protein was detectable at dilutions of at least 1/10,000, no sera showed reactivity with gp120 at dilutions of less than 1/50.

Surprisingly, there is little published data that actually proves the existence of antibody to gp120. Evidence from RIP assays that purports to show antibody to gp120 may be criticised on the grounds that steps were not taken to dissociate gp120 from gp41 before incubation with the antiserum. There are disulphide bridges between the two proteins which would remain intact in the detergents used for the immunoprecipitation, but which would be reduced on subsequent SDS-PAGE analysis of the immune precipitate. Co-precipitation undoubtedly occurs in all of the published RIP results, and the hypothesis that antibody to gp41 is largely responsible for the immune precipitation

of gp120 remains to be disproven. It is notable that both of the methods used for radiolabelling would lead to preferential incorporation of isotope into gp120 rather than gp41. Surface labelling with iodine would obviously favour the externally positioned gp120 proteins, while metabolic labelling with cysteine would also be more efficient for gp120 (the number of cysteine residues in gp120 greatly outnumbers those in gp41; Alizon *et al.*, 1986). This might explain the relatively greater intensity of the gp120 band compared to that of gp41 in published RIP assay results.

There is other circumstantial evidence that supports the idea that there is little antibody to gp120 produced on natural infection with HIV. Firstly, it would seem difficult to account for the generally low neutralising titres to HIV, if it is supposed that there are high titres of antibody to gp120. It has been suggested that such antibody may be directed away from the sites responsible for interaction with the T4 receptor. However, it is difficult to see how steric hindrance of the T4 receptor resulting from the attachment of antibody to neighbouring sites could be avoided, particularly in view of the relative sizes of gp120 and IgG. It is well known that antibody to those glycoproteins of HSV shown to be inessential *in vitro* may be neutralising; the mechanism presumably being steric hindrance of the proteins that mediate attachment or fusion.

Gnann *et al.*, (1987) produced synthetic polypeptides corresponding to parts of the amino acid sequence of p24, gp41 and gp120. All four of the gp120 polypeptides and all six of those corresponding to gp41 were shown to elicit an antibody response in rabbits. However, while antibody to 4 of the 6 polypeptides of gp41 was found in convalescent sera from HIV infected patients, none of the human sera reacted with

any of the four polypeptides corresponding to gp120. These results would indicate that there is less naturally occuring antibody to gp120 than gp41. However, only linear epitopes would have been present in the synthetic proteins, and it remains to be seen whether there is a similar lack of antibody to conformational epitopes of gp120.

Several studies have demonstrated the presence of antibody to non-structural proteins of HIV (Arya & Gallo, 1986; Knight *et al.*, 1987). Recent work investigating the role of the *sor* gene has shown that it is essential for the infectivity of the virus (Fisher *et al.*, 1987; Strebel *et al.*, 1987). There is the possibility that sor is present in the virion, although its detection in the IB assay with purified virus has not been reported. There may be other explanations for its non-detection; it may be present only in small amounts, or alternatively, it may have predominantly conformational epitopes that would be lost on solubilisation in SDS. However, careful scrutiny of the results of the IB assay does reveal the presence of a 23 KDa band in the IB assay on reaction with some sera (eg. J2; patient 4 of the seroconconversion study: panel c, figure 24). Significantly, the mouse antiserum, while showing marked reactivity with gag proteins (see above), does not react with the 23 KDa band, indicating that it is not merely an degradation product of p24.

In summary, convalescent sera show a wide range of reactivities with HIV encoded proteins. As with HSV, there are discrepancies between the results of the IB and published RIP assays. Little evidence presented conclusively proves that there are significant levels of antibody to gp120.

CONFIRMATION OF POSITIVE SERA BY IB

Reliable detection of antibody to HIV is important in determining past exposure to the virus, and hence public health measures to prevent the spread of HIV in the community. In particular, screening and confirmation should be able to give a positive or negative result for every serum tested; an equivocal result is unacceptable in view of the health implications of HIV. Tests therefore need to combine high sensitivity with specificity. Unfortunately, IB is impractical for widespread use, in view of the complexity of the procedure, and the experience required in interpretation. However, IB has a role in the confirmation of positive results from simpler screening tests, such as ELISAs, and in the investigations of samples showing equivocal results on initial testing.

IB has been used for this purpose in the Edinburgh Reference laboratory for the last two years. Over this time, several interesting sera have been investigated by the IB assay, as well as the routine confirmation of positive sera. Confirmation of positive sera has been relatively straightforward with few discrepancies between the ELISA results and those of the IB assay. In many cases (figure 22), borderline reactivity of certain sera in the Wellcome assay could not be confirmed in the IB assay or Pasteur EIA. All of these sera were referred by the BTS, apart from one which was of unknown origin. In view of the large number of donors screened by the BTS, such reactivities in the Wellcome assay can be seen to be very rare. Investigation of one of the sera revealed absent reactivity in several other sensitive tests for HIV antibody. It has been suggested by others that weak reactivity in the Wellcome assay could be mediated by serum anti-HRP antibodies, or non-specific enzyme inhibitors. There is

no evidence that suggests low levels of antibody to HIV. The Wellcome assay produces considerably fewer false positive that indirect ELISAs for HIV antibody. Early experience with the original Abbott assay, and those from Dupont and Organon indicated frequent false positive reactions. In many cases, increased serum stickiness from storage, heat inactivation or malaria could be attributed as causes. This latter cause is discussed in Biggar *et al.*, (1985a).

Other sera investigated showed different patterns of reactivity in the various tests for HIV antibody. Several sera showed weak reactivity in the Wellcome test, and were shown to have weak reactivity in the IB assay, typically producing a single p24 band. Subsequent sera from the same patients indicated that the patients had recently seroconverted for antibody. the results of these, and sera from several other patients with acute infection are discussed below. Several sera showed isolated reactivity with p17, and were referred after detection of weak reactivity in the Dupont or Abbott EIAs by the BTS. Unlike the sera from patients with acute infection, no change over time was seen in the reactivity with p17 in the two patients who were investigated further. No reactivity was seen in the competitive ELISAs for HIV (Wellcome, Abbott confirmatory test). Where possible, an enquiry was made into possible risk factors for HIV infection. Neither of the two donors investigated were in the at-risk age group, and neither had travelled to Africa. In view of these results, HIV infection was therefore ruled out as a cause of this reactivity in the IB and Dupont assays.

There have been several reports of isolated reactivity in the IB assay with p17 and pr55 (Courouce *et al.*, 1986), or with p24 and pr55 (Biberfeld *et al.*, 1986; van der Poel *et al.*, 1986, amongst others).

Variation in the relative amounts of precursor gag proteins between different sources of antigen may account for the failure to detect antibody to pr55 in the results presented here. The sera described by these investigators were described as being false-positive on the basis of failure to develop further bands on follow-up (ruling out seroconversion), absent risk factors, absent reactivity in alternative assay systems, and, in one study, absent infectivity (as shown by absence of seroconversion on follow-up of recipients of blood taken from donors showing reactivity to p25 and pr55). Lelie *et al.*, (1987b) compared the reactivity of sera previously shown to be false-positive (p24 and pr55 bands only) with acute phase sera from patients with primary infection with restricted reactivity to p24 only. The investigators used an IB assay with unheated antigen, and found some reactivity with a higher MW band, identified as gp120, in sera from patients seroconverting for antibody, but not in the false positive sera. These results agree with the finding above of slightly enhanced reactivity of J2 with gp120 on comparison of different heat treatment for HIV antigen.

None of the investigators have proposed reasons for the false positive reactions in the IB assay. The possiblity of cross-reactivity with viral or non-viral antigen needs to be considered. It is interesting to speculate on the presence of, so far, uncharacterised retroviruses in the community, with sufficient homology to cause cross-reaction of antibody elicited by infection. Such viruses may be non-pathogenic or may be associated with some of the diseases of obscure aetiology. There have been serious suggestions that rheumatoid arthritis and multiple schlerosis (MS) may be of retroviral aetiology. The latter suspicion is based of evidence of involvement of HTLV-I in tropical spastic paraparesis, a disease that resembles MS. Cross-reactivity of

HTLV-I, -II or HIV-2 in the IB assay are other possibilities to account for the false positive IB results, although the evidence to show cross-reactivity of HTLV-I and -II with HIV is conflicting. The experimental data presented by Sarngadharan *et al.*, (1985), to show extensive cross-reactivity of several proteins in the IB assay are almost certainly mis-interpreted. However, other evidence that demonstrates a lack of cross-reactivity between HTLV-I and HIV is more credible (Hattori *et al.*, 1985). Notwithstanding this, the patients described here, and in the reports quoted above, are not in the risk groups for these viruses.

It is, however, certainly possible that the donor who showed reactivity with p24 and pr55, weak reactivity in the Wellcome EIA, absent anti-"env" but very high levels of anti-"core" was infected with HIV-2. This patient had a history of travel in Africa; furthermore, it is been shown that there is cross-reactivity between the gag proteins of the two viruses in the IB assay (Vittecoq *et al.*, 1987). In the future, it be necessary to adopt screening methods for HIV-2 as well as HIV-1, not only for individuals who have lived in Africa, but amongst the risk groups for HIV-1 infection. Recent data has indicated the spread of HIV-2 amongst homosexuals and drug abusers in Europe.

SEROCONVERSION STUDIES

These experiments were designed in a similar way to those of HSV, although in this case, there were no RIP results to corroborate the findings. Overall the results showed good agreement about the time of appearance of antibody, apart from the Abbott EIA. The results from this latter test are non-specific and will not be considered further. In two patients (no. 1 and 8), the Wellcome EIA gave an equivocal result for a sera that was positive for antibody by the other tests. In another, (no. 2), there was a marked discrepancy in results, with only the IB and Abbott confirmatory assays detecting antibody in second and third sera from the patient. The results with the other tests for the first of these two sera were uniformly negative, while only the Dupont assay detected antibody in the second (ie. the third of the series). Undoubtedly, timing of samples is important; the relatively good agreement between tests in the other patients may simply indicate that sera were taken well away from the moment of seroconversion. The relatively large intervals beteen the first and second sera in the majority of patients would support this conclusion. However, patient 2 was suffering from acute myeloid leukaemia, and the disorders in immune function found in such patients may have led to an abnormal antibody response to infection. Certainly, comparison of the the bands produced in the IB assay with those produced by the other patients shows that reactivity was virtually confined to gag proteins and gp41 up to 176 days after the last negative serum. Other patients showed relatively broad antibody specificies by this time, even though the initial antibody response may have been confined to p24. The initial antibody response may have been oligoclonal, perhaps favouring reactivity to the critical epitopes of the IB and anti-"env" assay, rather than those of the other tests.

The results of testing many of the patients in the seroconversion study and those in the haemophiliac cohort by the IB assay, showed that antibody reactivity was confined to p24 or p24 and p17 (figures 24; 25). Such results tended to be found in those patients with low antibody levels by the other tests, and may indicate proximity to seroconversion. This is not necessarily evidence that the initial antibody response is qualitatively different from that found later in infection. Although antibody specificity appears to broaden in the weeks after seroconversion, this may be the result of differential sensitivity of the test for antibody to p24 relative to other proteins. The broadening of antibody specificity may be more apparent than real, and the difficulties of comparing antibody reactivity with different proteins becomes apparent once again.

Differential sensitivity of antibody tests accounts largely for the results of Allain *et al.*, (1986). This study describes the testing of sera from haemophiliacs around the time of seroconversion by the Abbott confirmation test. Results show that antibody to the core protein (recombinant p24) appear some time after antibody to env (recombinant gp41) during seroconversion. The results presented in the work described here indicate that these results may be merely an artefact, and result from the relatively greater sensitivity of the test for anti-"env" than anti-"core". The author found that the three of the four sera positive for anti-"env" and negative for anti-"core" were all reactive with p24 in the IB assay (patient 2, table 13; patients W5 and U1, table 15). The exception (W10, table 15) failed to develop antibody to anti-"core" or show reactivity with p24 in the IB assay, so this was not a case of delayed appearance of antibody. Conversely, no sera were found to be IB negative, yet reactive with env in the Abbott confirmatory assay. Similarly, Gaines *et al.*,

(1987) reported the simultaneous detection of anti-"env" antibody and reactivity with p24 in the IB assay in patients seroconverting following exposure to HIV. Thus, there is no evidence, yet, to corroborate the hypothesis advanced by Allain *et al.*, (1986) of differential antibody reponses on primary infection.

Several comparisons of different assays for antibody to HIV around seroconversion have been reported. Comparisons between groups are problematic for tests such as IB, IF and RIP assays, since the performance of the tests may vary between laboratories, or with time in the same laboratory as experience is gained. Gallo *et al.*, (1986) reported the equal sensitivity of IB and IF assays in the detection of antibody to HIV, although this was not found in this study. Gaines *et al.*, (1987) has recently presented comparative data on the performance of tests around seroconversion. This extensive study shows that IB, RIP and the Abbott confirmatory test were substantially in agreement about the time of seroconversion, although the targets of antibody were different in the IB and RIP tests. As might be expected, IB detects antibody to p24, while bands of 160, 120 and 24 KDa are found in the RIP assay. Whether the appearance of a 120 KDa band in the immune precipitate indicates an antibody response to gp120 has been discussed above. Several commercial EIAs were also used to test the sera, and the results showed that the IF assay and the Abbott, Dupont, Organon, Pasteur and Wellcome EIAs all detected antibody several weeks later than the three confirmatory tests. These results were obtained from very closely spaced sera, and do not contradict the findings made in this study; the greater agreement in results found here may have been related to the relative infrequency of samples from the patients.

Marlink *et al.*, (1986) and Ulstrup *et al.*, (1986) reported the seroconversion in HIV infection in small studies comprisisng two and three patients respectively. Both studies reported the earlier detection of antibody reactivity in the IB assay relative to a range of commercially available ELISAs. The latter author also found the IF assay to be significantly less sensitive than the IB assay for HIV-specific antibody. This perhaps illustrates the problem of comparing such tests between different laboratories, since the results are in marked disagreement to those of Gallo *et al.*, (1987). The findings of Oldham *et al.*, (1986) are more difficult to evaluate. 20 sera showing weak reactivity in a screening test for HIV antibody were tested by a range of assays, including IB and a newly-developed competition assay, as well as many of the commonly used commercial ELISA tests. The results show more frequent detection of antibody reactivity in the new assay than in the others, including IB. However, no attempt was made to establish why some sera appeared to give discrepant results. Proximity to seroconversion would be the most likely cause, yet no data was presented on the testing of follow-up samples from those patients showing initially weak reactivity in the assays. It is questionable whether such low reactivity, not in association with seroconversion, genuinely represents the presence of specific antibody to HIV.

Huisman *et al.*, (1987) presented data to suggest that the RIP assay may detect antibody at an earlier stage of infection than IB. The report describes the detection of antibody to p24 in the RIP assay some weeks before the appearance of p24 reactivity in the IB assay. These results are interesting, and may result from a greater sensitivity of the RIP assay for antibody. However, it is possible that the RIP may be detecting antibody in immune complexes. The

antigenaemia that precedes seroconversion may be sufficient to absorb the initially low levels of antibody elicited by infection. Soluble radiolabelled antigen added during incubation with the test serum might compete for antibody within the immune complex, and lead to a degree of incorporation of label into the immunoprecipitate. Subsequent analysis by SDS PAGE would reveal the presence of radiolabelled bands, and hence give a positive result. This mechanism relies on the antigen being in solution, and would be less likely to occur if it were immobilised on a solid phase, such as is found in the ELISAs and IB assay. Immune complex formation, and hence adsorption of the early immune response to HIV, may be the reason for the generally low rates of detection and low levels of HIV-specific IgM found in primary infection. It is possible that the transient IgM response may have been exhausted by the time that the antigenaemia is cleared, and seroconversion for IgG takes place. It is not impossible, therefore, that infection and hence the initial immune response takes place several weeks before virus-specific IgG is detected. This would agree with the clinical experience (see chapter 4), where the acute illness associated with initial infection is often found to precede the appearance of antibody by several weeks. Circumstantial evidence to support this hypothesis comes from consideration of the serology of HBV. Initial infection with this virus is associated with an antigenaemia. The antigenaemia comprises predominantly surface antigen, and while IgM to core may be readily detected in the acute phase of the illness, IgM to surface antigen is not found. Immunosorption of the initial immune response to HBV may be the reason for the lack of IgM.

That reliable detection of antibody in the early stages of infection is important is shown by local experience from the Glasgow BTS. A

donation was negative in the standard screen (Wellcome) yet transfusion of the blood led to seroconversion in the recipient. Subsequent analysis of the blood revealed the presence of HIV antigen. The serum was tested by IB and Abbott confirmatory test and found to be negative. That there is a transient antigenaemia before seroconversion is well established (see chapter 4), and was detected in several of patients studied in the work described here.

Patient 2 of the seroconversion series, and 5 of 11 last negative sera from patients in the haemophiliac cohort contained detectable antigen. The failure to detect antigen in some patients may be related to the frequency of sampling. While the interval between the last negative and first positive sera in the five patients where antigen was detected ranged from 6 to 50 days, the interval in those where antigen was not detected was from 55 to 177 days. It is difficult to go further than this in the statistical determination of the duration of antigenaemia. The data has been presented to Dr R. Prescott, Medical Statistics Department, University of Edinburgh, for analysis. No firm conclusions have been reached at present. Not only is the data for such an undertaking incomplete, but statistical analysis of the results from the 11 patients brings in the assumption that a uniform phenomenom is being studied. However, comparison between the available data from the patients indicates that this is not so. Figure 26 indicates that the antigenaemia in patient U2 can not be longer than 33 days, while that in patient U1 could not have been shorter than 40 days.

Two sera from patients in the haemophiliac cohort showed the simultaneous presence of antibody and antigen. Both sera showed reactivity with p24 in the IB assay. One was negative for anti-"core"

and positive for anti-"env". Antigen positive, anti-"core" negative and anti-"env" positive results were found in seven sera from haemophiliacs studied by Allain *et al.*, (1986) around seroconversion. However, in this study, a serum that was antigen positive and anti-"core" positive was also found. Three such sera were also found in the quoted study, two of which were from patients who had previously shown the previous pattern. One serum (from patient W6) was anti-"env" positive, anti-"core" negative and antigen negative. Furthermore, the serum was reactive with p24 and other gag proteins in the IB assay (figure 25). 11 such sera were found in the French study, although not tested by IB, three of which were taken from patients who had previously been antibody negative, antigen positive. Allain *et al.*, (1986) also reported an association between sampling interval and detection of antigen. In this study, there was a 25% prevalence in the group where the sampling interval averaged 5 months, and 50% in the group with a mean interval between samples of 3.5 months. Similar figures are reported by Goudsmit *et al.*, (1986), using similar assays (31%; 3 months mean interval). These figures indicate a similar incidence to those reported here. The cumulative results indicate a prevalence of 46% over 81 days mean interval. This is a very similar figure to that reported by others. If the prevalence is multiplied by the mean sampling interval, then an approximate duration of antigenaemia is obtained. This elementary statistical method gives the following results: this study, 37 days; Allain *et al.*, (1986), 1.25 and 1.75 months (38 and 53 days); Goudsmit *et al.*, (1986), 0.9 months (28 days). This is a surprisingly high figure and indicates that the prevalence of antigen carriers, who are antibody negative, may be relatively high in the community. Assuming very approximately, an average of 3 years for the duration of HIV infection in Britain, then it can be seen that between 2.5% and 4.8% of individuals infected with

HIV may be antibody negative. The high prevalence of such individuals in the community indicates the difficulty of preventing spread of HIV by screening blood donations for antibody. This data provides an explanation of the findings of Salahuddin *et al.*, (1984), who isolated HIV from 4 out 96 seronegative samples from high-risk patients. This study was carried out long before the development of the antigen assay, and before the existence of a transient antigenaemia on seroconversion was suspected.

The time to seroconversion after exposure could be studied in the haemophiliac cohort since the source of infection was later identified (Ludlam *et al.*, 1985). These results are not necessarily comparable to those of infection in other risk groups, since the route of infection differs. Nothing is known about the early events in the course of sexually spread infection, and there is the possibility of local viral replication at the site of infection for considerable lengths of time before dissemination and seroconversion. Infection from administration of whole blood would expose the recipient to a relatively large infectious dose, and would be expected to lead to a rapid seroconversion.

The results from the haemophiliacs indicate that the infectious dose may be relatively small. The prevalence of HIV infection in Edinburgh in 1983 was very low; this was the time when blood donations that were processed into the implicated batch of factor VIII were collected. It is therefore possible that only one or two HIV infected samples infected the final plasma pool of 500 donations used to prepare a batch of factor VIII. There is, therefore, a considerable dilution of virus compared to that found in whole blood. Lyophilisation of factor VIII after purification has been shown to inactivate up to two logs of

virus infectivity (MacDougal *et al.*, 1985), and this leads to a further reduction in infectious dose. Circumstantial evidence for the low dose of HIV received by the haemophiliacs comes from the observation that, of the 34 who received the infected material, only 18 were infected. By contrast, recipients of whole blood from seropositive donors show very high rates of infection, approaching 100%.

There appears to be some association between the numbers of bottles of factor VIII administered and the time to seroconversion. Paricularly notable is the relatively long interval observed in patients W4 and U1, who were both low users. There was no sign of infection in W4 at least 109 days after first exposure. The detection of antigen in the absence of antibody after 157 days in patient U1 is similar evidence for a relatively long incubation period. Patients with relatively higher [illegible] consumption such as U2 and U8 showed a more rapid seroconversion. It is possible that the statistical analysis currently being carried by Dr Cuthbert and Dr Prescott may be able to show a significant relationship. However, it is worth pointing out at this stage that the evidence for the infectivity of the implicated batch is circumstantial rather than positive (Ludlam *et al.*, 1985). Virus has not been detected in the remaining samples of the batch on testing in the antigen assay; similarly the material is antibody negative. These two lines of evidence do not rule out the presence of HIV in view of the possible low infectious dose, and because of the partial removal of antibody from the factor VIII fraction during manufacture. Alternatively, it is possible that the donor was antibody negative, yet infectious, in the early stages of primary infection.

The reports are reviewed in chapter 4 on the incubation time between exposure and seroconversion. In view of the different routes of infection, it is difficult to compare the data in this work with that of other investigators. Blood transfusion-associated infection is relatively well documented. However, little data on the interval between exposure and infection in haemophiliacs has been presented, in view of the relatively early infection of haemophiliacs by factor VIII of commercial origin. It is similarly difficult to establish the time of exposure in the homosexual and drug-abuser risk groups. The data presented here on the duration of antigenaemia and time to seroconversion in haemophiliacs is therefore relatively unique.

FOLLOW-UP PERIOD

The cohort was relatively intensively followed up and the regular supply of samples from all but one of the patients in the cohort presented a good opportunity for the serological characterisation of infection.

All patients show a continual rise in antibody levels to whole virus, as determined by the Dupont EIA, and to gp41, as found by measurement of antibody levels to env in the Abbott confirmatory assay (figure 27; 28). The quantitation of anti-"env" antibody was made difficult by the nature of the test. While direct binding ELISAs generally show a linear relationship between antibody concentration and optical density, no such simple relation exists in a competitive ELISA. Titration curves of J2 were comparable to those of other sera. Furthermore, preliminary results showed that antibody levels calculated from the ODs at different dilutions of test serum were

similar, provided that the OD fell in the range 0.15 to 0.8. In practice it proved necessary to test sequential sera from the same patient in the same batch, since inter-test variation was significant. In the future, it would be desirable to design a direct binding assay using recombinant gp41 for better quantification of anti-"env" levels. The technical problems with the test prevented testing of the mid-1987 samples, but the results of those up to 1986 showed that there were significant rises in antibody level in the patients in the cohort. There was, however, variation in the level of antibody finally achieved, although this did not correl ate with clinical progression. The final levels, and the rate of rise between the well and unwell groups did not appear to differ significantly, even if the patients with PGL are excluded from consideration.

The Dupont EIA uses purified virus as antigen in a direct binding assay for antibody to HIV. Antibody reactivity to any of the HIV proteins is therefore detected by this test. The relative amounts of gag proteins to env available for antibody binding was not known. Some qualifications concerning the use of OD to quantitate antibody levels are necessary at this stage. This discussion also applies to the quantitation of antibody levels to HSV.

The terms antibody level and antibody titre are distinct, and refer to different measurements. Titres are determined by serial dilution of test sera, and recording the dilution of serum at which reactivity becomes undetectable. There are several problems with this method. Firstly, the accuracy of the test depends on the dilution factor between serial dilutions. If two-fold dilutions are used, then the test can only distinguish between test sera that differ in antibody titre by a factor of two. Furthermore, the uncertainties of

measurement of very low ODs, and the relative arbitrariness of assigning a cut-off point below which sera are to be considered unreactive, leads to considerable inaccuracies in practice. The third problem concerns the nature of the information obtained from the titration. It can be seen that titration of sera measures the concentration of one antibody specificity alone, ie. the most abundant one. This may lead to problems in interpretation. For example, it was found that the titre of HSV-2 antisera with HSV-1 and HSV-2 antigen was similar; the explanation being that the most abundant antibody specificity happened to cross-react with HSV-1. Equal titres to antibody to type-homologous and heterologous is surely a counter-intuitive result.

Another method of titration would be to determine the dilution of serum that produced an OD of 0.5, or some other easily measured figure. The advantages would be greater accuracy, because of the greater values of the ODs recorded relative to the experimental error. Unfortunately, this method suffers from its inability to quantitate antibody reactivity to relatively scarce antigens on the solid phase. Reactivity with a minor component might be insufficient to produce the requisite OD, even if the antibody was present in excess. In summary, it is difficult to assign a single numerical value to a complex mixture of antibody of varying specificities, particularly in an ELISA with multiple antigens.

It was observed on titration of several control sera in the Dupont assay that there was an approximately linear relationship between antibody concentration and OD over a relatively large range. However, sera that lacked antibody to core proteins showed such a relation over a much more restricted range, typically between 0 and 0.2, above which

increasing the antibody concentration led to a less than expected increase in OD. It is possible that predominant reactivity with pol or env proteins, present in relatively lower amounts than gag proteins on the solid phase, accounted for these observations. In practice, a compromise solution was adopted. Test sera were screened at two dilutions, and antibody levels calculated from the OD produced by each. Where there was disagreement, and this was principally found in sera unreactive with gag proteins, the antibody level was calculated from the OD of the higher dilution. In plotting the graphs in figure 28, care was taken to ensure that the antibody levels of sequential samples were calculated from the ODs at the same dilutions. This was to ensure comparability between the results from the same patient. However, from the previous discussion, it can be seen that it is relatively meaningless to use these results to compare the antibody levels of different patients. The abundances of antibody to different proteins might vary between individuals leading to greater or lesser reactivity with the range of proteins on the solid phase.

Antibody levels in the Dupont EIA were seen to rise steadily over the first two years in all patients, whether well, or unwell. These results corroborate those of the anti-"env" assay. However, testing over the third year of infection showed significant changes between individuals. In particular, falls in antibody level were found in four patients, co-incident with the development of symptoms of ARC or AIDS. Patient U2 showed a sustained fall in antibody level in the months after AIDS was diagnosed. It would seem that antibody levels to whole virus have some prognostic value, although the disease may be severe before a fall is noted. ARC was diagnosed in U2 9 months after infection, at a time when antibody levels were rising. Similarly, antibody levels were seen to continue to rise after the diagnosis of

ARC (group IVa) in U4 and U6. Three patients showed marked increases in antibody level in the third year of infection (patients W4, W5 and W9). Whether this represents a favourable prognostic sign will require prospective studies of the cohort. At present, there is no clinical evidence to distiguish these patients from many of the others in the well group.

Weber *et al.*, (1987) has reported anti-"env" titres in sequential sera of two cohorts of homosexuals infected with HIV. In contrast to the findings here, no significant change was seen in titre in either well or unwell patients over the three years of study. It is difficult to account for the differences in these results with those presented here. One difference between the studies is that the time of seroconversion of the patients in the London cohort was not known, and therefore many of the patients may have been seropositive for several years before the study commenced. It is possible, therefore, that a much later stage in HIV infection was being studied in many of the patients. The titration method used was inaccurate, in that serial ten-fold dilutions of sera were tested. Therefore, only relatively large changes in antibody level could be detected by this method. In the study presented here, ten-fold rises only took place in the first year of infection. The proportionally smaller rises found subsequently possibly would not be detectable by the titration method used in the London study.

Reactivity in the IB assay, particularly with p24 showed marked variation between patients. Reactivity with the latter protein correlated well with reactivity of sera in the anti-"core" assay, and substantial agreement between the two measurements was found with all sera. However, both in the seroconversion studies, and in the

follow-up study, the IB assay was found to be more sensitive for antibody to core proteins than the specific ELISA. The IB results of the first positive, mid-1985, -1986 and -1987 sera from haemophiliacs showed that the most significant changes took place in the first year on infection. Surprisingly, there was little change in the reactivity of sera of most patients in subsequent years in the assay. This may be more artefactual than real. The IB test is very sensitive for antibody, and it requires very little antibody to produce a prominent band on substrate development. As a result, there is likely to be saturation of many of the bands by antibody positive sera. To compare the reactivity of sequential sera to many of the bands, it would be necessary to test serial dilutions in order to avoid the saturation effect. Fortunately for this study, levels of antibody to p24 were sufficiently low in many patients to enable comparison of reactivity with time at a standard dilution of 1/50.

The results showed that loss of reactivity to p24 took place in many of the patients between seroconversion and the mid-1985 sample. These results are corroborated by measurements of anti-"core" levels (tables 16 and 17). More detailed analysis of these patients (table 19) showed two main patterns of loss. Four patients showed loss of anti-"core" and p24 reactivity and remained unreactive with gag proteins for the remainder of the follow-up period. The three patients in part B of the table showed fluctuating levels of anti-"core". Fluctuating reactivity with p24 was also noted in the IB assay and the results of patient U4 are illustrated in figure 33. Comparison of the mid-1986 strip with that of 1987 confirms the result of the anti-"core" ELISA that reactivity fell away again subsequently.

Antigenaemia was found in some patients in the follow-up period,

particularly in the last year of the study. Six of the 17 patients now show persistent antigenaemia, with the suspicion of antigen in two more, on the basis of borderline, non-confirmable reactivity in the ELISA. That this may herald the development of antigenaemia is shown by patient U3. He showed this pattern of reactivity for 2 years before finally becoming confirmably antigen positive in the last month of the study. All of the patients with antigenaemia had shown previous falls in anti-"core" levels, and reduced reactivity with p24 in the IB assay. However, antigenaemia was found in one patient (W8) with fluctuating anti-"core" levels; a rise in antigen level initially ocurring against a background of rising anti-"core" levels. None of the patients with high anti-"core" levels showed antigenaemia. The converse holds true, with one exception. Only U6 of all the patients showing a fall in anti-"core" levels has shown no evidence of antigenaemia. The likely explanation for the results of patient W10, who has shown no anti-"core" reactivity at any stage of infection, is that the first positive sera was collected so long after seroconversion that anti-"core" and p24 antibody reactivity had already become undetectable. To support this hypothesis is the observation of the relatively rapid loss of anti-"core" reactivity in other patients after seroconversion. The first positive serum of W10 could have been collected at most 177 days after seroconversion; by this time, significant falls in reactivity had already occurred in patients U2 and U6. Therefore, patient W10 could perhaps be regarded as showing the same type of immune response as those in part A of table 19. Serum antigen was detected in this patient in mid-1987, although he remains well.

There was a marked correlation between antigenaemia and loss of antibody reactivity to core proteins, as can be seen from the results.

Loss of antibody tends to occur before the appearance of antigen, often by several years (U2, U3). Low levels of anti-"core", typically those seen in patients with fluctuating levels, may co-exist with antigenaemia. Finally, although sera may become completely unreactive in the specific ELISA, very few sera become completely unreactive with the p24 band in the IB assay. That the p24 band rarely disappears completely was noted by Groopman *et al.*, (1986), although this does not mean that p24 reactivity was the same for all patients. A large study by Paul *et al.*, (1987) found that 92% of sera in which antigen was found were negative for anti-"core". Of the remaining 8%, generally very low titres of antibody were found. Lange *et al.*, (1986b; 1986c) has also presented evidence to show that loss of reactivity to p24 in the IB and RIP assay is associated with the onset of antigenaemia. A strong association between antigenaemia and absence of reactivity in a specific ELISA for antibody to core proteins was reported by Goudsmit *et al.*, (1987a).

Further evidence to link the phenomenon of antigenaemia with loss of gag protein reactivity comes from the detection of immune complexes in HIV-infected patients. Morrow *et al.*, (1986) described their detection in patients with ARC and AIDS, and showed, by SDS-PAGE, the presence of p25 and pr55 proteins within the complexes. However, no data on the serological reactivity to gag proteins was presented. Lange *et al.*, (1987) found HIV antigen in immune complexes of HIV infected patients, and showed its association with lack of anti-"core" antibodies. Sera that were anti-"core" negative, and antigen negative in the conventional assay, were shown to have contained such immune complexes, while they were not found in sera that were anti-"core" positive. These findings are highly significant, and strengthen the association between anti-"core" and circulating antigen, either free

or complexed. They also provide some information on the mechanism of infection. The reason for the loss of anti-"core" antibody reactivity is not known. One suggestion is that there is clonal exhaustion of B cells, leading to reduction in HIV-specific immunoglobulin synthesis. A second suggestion is similar, and proposes that the continual exposure of HIV to the immune system leads to a state of tolerance. According to both of these theories, antigenaemia occurs as a result of the decline in specific immunity to the virus. The third explanation, and one supported by the findings of immune complexes, is that core antibody production continues throughout all stages of infection, but in some patients, there is such a rapid rate of virus replication that immunoadsorption of antibody takes place. Initially, immune complexes would form, but as the rate of virus replication increases still further, free antigen would appear, and become detectable in the conventional antigen test. The latter explanation would predict a relatively high rate of antigen production, and the question arises concerning the site in the body where this could take place. It does not tally with the relatively infrequent detection of HIV infection in T-lymphocytes, but does lend evidence to the possibility of widespread infection in non-lymphoid tissue, such as the brain, gut and lungs.

The relationship between antigenaemia and viraemia is not clear. The precise composition of the serum antigen is not known. Morrow *et al.*, (1986) reported the detection of gag proteins in immune complexes, but the bands were faint, and the failure to detect other proteins may have resulted from differential sensitivity of the IB method. Lange *et al.*, (1987) used an ELISA for gag protein in the analysis of the immune complexes; thus there was no data on the possible presence of other virion components. It is well established, on the basis of

numerous case reports, that antigen-negative blood is infectious. However, it is less clear whether cell-free blood products from antigen-negative donors are also infectious. The disappearance of antibody to p24 and other gag proteins that occurs in association with antigenaemia or immune complex formation provided some evidence that the antigenaemia comprises predominantly *gag* -encoded proteins, although it may merely reflect the fact that the immune response to gag proteins is relatively less effective than that to env proteins. The fall in antibody levels in the Dupont assay found in some patients on development of illness may reflect loss of antibody reactivity to proteins other than gag, and the fading of the p54, p51, gp41 and p33 bands between 1985 and 1987 in patients U2 and U3 in the IB assay smilarly supports the hypothesis that other proteins are found in the antigenaemia. However, both patients were profoundly unwell at the time of this loss of reactivity, and it may merely reflect HIV-induced immunological abnormalities.

It is well known that isolations may be successful with both antigenaemic and non-antigenaemic patients. In this laboratory (S. Rebus, personal communication), and elsewhere (Farthing *et al.*, 1987), there appears to be no correlation between antigenaemia and isolation rate. In particular, recent attempts to isolate virus from patient W8 have not proved successful, yet this patient has the highest antigen level of all of the patients in the cohort (250 ng/ml, recently recorded at 500 ng/ml). However, it was possible to isolate from this patient at the beginning of 1986, when antigen levels were lower (A. Lainson, personal communication). This is some evidence for the antigenaemia not representing a true viraemia.

CORRELATION BETWEEN SEROLOGICAL MARKERS AND CLINICAL PROGRESSION

The investigation of serological markers in cross-sectional and prospective studies of HIV-infected patients is motivated by the desire to monitor the course of infection and to predict the clinical outcome. In this study, a strong correlation between the presence of anti-"core" and lack of disease progression was noted. This conclusion requires the inclusion of patients with PGL (group III) in the well group. Current evidence indicates that such inclusion is not unjustified on clinical grounds, in that PGL does not appear to be prodromal in many of the patients in this group (see chapter 4). The patients with high levels of anti-"core" are W3, W4, W5, W6, W7, W9 and U5 (PGL).

However, the converse association between progression and loss of anti-"core" antibody does not apply. There were three well patients who showed loss of anti-"core" and antigenaemia, as well as one with PGL, who could also be considered to have not shown clinical progression. All of the patients in whom ARC or AIDS was diagnosed did show loss of anti-"core", with or without antigenaemia. A striking feature about this study is the rapidity with which most of the patients lost antibody to core proteins after seroconversion. Such loss was noted in the first year of infection in all but two patients, yet in many cases, symptoms of progression were only noted 2 years later (U3, U4, U6). Loss of anti-"core" would appear to be more valuable in the prediction of clinical outcome than the detection of antigenaemia, since the appearance of the latter is often delayed for up to two years after antibody loss (patient U2 and U3) or may remain absent (U6). However, improved tests, such as those that detect antigen in immune complexes, may reduce this interval (see above).

It is possible that the three well patients and the one with PGL, who all showed loss of anti-"core" and antigenaemia, may differ from the unwell patients only in the time course of progression. As mentioned, clinical progression was found in some patients up to two years after loss of anti-"core". It is possible, therefore, that a still longer time course of disease may be found on prospective study of those remaining well. Only follow-up studies could establish whether there was a difference in prognosis between these three patients and the seven with high levels of anti-"core". Were this to be found, then the anti-"core" and IB tests would be of major clinical and epidemiological importance in cross-sectional studies of infection. Furthermore, by differentiating between those likely to progress from those who are likely to remain well, early decisions about treatment could be made. Drug therapy could therefore commence at an early stage of infection, and would obviously have a better chance of success than the current policy of treating symptomatic patients.

There have been several other reports of the association between disease progression and anti-"core" levels or antigenaemia. Schuepbach *et al.*, (1985) noted a fall in the reactivity to p24 and other *gag* -encoded proteins in the sera of those developing ARC or AIDS. Biggar *et al.*, (1985b) tested sera from a patient who died of AIDS, and compared the results with those of HIV infected patients who remained well. They showed that, while antibody levels to EBV and CMV were maintained, reactivity with HIV proteins, particularly those encoded by *gag*, in the IB assay fell in the patient with AIDS. Lange *et al.*, (1986a) noted a fall in antibody reactivity with gag proteins in two patients who developed AIDS, while thirteen patients who remained well showed no such loss of reactivity. Lange *et al.*, (1986b) tested sequential samples from thirteen homosexuals infected

with HIV and showed that those from twelve of the patients, who remained well, remained antigen negative, anti-"core" positive in the specific ELISA and retained reactivity with p24 in the IB assay. Sera from the patient who developed AIDS, however, showed falling levels of anti-"core" and lost reactivity in the IB assay with p24. Antigen was detected co-incident with the diagnosis of ARC, but at least two years after the loss of reactivity with gag antibody reactivity. Lange *et al.*, (1986c) reported similar findings in a cohort of nine children with transfusion acquired HIV infection, although in this study, two patients with antigenaemia and loss of antibody remained well.

A strong association between clinical progression and antigenaemia was reported by Goudsmit *et al.*, (1986), and in a further study by the same investigators (Goudsmit *et al.*, (1987a), similar conclusions were reached. In this later study, nine of the eleven asymptomatic patients had anti-"core" and were antigen negative. Two well patients were, however, antigenaemic and anti-"core" negative. Of the eight patients with ARC and AIDS, 5 were antigenaemic, and anti-"core" negative. Two were antigen negative, and showed low titres of anti-"core". However, the eighth patient (ARC) was antigen negative, and had similar titres of anti-"core" to those of the well patients. Pan *et al.*, (1987) report an association between reactivity with p24 in the IB assay with lack of disesase progression in a large study of 103 individuals. Lack of reactivity with p24 in well patients was reported, as well the finding of strong p24 reactivity in a minority of patients with AIDS.

Paul *et al.*, (1987) reports the prevalences of antigenaemia and anti-"core" reactivity in a large number of patients who had AIDS, ARC or who were asymtomatic. Antigen was detected in 69%, 46% and 19% of

the AIDS, ARC and well patients respectively. Anti-"core" was found in 23%, 54% and 79% of patients in these groups. The finding of high prevalences of anti-"core" in the AIDS and ARC patients (23% and 54%) is surprising, and differs from the current study, and those described by the other investigators in the field. The results could have been improved by measurement of the titre of antibody in the ELISA. Weber *et al.*, (1987) showed a statistical association between titre of anti-"core" in a specific ELISA between the well and unwell groups in two cohorts of homosexuals. A similar association between disease progression and reactivity with p24 in the RIP assay was also shown for these patients. There was, in contrast, no statistically significant difference in neutralising titres of HIV between the two groups. In contrast, Wendler *et al.*, (1987), on the basis of a small study of neutralisation titres in 6 patients, three of whom had AIDS, reported an association between fall in neutralisation titre and progression. These, and other reports, have not resolved the issue of whether there is an association between neutralisation and onset of disease, and there is no information at all to indicate whether low neutralisation titres cause progression, or whether clinical disease causes the fall in antibody.

In summary, the studies of serological markers of progression in the Edinburgh haemophiliac cohort have proved comparable to those of other investigators. Follow-up has allowed a relatively intensive study of HIV infected individuals. A particularly rapid loss of anti-"core" antibody was seen in several patients, many of whom developed disease some years later. Prospective studies should show whether patients showing this pattern of loss but remaining well have a worse prognosis than those who remained anti-"core" positive. Antigen detection correlated less well with progression, although this may be accounted

for its relatively delayed appearance; evidence suggests that antigen may be in the form of immune complexes in the transition period between loss of anti-"core" and detection of free antigen.

The observed difference in serological and clinical outcome between the patients, in this cohort in particular, disposes of a number of theories concerning the pathogenesis of HIV infection. The measurement of antibody levels over a relatively long period indicates that all of the patients were genuinely infected. The possibility that some individuals, particularly haemophiliacs, may be immunised with inactivated virus rather than truly infected has been often mentioned. This study indicates that all of the patients, including all of those who remained well, show evidence of active infection three years after exposure. Furthermore, there was no correlation between clinical outcome and infectious dose, although there is some evidence that this influenced the time to seroconversion.

Another theory to explain the different outcome of infection between individuals supposes that different strains of HIV may be more or less pathogenic. While this may be true, the current study shows that serological responses and clinical outcome may vary when different patients are infected with the same strain of HIV. Similarly, the results show that progression is not related to continual re-infection with HIV. In all but one of the patients in the cohort, it is probable that the implicated batch was the single source of infection.

This cohort provided the ideal opportunity to study genetic differences in susceptibility to infection by HIV. Some connection between clinical outcome and HLA type has been found by Dr M. Steel, Western General Hospital, Edinburgh in this cohort. There remains the

possibility of establishing a correlation between phenotype and the rapid fall in antibody to anti-"core" after infection in some of the patients, since this may be less likely to be influenced by extraneous factors than disease progression. Eales *et al.*, (1987) reported the association between clinical outcome and the mobility of group specific component, the vitamin D receptor. This has been investigated in this cohort, both in terms of susceptibility to initial infection and to clinical progression, but no correlation has been found with these patients (Dr Ludlam, personal communication). At present, the reason for these differences between patients remains unresolved.

FUTURE WORK

Monitoring the cohort in the future using a similar range of tests as those described above is a priority. Clinically useful information about the course of infection may be obtained from such study, as well as helping to select patients for treatment, and monitoring its effect. Longer study will allow the significance of anti-"core" levels to be more fully assessed. The evaluation of new tests for HIV-specific antibody and antigen is facilitated by the collection of intensively studied sera taken around seroconversion in several individuals. That the precise time of exposure is known in many of the patients is very valuable in establishing the specificity of new assays. A report in press (Lelie *et al.*, 1987a) indicates the superiority of three new ELISA tests over their predecessors in sensitivity and specificity. Evaluation of these tests with the range of sera available in this laboratory may prove an interesting exercise.

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ABBREVIATIONS

ACG	Acycloguanosine; Acyclovir
ADCC	Antibody dependent cell mediated cytotoxicity
AIDS	Acquired immunodeficiency syndrome
ARC	AIDS-related complex
ARV	AIDS-related virus
AZT	3'-azido-3'-thymidine; Retrovir
bis	NN-methylene-bis-acrylamide
bp	Base pair
BTS	Blood transfusion service
CAEV	Caprine arthritis encephalitis virus
CaMV	Cauliflower mosaic virus
CDC	Centres for Disease Control
CMC	Cell mediated cytotoxicity
CTC	Cytotoxic T cell
CMV	Cytomegalovirus
Da	Dalton
DE	Delayed early
DNA	Deoxyribonucleic acid
DOC	Deoxycholic acid, sodium salt
DTH	Delayed type hypersensitivity
EBV	Epstein-Barr virus
EIA	Enzyme immunoassay
EIAV	Equine infectious anaemia virus
EL	Early late
ELISA	Enzyme linked immunosorbent assay
EM	Electronmicroscopy
EMEM	Eagle's minimum essential medium
env	Envelope
FITC	Fluorescein isothiocyanate
gag	Group antigen
GM	Growth medium
GUM	Genitourinary medicine
HBLV	Human B-lymphotropic virus
HBV	Hepatitis B virus
HCMV	Human cytomegalovirus
HIV	Human immunodeficiency virus (type 1)
HIV-1, 2	Human immunodeficiency virus type 1, 2
HLA	Human leucocyte antigen
HPA	*Helix pomatia* lectin
HRP	Horseradish peroxidase
HSV	Herpes simplex virus
HSV-1, -2	Herpes simplex virus types 1, 2
HTLV-I, II, IV	Human T-lymphotropic virus types I, II, IV
IB(A)	Immunoblotting (assay)
ICP	Infected cell polypeptide
IE	Immediate early
IF(A)	Immunofluorescence (assay)
IgG	Immunoglobulin G
IgM	Immunoglobulin M
K	Killer
KDa	Kilodalton
LAV	Lymphadenopathy associated virus
MABIA	Monoclonal antibody binding inhibition assay
MCP	Major capsid protein
MDBP	Major DNA binding protein
MHC	Major histocompatibility complex
moi	Multiplicity of infection

mRNA	Messenger RNA
MW	Molecular weight
NBCS	New born calf serum
NK	Natural killer
nm	Nanometres
OD	Optical density
OPD	o-phenylene diamine
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
PBS	Phospate buffered saline
pfu	Plaque forming unit
PGL	Persistent generalised lymphadenopathy
PHA	Phytohaemagglutinin
PMSF	Phenylmethylsulphonyl fluoride
pol	Polymerase
PPV	Progressive pneumonia virus
RIP(A)	Radioimmunoprecipitation (assay)
RNA	Ribonucleic acid
RT	Reverse Transcriptase
SDS	Sodium dodecyl sulphate
SIV	Simian immunodeficiency virus
STLV-III	Simian T lymphotropic virus type III
TDCA	Taurodeoxycholic acid, sodium salt
TK	Thymidine kinase
TL	True late
ts	Temperature sensitive
TX100	Triton X-100
U	Unit; 1000 mU
VP	Viral protein
VV	Visna virus
VZV	Varicella zoster virus

PBL Peripheral blood lymphocyte

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Detection of Antibody to Viral Proteins Following Primary Infection With Herpes Simplex Virus

P. Simmonds, I.W. Smith, and J.F. Peutherer

Department of Bacteriology, Edinburgh University Medical School, Edinburgh, Scotland

Sera from patients with primary genital infection with herpes simplex virus (HSV) were tested by immunoblotting (IB) and radioimmunoprecipitation (RIP) analysis to determine the protein targets of antibody elicited by infection. The two tests detected antibody to different antigens: IB primarily detected reactivity with p40, a phosphorylated capsid protein, and RIP detected antibody to glycoprotein B, a viral envelope component. Furthermore, RIP detected antibody at an earlier stage of infection then IB. A nondenaturing version of IB was developed and used to investigate the role that the solubilisation of antigen plays in the sensitivity of each test for antibodies with different specificities.

Key words: antibody response, antigenic targets, nondenaturing immunoblotting

INTRODUCTION

The humoral antibody response to infection with herpes simplex virus (HSV) has been studied by a variety of serological techniques. Radioimmunoprecipitation (RIP) and immunoblotting (IB) are two methods capable of showing the protein targets of antibody elicited by human infection or animal inoculation with HSV. Sera from patients with previous infection with HSV 1 or 2 contain antibodies reacting with a range of HSV-specified proteins and glycoproteins [Eberle and Mou, 1983; Gilman et al, 1981; Teglbjaerg et al, 1986]. However, the sera from patients with recent primary infection of less than a month's duration contain antibodies reactive with a small subset of HSV proteins. IB reveals predominant reactivity with p40 [Eberle et al, 1984; Bernstein et al, 1984; Eberle et al, 1985], a phosphorylated protein associated with the nucleocapsid [Braun et al, 1984]. Other workers using RIP assays have found that the early humoral antibody response is directed against gB, gC-1, and the major capsid protein (MCP) of HSV 2 [Ashley and Corey, 1984; Ashley et al, 1985; Zweerink and Corey, 1982].

The different results obtained by the two methods may be accounted for by the differing solubilisation conditions used in the preparation of the antigen for each assay. The IB assay uses antigen solubilized in sodium dodecyl sulphate (SDS). This is a

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Address reprint requests to P. Simmonds, Department of Bacteriology, University of Edinburgh, Teviot Place, Edinburgh, EH8 9AG, Scotland.

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powerful detergent that solubilises the majority of cellular and viral proteins, but with the resultant loss of a considerable proportion of the antigenic determinants. The antigen for RIP assays is produced by milder solubilisation conditions that preserve proteins in a state approaching their native configuration. However, a significant proportion of viral proteins such as the MCP of HSV 1 are insoluble in the detergents commonly used in RIP analysis (personal observation). RIP methods also have the disadvantage that spurious results may arise from coprecipitation of proteins present in partly solubilised aggregates of cellular membranes [Cross, personal communication].

Alternatives to conventional IB have been described. Snowden and Halliburton [1985] describe the immunoblotting of unheated herpes proteins in their studies of the serological relatedness of gB of HSV with other herpes viruses. Unheated gB or the homologous proteins of other herpesviruses appear broadly cross-reactive, in contrast to the apparent species specificity of denatured gB.

Another approach is the use of detergents other than SDS. Helenius and Simons [1975] and Helenius et al [1979] review the numerous detergents that have been characterised. Dewald et al [1975] describe the solubilisation and subsequent electrophoresis of membrane-associated enzymes in Triton X-100.

The aim of the work was to compare the results of testing sera of patients with primary genital HSV 1 and 2 infection by both RIP and IB methods. A nondenaturing version of IB was developed and used to investigate these sera in order to evaluate the importance of the solubilisation conditions of the HSV antigen used in these tests. In view of the reported cross-reactivity of rabbit antisera to gB of HSV with other members of the herpes group [Snowden et al, 1985], patient sera were also tested for the presence of antibodies to varicella zoster virus (VZV), Epstein-Barr virus (EBV), and human cytomegalovirus (HCMV).

MATERIALS AND METHODS

Cells and Virus

Vero cells grown in Eagle's MEM (Gibco) supplemented with 5% inactivated newborn calf serum (Gibco) were used in all experiments; 1657 [Peutherer, 1970] and MS [Plummer, 1964] were the HSV 1 and HSV 2 strains used for the production of antigen.

Control Sera

Rabbit anti-HSV 1 and anti-HSV 2 sera, both prebleed and immunised, were available [Peutherer, 1970]. Three negative human sera collected from patients, aged five to ten, for reasons unrelated to HSV infection, and a positive serum from a laboratory worker with recurrent labial HSV 1 infection were also used. The following monoclonal antibodies (mcAbs) were a kind gift from Dr. A. Cross, MRC Virology, Glasgow (specificities in parentheses): 1892 (gD-1, gD-2), 2462 (gC-1), 2975/22 (gB-1, gB-2), 3104/23 (gE-1), and 5010 (p40, HSV 1 and 2). LP14 (gD-1, gD-2) and AP2 (MCP, HSV 1 and 2) were mcAbs kindly supplied by Dr. A. Minson, Cambridge University.

Patient Sera

Sera were obtained from patients attending the genitourinary medicine (GUM) clinic at the Royal Infirmary of Edinburgh with symptoms of primary genital HSV infection. At their first visit to the clinic, a first serum sample was obtained and a genital swab submitted for HSV isolation. The duration of symptoms prior to the visit was noted.

The second serum was collected at a subsequent visit to the clinic (8–18 days later). Patients were considered to have a primary HSV infection if the first serum was seronegative by immunoblotting, the second serum seropositive, and HSV isolated. Isolates were typed by restriction enzyme analysis by other workers in the laboratory. Five patients with primary HSV 1 and five with HSV 2 infection were selected for the study (nine female, one male).

Immunoblotting

Confluent monolayers of vero cells were overlaid with 10 pfu/cell HSV 1 or HSV 2 in a small volume of Eagle's medium or similarly mock infected. After absorption of 1 hour at 37°C, the inoculum was replaced with Eagle's medium. Cells were harvested with glass beads after 20 hours at 37°C, pelleted by centrifugation at 1,000 *g* for 5 minutes, and washed twice with Dulbecco A buffered-salt solution (DSS). After resuspending in sample buffer containing 1% SDS and 1% 2-mercaptoethanol [Laemmli, 1970] at a cell concentration of 5×10^6 cells/ml, the cell lysate was held at 100°C for 3 minutes. The subsequent polyacrylamide gel electrophoresis (PAGE) with a discontinuous buffer system was performed as described by Laemmli [1970]. Two ml of sample was applied to the top of a 17 cm wide, 10% polyacrylamide separating gel, cross-linked with 0.27% N,N'-methylene-bis-acrylamide. After electrophoresis, the gel was blotted overnight at 4°C in tris-glycine buffer (0.025 M tris, 0.192 M glycine, pH 8.3) at 1.5 V/cm on nitrocellulose sheets (Schleicher and Schull, Dassel, West Germany), by the method of Towbin et al [1979]. The nitrocellulose sheets were blocked in 3% gelatin (Sigma, G-2625, St. Louis, MO) in tris-buffered saline (0.5 M NaCl, 0.02 M Tris, pH 7.5; TBS) for 30 minutes. This and all subsequent steps were performed at room temperature on an orbital shaker (Luckham, Burgess Hill, UK); 2.5 mm strips of nitrocellulose bearing cellular and viral proteins were cut from the sheet and incubated with human or rabbit serum (1:20 dilution) or mcAb (1:100) for 2 hours. All antibody dilutions were made in TBS containing 1% gelatin. Strips were washed in two changes of TBS containing 0.05% Tween-20 (BioRad, Richmond, CA). Bound antibody was detected by incubation in horseradish peroxidase conjugated antispecies IgG (Sigma) at a dilution of 1:250 for 2 hours. Substrate was 4-chloro-1-napthol (BioRad) prepared according to the manufacturer's instructions.

Radioimmunoprecipitation

Antigen was prepared as for IB with the following modifications: the serum concentration was reduced to 0.5%, at 3 hours p.i.; monolayers were washed in DSS and the medium replaced with Eagle's methionine free medium (Gibco, Paisley, Scotland, UK) containing 50 μCi/ml [^{35}S]methionine (Amersham, >800 μCi/mMol, Amersham, UK). Cells were harvested at 20 hours, washed twice with DSS, and resuspended at a cell concentration of 10^7 cells/ml in RIP buffer (1% Triton, 1% sodium deoxycholate in 0.1 M Tris-HCl, pH 7.5) on ice; 0.1 mg/ml DNAase II (Sigma, D4138) and 0.02 M phenylmethylsulfonyl fluoride (PMSF) were present in the RIP buffer. After 30 minutes on ice, the cell lysates were centrifuged at 100,000 *g* for 1 hour to remove insoluble material. The supernate was aliquoted and stored at −70°C. To avoid competition for antigen by IgM present in the patient sera, RIP analysis was performed in reverse, in a manner resembling a "G" capture assay. Ten μl of patient or rabbit serum or 5 μl mcAb was diluted in 400 μl RIP buffer: 25 μl of a 50/50 v/v suspension of sepharose-protein A (Sigma) in PBS was added and the mixture incubated, keeping the sepharose in suspension for 2 hours at room temperature. The sepharose-protein A was then washed in PBS containing 0.05% Tween-20 (PBST); 25 μl of HSV or mock-infected cell

lysate in 375 μl RIP buffer was then added and incubated for 2 hours. The sepharose-protein A was washed in PBST and resuspended in 100 μl SDS-PAGE sample buffer and heated for 3 minutes at 100°C. Ten μl was removed from the sample at this stage and spotted on a glass-fiber disc for liquid scintillation counting. The remainder of the sample was analyzed by PAGE, as for IB. Autoradiography was performed for 10 days at −70°C on a blot of the gel in order to preserve the sharpness of the bands and to increase the sensitivity of detection. Coomassie blue staining of the gel after blotting showed transfer of proteins to be complete, apart from very weak bands of size greater than 200 KDa.

Molecular Weight Determination

Seven marker proteins were run on SDS-PAGE gels as described above and stained by coomassie blue. They were as follows (molecular weights in parentheses): carbonic anhydrase (29 KDa), ovalbumin (45 KDa), bovine serum albumin (66 KDa), lactate dehydrogenase (36.5 KDa), glutamate dehydrogenase (55.4 KDa), phosphorylase B (97.4 KDa), and α macroglobulin (170 KDa). A plot of Rf value against log. molecular weight was an approximate straight line in the range 30 KDa to 120 KDa, and was used for molecular weight determination of viral proteins.

Nondenaturing Immunoblotting

Antigen preparation was essentially as described in the RIP method, except that the proteins were not radiolabeled, and 1% taurodeoxycholic acid, sodium salt (Sigma; TDCA) was used in place of the Triton and sodium deoxycholate. Electrophoresis of the TDCA solubilised material was as described for IB with the following modifications: the acrylamide concentration was reduced to 7%; all steps were done in a cold room (0–4°C); and the 0.1% SDS in the gel and electrode buffers was replaced with 0.5% TDCA (1% in stacking gel). Blotting was performed in the absence of detergent in tris-glycine buffer, pH 8.3, initially with nitrocellulose sheets on the anodic and cathodic sides of the gel; as little protein migrated toward the cathode, this latter precaution was discontinued. Coomassie blue staining of the gel after blotting showed transfer of proteins to be essentially complete. During development of this method, other detergents were used in place of TDCA. Triton X-100 and 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane-sulfonate (CHAPS) were tested using the same protocol as described above. The testing of sera with blotted antigen was carried out as described in the IB section.

HCMV, EBV, and VZV Antibody Measurement

HCMV antibodies were estimated by an indirect ELISA technique, using glycine extracted CMV-infected cells as antigen. Acetone-fixed Raji cells were used in an immunofluorescent (IF) technique for the detection of EBV IgG antibody. VZV antibodies were measured in a standard complement fixation test.

RESULTS

Specificity of IB and RIP Assays

There was no significant reactivity of any of the sera with mock-infected material in either assay (data not shown). The specificity of the two methods was assessed by testing preimmune and immunized rabbit antisera and negative and positive human sera with type-homologous HSV antigen by both methods (Table I, Figs. 1, 2). Both preimmune

TABLE I. Reaction of Control Sera in IB and RIP Assays

Serum	cpm[a]	RIP bands[b]	IB bands
Prebleed rabbit	22	None	None
HSV 1 Immunised rabbit	21,870	gC[c]**gB** 80 K gD/gG	150–140 K **130 K 108–104 K** gC 78 K 75 K 67 K **p40**
Prebleed rabbit	134	None	None
HSV 2 Immunised rabbit	13,770	MCP **130 K 120 K gB** 95 K 80 K 72 K gD 38 K	150 K 130 K **120 K** 110 K **73 K** 69 K gD p40
Negative human	151	None	None
Negative human	108	None	None
Negative human	218	None	None
Convalescent human	13,112	gC gB 80 K gD/gG p40	150–140 K 130 K **108–104 K** 78 K 75 K gD/gG p40

[a]Counts on liquid scintillation counting of 1/10th of immune precipitate.
[b]Name of protein given, or MW in KDa if not identified.
[c]Prominent bands in **boldface.**

rabbit sera failed to react in either method producing no detectable bands (Fig. 1, lanes 3, 11; Fig. 2, lanes 1, 10) and no significant activity on scintillation counting. All three negative human sera were unreactive against HSV 1 and HSV 2 antigen (Fig. 1, lane 5, Fig. 2, lanes 3–5, 12–14). By contrast, the immunised rabbit sera and the adult convalescent serum produced elevated counts in the RIP assay and numerous bands in both IB and RIP assays, reflecting broad polypeptide specificities (Fig. 1, lanes 4, 6, 12; Fig. 2, lanes 2, 6, 11).

Identification of Proteins in the RIP Assay

Figure 1, lanes 7–10, 13, shows the reaction of mcAbs in the RIP assay; gD-1 (lane 7) and gD-2 (not shown) both appear as a broad band of approximate MW 66 KDa, and the band is distorted by large amounts of comigrating IgG. There is also a weaker band of MW 121 KDa in lane 7, probably the result of coprecipitation of gC-1. Richman et al [1986] showed that a mcAb, LP10, precipitated gG-1, producing a band of similar mobility and appearance to gD-1 in their RIP assay. Bis was used as a cross-linker in the PAGE analysis of the precipitate. Therefore, bands in this area cannot be positively identified as either gD-1 or gG-1 in the RIP and IB assays used in this work, and will be referred to as gD/gG. gC-1 (lane 8) appears as a prominent band of MW 121, with weaker bands at 75 K and 45 K; gB-1 (lane 9) has two prominent bands of size 118K and 112K, with two lower molecular weight bands of 56 K and 50 K. These probably represent cleavage products. gB-2 (lane 13) has a major band of 110 K and similar lower molecular weight bands; gE-1 was not detectable in the RIP assay using mcAb 3104/23. McAb 5010 (p40) produced weak bands of MW 47.5, 45 and 42.5 KDa with HSV 1 antigen (lane 10), and no bands with HSV 2. AP2 (MCP) produced faint bands of apparent MW 158 KDa with HSV 1 and 2 (data not shown).

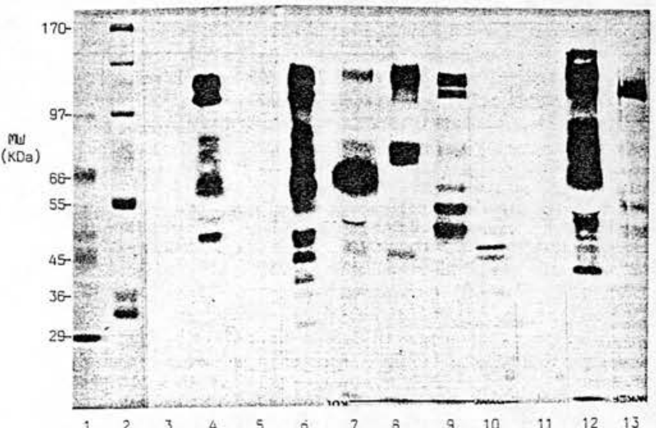

Fig. 1. Reaction of control sera in the radioimmunoprecipitation (RIP) assay. Lanes **1** and **2**, standard proteins (molecular weights in brackets), coomassie blue stain; lane 1, carbonic anhydrase (29 kilodaltons (KDa)), ovalbumin (45 KDa), bovine serum albumin (66 KDa); lane 2, lactate dehydrogenase (36.5 KDa), glutamate dehydrogenase (55.4 KDa), phosphorylase (97.4 KDa), and macroglobulin (170 KDa); lanes **3–10**, reaction of control sera with herpes simplex virus type 1 (HSV 1) antigen in RIP assay; lanes 3 and 4, prebleed and HSV 1 immunised rabbit antisera; lanes 5 and 6, negative and convalescent adult human sera; lane 7, monoclonal antibody (mcAb) 1892 (gD); lane 8, 2462 (gC); lane 9, 2975/22 (gB); lane 10, 5010 (p40); lanes **11–13**, reaction of control sera with HSV 2 antigen; lanes 11 and 12, prebleed and HSV 2 immunised rabbit antisera; lane 13, mcAb 2975/22 (gB).

Identification of Proteins in the IB Assay

Only LP10 (gD), 2462 (gC-1), and 5010 were reactive with denatured protein in the IB assay. gD-1 (Fig. 2, lane 7) and gD-2 (not shown) appear as bands of approximate MW 57 KDa. McAb 5010 reacts with p40 of both HSV 1 and 2 (lanes 9, 16), whereas 2462 (gC) produces bands of 102, 100, and 90 KDa (lane 8). It can be seen that proteins have different apparent MWs in the two systems.

TDCA IB

Preliminary experiments were carried out in order to find a suitable detergent. Figure 3, lane 1 shows the resolution achieved by electrophoresis of cellular proteins in the presence of TDCA. Triton and CHAPS caused smearing of proteins and were, therefore, not used further. All subsequent experiments described in this section use TDCA. Water-soluble marker proteins do not fractionate solely on the basis of size in this gel system, unlike SDS-PAGE. Carbonic anhydrase (29 KDa) had an Rf value of 0.7, ovalbumin (45 KDa) had an Rf value of 0.31, and bovine serum albumin (66 KDa) had a value of 0.72 (data not shown). Therefore, bands cannot be ascribed a molecular weight, so are referred to by their Rf values. Lanes 2–8, Figure 3 show the results of testing the various control sera by this method. The reaction of convalescent adult serum with HSV-1

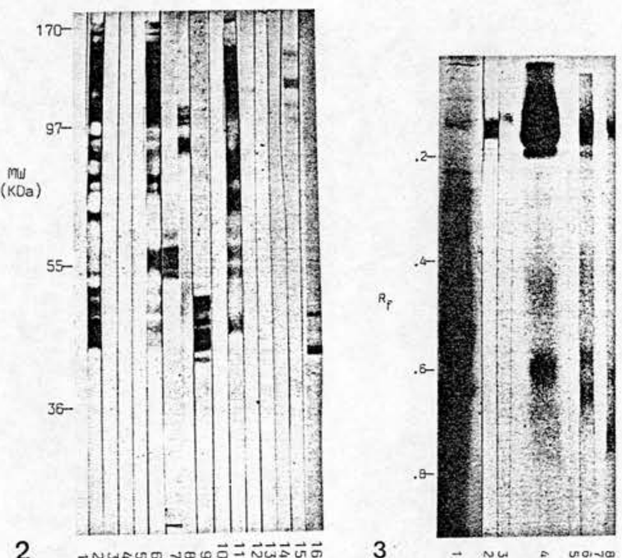

Fig. 2. Reaction of control sera in immunoblotting assay. Lanes **1–9**, herpes simplex virus type 1 (HSV 1) antigen; lanes 1 and 2, prebleed and HSV 1 immunised rabbit antisera; lanes 3–6, three negative and a convalescent adult human sera; lane 7, monoclonal antibody (mcAb) LP14 (gD); lane 8, 2462 (gC); lane 9, 5010 (p40); lanes **10–16**, HSV 2 antigen, lanes 10 and 11, prebleed and HSV 2 immunised rabbit antisera; lanes 12–15, three negative and a convalescent adult human sera; lane 16, mcAb 5010 (p40).

Fig. 3. Reaction of control sera in taurodeoxycholic acid immunoblotting assay. Lane **1**, coomassie blue stain of herpes simplex virus (HSV 1) infected cell extract; lanes **2** and **3**, monoclonal antibody (mcAb) 2975/22 (gB) with HSV 1 and HSV 2 antigen; lane **4**, convalescent adult human serum, HSV 1 antigen; lanes **5** and **6**, prebleed and HSV 1 immunised rabbit antisera, HSV 1 antigen; lanes **7** and **8**, prebleed and HSV 2 immunised rabbit antisera, HSV 2 antigen.

antigen is shown in lane 4; the development of antibody in the two rabbits following HSV 1 and 2 immunisation is shown in lanes 5–8. Two of the three negative human sera were unreactive in this assay. However, one negative human serum reacted weakly with gB-1 and gB-2 (data not shown). The only monoclonal antibodies reactive in this assay were 1892 (gD) and 2975/22 (gB). gB can, therefore, be located in the prominent band of Rf value 0.15 for HSV 1 and 1.3 for HSV 2 (lanes 2, 3); gD-1 migrates to a distance of 0.65 (data not shown).

TABLE II. Reaction of Patient Sera in RIP, TDCA IB, and IB Assays

				First sera				Second sera				
Patient	Isolation[a]	HCMV[b]	Symptoms[c]	cpm[d]	RIP bands[e]	TDCA IB bands	IB bands	t[f]	cpm	RIP bands	TDCA IB bands	IB bands
1	1	−	7	743	gB	0.03 gB[g]	None	9	3,159	gB	0.03 gB	gD/gG p40
2	1	−	3	40	None	None	None	12	3,104	gB	0.03 gB	112 K p40 112 K 85 K 70 K
3	1	−	NK	115	None	None	None	11	2,791	gB	0.03 gB	gD/gG p40
4	1	+	NK	217	None	None	None	13	3,644	gB	0.03 gB	112 K p40
5	1	−	4	827	gB	gB	None	15	7,939	gC gB	0.03 gB gD	112 K p40
6	2	+	7	0	130 K gB	gB	None	10	2,386	MCP 130 K gB 95 K 38 K	gB	107 K p40
7	2	+	6	93	130 K gB	gB	None	10	2,181	MCP 130 K gB 95 K 32 K	gB	107 K p40
8	2	−	4	438	130 K gB	gB	None	8	1,789	MCP 130 K gB 95 K	gB	p40 >150 K 150–130 K 107 K 82–66 K gD
9	2	+	3	108	None	gB	None	18	3,832	MCP 130 K gB 95 K	gB 0.63	p40
10	2	+	5	14	130 K gB	None	None	8	769	MCP 130 K gB 95 K	gB	103 K p40

[a]Isolation type-HSV 1 or HSV 2.
[b]HCMV antibody status; all sera were positive for EBV except patient 10; no sera had significant levels of Cf activity to VZV.
[c]Figures refer to the reported duration of symptoms in days; NK = not known.
[d]Counts on liquid scintillation counting 1/10th of immune precipitate.
[e]Name of protein given, or mw in KDa if not identified.
[f]Number of days between collecting first and second sera.
[g]Prominent bands in boldface.

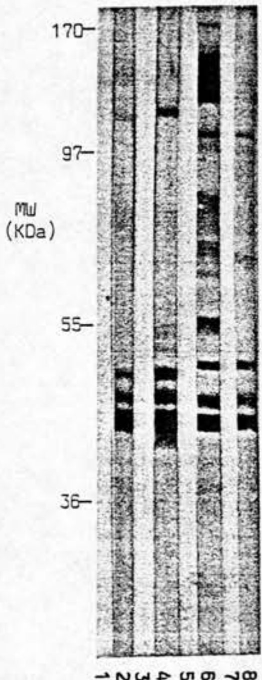

Fig. 4. Reaction of first and second patient sera in immunoblotting assay. Lanes 1–4, herpes simplex virus type 1 (HSV 1) antigen; lanes 5–8, HSV 2 antigen; lanes 1 and 2, first and second sera of patient 2; lanes 3 and 4, sera of patient 5; lanes 5 and 6, patient 9; lanes 7 and 8, patient 7.

Antibody Response in Patients With Primary Genital HSV Infection

These results are presented in Table II. None of the first sera had detectable antibody by IB (Fig. 4, lanes 1, 3, 5, 7), but six out of ten were positive by RIP and TDCA IB (although patients 9 and 10 gave conflicting results by the latter two methods). The counts detectable in the immune precipitate obtained from weakly positive sera do not correlate with detection of bands by SDS-PAGE. The target of antibody in the first sera, as measured by RIP, was gB (Fig. 5, lane 3); in addition, type 2 infections elicited antibody to another protein of MW 130 K (Fig. 5, lane 7). TDCA IB revealed an antibody specificity to gB in the six positive first sera (Fig. 6, lanes 3, 7). Two patients with HSV 1 infection also developed antibody to a protein of Rf value 0.03 (data not shown).

All second sera were positive for antibody to HSV by all three tests. Sera were

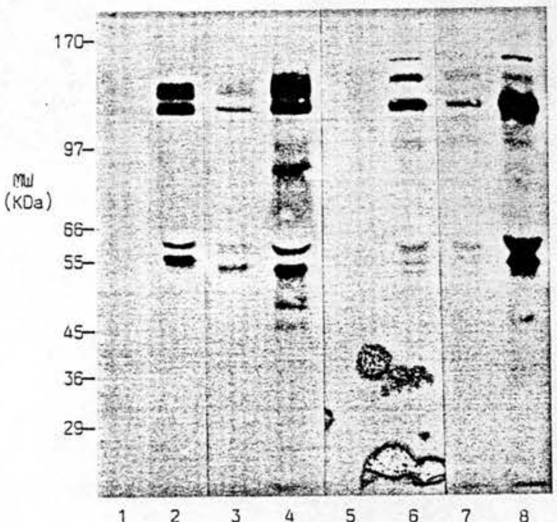

Fig. 5. Reaction of patients' sera in radioimmunoprecipitation assay. Legend as for Figure 4.

strongly reactive with p40 in the IB assay (Fig. 4, lanes 2, 4, 6, 8), with weaker and more variable reactivity with gD/gG and proteins of MW 107 K and 112 K in particular. Only the sera from patients 3 and 9 had broad polypeptide specificities, but as can be seen in lane 6, patient 9 reacted most strongly with p40; the same is true for patient 3 (data not shown). Second sera from all patients showed elevated counts in the RIP assay and prominent reactivity with gB in both HSV 1 and 2 infection (Fig. 5, lanes 2, 4, 6, 8). The second serum of patient 5 also reacted with gC (lane 4). Sera from patients with type 2 infection showed additional specificities to MCP, the 130 K protein and weak reactivity with a 95K band. TDCA IB revealed antibody reactivity with gB in all sera (Fig. 6, lanes 2, 4, 6, 8), whereas type 1 sera also reacted with a protein of Rf value 0.03. Two patients (5 and 9) showed weak reactivity with faster migrating bands (lanes 4, 6).

Nine out of ten first sera were positive for EBV antibodies, five were positive for HCMV, and none had significant levels of complement fixing activity with VZV. The presence of antibody to other human herpes viruses does not correlate with the detection of HSV-specific antibody in the first sera.

The duration of symptoms prior to attending the GUM clinic is recorded in Table II. These data were not available for two cases, but the first sera from these two patients was negative by all three tests, providing laboratory evidence that the patients had had primary infections. Two patients had symptoms for three days before attending the clinic, and both first sera were seronegative by RIP. One was negative by TDCA IB and the other was

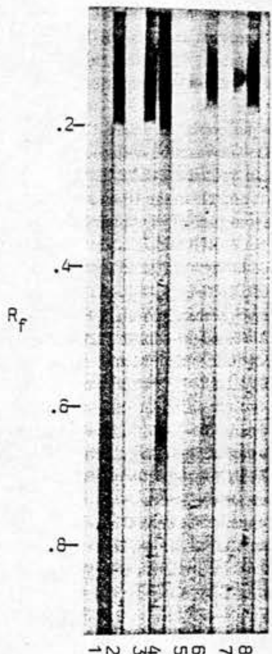

Fig. 6. Reaction of patients' sera in the taurodeoxycholic acid immunoblotting assay. Legend as for Figure 4.

weakly reactive in this test. The first sera from the six patients with symptoms for four days or more were all positive by RIP, and five of the six were positive by TDCA IB.

DISCUSSION

The preimmunised rabbit sera and the three negative human sera showed little detectable nonspecific reactivity in the IB and RIP assays. Nonspecific reactions in the RIP assay are commonly observed with glycoproteins [Zweerink and Corey, 1981; Cross, personal communication]. In particular, detergent-solubilised gE has been shown to have Fc binding activity [Baucke and Spear, 1979]. Nonspecific Fc binding activity may have been avoided by the different methodology of the RIP assay described here. All antibody is attached by the Fc region to protein A during the incubation with HSV antigen, possibly preventing the unwanted reaction with gE. The reason for the slight reaction of one of the negative human sera with gB in the TDCA IB assay is not clear at present.

McAbs of known specificity were of some help in the identification of bands in the three assays. They also revealed that several HSV proteins appeared as more than one band, complicating the subsequent analysis. gB-1 appears as two main bands of MW 112 and 118 KDa. These are probably the precursor and mature forms of the polypeptide, respectively. The two lower molecular weight bands of 56 and 50K (also observed with gB-2) are probably cleavage products, since they are not observed in the RIP assay of Ashley et al [1985], who performed all incubations at 4C. p40 and the MCP appear as faint bands in the RIP assay on reaction with the appropriate mcAbs, probably reflecting the low solubility of these proteins in the detergents employed. McAbs allowed the identification of p40, gC-1, and gD in the IB assay. p40 of both HSV 1 and 2 reacted with mcAb 5010, producing a complex of very prominent bands. gD and gC-1 had different apparent molecular weights in RIP and IB assays. The lower apparent MW of gD in the IB assay may be accounted for by the absence of comigrating IgG that distorts the band in the RIP assay. The 121 KDa form of gC-1 found in the RIP assay is not detected in the IB assay; instead there are lower molecular weight forms. The reasons for this difference are unclear at present. There was slight coprecipitation of gC-1 by 1892 (gD), but overall, proteins appeared to precipitate independently in the RIP assay. The identification of proteins is further complicated by the anomalous migration of glycoproteins in SDS-PAGE. Their migration is influenced by the cross-linker used in the gel system. For example, gG-2 has an apparent MW of 92K when Bis is used [Marsden et al, 1984], whereas experimenters using diallyltartardiamide find gG-2 to have a MW of 130 [Roizman et al, 1985; Olofsson et al, 1986]. For this reason, it is difficult to compare results obtained by different investigators.

Detergents for immunoblotting should be of low micellar size to allow free migration of detergent-solubilised material in the gel, be soluble throughout the range of pH values used in discontinuous gel buffer systems, and bind to a wide range of proteins. These properties are to be found in TDCA, which has the advantage over the more commonly used deoxycholic acid in being freely soluble below pH 7. In contrast to triton, TDCA has a micellar size similar to SDS, and, therefore, should not restrict the mobility of solubilised proteins during electrophoresis. PAGE with TDCA solubilised material gave moderate resolution of several cellular and viral proteins. When blots of such gels were used in an IB assay, several viral antigens could be detected by the rabbit antisera. Two of these could be identified as gB and gD by mcAbs. Although TDCA is negatively charged, it does not cause fractionation of proteins purely on a size basis, as the experiment with marker proteins shows. The low migration rate of gB on a 7% acrylamide gel (Rf values 1.5 (HSV 1) and 1.3 (HSV 2)) suggests that gB is present in an oligomeric form in this system, perhaps similar to the dimeric form observed by Snowden and Halliburton, [1985] in their modification of IB. These authors report the cross-reactivity of gB-1, gB-2, and the homologous proteins of EHV-1 and BMV. The lack of correlation between antibody to HCMV, EBV, and VZV and antibody to gB in any of our three assay methods discounts the possibility of cross-species antibody reactivity influencing our results.

RIP and conventional IB methods use different detergents to solubilise the antigen. SDS, used in IB, solubilises almost all protein, but disrupts the tertiary structure of the proteins, leading to a loss of a proportion of possible epitopes. RIP assays generally use milder detergents that preserve antigenic determinants formed by the tertiary structure of the protein; however, the solubilisation of viral proteins is incomplete, and only a subset of proteins can be studied. This difference in solubilisation undoubtedly contributes to the difference in results of the IB and RIP methods found in this paper. However, there are

several methodological differences between the two assays that may also contribute. Depending on the method, the antigen is either in solution or bound to a solid phase. In the RIP method, the sensitivity of the test is a function of the specific radioactivity of the antigen, whereas in IB assays, the sensitivity depends on the amount of antigen available for reaction and the sensitivity of the second antibody and substrate to detect bound antibody. Coprecipitation of proteins in the RIP assay is a cause of nonspecificity that is irrelevant to IB. Proteins incorporate radioactivity with differing efficiencies during the labeling procedure required for the RIP assay. For example, Ashley et al [1985] found that gG was labeled poorly by [^{35}S]methionine, and antibody to it could not be detected in their RIP assay.

TDCA IB is methodologically similar to conventional IB; the only difference being the choice of detergent. It, therefore, gives an opportunity to assess the importance of solubilisation conditions of the antigen independently of all other considerations. Both RIP and TDCA IB detected gB antibody in several of the first sera, whereas all were negative by SDS IB. All second sera were positive by all three tests, but the targets were different. Sera tested by SDS IB showed major reactivity with p40, with weaker and more variable reaction with other proteins, particularly with gD (or possibly gG-1) and a protein of MW 107K. Second sera from patients with HSV 1 infection by RIP had antibody to gB and in one case to gC, whereas type 2 sera reacted with gB-2, MCP, and the 130 K protein. At this stage the identity of the 130 KDa protein cannot be ascertained. gG-2 would be expected to appear at around 90–100 K since Bis was used as a cross-linker. ICP 8 has an apparent MW of 128 K, and this is a possible candidate. TDCA IB detected predominantly gB reactivity in the second sera. Overall, IB contrasts with the RIP and TDCA IB in its detection of antibody to p40, and its failure to detect antibody to gB. The failure of RIP and TDCA IB to detect antibody to p40 may result from poor solubility of p40 in mild detergents (as shown by the weak reaction of mcAb 5010 in the RIP assay and its nonreactivity in the TDCA IB assay). The epitopes of gB are presumably sensitive to denaturation by SDS, leading to the failure of SDS IB to detect gB antibody in any of the first sera. Overall, the results of RIP and TDCA IB are in agreement and contrast with those of conventional IB. Therefore, the discrepancy in SDS IB and RIP results observed in this paper and by previous workers studying antibody responses to primary HSV infection may be accounted for by the solubilisation conditions of the antigen, and not by the other methodological differences between the two assays.

There is a possible objection to this conclusion. Protein A used in the RIP assay does not react with IgG3, whereas the enzyme-linked antihuman IgG used in the IB assay would be expected to react with all the subtypes of human IgG. That this may lead to the detection of different apparent antibody specificities is refuted, first, by the evidence of Eberle et al [1985], who obtained similar results in IB with protein A and antihuman IgG conjugate, and second, by the detection of gB antibodies by TDCA IB using the same conjugate as used in conventional IB.

The detection of antibody in the first sera by RIP and TDCA IB assays was dependent on the duration of symptoms prior to attending the GUM clinic. The first sera from the two patients with symptoms for only three days were negative by RIP assay and no more than weakly positive by TDCA IB, whereas the sera from patients with symptoms of four or more days duration were reactive in the RIP assay (five out of six positive by TDCA IB). Second sera taken from two patients (nos. 5 and 9 at 19 and 21 days after onset of symptoms) had broader polypeptide specificities in the IB and TDCA IB tests and higher counts than second sera taken from the other six patients with symptoms of shorter

duration. Although the numbers of patients in this trial were small, the results indicate that antibody elicited by HSV infection is detectable within four days of the onset of symptoms by RIP and TDCA IB methods. All first sera were negative by SDS IB, indicating that antibody to p40 becomes detectable at a later stage, more than seven days after the onset of symptoms in this group of patients.

Further work will be directed at improving the TDCA IB method so that a greater range of antibody specificities may be detected, and to collect more exact information on the sequence of events around the time of seroconversion.

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19

LAV/HTLV-III Antibody Testing: Confirmation Methodologies and Future Prospects

P. SIMMONDS, J. F. PEUTHERER, and D. B. L. MCCLELLAND*
Edinburgh and South-East Scotland Regional Blood Transfusion Service and Edinburgh University Department of Bacteriology, Edinburgh, Great Britain*

Routine testing of all blood donations for antibody to LAV/HTLV-III was introduced throughout the United Kingdom on 14 October 1985, using screening tests manufactured by Wellcome Diagnostics (UK) and Organon-Teknika Ltd. In England and Wales, confirmatory testing was undertaken by several centres of the Public Health Laboratory Services, which used a varying range of confirmatory tests. In Scotland two regional virology laboratories undertake all confirmatory work for the Blood Transfusion Service using immunoblotting as the primary confirmatory procedure.

SCREENING PROGRAMME RATIONALE

The intentions of the donor assessment protocol are to ensure that no donor is informed of a positive test result until this has been confirmed by a positive immunoblot, and secondly to ensure the immediate and permanent removal of any suspect donation from the blood supply. In addition, we feel it is vital to have such a schema to ensure that no donor is left in an 'unclassified'

position due to an unconfirmed positive screening test result. This latter situation could lead to repeated calls to donate followed by immediate discarding of the blood, which is clearly not an acceptable position.

RESULTS

Table 1 summarizes the United Kingdom donor testing results from October 1985 to the end of February 1986. This emphasizes the very low prevalence in the United Kingdom overall. The prevalence of LAV/HTLV-III antibody in the West of Scotland is identical to that in the UK as a whole. However, in the East (accounted for by four different blood collecting centres) the prevalence is six times higher. It is important, therefore, to determine whether these substantial differences can be explained by factors related to the performance of screening or confirmatory testing, or whether they are due to donor factors such as a high prevalence of infection in the community, or a tendency for donors in high-risk groups to disregard the prohibition on blood donation.

Table 1 Donor testing, October 1985–February 1986 (United Kingdom)

		Scotland		
	All UK	All	East	West
Tested	1,052,086	122,667	63,370	59,297
Confirmed positive	19	8	7	1
Prevalence	1 in 55,372	1 in 15,333	1 in 9,053	1 in 59,297

With respect to Scotland, the evidence favours the interpretation that the differences between East and West are due to the large difference in prevalence of infection between certain parts of the country. Sixty-five per cent of intravenous drug abusers (IVDA) in the East are LAV/HTLV-III antibody test positive, in contrast to 4.5% of IVDA in the West.[2] Eight antibody-positive donors in Scotland were found to be IVDA.

In the Scottish Blood Transfusion Services experience to date, the Wellcome competitive ELISA has yielded no repeatably positive results other than those which have eventually been confirmed by immunoblotting. Thus our two reference laboratories have received referred samples from eight donors and all have been strongly immunoblot-positive. This has led to a level of concern about the sensitivity of the screening test, and we have therefore concentrated in the past few months on assessing the relative sensitivity of various screening ELISAs and of our immunoblot confirmation procedure to try and determine whether greater sensitivity is needed to reduce any risk of false-negative results. Initial studies on about 10,000

donors and on high-risk patients indicated, as have other reports, that the Wellcome screening assay is at least as sensitive as other screening tests. We have recently concentrated on the serological events which occur around the time of seroconversion, as it is here that we feel the risk of a failure in a donor screening test may be greatest, either because the initial response may contain very low levels of antibody or because the specificity of the early antibody response could be very restricted. For example, if the very earliest antibody response is to a large envelope glycoprotein, certain assays could fail to detect this.

The immunoblot procedure which has been used throughout this work is based on the methods of Laemmli[3] and Towbin *et al.*[4] We are aware of a number of modifications which are likely to increase sensitivity and these are being assessed. Tables 2 and 3 illustrate some of the results obtained from serial samples from donors and transfusion recipient. Table 2 includes the two subjects in whom, on one or more occasions the immunoblot was clearly positive when the screening ELISAs were equivocal or non-reactive. In the case of the blood donor, at the time the blot became positive there was detectable reactivity in the Wellcome assay, but this was only noted by the use of a more critical method of interpreting the results than that which is recommended by the manufacturer. In the case of the blood (platelet) recipient, there is a series of samples consistently positive by immunoblot (reacting only in the p19 and p25 region) and consistently non-reactive by the ELISA tests.

Table 3 shows a similar study on two recipients of coagulation factor products. In each of these subjects, reactivity observable in the screening assay at the same stage as antibody was detected by the immunoblot. These early data show that reactivity are no grounds to be complacent about the performance of either donor screening or confirmatory testing methods.

DEVELOPMENT OF IMPROVED TESTS

Some possible lines of future technical development include improved immunoblots, radioimmune precipitin assays (RIPA), competitive immunoassays (capture or competition) and flow cytometry detection of viral coded cell surface antigens. We believe it is important that more work be done on optimizing the immunoblot procedure and in particular to compare it directly with the RIPA, since there is evidence from various sources that the RIPA tests perform better in detecting antibodies to the high molecular weight components of the envelope. It is important to know if and when these antibodies may be missed by the immunoblot, since without this information it is not known whether the present confirmatory procedure is partly blind.

Table 2 Serological response to LAV/HTLV-III infection

Time (weeks)	Edinburgh BTS Wellcome EIA	Edinburgh Ref. Lab Wellcome EIA	Immuno-fluorescence	Pasteur EIA	Western blot MW (KDa) of bands reactive with sera						
					19	25	31	41	55	65	120
Blood donor											
−36	−	−	−	−	−	−	−	−	−	−	−
0	−	−	−	−	−	−	−	−	−	−	−
12	+/−	+/−	wp	+	−	++	−	−	−	−	−
28	+	+	+	+	−	+	−	+	−	+	−
40	+	+	+	+	−	+	+	+	++	++	−
Blood recipient (leukaemia patient)											
0	−	−	−	−	−	−	−	−	−	−	−
2	−	−	−	−	+	+	−	−	−	−	−
2.5	−	−	−	−	+	+	−	−	−	−	−
5	+	+	wp	+	+++	+++	−	−	−	−	−
13	+	+	+	+	+++	+++	−	+	−	+	−

Time = Time elapsed since last serum negative by all tests.
wp = Weak positive (immunofluorescence).
Western blot reactivity graded by eye on scale −, +, ++, and +++.

Table 3 Serological response to LAV/HTLV-III infection

Time (weeks)	Edinburgh BTS Wellcome EIA	Edinburgh Ref. Lab Wellcome EIA	Immuno-fluorescence	Pasteur EIA	Western blot MW (KDa) of bands reactive with sera						
					19	25	31	41	55	65	120
Blood product recipient A											
0	−	−	−	ND	−	−	−	−	−	−	
6	+	+	wp	ND	−	+	−	−	−	−	−
12	+	+	+	ND	+	+	−	−	−	+	−
13	+	+	+	ND	+	+	−	+	+	+	−
22	+	+	+	ND	+	++	++	++	+	++	−
Blood product recipient B											
0	−	−	−	ND	−	−	−	−	−	−	−
7	+	+	+	ND	+	+	−	−	−	−	−
22	+	+	ND	ND	+	+++	+++	+	+	−	
29	+	+	ND	ND	++	+++	++	+	+	+	−

Time = Time elapsed since last serum negative by all tests.
ND = Not done.
wp = Weak positive (immunofluorescence).
Western blot reactivity graded by eye on scale −, +, ++, and +++.

Secondly, the appearance of alternative competition immunoassays using well-characterized core and envelope antigens produced by recombinant DNA methods or by synthesis will provide an excellent tool for investigating the fine specificity of antibodies detected by other methods; and by using a range of antigens and monoclonal antibody probes, competitive assays will probably help to define a small number of epitopes which are essential for screening tests. Eventually such assays are likely to become routine confirmatory procedures, or indeed largely remove the need for secondary tests.

We should be preparing to respond to a growing need to give additional information about infectivity of blood donors who are found to be antibody-positive. While direct antigen assays on body fluids probably may not for some time be sufficiently sensitive for this application, it is desirable to pursue rapid improvements in immunoassay methods for detecting virally coded antigens. It may prove very useful to have a 'user-friendly' system which permits the establishment of a short-term culture of whole blood in the presence of a suitable lymphocyte stimulant, followed by a simple procedure to detect antigen or other viral product in the supernatant.

Finally, the safety of blood and blood products will only be assured by a combination of technical excellence and good management. I wish to conclude by suggesting that there is a continued need for national and international agreement about what confirmatory procedures are of most value, about which laboratories should do them, and about how the results should be interpreted. Additionally, there should be an effective system of national quality assessment of testing laboratories and of commercially produced test kits for screening and confirmation. Perhaps most important, individual laboratories should operate and report daily QA schemes which are correctly designed to test the overall performance of the whole testing facility. There must be properly established ways for the results of these activities to be reported, and to make sure that effective corrective action is taken when things are not going right.

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DISCUSSION

Dr Deinhardt

Regarding the plea of Dr McClelland, I wanted to mention that at the last meeting of the WHO collaborating centres on AIDS exactly the same plea for standardization of reagents, of sera and of methodology was made. In the meantime one standard reference serum containing antibodies against the major viral antigens was provided by the USA, and 10,000 ampoules of a reference serum have been prepared and lyophilized for the German Association against Viral Diseases by Dr Habermehl in the Federal Republic of Germany. In addition, a standard antigen for immunoblots as well as immunoblot strips has been prepared, and can be obtained for standardization in limited amounts. These reagents have been evaluated by more than 20 laboratories both in Europe and the USA. This is obviously only a small beginning, but you should know that these materials are already available.

Dr McClelland

May I just enquire about the contact and access to these materials. Would that be through yourself Professor Deinhardt?

Dr Deinhardt

Yes, or through Dr Assaad or Dr Petricciani at WHO.