Analysis of antigen presentation using particulate carrier systems

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ANALYSIS OF ANTIGEN PRESENTATION USING PARTICULATE CARRIER SYSTEMS

submitted by Joanne Clare Griffiths

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ABSTRACT

Particulate carrier systems have been developed to produce hybrid particles that present viral proteins in a multivalent form as potential recombinant vaccines. The presentation of epitopes from human immunodeficiency virus (HIV) and simian immunodeficiency virus (SIV) has been evaluated using two different carrier systems.

The first system exploits the self assembly properties of a protein (p1) encoded by the yeast retrotransposon Ty. Expression of p1-fusion proteins in yeast results in the formation of hybrid Ty virus-like particles (Ty-VLPs) containing multiple copies of the added antigen. The ability of hybrid Ty-VLPs carrying the major neutralizing epitope of HIV (the V3 loop) from different virus isolates to elicit production of virus-neutralizing antibodies has been investigated. High titre neutralizing antibodies were induced by Ty-VLPs carrying the V3 loop from the HXB2 clone of virus isolate IIIB following immunization in either Freund's adjuvant or aluminium hydroxide. Ty-VLPs carrying the V3 loop of isolate MN only induced high titre neutralizing antibodies following immunization with Freund's adjuvant. Hybrid Ty-VLPs containing an equivalent region from SIV were also investigated. However, this region was only weakly immunogenic and did not elicit high titre neutralizing antibodies, suggesting that the V3 loop of SIV is not the principal neutralizing determinant of SIV.

The Ty protein p1 is functionally analogous to the core proteins encoded by the gag gene of retroviruses such as HIV. The second carrier system evaluated therefore addressed the use of gag-encoded proteins to present the V3 loop. This system has the potential advantage of eliciting both anti-core and anti-envelope responses. Modification of the GAG protein was required to produce hybrid GAG:V3 particles in both yeast and insect cell expression systems. Immunogenicity studies of hybrid GAG:V3 particles demonstrated that although the humoral response to the V3 loop was weak, substantial anti-GAG antibodies were induced.

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CONTENTS

CHAPTER 1

INTRODUCTION

1.0 INTRODUCTION

This project forms part of the vaccine programme at British Bio-technology Limited. The main aim of this programme is to produce a candidate vaccine against the aetiologic agent of Acquired Immunodeficiency Syndrome, the human immunodeficiency virus (HIV). The introduction will discuss current vaccine technologies, the development of novel antigen presentation systems and the primary targets of this investigation, the human immunodeficiency virus and the simian immunodeficiency virus.

1.1 IMMUNE RESPONSES TO VIRAL INFECTION

The immune response to viral infection is extremely complex and the precise mechanisms that occur for successful recovery from infection with some viruses and not others are not yet fully understood. The function of the immune system is to eliminate free virus and to destroy virally infected cells. The immune response is mediated by recognition of antigenic sites or epitopes of viral proteins. B cell epitopes are recognised by antibody and T cell epitopes are only recognised by the appropriate T cell after antigen presentation. This section will provide a brief summary of the cell types that are involved in eliciting the immune response.

The immune response involves cooperation between the cell types of the immune system, outlined in Figure 1.1. The first cell type is the antigen presenting cell that processes the virus into components for recognition by the other cells. Antigen presenting cells include macrophages, dendritic cells and B cells. Lymphocyte activation is a key reaction in the immune response and both T and B lymphocytes respond to the binding of antigen by differentiating into active lymphoblasts and dividing into a clonal population, some cells of which provide immunological memory.

T lymphocytes include helper, cytotoxic, suppressor and delayed-type hypersensitivity

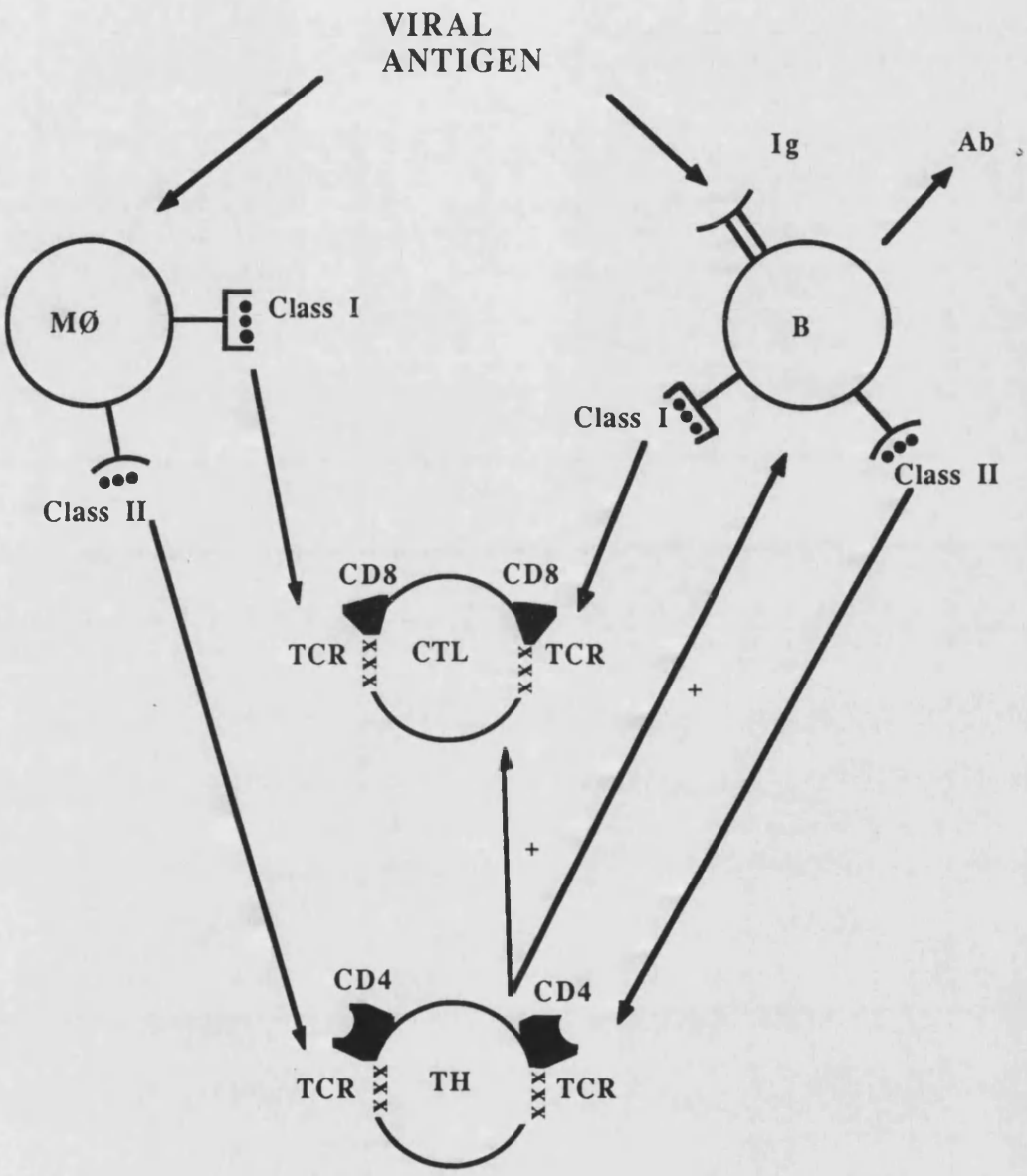


Figure 1.1 Schematic representation of the basic mechanisms of the immune response

MØ= macrophage; B= B cell; CTL= cytotoxic T cell; TH= T helper cell
TCR= T cell receptor; Ig= immunoglobulin; Ab= Antibody

cells. T cells can only recognise antigen when presented in conjunction with the major histocompatibility (MHC) self antigen. Antigen presentation by MHC is restricted by HLA type which results in differential recognition of antigens. The MHC in man consist of three loci (HLA-A, B and C) for class I antigens and three (HLA-DP, DQ and DR) for class II. T helper cells recognise antigen in association with class II MHC antigens that are present on macrophages and related cells. Recognition is mediated through the T cell receptor and CD4 molecules. T helper cells provide help for the activation of B cells and the generation of specific cytotoxic T cells. Cytotoxic T cells recognise antigen in association with class I MHC antigens that are present on most cells and in this way can recognise and destroy virally infected cells. Recognition is mediated through the T cell receptor and CD8 molecules. Peak cytotoxic T cell activity occurs about a week after viral infection and is therefore an early defence mechanism. Memory cytotoxic T cells are thought to exist for life.

B cells are responsible for antibody production. B cells bind antigen that contain antigenic determinants complementary to their receptors (antibody) and, after receiving the appropriate help from T cells, divide and differentiate into antibody secreting cells. Early in the response, in the presence of large amounts of viral antigen, B cells can be activated that produce low affinity antibodies due to the poor fit of receptor to antigen. Later in infection selection of B cells occurs that secrete high affinity antibodies and memory B cells are also generated. There are five different immunoglobulin classes of antibody. Multivalent IgM antibodies are produced early in infection and are later replaced by the major circulating IgG antibodies. IgA antibodies are important in establishing mucosal immunity. IgD antibodies are present on the surface of B cells and it is thought that together with IgM act as antigen receptors for lymphocyte activation and suppression. IgE antibodies cause an acute inflammatory reaction by recruitment of effector cells involved in protecting external mucosal surfaces. Antibodies act on the

virus via several different mechanisms. Antibodies that bind with high affinity to certain epitopes on the viral envelope inhibit infectivity and therefore neutralize the virus. These epitopes are known as neutralization epitopes. Activity of neutralizing antibodies may be enhanced by complement activation. Antibody-complement mechanisms may also be involved in the destruction of virally infected cells. Antibody dependent cell-mediated cytotoxicity (ADCC) is mediated by the antibody bound to a viral antigen on the surface of an infected cell. Effector cells such as killer cells and macrophages then target the infected cell.

Recovery from viral infection, induction of immunological memory and therefore rapid clearance of further infection are due to a combination of the responses outlined above. However the exact mechanisms that lead to a successful immune response are not known and are likely to vary from virus to virus and host to host.

1.2 VIRAL VACCINES

With one exception, successful viral vaccines to date that have been administered to humans are viruses themselves, presented either as live (attenuated or closely related non-pathogenic) or killed viruses. The majority of live attenuated viruses have been produced by passage of the virus in cell culture until an avirulent mutant emerges. These vaccines have the advantage of presenting the virus in the "natural" form to induce protective immunity but also have certain disadvantages which include possible reversion to virulence and the presence of contaminants introduced into the vaccine preparation from the cell culture. Killed virus vaccines are prepared by inactivating the virus using, for example, formalin. Such vaccines are generally considered safe as the virus is non-infectious, but have certain disadvantages including the need to be injected in large amounts and the requirement of boosting to generate an effective immune response.

Smallpox vaccine

Variola virus, the causative agent of smallpox, is the only virus that has been eradicated from the world's population by vaccination (reviewed by Fenner, 1982). Vaccinia virus was used for vaccination against smallpox and was introduced by Jenner (1798). The origin of vaccinia virus is unknown, although the first vaccine preparations were isolated from infected cows. Subsequently, vaccinia virus was isolated from calves or sheep, or grown in chick embyros. The vaccine programme to eradicate smallpox began in 1967 and the last case was reported in 1977 (Fenner, 1982).

Poliomyelitis vaccine

The first poliomyelitis vaccine was a formalin killed preparation of the three immunological subtypes, grown in cultures of monkey testicular or kidney tissue and was shown to induce levels of antibody comparable to the natural infection (Salk 1953). Sabin (1957) developed the live attenuated oral vaccine which consists of attenuated strains of the three subtypes. Originally the vaccine strains were prepared by culturing in monkey kidney cells and are now grown in human diploid cell cultures. As the vaccine strains infect, multiply and subsequently are excreted from the vaccinee, they are spread into the community giving the advantages of infecting and therefore protecting non-vaccinated hosts and also of replacing wild-type viruses. However extensive genetic changes have been shown to occur on replication in the vaccinee or contact host (Kew et al., 1981; Minor et al., 1986) and reversion to virulence sometimes occurs, particularly in the type 3 virus. Successful vaccination programmes have resulted in only rare cases of poliomyletitis being observed in developed countries (reviewed by Sabin (1985)).

Other live attenuated vaccines that are routinely administered include vaccines against measles, rubella and mumps (reviewed by Melnick (1986)).

Hepatitis B vaccine

The hepatitis B vaccine is the only currently available sub-unit vaccine. In an infected host the hepatitis B surface antigen (HBsAg) occurs as 22nm particles. The first vaccine preparations were HBsAg particles purified from the plasma of infected individuals and were found to induce protective immunity (Szmuness et al., 1980). HBsAg particles have now been produced as recombinant proteins in yeast (Valenzuela et al., 1982). These particles represent the first recombinant vaccine to be administered to humans.

Outlined above are examples of successful vaccine strategies currently in use. Due to the mechanisms of HIV infection and the long latency period it is unlikely that a vaccine could be developed using a live attenuated strain or killed virus preparation. Avirulence of attenuated vaccine strains are confirmed using animal models. No such model exists for HIV as although chimpanzees can be infected they do not develop disease symptoms. The possibilty of reversion to virulence also makes an attenuated strain unfeasible for an HIV vaccine. Killed virus preparations are also unlikely to meet safety requirements due to concerns about incomplete inactivation and about the ability of the "non-infectious" virus to still retain integration properties. Suitable HIV vaccine candidates are therefore likely to be recombinant sub-unit preparations. However, viral components that would induce protective immunity in man have yet to be defined. Analysis of the immune response in infected individuals progressing towards disease has focussed attention on the envelope and core proteins of the virus and presentation systems that express these proteins have been developed, which are included in the discussion in section 1.3.

1.3 PRESENTATION SYSTEMS

Several expression systems are now being exploited for the production of potential sub-unit vaccines. These include the production of monomeric proteins using a variety of expression technologies and systems which utilize viruses to express heterologous antigens either as part of a replicating recombinant virus, or as a purified antigen from infected cells. Antigen carrier systems have been developed where the antigen of interest is presented in a multivalent form as a particulate fusion protein. Examples of these expression systems are given below with particular reference to expression of HIV proteins.

1.3.1 Production of monomeric proteins in non-replicating systems

Recombinant HIV envelope proteins have been expressed in a variety of cell types. Envelope proteins produced in yeast elicited production of anti-envelope antibodies (Barr et al., 1987). Recombinant envelope glycoprotein gp120 produced in mammalian cells has been shown to elicit a protective immune response on HIV challenge in vaccinated chimpanzees (Berman et al., 1990).

1.3.2 Replicating expression systems

Polioviruses have been exploited as antigen carriers. The first poliovirus chimaera was made by inserting a defined region of type 3 poliovirus into the type 1 Sabin vaccine strain. This chimaera induced the production of type 3 neutralizing antibodies (Burke et al., 1988). Poliovirus/HIV chimaeras have also been produced. Evans et al. (1989) showed that a chimaera containing a sequence from the HIV envelope glycoprotein gp41 induced the production of broadly cross-neutralizing HIV antibodies. A poliovirus chimaera with a sequence inserted from the human papillomavirus type 16 major capsid protein induced the production of antibodies against the inserted sequence (Jenkins et al., 1991).

Recombinant vaccinia viruses have been constructed that express proteins from other viruses, in particular HIV proteins. Viruses that express the gp160 envelope glycoprotein have been made and have been shown to elicit an anti-gp160 response following infection of experimental animals (Chakrabarti et al., 1986; Hu et al., 1986). However, vaccinated chimpanzees were not protected when challenged with HIV (Hu et al., 1987). Clinical trials of recombinant viruses that express gp160 have been carried out and vaccinated individuals generated both humoral and cellular anti-gp160 responses (Zagury et al., 1988; Cooney et al., 1991). Recombinant vaccinia viruses have also been produced that express the GAG and GAG-POL precursor proteins of HIV. Karacostas et al. (1989) demonstrated that when mammalian cells were infected with a recombinant vaccinia virus that encoded the GAG-POL precursor protein, virus-like particles were produced that contained reverse transcriptase and processed GAG proteins. Particles have also been produced when the GAG precursor alone was expressed from a recombinant vaccinia virus carrying the gag gene of HIV (Shioda and Shibuta, 1990). Vaccina-GAG recombinant viruses have been shown to elicit anti-GAG humoral and cellular responses following infection of experimental animals (Hu et al., 1990). Coinfection of mammalian cells with recombinant viruses carrying gag and protease genes and the env gene of HIV resulted in the formation of particles containing GAG and envelope proteins (Haffar et al., 1990).

Recombinant adenoviruses have also been produced that express heterologous viral proteins. Infection of mammalian cells with a recombinant adenovirus expressing the hepatitis B surface antigen resulted in the formation of 22-nm particles (Davis et al., 1985). Protection from hepatitis B challenge has been demonstrated in a chimpanzee infected with this recombinant adenovirus (Lubeck et al., 1989). HIV proteins have also been expressed using recombinant adenoviruses. Dewar et al. (1989) demonstrated that when human cells were infected with a recombinant adenovirus expressing gp160,

this precursor protein was processed to the envelope glycoproteins gp120 and gp41. Animals infected with the recombinant adenovirus generated anti-envelope antibodies. Coinfection of cells with recombinant adenoviruses carring the gag and pol genes of HIV resulted in the formation of virus-like particles (Vernon et al., 1991). Macaques immunized with a recombinant adenovirus encoding the HIV capsid protein p24 generated anti-p24 antibodies (Prevec et al., 1991).

An expression system that exploits the Autographa californica nuclear polyhedrosis virus which infects insects has been developed. This baculovirus system will be discussed in detail in chapter 7. Recombinant baculoviruses that express HIV proteins have been produced. Gheysen et al. (1989) showed that infection of insect cells with a recombinant baculovirus carrying the gag gene of HIV resulted in formation of virus-like particles that budded from the cell surface. The envelope glycoprotein gp160 has also been expressed using this system (Wells and Compans, 1990).

A further live recombinant vaccine vehicle has been produced that expresses HIV proteins. This system utilizes the human tuberculosis vaccine Mycobacterium bovis bacillus Calmette-Guerin (BCG). BCG recombinants have been produced that express the gag, pol, and env gene products of HIV. Mice immunized with BCG-HIV GAG and BCG-HIV ENV generated low levels of anti-HIV antibodies and cell-mediated responses were demonstrated in the mice immunized with BCG-HIV GAG (Aldovini and Young, 1991).

1.3.3 Particulate antigen carrier systems

Both hepatitis B core and surface antigens have been used to produce hybrid particles carrying foreign proteins. Clarke et al. (1987) demonstrated that a fusion protein comprising the hepatitis B core protein and a peptide sequence from the foot and mouth

disease virus (FMDV), produced by expression using the vaccinia system, elicited strong anti-FMDV responses. Hybrid FMDV/hepatitis B core particles have also been produced in yeast (Beesley et al., 1990). Recombinant hepatitis B core particles have been constructed that carry gp41 of HIV and gp51 of bovine leukemia virus, although the immunogenicity of these hybrid particles was reported to be poor (Borisova et al., 1989). Hybrid hepatitis B surface particles containing a poliovirus neutralization epitope induced poliovirus neutralizing antibodies (Delpeyroux et al., 1982) and induction of HIV neutralizing antibodies by recombinant hepatitis B surface antigen particles carrying a fragment of gp120 has been demonstrated (Michel et al., 1988).

The self assembly properties of the tobacco mosiac virus coat protein (TMVCP) has also been exploited. A TMVCP fusion has been produced that carries an antigenic epitope from poliovirus type 3. The TMVCP-polio 3 fusion protein assembled into virus-like rods and induced the production of polio virus neutralizing antibodies when injected into animals (Haynes et al., 1986).

Another approach for the production of sub-unit vaccine candidates has been the production of immunostimulating complexes or ISCOMs (reviewed by Morein et al., 1987). ISCOMs are produced by the incorporation of the antigen of interest with the adjuvant Quil A into cage-like structures. ISCOMs have been shown to elicit protective immunity against several animal pathogens, for example, gp85 of feline leukemia virus in ISCOMs induces protection in cats (Osterhaus et al., 1985).

The antigen presentation system developed at British Bio-technology that exploits the self-assembly properties of p1, a protein encoded by the yeast retrotransposon Ty, is discussed in detail in section 1.4.

1.4 THE YEAST RETROTRANSPOSON, Ty

The term Ty stands for transposon of yeast. Ty elements were originally described by Cameron et al. (1979), and are a family of dispersed, repetitive DNA sequences present at about 35 copies per haploid genome of Saccharomyces cerevisiae.

Comparisons with retro-elements such as mammalian and avian retroviruses and the mobile repetitive copia-like elements of Drosophila have revealed notable similarities. These similarities include the way these elements move from one genomic location to another via an RNA intermediate and a reverse transcriptase reaction (Mellor et al., 1985a). As a result of this mode of transposition, mobile DNA elements, including Ty, have been termed retrotransposons.

Most Ty elements are approximately 5.9kb in length, which can be sub-divided into a unique 5.2kb internal region flanked by 340bp terminal repeats or delta sequences (Cameron et al., 1979). The major Ty RNA is a 5.7kb transcript that initiates in the left delta and terminates in the right delta such that the RNA has a 50 nucleotide terminal redundancy (Elder et al., 1983). This RNA is both the major message and the intermediate in Ty transposition via a reverse transcriptase reaction (Dobson et al., 1984) (Figure 1.2).

The transcriptional unit of Ty is divided into two overlapping reading frames, TYA and TYB, that are analogous to retroviral gag and pol genes, respectively. Overexpression of all or part of the transcriptional unit using high-efficiency yeast expression vectors has allowed the identification of several Ty-encoded proteins (Mellor et al., 1985b). TYA encodes a 50kD protein, p1, which is produced by simple translation from the mRNA. Protein p1 is subsequently processed via a Ty-encoded protease to a 45kD protein called p2 and minor products p4, p5 and p6 (Mellor et al., 1985a; Adams et al.,

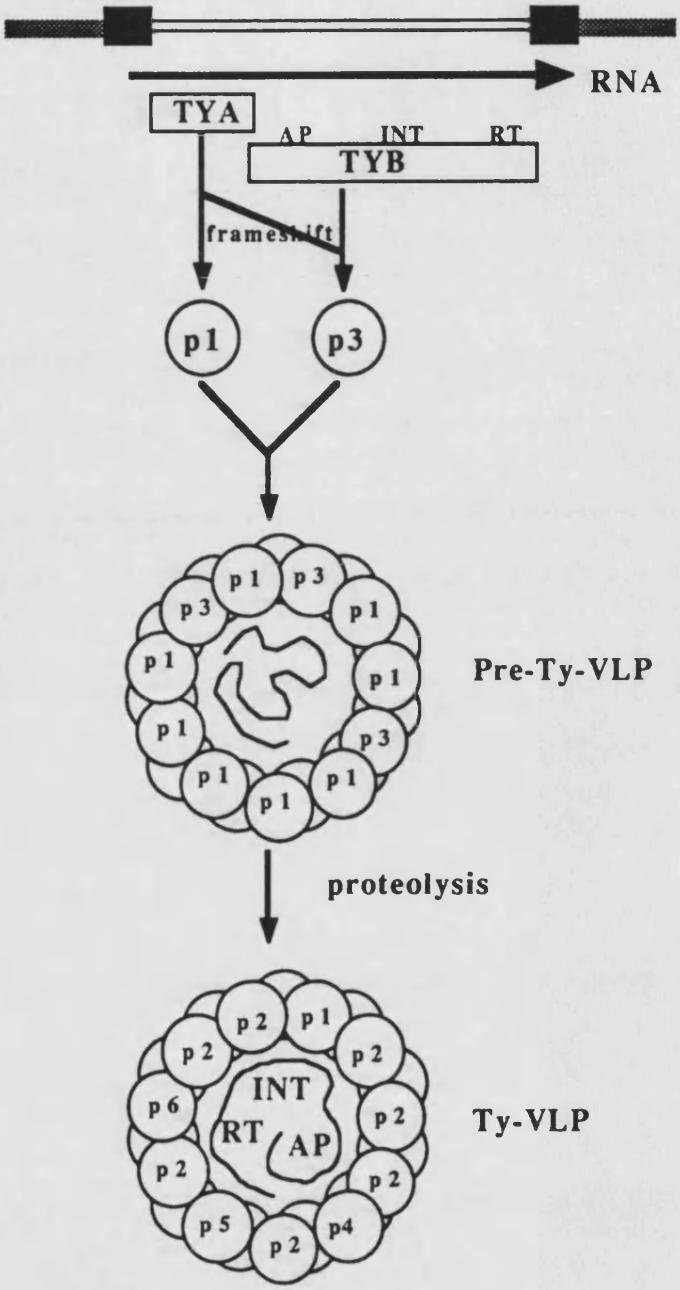


Figure 1.2 Genome organization of Ty and particle formation

AP= acid protease; RT= Reverse transcriptase; INT= Integrase

1987a). TYB is expressed from the mRNA as a 190kD TYA:TYB fusion protein, p3. This process requires a specific ribosomal frameshifting event in order to avoid the termination codon in TYA and shift translation into the +1 reading phase of TYB (Wilson et al., 1986). Protein p3 is also proteolytically processed to release the protease, reverse transcriptase and integrase enzymes (Figure 1.2) (Adams et al., 1987a).

The replicative transposition cycle of Ty elements involves the packaging of Ty RNA into virus-like particles (VLPs) where reverse transcription then generates a double stranded DNA copy (Figure 1.3). VLP assembly is thought to be a two stage process. First, p1 and p3 form an initial complex stabilized by both protein-protein interactions and protein-RNA interactions to produce a pre-Ty-VLP. Second, cleavage of p1 and p3 results in a morphological change and increased stability of the particle. Wild-type Ty-VLPs are approximately 50nm in diameter and when examined under the electron microscope can be seen to have an electron-dense core surrounded by an electron-luscent shell (Figure 1.4). The mature VLPs are composed mainly of the proteolytic cleavage product p2 but also contain reverse transcriptase, protease, integrase and a tRNA primer. It is therefore likely that Ty-VLPs contain all the components neccesary for replication of the element and subsequent integration of the dsDNA copy into the chromosome at a new site (Figure 1.3) (Mellor et al., 1985a; Adams et al., 1987a).

1.4.1 Formation of hybrid Ty-VLPs

The production of hybrid Ty-VLPs is based on the observation that the protein p1, the primary translation product of the TYA gene, can assemble into Ty-VLPs in the absence of proteolytic maturation (Adams et al., 1987a). This led to the suggestion that it may be possible to add other protein sequences to the TYA gene, yet retain the

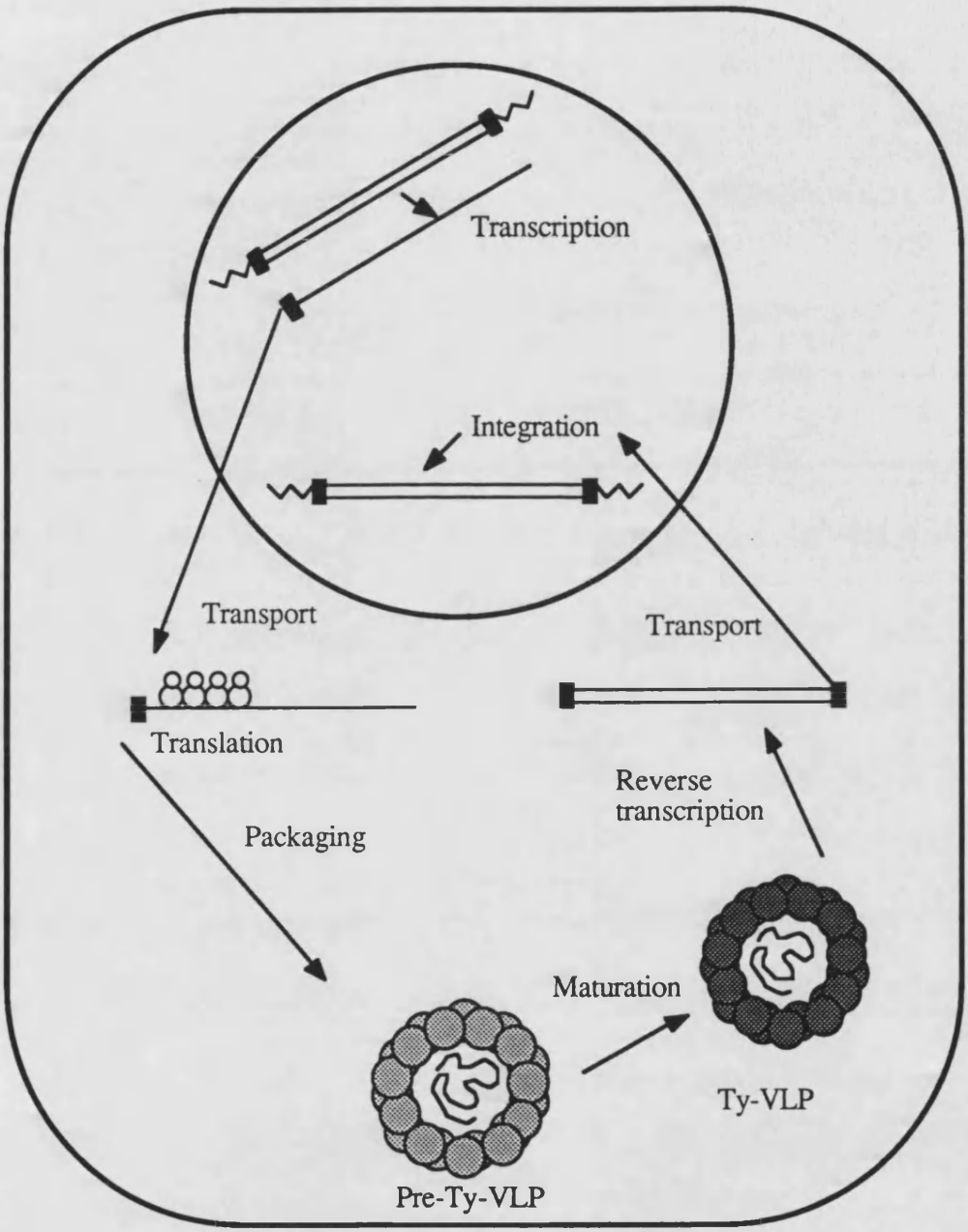


Figure 1.3 Transposition cycle of Ty

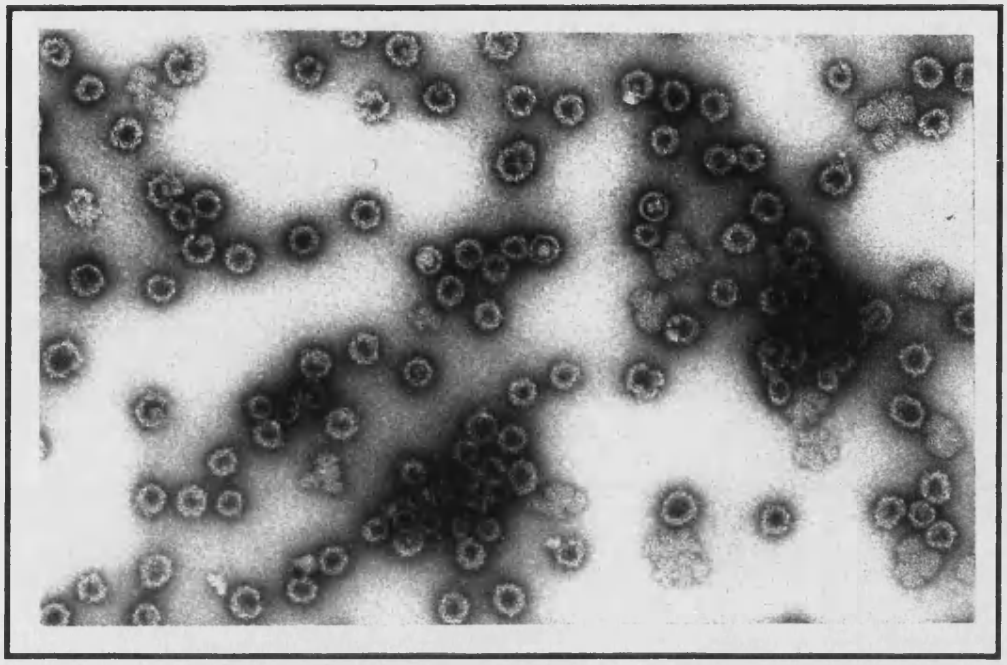
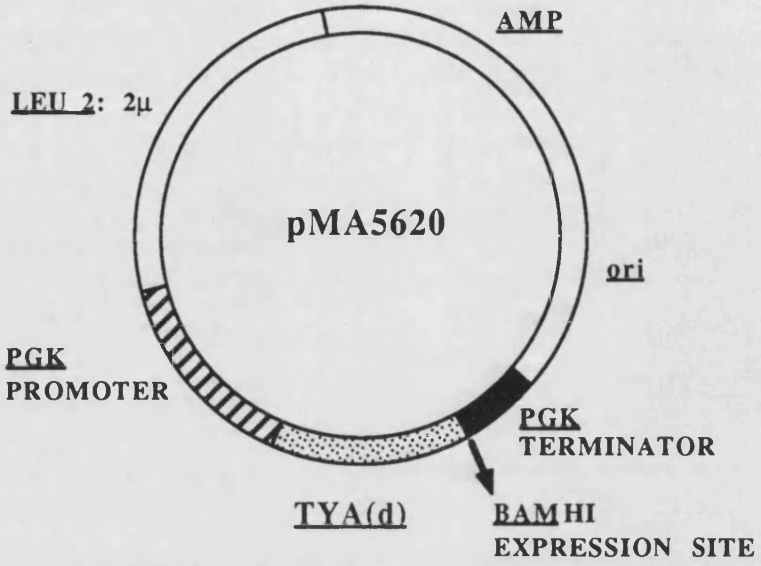


Figure 1.4 Transmission electron micrograph of "wild type" Ty-VLPs The particles were stained with uranyl acetate

particle forming properties of p1.

Specialized expression vectors have been designed in order to express Ty fusion proteins in yeast (Adams et al., 1987b). Plasmid pMA5620 is an E.coli/yeast shuttle vector which contains DNA origins of replication that allow replication in both cell types, plus an ampicillin gene for selection in E.coli and a LEU2 gene for selection in leucine auxotrophic yeast strains. The promoter of the yeast phosphoglycerate kinase gene (PGK) (Mellor et al., 1983) drives expression of the first 381 codons of the TYA gene (TYA(d)). At codon 381 is a BamHI restriction enzyme site which allows the insertion of antigen coding sequences of interest at the 3'end of the TYA(d) gene. This BamHI site is followed by translation termination codons and a transcription terminator (Figure 1.5).

Occasionally, constitutive expression of a particular p1-fusion protein from the PGK promoter causes the yeast cells to grow very slowly. In order to circumvent this problem an inducible system has been developed in which the PGK upstream activation sequence (UAS) has been replaced by the UAS of the GAL1-10 promoter to generate plasmid pOGS40 (Figure 1.5) (Kingsman et al., 1991). Yeast cells containing plasmids with p1-fusion genes under the control of this PGK-GAL hybrid promoter are grown to high cell density on media containing glucose. Expression is then induced by the addition of galactose to the media. Galactose inducibility is mediated by an alteration in the functional interaction between the yeast GAL4 and GAL80 proteins. However the utility of the galactose induction system is limited by the low levels of GAL4 protein present in yeast cells. Therefore pOGS40 derivatives are cotransformed into yeast cells with plasmid pUG4IS which contains the structural gene for GAL4. This plasmid contains the URA3 gene for selection in uracil auxotrophic yeast strains.



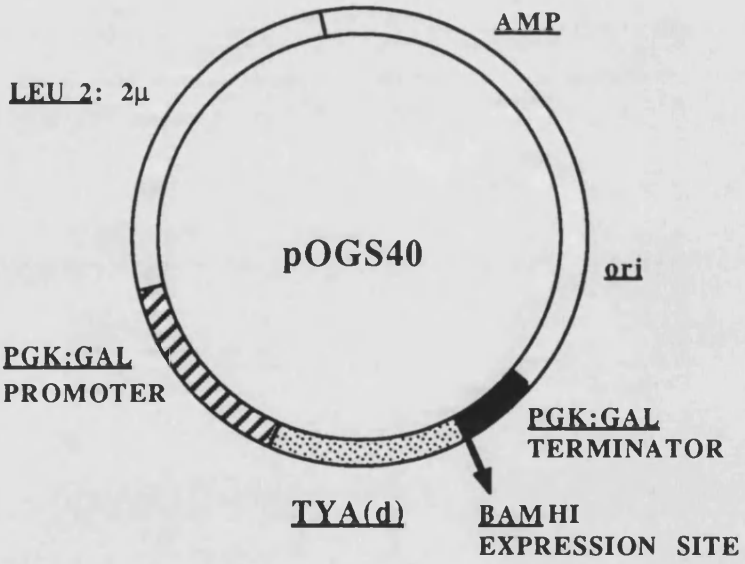


Figure 1.5 VLP expression vectors

Cotransformation of yeast cells in this way ensures that the galactose induction system works efficiently.

Expression of p1-fusion proteins in yeast results in the formation of hybrid Ty-VLPs (Adams et al., 1987b; Kingsman & Kingsman, 1988). Purified hybrid particles appear quite different to "wild type" particles. Figure 1.6 compares the particles produced when the TYA(d) gene alone is expressed with hybrid HIV:Ty particles which are discussed in detail in chapter 3.

1.5 HUMAN IMMUNODEFICIENCY VIRUS

The HIV retroviruses belong to the subfamily lentiviruses, so called because of the long incubation period between infection and overt disease. The human lentivirus HIV-1 was discovered in 1983 (Barre-Sinoussi et al., 1983; Gallo et al., 1983) and HIV-2 in 1986 (Clavel et al.,1986). Other examples of lentiviruses include simian immunodeficiency virus (SIV) (Daniel et al., 1984) and feline immunodeficiency virus (FIV) (Pederson et al., 1987).

HIV-1, HIV-2 and SIV are thought to have evolved from a common ancestral retrovirus showing tropism for cells expressing the CD4 receptor. HIV-1 and HIV-2 show 50-60% similarity in primary DNA sequence. HIV-2 is more closely related to SIV, with a sequence homology of about 70%. The molecular diversity of HIV-1 has been extensively studied and many molecular clones characterised. Multiple variants are often found in one patient, suggestive that biological variation exists in vivo (Fisher et al., 1988).

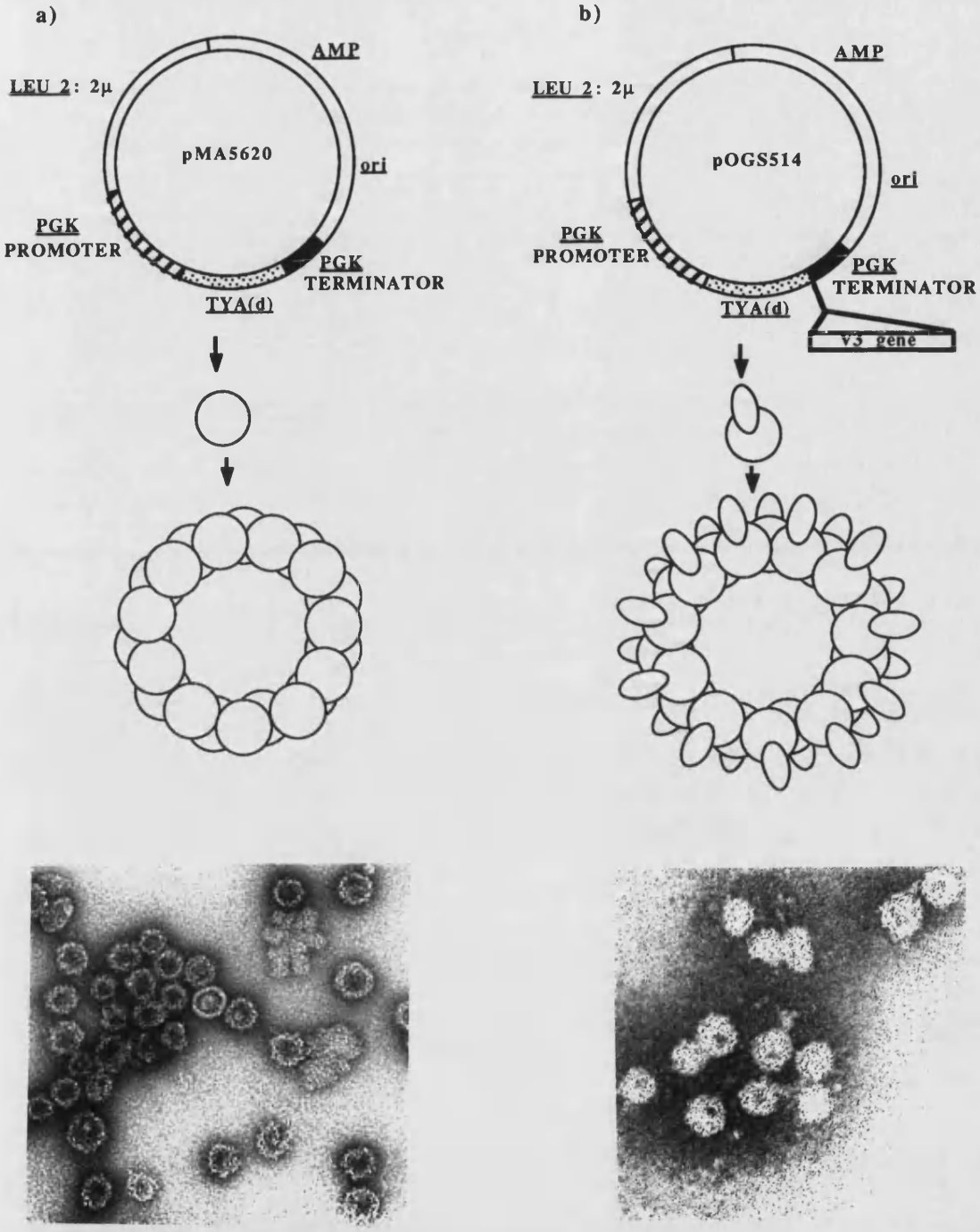


Figure 1.6 Construction and formation of Ty-VLPs. Panel (a) shows the plasmid pMA5620 above an electron micrograph of particles purified from yeast transformed with pMA5620. Panel (b) shows the construction of a TYA(d):HIV fusion gene (pOGS514) above an electron micrograph of hybrid HIV V3 loop:Ty-VLPs purified from yeast transformed with pOGS514.

1.5.1 HIV genetic organization

The HIV provirus is about 9.7 kilobases long with LTRs of 634 base pairs (Ratner et al., 1985) (Figure 1.7). The gag, pol, and env genes code for polyprotein precursors. Gag and env code for the structural proteins of the core and envelope proteins and pol encodes three enzymes, reverse transcriptase, integrase and protease, essential for viral replication. There are also additional open reading frames that code for the regulatory proteins (tat, rev, nef, vpr), and virion infectivity factor (vif) and vpu protein (vpu) that increase infectivity and facilitate export from the cell (reviewed in Haseltine, 1991) (Figure 1.7).

The gag gene

The gag gene encodes a polyprotein precursor, Pr55, which is cleaved by the viral protease into the core proteins p24, p17 and p15 (Figure 1.7). p15 is also cleaved to produce proteins p7 and p6. p7 is a nucleic acid binding protein and is thought to be responsible for condensing the viral RNA within the core (Delassus & Wain-Hobson, 1988). p24 is phosphorylated and is found within the core of the virion (Veronese et al. 1988). The p17 matrix protein is found just inside the viral envelope (Gelderblom et al. 1987) (Figure 1.8).

The pol gene

The pol gene is expressed as a GAG:POL fusion polyprotein (Pr160) via a ribosomal frame-shifting event in the gag-pol overlap region (Jacks et al., 1988; Wilson et al., 1988) (Figure 1.7). In mature virions the gag and gag:pol gene products are present in a ratio of about 20:1 (Veronese et al., 1986). The POL precursor is cleaved to produce protease, reverse transcriptase (RT) and endonuclease (or integrase) (Figure 1.7) (Varmus, 1988). The RT also possesses ribonuclease H activity (Varmus, 1988).

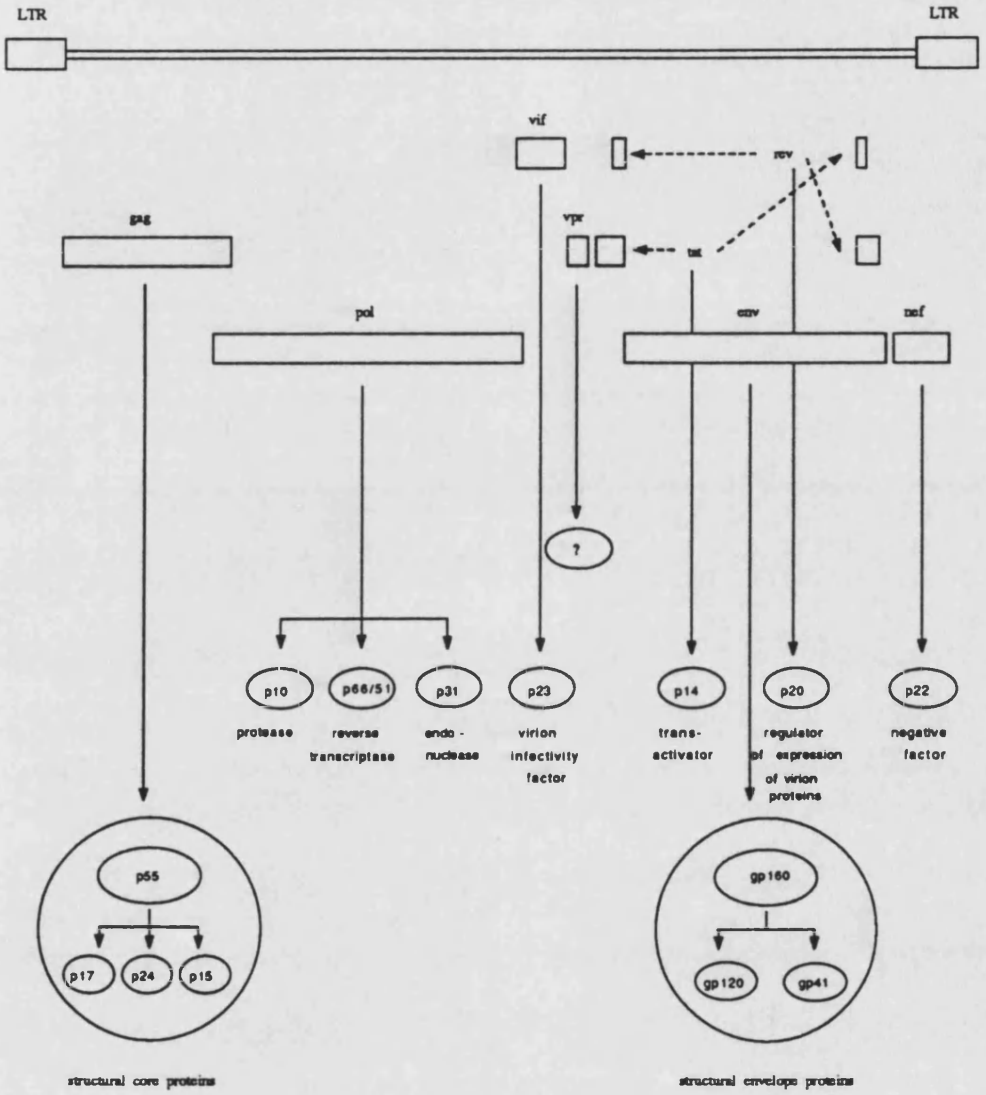


Figure 1.7 HIV genetic organisation and protein formation

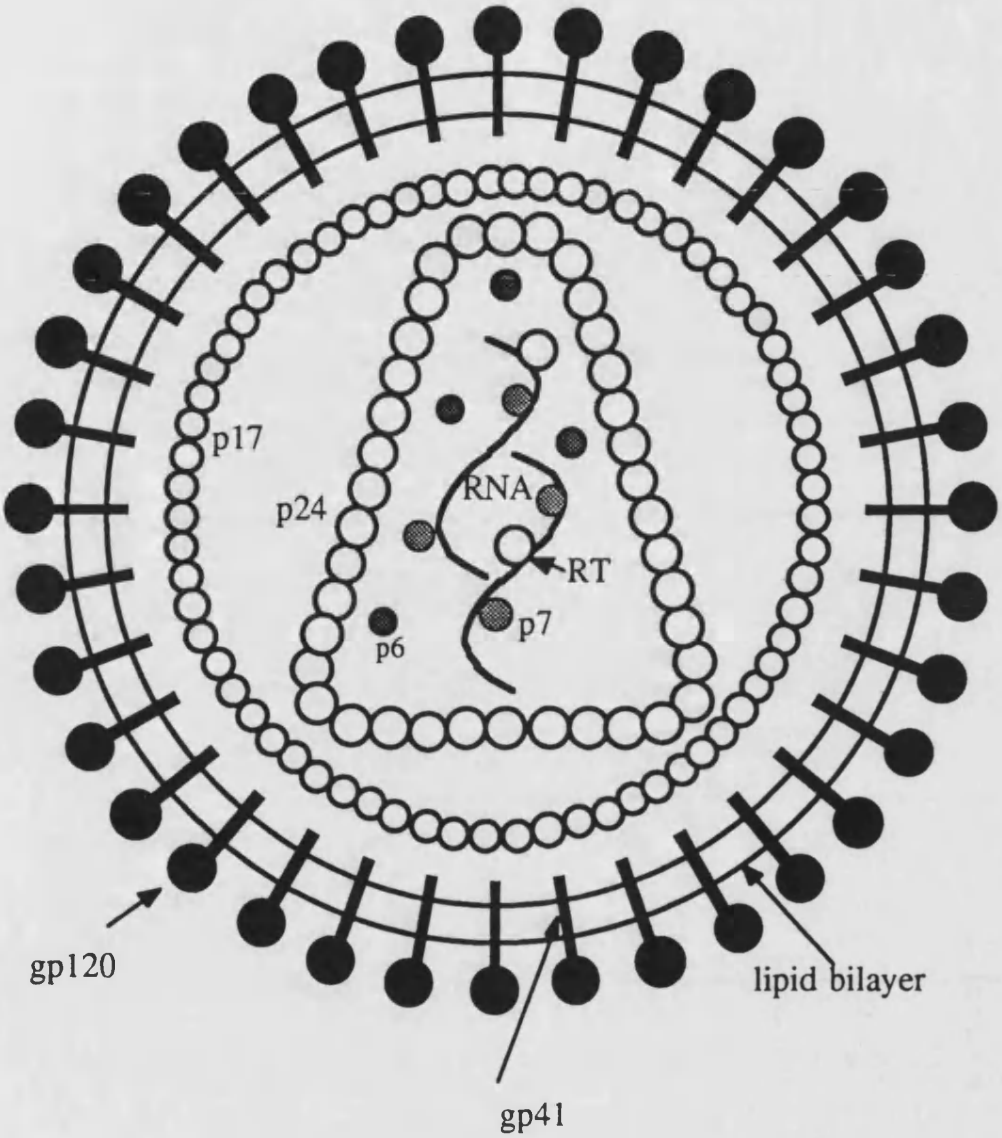


Figure 1.8 Schematic diagram of an HIV virion

The env gene

The polyprotein encoded by the env gene (gp160) is heavily glycosylated (Robey et al., 1985). gp160 is cleaved to produce the envelope surface protein gp120 and the transmembrane protein gp41 which remain attached via non-covalent interactions. gp120 and gp41 form spikes on the suface of the virion (Figure 1.8) (Gelderblom et al., 1987). Extensive genetic diversity exists between the envelope proteins of different isolates of HIV, particularly within hypervariable regions of the gp120 protein (Modrow et al., 1987).

1.5.2 HIV viral life cycle

The HIV viral life cycle is shown in Figure 1.9. The virus attaches to T helper lymphocytes (Dalgleish et al., 1984) and other CD4 positive cells, e.g., macrophages and dendritic cells, via the CD4 receptor. Entry occurs via fusion of cell and virus membranes and is mediated by the envelope transmembrane glycoprotein, gp41 (Stein et al., 1987). After entry into the cell the virus is uncoated in the cytoplasm. The RT produces hybrid RNA/DNA molecules followed by double-stranded linear DNA molecules which contain two copies of the long terminal repeat. This DNA is translocated into the nucleus and integrated into the viral genome, catalysed by the viral integrase. The HIV provirus may then remain latent for several years. Following cellular activation, by as yet unclear mechanisms, viral transactivators and host transcription factors lead to HIV gene expression (Nabel et al., 1987). Full length viral RNA directs the synthesis of the GAG and POL polypeptide precursors and the ENV precursor is made from singly spliced messenger RNA (Haseltine, 1991). These precursors are then assembled into viral particles together with two copies of single stranded genomic HIV RNA. The virion matures during budding and release from the host cell (Figure 1.9) (Varmus, 1988). Transmission electron microscopy demonstrates that the mature virion is approximately 100nm in diameter and has an elongated core

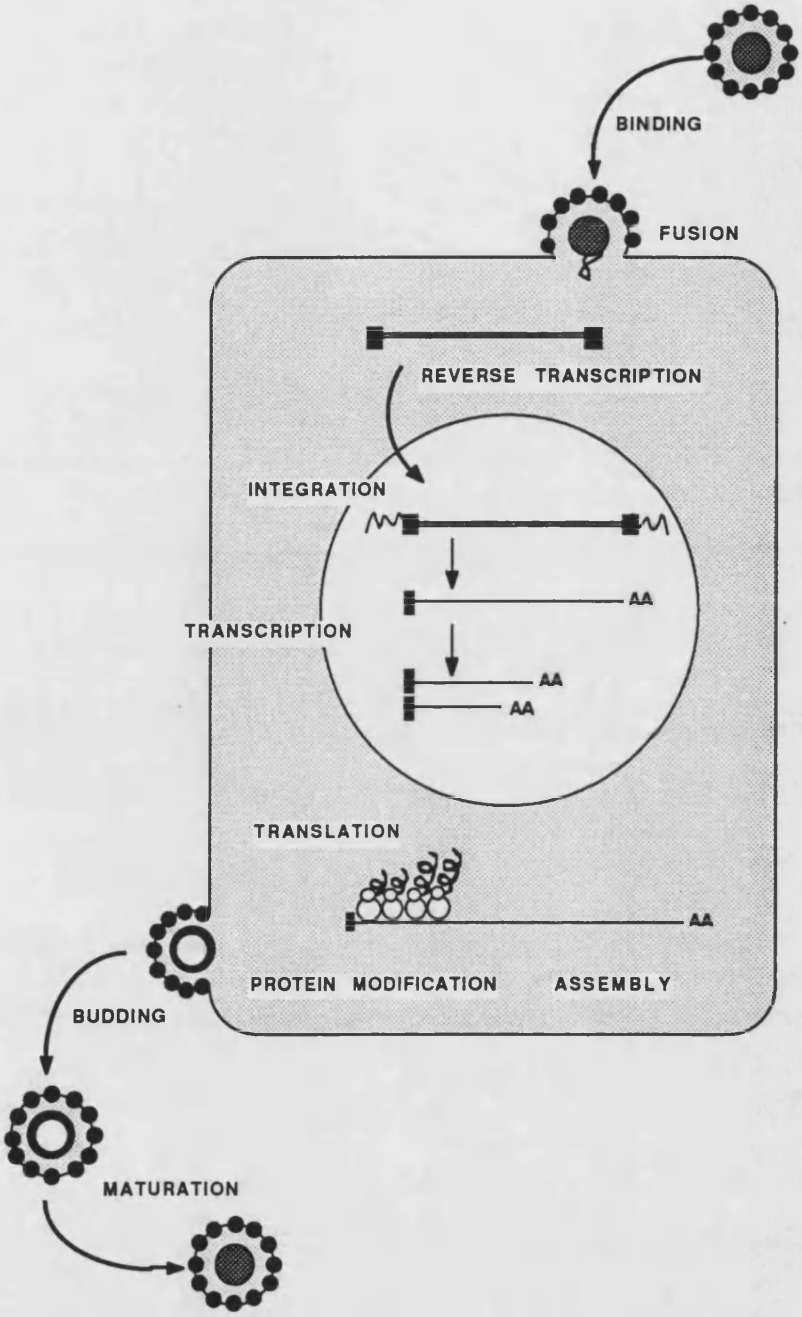


Figure 1.9 Infection cycle of HIV

(Figure 1.10; open arrow). Figure 1.10 (closed arrows) also shows virions budding from the cell surface; the particle seen on the left is at an early stage of budding and the particle on the right is nearer the stage of release.

1.5.3 Functional and immunogenic regions of the envelope glycoproteins

The precursor envelope glycoprotein, gp160, is proteolytically cleaved intracellularly to the gp120 and the gp41 transmembrane proteins (Willey et al., 1988). McCune et al. (1988) have shown that endoproteolytic cleavage is required for the production of infectious virions.

A number of functional and immunogenic regions of the envelope glycoproteins have been identified, which are outlined below. Computer assisted analysis of gp160 has identified conserved and variable regions (Modrow et al., 1987).

1.5.3.1 Functional regions

The functional domains include the region responsible for the binding of gp120 to the immunoglobulin-like domain of the CD4 receptor (Lasky et al., 1987). Using monoclonal antibodies that blocked the CD4-gp120 interaction, the binding region was found to be within amino acids 397-439. Further analysis has shown that changing amino acid residues, 257, 368, 370 and 457 resulted in a significant reduction of CD4 binding (Olshevsky et al., 1990). The CD4 binding region is, therefore, composed of several, discontinuous regions of gp120. Once the envelope glycoprotein is bound to CD4, the virus enters the cell by envelope fusion of the cell membrane. A hydrophobic amino-terminal domain is thought to provide at least one fusion site necessary for viral entry (Gallaher et al., 1987; Bosch et al., 1989). Fusion can also occur between virus infected cells expressing gp120 and gp41 and uninfected CD4 positive T cells (Lifson

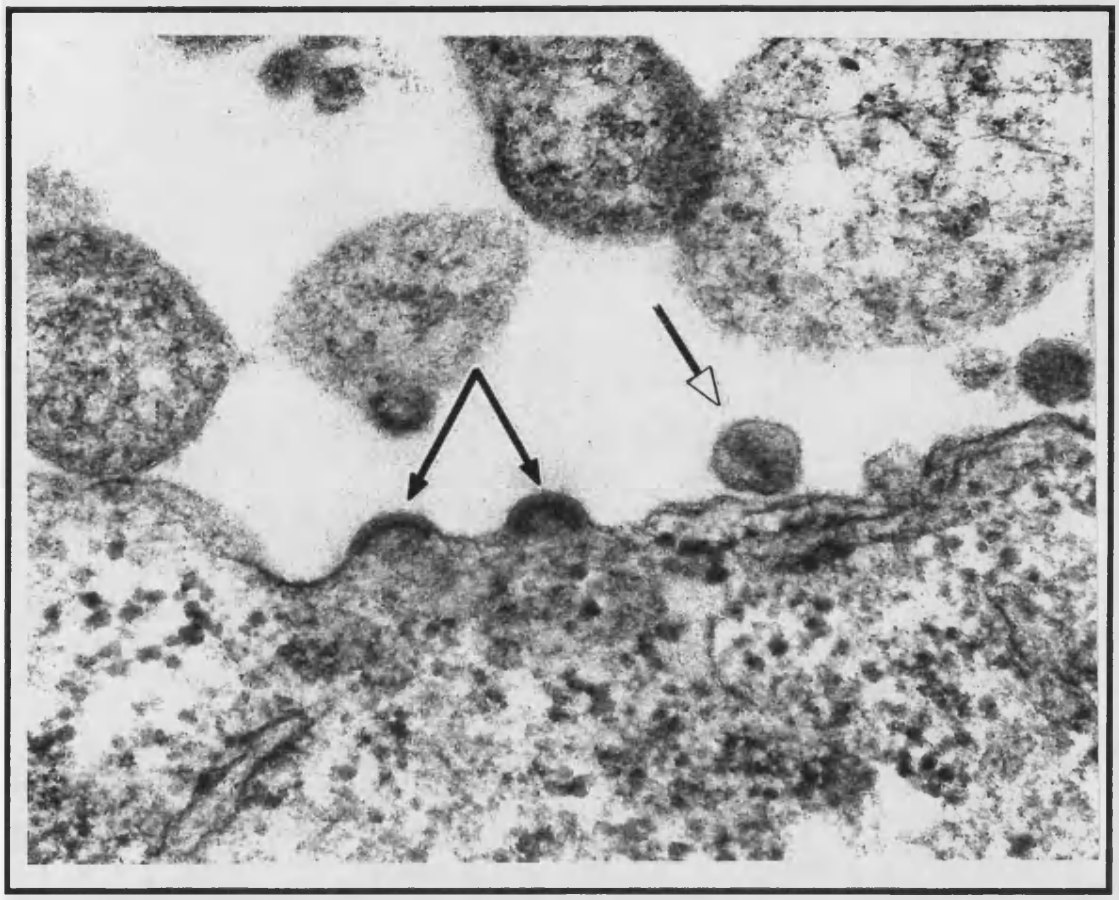


Figure 1.10 Transmission electron micrograph of an HIV infected cell

The closed arrow indicates a mature budded virion
The open arrows indicate budding particles

et al., 1986; Sodroski et al., 1986). This results in the formation of synyctia, i.e., multinucleated giant cells. This type of fusion can allow HIV to pass directly from cell to cell. Studies have shown that other regions of gp120 are also involved in the events following CD4 binding. Freed et al. (1990) demonstrated that the amino terminus of gp41 is involved in cell to cell fusion. Furthermore, mutational analysis has shown that susbstitutions of single amino acids 266, 267 and 268 led to non-infectious virions (Willey et al., 1988). The V3 loop, which is the principal neutralizing determinant of HIV (see section 1.5.3.2 below), is also thought to be an important functional region of the virus. The V3 loop has a similar sequence to a protease inhibitor, trypstatin (Hattori et al., 1989) and it has been shown that trypstatin inhibits synyctia formation in HIV-1 infected cell cultures. From this evidence, it has been speculated that, after CD4 binding, the V3 loop is proteolytically cleaved and that cleavage is essential for viral infection of the cell (Stephens et al., 1990). Clements et al. (1991) have shown that thrombin, tryptase and an endosomal aspartic protease, cathepsin E, cleave the V3 loop and further suggest that cleavage of the V3 loop occurs during the cell fusion reaction. Freed et al. (1991) have suggested that the V3 loop is a fusion domain.

1.5.3.2 B cell epitopes

B cell epitopes have been identified on both gp120 and gp41.

Neutralizing epitopes

The major neutralizing epitope of HIV is located in a hypervariable region of the envelope glycoprotein gp120 (the third variable domain, V3) found between amino acids 296-331 in HIV-1 isolate IIIB (Goudsmit et al, 1988; Palker et al, 1988; Rusche et al, 1988). The V3 region is a loop structure which is defined by two flanking cysteine residues linked by a disulphide bond (Leonard et al., 1990).

When recombinant gp160 preparations are used to immunize experimental animals the neutralizing antibodies are directed against this loop (Rusche et al, 1988). Antibodies to this domain neutralize the virus post-CD4 binding (Skinner et al, 1988a). Using synthetic peptides the neutralizing determinant of the V3 loop of HIV isolate IIIB has been mapped to the 8 amino acids found at the tip of the loop (Javaherian et al., 1989). It has subsequently been shown that neutralizing antibodies can be raised against hexamer peptides coding for a GPGRAF sequence at the tip of the loop (Javaherian et al., 1990). HIV human positive sera have been shown to have antibodies that bind to this epitope (Goudsmit et al., 1988; Goudsmit et al, 1989). Human monoclonal neutralizing antibodies to the V3 loop have been generated (Scott et al., 1990; Gorny et al., 1991), demonstrating that anti-V3 neutralizing antibodies are raised in a natural infection of HIV. In addition, studies of in utero transmission of HIV have shown that mothers who have high affinity/avidity antibodies to this region are less likely to transmit HIV to their children (Devash et al., 1990a) and decline of anti-V3 antibodies has been observed during the development of disease in HIV positive individuals (Neurath et al., 1990). Berman et al., 1990 showed that chimpanzees immunized with recombinant gp120 were protected from a challenge with the homologous strain of HIV-1. The protection was correlated with the induction of high titre neutralizing antibodies against the V3 loop. However, direct evidence that neutralizing antibodies raised against the V3 loop confer protection comes from the work of Emini et al. (1992) who showed that chimpanzees passively immunized with a human/mouse chimaeric V3 loop monoclonal antibody were protected from challenge with live HIV.

Neutralizing antibodies to this dominant loop are type-specific, i.e., they only neutralize the virus isolate against which they were raised. Therefore interest in designing sub-unit vaccines based on this epitope has led to serological grouping of HIV-1 strains. It has been shown that there is a prevalence of antibodies in infected individuals

which react with the MN isolate (Devash et al., 1990b) and antibodies that recognised the MN V3 loop have been detected in sera from Africa and the Americas (Carrow et al., 1991). Therefore the MN V3 loop epitope should be investigated as a sub-unit of a potential vaccine.

Neutralizing antibodies have been raised against other domains of gp120 including amino acids 254-274 (Ho et al., 1988). However, this region is naturally immunosilent on gp120 and, as this result could not be repeated, this epitope is no longer considered to be a neutralization epitope (Ronco et al., 1991). Kennedy et al. (1986) showed that antibodies raised against the region encoded by amino acids 503-532 had neutralizing activity. Conformation-dependent gp120 neutralizing antibodies have been identified in human sera, although these antibodies arise later in the course of infection and are of low titre. Steimer et al. (1991) demonstrated that conformation dependent neutralizing antibodies blocked CD4-gp120 binding. A broadly reactive human monoclonal antibody has also characterized a discontinuous epitope in gp120 as binding of this antibody was affected by changes in amino acids 256, 257, 360-370, 421 and 470-484 (Thali et al., 1991). Berkower et al. (1991) also identified human neutralizing antibodies that recognised a conformation-dependent epitope that mapped to residues 342-511 of gp120. Ho et al. (1991) generated monoclonal antibodies against gp120, one of which identified another discontinuous epitope and had the ablity to cross-neutralize viruses.

Another neutralizing domain is located in a conserved site, within gp41, between amino acids 735-752 (Chanh et al., 1986; Dalgleish et al., 1988). Evans et al (1989) showed that when this region was inserted as a surface component of poliovirus, broad cross neutralizing antibodies were elicited. The polio chimaera could also remove neutralizing antibodies from human HIV positive sera.

Human monoclonal antibodies that map to the N-terminal two-thirds of gp41 have enhanced the infection of HIV-1 in vitro (Robinson et al., 1990a). Further studies have mapped the regions on gp41 to which these antibodies bind; amino acids 586 to 620 (Robinson et al., 1990b) and amino acids 579 to 613 and 644 to 663 (Robinson et al., 1991). These studies therefore suggest that selected epitopes and not whole proteins should be incorporated into vaccine design.

Antibody mediated cellular cytoxicity (ADCC)

It has been shown that anti-gp120 antibodies present in human sera can mediate ADCC (Shepp et al., 1988; Lyerly et al., 1987). Antibodies that mediate ADCC appear to be broadly cross reactive between HIV isolates (Lyerly et al., 1988). It has been suggested that ADCC may inhibit HIV-1 infection in vivo (Sawyer et al., 1990) and reductions of ADCC titres correlating with disease progression have been reported (Ljunggren et al., 1987). A monoclonal antibody, raised to the V3 loop has been found to mediate ADCC (Broliden et al., 1990). A human monoclonal antibody has also been shown to recognise a conformational ADCC epitope on gp120 (Koup et al., 1991).

1.5.3.3 T cell epitopes

T helper cell epitopes

Several T helper cell epitopes have been identified on gp120. The CD4 binding region contains a sequence that is recognized in conjunction with MHC Class II antigens by CD4 positive T helper cells (Cease et al., 1987). In the same study a T helper epitope was also confirmed between amino acids 112-124. Using synthetic peptides that code for the V3 loop as immunogens, Palker et al. (1989) have demonstrated lymphoproliferative responses to this region. HIV envelope-specific helper T cell responses from infected individuals have also been reported (Schrier et al., 1989). Volunteers immunized with various recombinant gp160 preparations, for example a

baculovirus rgp160 (Tacket et al., 1990) and a recombinant vaccinia-gp160 (Berzofsky et al., 1988), have developed HIV-specific lymphoproliferative responses. T cell epitopes have also been identified on gp41 (Schrier et al., 1988; Ahearne et al., 1988). However, a region has also been identified on gp41 that is immunosuppressive to T cell stimulation (Ruegg et al., 1989).

Cytotoxic T lymphocytes (CTL)

Several CTL epitopes have been identified on the envelope glycoproteins. Clerici et al. (1991) demonstrated that in human HIV positive individuals four of the regions on gp120 recognised by T helper cells were also recognised by class I restricted CTL. These regions included the V3 loop. Takahasi et al. (1988) have demonstrated the recognition of the V3 loop by CD8 positive class I MHC restricted murine CTL. Takahasi et al. (1992) have also shown that a murine CTL line that recognised the MN V3 loop showed cross-reactivity between isolates.

1.5.3.4 Epitope mimicry

Molecular mimicry by viruses can stimulate the immune system to recognize self proteins as foreign antigens, thereby evoking attack on normal cells (Oldstone 1987). Several regions of gp120 and gp41 have been shown to have homology with proteins present on the surface of cells. These include homology to IL2 found in the C-terminus of gp41 (Reiher et al., 1986). Homology to the MHC class II domain HLA-DR within gp41 has been reported (Golding et al., 1988). These studies provide further evidence that whole envelope glycoproteins used as immunogens could have harmful effects in humans.

A number of B and T cell epitopes have therefore been identified on the envelope glycoproteins that could be considered for inclusion in a vaccine candidate. However,

from reports in the literature to date, the V3 loop represents an attractive candidate for inclusion into a potential vaccine as it is a "combined" neutralizing, T helper cell and CTL epitope.

1.5.4 Functional and immunogenic regions of the gag encoded proteins

1.5.4.1 Functional regions

The GAG precursor Pr55 of HIV is modified by proteolytic cleavage into the proteins p17, p24 and p15 (p7/p6) (Wellink and von Kammer, 1988). Cleavage is thought to occur post budding of the virus from the cell (Kohl et al., 1988). A further modification of Pr55 is the addition of a myristic acid moiety to the N-terminal glycine residue (Henderson et al., 1988). The targeting of other retroviral gag encoded precursors at the cell membrane has been shown to be dependent on myristilation (Schultz and Oroszkin, 1983). Studies on GAG precursor protein expressed using a recombinant baculovirus system show that myristilation is also essential for HIV GAG Pr55 to associate with the plasma cell membrane (Gheysen et al., 1989).

1.5.4.2 B-cell epitopes

Studies of the immune response in infected humans have shown that there is a strong B cell response to p24, the core protein of HIV (Casey et al., 1985; Samgadaharan et al., 1985). Correlation between the decline of anti-GAG antibodies and the onset of disease symptoms has been reported (Schubach et al., 1985; Lange et al., 1986; Weber et al., 1987). However, immunodominant B cell epitopes have not yet been specifically mapped on the gag encoded proteins. Anti-GAG monoclonal antibodies have defined antigenic determinants on p17, p24 and p15 (Niedrig et al., 1989; Marcus-Sekura et al., 1990).

1.5.4.3 T-cell epitopes

Examination of lymphoproliferative responses from HIV infected humans or immunized animals has identified several T helper cell epitopes on p24 (reviewed by Mills et al., 1989). In a study of HIV positive humans Schrier et al.(1989) demonstrated lymphocyte proliferative responses against synthetic peptides corresponding to five GAG sequences. These sequences coded for amino acids 22-29, 228-235, 282-301, 439-446 and 466-473. Using hybrid p24:Ty-VLPs as immunogens Mills et al. (1990) have defined two T helper cell epitopes, corresponding to amino acids 235-249 and 265-279. Epitope recognition was found to be MHC class II restricted by CD4 positive cells.

Cytotoxic T lymphocytes specific for GAG have been identified in HIV seropositive individuals. In all cases the CTL have been MHC class I restricted CD8 positive cells (Nixon et al., 1988; Walker et al., 1987; Riviere et al., 1989). Nixon et al., (1988) have demonstrated that HLA-B27 restricted CTL recognise a peptide corresponding to amino acids 265-279. Thus this region has been shown to be a combined T helper cell and CTL epitope. This region is highly conserved between HIV-1 isolates. CTL epitopes on GAG that are restricted by other HLA types have also been identified (reviewed in Nixon and McMichael, 1991).

Studies on the immune response to gag encoded proteins therefore suggest that these proteins should be investigated for inclusion in a vaccine candidate against HIV.

1.6 SIMIAN IMMUNODEFICIENCY VIRUS (SIV)

Candidate vaccines are normally evaluated in challenge experiments using animal models. However the only animals that can be infected by HIV are chimpanzees, baboons and gibbon apes and in experiments reported in the literature no disease

symptoms have been induced in these species following infection. These animals are in very limited supply and it is therefore not feasible to use them for screening vaccine candidates. However, simian immunodeficiency virus (SIV) has been shown to cause disease syndromes in macaques similar to human AIDS (Letvin et al., 1985). An alternative approach may therefore be to develop vaccine candidates against SIV that could be used as a model for the design of an HIV vaccine candidate.

SIV was first isolated from rhesus macaques in 1985 (Daniel et al., 1985). An isolate of SIV, SIVmac 142, has been cloned and sequenced (Chakrabarti et al., 1987) which showed that SIV has a genome organization similar to that of HIV-1 and HIV-2. The differences include a premature translation termination signal in env resulting in a truncated form of the transmembrane protein (Hirsch et al., 1987). SIVmac has morphology, growth properties and tropism for CD4 positive cells similar to those of HIV (Kannagi et al., 1985; Beneviste et al., 1986). Serologic cross reactivity against the gag proteins of HIV and SIV has been demonstrated (Beneviste et al., 1986), whereas cross reactivity of the envelope glycoproteins is weak. Immunodominant regions of SIV gag and env proteins have not yet been specifically mapped. In particular, the major neutralization epitope remains to be identified. However, monoclonal antibodies raised against the SIV envelope have been shown to have neutralizing activity and suggest that conformation-dependent epitopes exist on gp120 (Kent et al., 1991a). A monoclonal antibody that recognises amino acids 106 to 110 in the transmembrane protein has been shown to have strain-specific neutralizing activity (Kodama et al., 1991). Anti-envelope antibodies have been detected in experimentally infected macaques (Shafferman et al., 1989) and T cell proliferative responses reported (McGraw et al., 1990), although important epitopes have not yet been defined. A GAG CTL epitope corresponding to amino acids 171-195 has been identified (Yamamoto et al., 1990).

Using inactivated SIV as an immunogen, protection of macaques against SIV has been achieved (Desroisers et al, 1989; Murphey-Corb et al., 1989; Carlson et al., 1990). However, it is not yet clear which components of the whole virus preparation were responsible for the protection. Hu et al. (1992) demonstrated that protection of macaques against SIV infection can be achieved using a gp160 envelope based vaccine preparation. The animals were immunized with a recombinant vaccinia virus expressing gp160 and boosted with gp120 purified from insect cells. Following the boost the neutralization titres increased and the animals were protected from a subsequent challenge with homologous virus. However the epitopes on gp120 responsible for eliciting this protective response have not yet been mapped. There is therefore considerable interest in dissecting the protective response to identify key components that can be incorporated into a model to aid in the design of HIV vaccine candidates.

1.7 AIMS

The first phase of this project concentrated on the production of hybrid HIV:Ty-VLPs, and in particular VLPs that carry HIV envelope proteins. It has previously been reported that hybrid HIV:Ty-VLPs elicit an anti-HIV immune response (Adams et al., 1987b). This was shown by the detection of serum antibodies in animals immunized with the hybrid HIV:Ty-VLPs. However, it is generally believed that one of the requirements of a vaccine is that it should be able to induce the immune system to produce neutralizing antibodies. The initial aim of this project therefore was to investigate the presentation of the major neutralizing epitope of gp120, the V3 loop, by hybrid HIV:Ty-VLPs.

For a vaccine to induce protective immunity it is likely that both the humoral and cellular arms of the immune system need to be primed. The B cell population (the humoral arm) needs to contain effector cells that can produce virus neutralizing

antibodies and antibodies that mediate antibody-dependent cellular cytotoxicty (ADCC). A sub-population of memory B cells are also required so that there is rapid recall when an infection takes place. Induction of the cellular side of the immune system requires priming of T helper cells and cytotoxic T lymphocytes and the generation of memory T cells.

Although it has been shown that the V3 loop can stimulate lymphoproliferative responses in addition to inducing neutralizing antibodies (Palker et al., 1988; Takahasi et al., 1988), it is unlikely that the immune response against this epitope alone will confer protective immunity against HIV-1. As stated previously the V3 loop is hypervariable and to date the neutralizing antibodies generated against have been type specific. Many different isolates of HIV-1 have been identified and an HIV vaccine needs to be effective in generating immunity against all isolates. To generate this broad cross reactive response it is likely that in addition to the V3 loop, epitopes from other viral proteins need to be incorporated into the vaccine design.

A strong candidate for inclusion in an HIV vaccine are the core proteins of the virus encoded by the gag gene. As outlined above, HIV infected individuals generate a strong immune response to the core proteins of HIV. Furthermore, cellular immunity against other viral core proteins, for example the influenza A virus nucleoprotein, has been shown to play an important role in recovery from viral infections (Townsend et al., 1984; Yewdell et al., 1985; McMichael et al., 1986). It has also been demonstrated that in the case of hepatitis B virus, helper T cells specific for the hepatitis core antigens can "help" B cells to produce neutralizing antibodies against the envelope as well as against the core antigens (Milich et al., 1987).Taken together these observations suggest that incorporation of the gag encoded proteins of HIV into a combination vaccine may therefore be advantageous.

The major Ty structural protein p1 is functionally analogous to the core proteins of retroviruses such as HIV (Adams et al., 1987a). An attractive way forward for further development of a vaccine candidate against HIV was therefore to see if gag encoded proteins could be exploited as an antigen carrier system as an alternative to the Ty protein p1. The immune response generated against such a gag hybrid would then be directly relevant in terms of evoking immunity against HIV. The second phase of this project investigated the production of hybrid GAG:V3 VLPs. This GAG:V3 hybrid would combine the neutralizing epitope of gp120 with the T helper cell and CTL epitopes of GAG and therefore may be an attractive vaccine candidate against HIV.

CHAPTER 2

MATERIALS AND METHODS

2.0 INTRODUCTION

All the common reagents used in the methods described below were purchased from Sigma or BDH. The enzymes used in the molecular biology methods were purchased from Northumbria Biologicals Limited. To facilitate description, methods have been presented in a protocol format.

2.1 *Escherichia coli* STRAIN

HW87 = (*ara*D139, (*ara-leu*)del7697, (*lac*IPOZY)del74, *gal*U, *gal*K, *hsd*R, *rps*L, *srl*, *rec*A56).

2.2 YEAST STRAIN

Saccharomyces cerevisiae BJ2168: (*MATa*, *ura3*, *leu2-3,112*, *trp1*, *gal2*, *pep4-3*, *prc1-407*, *prb1-1122*).

2.3 *E. coli* MEDIUM

2 x YT medium

1.6% (w/v)	Tryptone, Difco
1%(w/v)	Yeast extract, Difco
0.25%(w/v)	NaCl
2 %(w/v)	Agar (solid medium)

Sterilize by autoclaving. Add 50mg/ml carbenicillin as required.

2.4 YEAST MEDIA

Transformed yeast cells are maintained in selective media. The yeast strain BJ2168 is

auxotrophic for leucine, uracil and tryptophan synthesis. Cells transformed with pMA5620 (LEU2) derivatives (described in chapter 1, section 1.4) are grown in the presence of uracil and tryptophan. Cells transformed with pUG41S (URA3) and pOGS40 (LEU2) derivatives are grown in the presence of tryptophan.

YEPD

2%(w/v)	Peptone, Difco
1% (w/v)	Yeast extract
2% (w/v)	Glucose

Sterilize by autoclaving.

Regeneration agar

1M	Sorbitol
1% (w/v)	Yeast extract
1% (w/v)	Glucose
0.67% (w/v)	Yeast Nitrogen Base (YNB)
3% (w/v)	Agar

Sterilize by autoclaving. Add filter sterilized solution of 1% uracil/ tryptophan or 1% tryptophan as appropriate to give a final concentration of 50mg/ml.

SC-glu

1% (w/v)	Glucose
0.67% (w/v)	YNB
2%(w/v)	Agar (solid medium)

Sterilize by autoclaving.

SC-glu/gal

0.3% (w/v)	Glucose
1% (w/v)	Galactose
0.67% (w/v)	YNB

Sterilize by autoclaving.

Add filter sterilized solution of 1% uracil/tryptophan or 1% tryptophan as appropriate to give a final concentration of 20mg/ml.

2.5 VECTOR PREPARATION

1) Incubate 10μg of vector DNA for 2 hours at 37°C with 2μl of appropriate enzyme, 10x Universal buffer and H_2O to 20μl.

2) Dephosphorylate by incubating DNA with 1μl of calf intestinal phosphatase in Universal buffer for 1 hour at 37°C.

3) Add an equal volume of a 1:1 phenol/chloroform mix, then vortex and microfuge for 5 minutes. Remove the upper layer containing the DNA into a new eppendorf tube.

4) Repeat step 3 twice.

5) Ethanol precipitate by adding 2.5 volumes of ethanol and 3M Na acetate to give a final concentration of 0.3M and then incubate at -70°C for 10 minutes.

6) Microfuge for 5 minutes, then remove the supernatant.

7) Add 200μl of 70% ethanol and vortex.

8) Repeat step 6.

9) Resuspend in 30μl of TE.

1 x TE

10mM Tris

1mM EDTA

pH to 7.4 with HCl

10x Universal Buffer

330mM	Tris
660mM	Potassium acetate
100mM	Magnesium acetate
40mM	Spermidine
5mM	Dithiothreitol

pH to 7.9 with HCl

2.6 OLIGONUCLEOTIDE CLONING

1) Dry down 10pmol of each strand of complementary DNA from aqueous solution.

2) Phosphorylate each strand separately at 37°C for 30 minutes by resuspending in 2µl 10 x kinase buffer, 1µl 15mM ATP, 1µl T4 polynucleotide kinase and 16µl H_2O.

3) Anneal the complementary strands by mixing 10µl of each and cool slowly from 90°C to room temperature.

4) Ligate a 3 to 5 fold molar excess of insert to the vector with 2µl ligase buffer, 1µl T4 DNA ligase, H_2O to 20µl at 16°C for four hours.

5) Transform 100µl of competent (calcium chloride treated) E.coli cells by incubating with half of the ligation mix on ice for 30 minutes. Heat shock the mixture at 42°C for 1 minute and then incubate in 1ml 2xYT broth for 60 minutes.

6) Pellet the cells by microfuging for 1 minute. Resuspend in 100µl 2xYT broth and spread onto 2xYT plates containing 50µg/ml carbenicillin. Incubate at 37°C overnight.

10x Kinase Buffer		**10x Ligase Buffer**	
500mM	Tris-HCl (pH7.4)	250mM	Tris-HCl (pH7.4)
100mM	$MgCl_2$	200mM	Dithiothreitol
50mM	Dithiothreitol	50mM	$MgCl_2$
1mM	Spermidine	10mM	ATP

2.7 PREPARATION OF E.coli COMPETENT CELLS

1) Inoculate 5mls 2xYT broth with a single E.coli colony. Grow, with shaking, at 37°C overnight.

2) Inoculate 50ml 2xYT broth with 0.5ml of the pre-culture and grow as above to exponential phase (approximately 1.5 hours). The cells are in exponential phase when an $O.D_{600}$ of 0.4 is reached.

3) Pellet the cells at 3 krpm for 5 minutes (Sorvall RT6000B centrifuge) and resuspend in 10mls ice cold 100mM calcium chloride.

4) Incubate on ice for 10 minutes.

5) Pellet the cells at 3 krpm for 5 minutes (Sorvall RT6000B centrifuge) and resuspend in 2ml 100mM calcium chloride.

6) Leave on ice for up to 24 hours.

2.8 ALKALINE LYSIS MINI-PREPARATION OF DNA

1) Inoculate 5ml 2xYT broth and incubate, with shaking, overnight at 37°C.

2) Pellet the cells by microfuging for 1 minute and resuspend in 100µl of Solution 1. Incubate at room temperature for 5 minutes.

3) Add 200µl of Solution 2, and incubate as above.

4) Add 150µl of Solution 3. Incubate on ice for 5 minutes.

5) Pellet the cell debris by microfuging for 2 minutes. Remove 400µl of the supernatant.

6) Add 400µl of a 1:1 phenol/chloroform mix. Vortex, then microfuge for 5 minutes. Remove the upper layer containing the DNA.

7) Add 1ml of ethanol and incubate at -70°C for 5 minutes.

8) Microfuge for 5 minutes, remove the supernatant and resuspend the pellet in 10µl TE.

Solution 1		Solution 2		Solution 3	
20mM	Glucose	200mM	NaOH	28%(v/v)	Acetic acid
25mM	Tris	35mM	SDS	2.3M	KOH
10mM	EDTA				
Lysozyme 2mg/ml					

2.9 LARGE-SCALE ALKALINE LYSIS DNA PREPARATION

1) Inoculate 500ml 2xYT broth with the appropriate E.coli colony. Incubate, with shaking, overnight at 37°C.

2) Harvest cells by centrifuging for 5 minutes at 6krpm (Sorvall RC5B centrifuge, Sorvall GSA rotor) and resuspend in 50ml of Solution 1. Incubate at room temperature for 5 minutes.

3) Add 100ml of Solution 2 and incubate as above.

4) Add 75ml of Solution 3 and incubate for 15 minutes on ice.

5) Pellet the cell debris by centrifuging for 5 minutes at 6krpm (Sorvall RC5B centrifuge, Sorvall GSA rotor) and filter the supernatant through glass wool.

6) Add 0.6 volumes of isopropanol and precipitate for 30 minutes on ice.

7) Pellet the DNA by centrifuging at 6krpm (Sorvall RC5B centrifuge, Sorvall GSA rotor) and resuspend in 5ml TE.

8) Add 1g/ml of caesium chloride (BRL) and 200µl of10mg/ml ethidium bromide.

9) Stand on ice for 15 minutes, then pellet debris by centrifuging at 9krpm (Sorvall SA600 rotor).

10) Fill "quick-seal" tubes (Beckman) with the DNA/CsCl/EtBr solution and seal.

11) Spin the gradients at 54Krpm overnight or 65Krpm for 4 hours (Beckman L8-70M ultracentrifuge, Beckman VTI65 rotor).

12) Withdraw the plasmid DNA band by first removing the top of the tube and then inserting a 21g needle attached to a 2ml syringe underneath the band. The

plasmid DNA band is the lower of the 2 bands in the tube.

13) Extract the ethidium bromide by adding 3 volumes of water-saturated butanol, vortex gently, and remove the upper butanol layer. Repeat until there is no colour remaining in the DNA solution.

14) Ethanol precipitate the DNA by adding 2 volumes of H_2O and 2.5 volumes of ethanol, and incubate at -70°C for 30 minutes.

15) Pellet the DNA by centifuging at 9 krpm for 20 minutes (Sorvall SA600 rotor).

16) Remove the supernatant, and add 10ml 70% ethanol. Vortex, and repeat step 15.

13) Resuspend in 200µl of TE.

14) Determine the DNA concentration by adding 5µl of the DNA solution to 1ml H_2O and then reading the OD_{260} of this solution. The $ODx10^4$ = µg/ml.

15) Adjust the DNA concentration to 1mg/ml.

2.10 PLASMID SEQUENCING

Plasmid sequencing is carried out as described by Biggin et al., (1983). The procedure is described briefly below:

1) Denature the DNA by incubating at room temperature for 5 minutes with 1M NaOH/1mM EDTA.

2) Centrifuge the DNA through a Sephacryl S3000 dialysis column at 3 krpm for 4 minutes (Sorvall RT6000B centrifuge).

3) Anneal the DNA to the primer, at 70°C for 2 minutes, followed by 37°C for 30 minutes.

4) For each sample carry out 4 reactions as outlined below in a microtitre plate:

	A	G	C	T
DNA	4µl	4µl	4µl	4µl
dNTPs	1µl	1µl	1µl	1µl
ddATP	1µl	-	-	-
ddGTP	-	1µl	-	-
ddCTP	-	-	1µl	-
ddTTP	-	-	-	1µl
Klenow enzyme	1µl	1µl	1µl	1µl
^{35}S-dATP (10mCi/µl)	0.5µl	0.5µl	0.5µl	0.5µl

Incubate at 42°C for 15 minutes.

The dNTPs (Pharmacia) are used at 0.5mM diluted in TE, and the ddNTPs (Pharmacia) are used at 10mM, made up in 50mM Tris, 1mM EDTA, pH7.5.

5) Incubate with chase solution (0.5mM all 4 dNTPs) at 42°C for 5 minutes.

6) Add formamide containing running dyes and denature at 80°C for 15 minutes.

7) Before loading pre-run the polyacrylamide gel at 45W for 15 minutes. Load 1.5µl of each sample and electrophorese at 45W for 1.5 hours.

8) Fix the gel in 10%methanol/10%acetic acid for 15 minutes, then remove the gel from the glass plate onto 2 sheets of Whatman 3MM paper. Cover the gel with cling film.

9) Vacuum dry, then remove the cling film and the bottom sheet of 3MM paper.

10) Expose the gel to autoradiograph film overnight and develop using a Kodak PP Xomat processor.

Formamide dyes

100ml	formamide
0.1g	Xylene Cyanol FF
0.1g	Bromophenol blue
2ml	0.5mM ETDA

The sequencing gel is poured using the following solutions. Take 50ml of the top gel solution and add 75µl of 25% ammonium persulphate and 75µl of TEMED. To 7ml of the bottom gel solution add 11µl of 25% ammonium persulphate and 11µl of TEMED. Six ml of the bottom gel is then poured between the gel plates, followed by 30ml of the top gel.

Top gel

147g	Urea
48ml	40% (w/v) Acrylamide
16ml	10 x TBE
H_2O to 320ml	

Bottom gel

20.7g	Urea
6.75ml	40% (w/v) Acrylamide
22.5ml	10 x TBE
4.5g	Sucrose
0.15ml	1% (w/v) Bromophenol blue

10xTBE

108g(w/v)	Tris
55g (w/v)	Boric acid
7.44g(w/v)	EDTA
H_2O to 1l	

Stock acrylamide: 38% (w/v) acrylamide plus 2% (w/v) bis-acrylamide.

Gel solutions are stored at 4°C.

2.11 PURIFICATION OF DNA FRAGMENTS

1) Digest 10µg of plasmid DNA with appropriate enzymes in Universal buffer for 2 hours at 37°C.
2) Electrophorese the fragments through the appropriate percentage low melting point agarose gel at 100V for 40 minutes.
3) Excise the fragment of interest.
4) Melt the gel slice in TE plus 5µg of tRNA.
5) Add 300µl of TE, vortex and then add 500µl of a 1:1 phenol/chloroform mix. Vortex and microfuge for 5 minutes.
6) Transfer the upper layer containing the DNA to a new tube and add 500µl of the phenol/chloroform mix. Vortex and microfuge for 5 minutes.
7) Repeat step 6.
8) Ethanol precipitate the DNA by adding 2.5 volumes of ethanol and 3M Na acetate to give a final concentration of 0.3M and incubate at -70°C for 10 minutes.
9) Pellet the DNA by microfuging for 5 minutes and then vortex in 500µl of 70% ethanol.
10) Pellet the DNA by microfuging for 5 minutes and resuspend in 20µl of TE.

Purified fragments are inserted into the vector DNA as described in section 2.6 (from step 4).

2.12 TRANSFORMATION OF S.cerevisiae BJ2168

1) Inoculate 100ml YEPD medium with 1 colony of the yeast strain BJ2168. Grow overnight, with shaking, at 30°C.
2) Harvest the cells at 1.5 x 10^7cells/ml. The cell count is determined using a haemocytometer.

3) Wash in 10ml of 1M sorbitol.

4) Sphaeroplast in 10ml 1M sorbitol plus 2% (v/v) glusulase (DuPont) at 30°C for 2 hours.

5) Wash the cells twice in 10ml of 1M sorbitol/10mM calcium chloride/10mM Tris (STC).

6) Resuspend the cells in 1ml of STC.

7) Add 2µg DNA to 100µl of competent yeast cells, and incubate at room temperature for 10 minutes.

8) Add 10 volumes of PEG 4000 and stand at room temperature for 10 minutes.

9) Microfuge the mixture for 40 seconds, remove the supernatant and resuspend the cells in 1ml of 1M sorbitol.

10) Add the transformed cells to 18ml regeneration agar (49°C) with the appropriate amino acids added (see section 2.4). Pour the agar into a petri dish.

11) Incubate the petri dishes at 30°C for 2-3 days.

12) When the colonies are sufficiently large, pick using a sterile inoculating loop and streak onto the appropriate selection plates (see section 2.4).

2.13 PURIFICATION OF Ty-VLPs

Unless otherwise stated all manipulations are carried out at 4°C.

1) Inoculate 50ml of SC-glu media containing the appropriate amino acids with a single colony yeast transformant. Grow, with shaking, overnight at 30°C.

2) Transfer to 1l of SC-glu media plus amino acids and incubate as above.

3) Transfer 125ml of this pre-culture to 2 x 1l of SC-glu medium plus amino acids and incubate as above.

4) Transfer 125ml of the culture in (3) to 16 x 1l of SC-glu medium if expression is driven from the PGK promoter or 16l SC-glu/gal medium if expression is driven from the PGK-GAL promoter, plus amino acids. Incubate overnight at

30°C with shaking.

5) Harvest the cells at >2x 10^7 cells/ml.

6) Wash the cells twice in H_2O and twice in TEN buffer. The cells are washed by resuspending in 200 mls and then centrifuging at 3krpm for 5 minutes (Sorvall RT6000B centrifuge). After the final wash resuspend the cells in 200mls of TEN buffer.

7) Homogenise the cells by bead beating with 6oz of glass beads (40 mesh) in the presence of protease inhibitors aprotonin, antipain, chymostatin, leupeptin and pepstatin each at 1.25µg/ml and phenylmethylsulfonyl flouride at 50µg/ml. The cells are homogenized using a bead beater (Biospec products) for 30 seconds on, alternating with 30 seconds off for a total of 10 minutes. The apparatus is then left to cool for 15 minutes, and the 10 minute homogenization procedure is repeated.

8) Pellet the cell debris at 3 krpm for 5 minutes (Sorvall RT6000B centrifuge).

9) Remove the supernatant and centrifuge at 9 krpm for 20 minutes (Sorvall RC5B centrifuge, Sorvall SA600 rotor).

10) Remove the supernatant and pipette 30 mls into each of 6 Ultra clear centrifuge tubes (Beckman, 25x89mm). Place 2mls of 60% sucrose/TEN solution into the bottom of each tube using a sterile pasteur pipette. Centrifuge the tubes at 28 krpm for 90 minutes (Beckman L8-70M ultracentrifuge, SW28 rotor).

11) Pipette off the supernatant and pool the sucrose cushions. Dialyse the sucrose cushion overnight against 1l of pre-cooled TEN buffer.

12) Centrifuge the dialysate at 9 krpm for 20 minutes (Sorvall SA600 rotor).

13) Load the supernatant onto a 5-20% (w/v) sucrose/TEN gradient with a 2ml 60% (w/v) sucrose cushion. The gradients are made in SW28 tubes by freezing 30ml 12.5% sucrose/TEN at -70°C, then thawing overnight at 4°C. The 60% sucrose/TEN cushions are added prior to the sample using a pasteur

pipette. Centrifuge the gradients for 6 hours at 25 krpm (SW28 rotor).

14) Repeat step 11.

15) Centrifuge the dialysate at 9 krpm for 20 minutes (Sorvall SA600 rotor).

16) Filter supernatant through a 3.5μm membrane (Millipore) and then through a 0.45μm membrane.

17) Concentrate the sample to 16mls using a Millipore CX-30 ultrafiltration unit attached to a Millipore CX Agitator.

18) Load the sample onto a 5 x 100cm Sephacryl S-1000 (Pharmacia) column that has previously been equilibrated in TEN buffer. Collect 30 minute fractions at a flow rate of 16ml/hr in TEN buffer (8ml per fraction).

19) Determine peak fractions by SDS-polyacrylamide gel (SDS-PAGE) analysis (see section 2.14). Pool the peak fractions and concentrate to 1mg/ml as described in step 17. Protein concentrations are determined by a Bradford dye-binding (BIORAD) assay according to the manufacturers instructions.

20) The particle preparation is then exchanged from TEN buffer to phosphate buffered saline (PBS) by dialysing overnight against 1l of pre-cooled PBS.

1 x TEN buffer		**1 x PBS buffer**	
10mM	Tris-HCl (pH7.4)	0.8% (w/v)	NaCl
2mM	EDTA	0.02% (w/v)	KCl
140mM	NaCl	0.115% (w/v)	Disodium hydrogen phosphate
		0.02% (w/v)	Potassium dihydrogen phosphate

Protease inhibitors

The protease inhibitors antipain, chymostatin, leupeptin, and pepstatin are made up to 1.25μg/ml in dimethylsulphoxide and aprotinin is made up to 1.25μg/ml in H_2O. Aliquots (50μl) are stored at -20°C and 1 aliquot per VLP preparation is used. Phenylmethylsulfonyl fluoride is made up fresh to 50μg/ml in ethanol and 1ml per preparation is used. All the protease inhibitors are purchased from Sigma.

2.14 SDS-PAGE

A 0.25x volume of 5x denaturing buffer is added to the sample and the mixture is boiled for 2 minutes. SDS-PAGE gels are made using the following solutions. For 2 gels take 10ml of resolving gel and add 100μl of 10% Ammonium persulphate and 15μl TEMED. Pour this solution between the gel plates leaving approximately 3cm for the stacking gel. Take 5ml of stacking gel and add 50μl of 10% Ammonium persulphate and 15μl TEMED. The stacking gel is poured after the resolving gel has set.

Resolving gel (10%)		**Stacking gel (5%)**	
10ml	30% (w/v) Acrylamide	1.67ml	30% (w/v) Acrylamide
8ml	1% (w/v) Bis-acrylamide	1.3 ml	1% (w/v) Bis-acrylamide
11.2ml	1M Tris-HCl pH8.7	1.25ml	1M Tris-HCl pH6.8
150μl	20% (w/v) SDS	50μl	20% (w/v) SDS
400μl	H_2O	5.7ml	H_2O

Gel solutions are stored at 4°C.

Running buffer		Denaturing buffer x 5	
190mM	Glycine	3.85% (w/v)	Dithiothreitol
100mM	Tris	5% (w/v)	SDS
3mM	SDS	80mM	Tris-HCl pH6.8
		25% (v/v)	Glycerol
		0.3% (w/v)	Bromophenol blue

The denatured samples are loaded onto the gel and electrophoresed at 200V for 45 minutes using a BIORAD mini-gel apparatus.

2.15 WESTERN BLOTTING

1) Electrophorese the samples through a 10% SDS/PAGE gel at 200V for 45 minutes.
2) The proteins are transferred to nitrocellulose (0.45µm, Anderman) using a Biorad Mini Trans-Blot Electrophoretic Transfer Cell. The transfer apparatus is assembled as a "sandwich" as follows: sponge-2pieces of 3MM paper (Whatman)-gel-nitrocellulose-2x3MM paper-sponge. The proteins are transferred at 100V for 1 hour.
3) Remove the nitrocellulose filter from the transfer apparatus and incubate in blocking buffer for 1 hour at room temperature.
4) Incubate the filter in the primary antibody (diluted 1/100-1/1000 in blocking buffer) for 30 minutes, at room temperature with agitation.
5) Wash the filter 3 times (2 minutes per wash) in blocking buffer.
6) Incubate the filter in horseradish peroxidase conjugated anti-species antibody (Sigma), diluted1/1000 in blocking buffer, as above.
7) Wash the filter 3 times (2 minutes per wash) in blocking buffer.
8) Develop the filter by immersing in 25ml of developing solution for 1 minute.
9) Wash the filter in H_2O to stop the reaction.

Transfer Buffer		Blocking buffer	
190mM	Glycine	0.5% (w/v)	Casein
25mM	Tris	150mM	NaCl
20%(v/v)	Methanol	0.05% (v/v)	Triton-X-100
		20mM	Tris pH 7.4

Developing Solution

25ml	50mM phosphate buffer pH 7.4
400µl	2.5% (w/v) 3'3 Diaminobenzidine tetrahydrochloride in PBS
1ml	1% (w/v) Cobalt chloride
1ml	1% (w/v) Ammonium nickel sulphate
50µl	Hydrogen peroxide

2.16 IMMUNOGOLD ELECTRON MICROSCOPY

1) Load 10µl of sample (at 0.2mg/ml) onto a formavar-coated electron microscope grid.
2) Leave for 30 seconds and then draw off liquid with filter paper. If the sample is not to be immunogold labelled, it can be stained at this point with water saturated uranyl acetate (UA).
3) To block the grid load 10µl of 5% normal goat serum (in TBS/BSA) and incubate for 1 hour at room temperature.
4) Remove the blocking buffer. Load 10µl of primary antibody (diluted 1/100 in blocking buffer) onto the grid and incubate at room temperature for 2 hours.
5) Wash the grid 5x with 10µl 1% normal goat serum (in TBS/BSA).
6) Load 10µl of secondary antibody (anti-species gold-labelled IgG (Sigma); diluted 1/160 in bocking buffer) onto the grid and incubate for 1 hour at room

temperature.

7) Wash the grid 5x with 10µl of H_2O. Dry the grid by removing the excess liquid with filter paper, then leaving to air dry for 5 minutes.

8) Stain with 10µl saturated UA for 30 seconds, dry the grid as above and examine in a transmission electron microscope.

TBS/BSA buffer

50mM	Tris, pH7.4
0.9%(w/v)	NaCl
0.1%(w/v)	Bovine serum albumin
20mM	Sodium azide

2.17 FIXED CELL IMMUNOFLUORESCENCE

1) Wash the cell suspension 3x using PBS.

2) Resuspend the cells at a concentration of 1 to 1.5 $x10^6$ cells/ml in PBS.

3) To each well of a 12 well teflon coated slide add 10µl of cell suspension, ensuring complete coverage of the well and leave to air dry.

4) Place the slides in 1:1 (v/v) acetone/methanol and leave at room temperature for 20 minutes, then air dry.

5) Add 15µl of each antibody dilution (diluted in PBS) to the appropriate well.

6) Place the slides in a sandwich box containing a moist tissue and incubate at 37°C for 30 minutes.

7) Wash the slides twice in PBS and once in distilled H_2O.

8) Add 15µl per well of FITC conjugate (Sigma) diluted in PBS containing 2 drops 0.25% Evans blue per 2ml volume.

9) Repeat step 6.

10) Wash the slides twice in PBS then twice in distilled H_2O.

11) Mount using 1 drop per well of mounting fluid, overlay with a cover slip and seal the edges of the coverslip with nail varnish.

12) Examine the slides using a Nikon Labophot microscope fitted with a UV light source and a 495nm filter.

Mounting fluid

0.42% (w/v)	Glycine
0.012% (w/v)	NaOH
0.51% (w/v)	NaCl
0.03% (w/v)	NaN_3
70% (v/v)	Glycerol

2.18 ELISA (see Figure 2.1)

1) Sensitise plates (NUNC Immunoplate II) with antigen at a pre-determined concentration (usually 5µg/ml) by incubating overnight at 4°C with 50µl/well, in 50mM sodium carbonate buffer, pH 9.5.

2) Wash the wells twice with PBS and block at 4°C with 100µl PBS-casein (2%) for 1 hour.

3) Wash the wells twice with PBS-Tween (1%).

4) Dilute primary antibody in PBS-casein (0.5%)-Tween (0.1%) and add 50µl to each well.

5) Incubate at 4°C for 2 hours.

6) Wash wells 3x with PBS-Tween.

7) Add 50µl/well horseradish peroxidase labelled secondary antibody (Sigma) at 1/1000 in PBS-casein (0.5%)-Tween (0.1%).

8) Incubate as above.

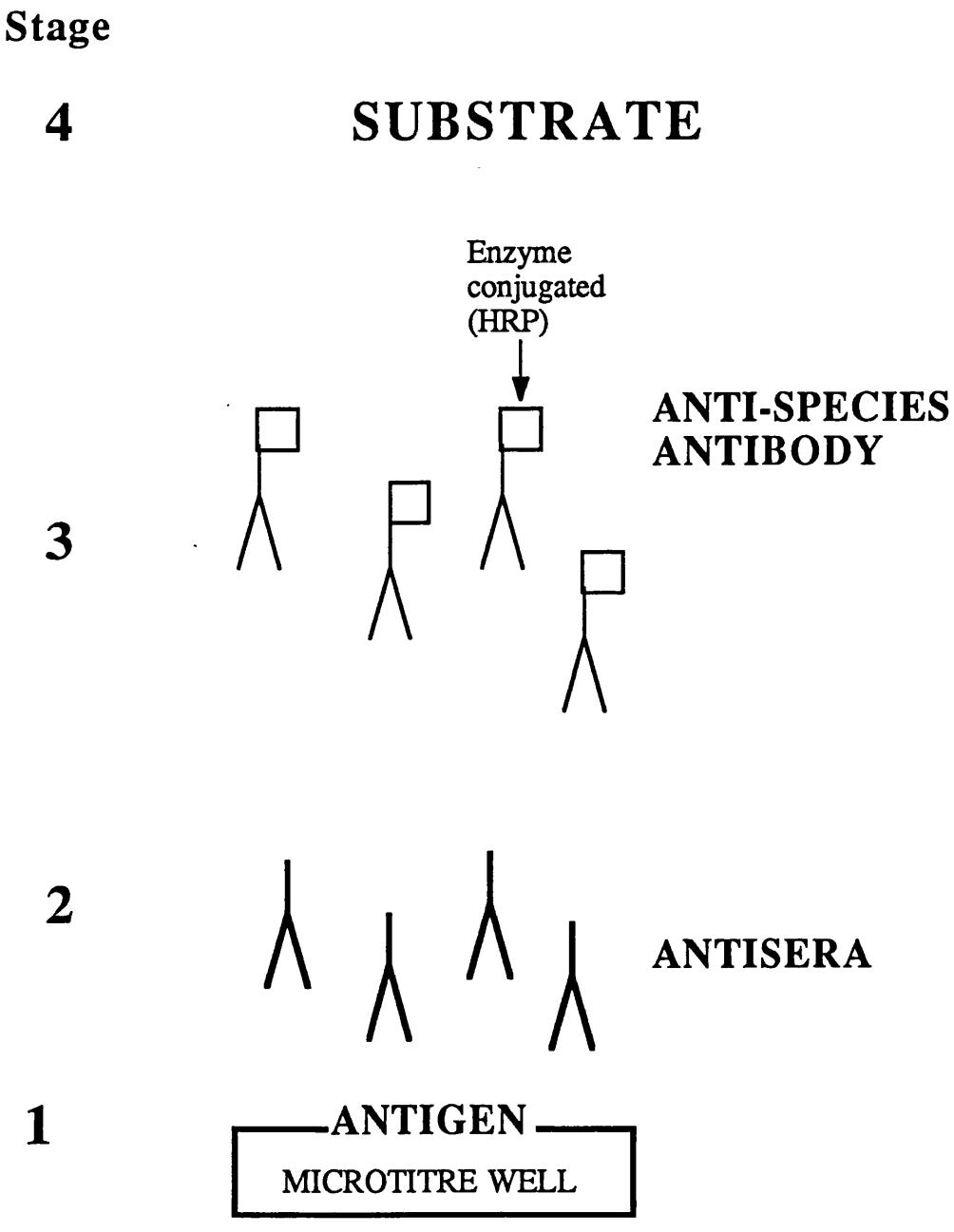


Figure 2.1 Schematic diagram of an ELISA assay

9) Wash the wells 5x with PBS-Tween.

10) Add 50µl/well enzyme substrate, 0.01% 3,3,,5,5,-tetramethylbenzidine plus 0.03% hydrogen peroxide in Na acetate, pH6.0

11) After 5 minutes stop the reaction with 25µl/well 2.5M H_2SO_4.

12) Read the O.D. at 450 nm.

The methods described in sections 2.19 to 2.22 are carried out in a Category III containment facility and all manipulations are carried out in the appropriate microbiological safety cabinet. Due to the extensive training required to work on live HIV, the preparation of HIV virus stocks and some of the HIV neutralization assays were carried out by Dr. Eleanor Berrie and Mrs Linda Holdsworth.

2.19 MAMMALIAN CELL CULTURE

The cells maintained in culture for HIV virus preparation and infection inhibition assays are H9 cells and C8166 cells. H9 cells were cloned from a T cell line derived from an adult with lymphoid leukaemia that were selected for their high growth rate and virus production (Popovic et al., 1984). These cells are used for the production of virus stocks. The C8166 T cell line was cloned from a line derived by fusion of cord blood cells with an HTLV-I producing line from an adult T-cell leukemia-lymphoma patient (Salahuddin et al., 1983). These cells are used in infection inhibition assays as a rapid cytopathic effect is seen following infection with HIV. The cells are cultured in RPMI 1640 medium (Sigma) supplemented with 10%(v/v) foetal calf serum (FCS), penicillin/streptomycin 100 IU/ml, 2mM glutamine (growth medium).

2.19.1 Storage of mammalian cells

Stocks of cells are stored under the gaseous phase of liquid nitrogen (-100°C) and are frozen using the following procedure:

1) Prepare a cell suspension of $1x10^6$ cells/ml in ice cold growth medium.
2) Place the vial containing the cell suspension in melting ice and add dropwise an equal volume of ice cold medium containing 20% FCS/20% dimethylsulphoxide (DMSO).
3) Dispense 1ml volumes into pre-cooled cryotubes and freeze overnight in the top tray of the liquid nitrogen storage vessel.
4) Store the cryotubes in the gaseous phase of the nitrogen.

2.19.2 Cell culture propagation

The cells are revived using the following procedure:

1) Thaw the cells quickly by immersion of the vial in pre-warmed water (37°C).
2) Slowly add a small volume of pre-warmed medium (37°C) to the cells. Transfer the cells dropwise to a tube containing 10ml pre-warmed growth medium and centrifuge at 1krpm for 10 minutes (Beckman GPKR centrifuge).
3) Discard the supernatant. Resuspend the cell pellet in 1 ml of growth medium. Remove an aliquot and count the cells using a haemocytometer.
4) Transfer the cells to 25 cm^2 tissue culture flask and add growth medium to give a cell concentration of 3 to 5 $x10^5$ cells/ml. The cells are cultured in a CO_2 incubator at 37°C with a 5 to 8% CO_2 /95% air mixture.

2.19.3 Culturing of cells

All cells are routinely cultured in $80cm^2$ plastic tissue culture flasks. The cells are maintained at 3-$5x10^5$ cells/ml and are diluted with fresh medium twice a week as follows:

1) Leave the tissue culture flasks standing in the microbiological safety cabinet for 5 to 10 minutes to allow the cells to settle.

2) Remove the spent tissue culture fluid using a pipette, leaving behind 5 to 8 mls of concentrated cell suspension.

3) Add 1/4th or 1/5th of the cell suspension to fresh tissue culture flasks (as many as are required).

4) Add fresh growth medium to give a final volume equivalent to the original starting volume.

2.20 HIV VIRUS PREPARATION BY CO-CULTURE

1) Mix 1 part of infected cells with 3 parts of uninfected H9 cells. Transfer the cells to a $80cm^2$ tissue culture flask.

2) Add growth medium to give a final cell concentration of $1x10^6$ cells/ml. Incubate in a 37°C CO_2 incubator for 4 days.

3) Clarify the supernatant by centrifuging at 2krpm for 10 minutes at 4°C (Beckman GPKR centrifuge).

4) Filter the supernatant on ice using a 0.45µm disposable filter.

5) Dispense in cryotubes and store in the gaseous phase of a nitrogen freezer.

6) Resuspend the cells in the original volume of medium and add an equal quantity of uninfected cells. Repeat steps 2 to 5.

2.21 TITRATION OF VIRUS STOCK

HIV virus titrations are carried out using C8166 cells:

1) Prepare a cell suspension containing $7x10^5$ cells/ml.

2) Dispense 150µl of the cell suspension to each well of a 96 well microtitre plate.

3) Make dilutions of the virus stock in cold growth medium. Keep the virus dilutions on ice.

4) Inoculate 50µl of the appropriate virus dilution to a minimum of 5 replicate wells.

5) Place the plate in a sandwich box with a moist tissue and incubate at 37°C in a CO_2 incubator.

6) After 48 hours examine the cells daily and score for cytopathic effects.

7) Terminate the experiment after 7 days.

8) Estimate the titre ($TCID_{50}$) of the virus stock using the method of Karber (described in Clinical Virology Manual, Specter and Lancz).

2.22 HIV INFECTION INHIBITION ASSAY (see Figure 2.2)

Virus-neutralizing antibody titres are measured using a syncytial inhibition assay based on a method described by Kinney-Thomas et al. (1988).

1) Heat-inactivate serum samples for 30 minutes at 56°C.

2) Make doubling dilutions of the sera in "V" bottomed microtitre plates, using growth medium as the diluent.

3) Add 50µl of virus containing 1000 $TCID_{50}$ to each well containing the appropriate serum dilution.

4) Prepare "virus controls" by diluting the virus stock to give dilutions with 1000, 100 or 10 $TCID_{50}$/50µl. Make replicates of each virus control.

5) Incubate the plates at 37°C in a CO_2 incubator for 60 minutes.

6) During the last 30 minutes of the incubation prepare the cells. Harvest C8166 cells and resuspend in fresh growth medium to give a final cell concentration of $7x10^5$ cells/ml.

7) Seed 150µl of the cell suspension into microtitre wells.

8) Transfer 2x50µl (i.e. replicate samples) of the virus/serum mixture to the cell plate.

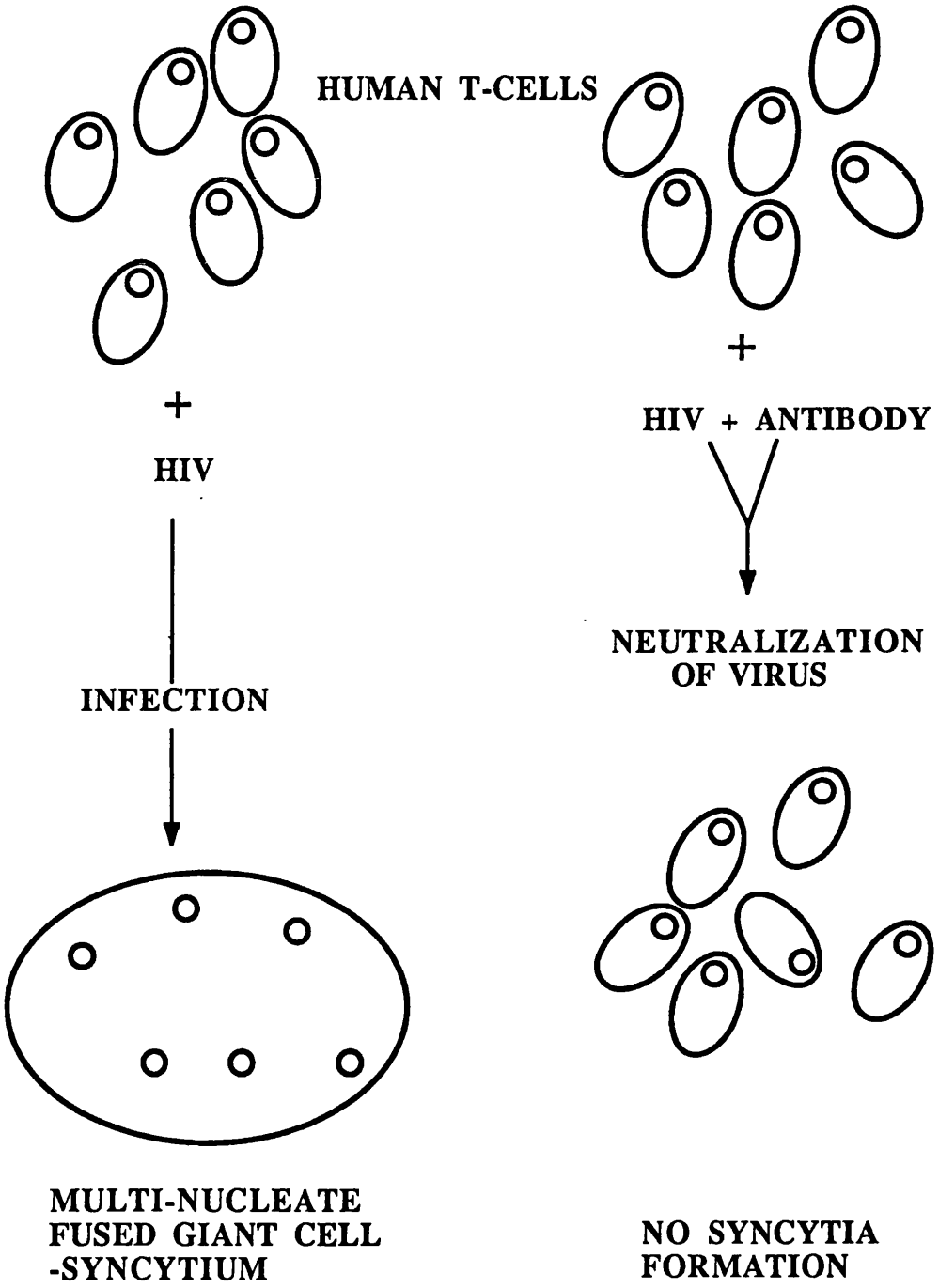


Figure 2.2 HIV infection inhibition assay

9) Place the plates in a sandwich box containing a moist tissue and incubate at 37°C in a CO_2 incubator for 48 hours.

10) Record the degree of cytopathic effect (i.e. syncytium formation) seen in each well.

11) Determine the 90% neutralization titre which is defined as the reciprocal of the serum dilution at which there is the same cytopathic effect seen as for the 100 $TCID_{50}$ "virus controls".

2.23 INSECT CELL CULTURE

The Lepidopteran insect cell line used is Spodoptera frugiperda IPLB-sf-9AE (Sf9). These cells are a cloned version of the cell line SF21 originally derived from insect pupal ovaries (Vaughn et al., 1977). The cells can be grown as a monolayer, or suspension culture when a larger number of cells are required. The cells have a doubling time of 18 to 24 hours.

The cell culture medium is TC100 (Flow laboratories) containing 10% (v/v) FCS plus 50 IU/ml penicillin, 50 mg/ml streptomycin.

2.23.1 Maintenance of insect cells

Low passage number cells are stored in liquid nitrogen using the following procedure:

1) Harvest the cells in the exponential phase of growth at a cell density between 1-1.5x10^6 cells/ml.

2) Add DMSO to a give a final concentration of 10%.

3) Fill cryotubes with 1ml of cell suspension and chill on ice.

4) Place the tubes in the gaseous phase of liquid nitrogen for 2 hours.

5) Store the tubes in the liquid phase of the nitrogen.

To revive the cells the following procedure is used:

1) Thaw a vial at room temperature for 1 to 2 minutes.

2) Transfer the cells to a $25cm^2$ tissue culture flask containing 8ml culture medium.

3) Leave the cells to settle at 27°C for 3 to 4 hours, then discard the medium and replace with fresh medium.

4) Leave the cells at 27°C for 2 to 3 days, until confluent. Discard the medium, add 3ml of fresh medium and scrape off the cells with a sterile scraper. Transfer the cells to a 50ml glass culture flask containing 20 ml of medium and incubate on a stirring platform at 27°C for 2 to 3 days.

2.23.2 Passaging of insect cells

1) Count the cells using a haemocytometer and calculate the concentration per ml.

2) Seed new cultures in medium as follows:
   $1x10^5$ cells/ml if required in 72 hours.
   $2x10^5$ cells/ml if required in 48 hours.
   $4x10^5$ cells/ml if required in 24 hours.

3) Incubate the cell culture on a stirring platform at 27°C.

2.24 PREPARATION OF INFECTIOUS AUTOGRAPHA CALIFORNICA NUCLEAR POLYHEDROSIS VIRAL (AcNPV) DNA

1) Infect a 500ml culture of Sf9 cells at $1.5 x10^6$ cells/ml at a multiplicity of infection (moi) of 0.1 plaque forming units (pfu) per cell. Incubate at 27°C on a stirring platform for 3 days.

2) Clarify the supernatant by centrifuging at 3krpm for 5 minutes (Sorvall RT6000B centrifuge).

3) Centrifuge the culture medium at 24krpm for 1 hour to pellet the virus particles

(SW28 rotor, Beckman L8-70M ultracentrifuge).

4) Resuspend the pellets overnight in 1ml of TE.

5) Layer the virus onto 10 to 50% (w/v) sucrose gradients (in TE). Centrifuge the gradients at 24krpm for 1 hour at 4°C (SW28 rotor).

6) Withdraw the virus band, then pellet at 24krpm for 1 hour at 4°C (SW28 rotor).

7) Resuspend the pellet overnight in 2ml of TE.

8) Add 200µl TE and 100µl sarkosyl solution to 200µl of virus suspension. Incubate at 60°C for 30 minutes.

9) Layer onto a 5ml cushion of 54% (w/v) caesium chloride/TE containing 25µg/ml of ethidium bromide. Centrifuge at 35krpm overnight at 20°C (VTI65 rotor, Beckman L8-70M ultracentrifuge).

10) Harvest the DNA band and extract the ethidium bromide 3 times with an equal volume of water saturated butanol.

11) Dialyse the DNA overnight against 1l of TE at 4°C.

12) Store the DNA at 4°C.

Sarkosyl solution

20%(v/v) sarkosyl

10mM EDTA

10mM Tris-HCl, pH7.8

2.25 CO-TRANSFECTION OF INSECT CELLS TO GENERATE RECOMBINANT VIRUS

1) Seed 3 35mm dishes with $1.3x10^6$ cells each.

2) Prepare a 100ng/µl solution of plasmid DNA in sterile H_2O.

3) Dilute 24µl of lipofectin (BRL) with 12µl H_2O (in a bijou tube).

4) Add 100ng viral DNA (See 2.24) to 0ng, 200ng (2µl) or 500ng (5µl) of plasmid DNA. Make the solutions up to 12µl with sterile H_2O (in bijoux tubes).

5) Add 12µl of lipofectin mix to each of the DNA solutions, mix gently and leave at room temperature for 15 minutes.

6) Remove the medium from the cells, wash 3 times with FCS-free TC100. Add 1ml FCS-free TC100.

7) Pipette the lipofectin/DNA mixtures dropwise onto the cells and leave at 27°C for 4 to 5 hours. Add 1ml TC100 medium containing 10% FCS and antibiotics. Incubate at 27°C for 48 hours.

8) Remove the supernatant from the dishes. Microfuge for 30 seconds, decant and store at 4°C.

9) Screen for recombinant virus formation in a Brown-Faulkner plaque assay (see below).

2.26 TITRATION OF VIRUS IN A PLAQUE ASSAY

The plaque assay is based on the method of Brown and Faulkner (1977).

1) Prepare 3% (w/v) low-gelling temperature (LGT) agarose in H_2O. Sterilize by autoclaving. Maintain at 45°C.

2) Seed 35mm dishes with 1.4 to $1.5x10^6$ cells.

3) Remove the medium from the dish and pipette 100µl virus (diluted as

appropriate in medium) onto the centre of the dish. Leave at room temperature for 1 hour, gently agitating the dish occasionally.

4) Remove the virus inocula and add 2ml of agarose/medium (50% LGT/50% medium) overlay. Stand at room temperature for 15 minutes to solidify.

5) Add 1ml of medium. Place the dishes in a sandwich box containing a moist tissue and incubate at 27°C for 3 days.

6) Discard the medium and then add 1ml 6% neutral red stain solution in PBS. Leave for 1 hour and then discard the stain solution. Leave dishes overnight at room temperature for the plaques to clear.

7) Calculate the titre of the virus by counting the number of plaques at each virus dilution, then multiply this by the virus dilution. Take the average over the range of dilutions to give the virus titre, expressed as plaque forming units (pfu)/ml.

2.27 PREPARATION OF RECOMBINANT BACULOVIRUS STOCKS

After plaque purification the titre and volume of the recombinant baculovirus is increased to provide a working virus stock. This is achieved by a 3 stage infection procedure.

1) Seed 35mm dish with $1.2x10^6$ cells. Discard the medium and infect with 150µl of a plaque which has been picked and vortexed in 500µl medium. Incubate for 1 hour at room temperature.

2) Add 2 ml of medium and incubate at 27°C for 5 to 6 days.

3) Clarify the supernatant by centrifuging at 3krpm for 5 minutes (Sorvall RT6000B centrifuge). Store the virus (now at passage number 3) at 4°C.

4) Seed an $80cm^3$ tissue culture flask with $8x10^6$ cells. Discard the medium and infect with 250µl of passage 3 virus for 1 hour at room temperature. Add 25ml

medium and incubate at 27°C for 5 to 6 days.

5) Clarify supernatant by centrifuging at 3krpm for 5 minutes (as above). Store the virus (passage 4) at 4°C.

6) Titre the passage 4 virus stock using the Brown-Faulkner plaque assay described in section 2.26.

When the virus titre has been determined prepare a high titre virus working stock as follows:

7) Seed a 500ml vessel with 250ml of cells at a density of 2.5×10^5 cells/ml. Incubate on a stirring platform at 27°C for 24 hours.

8) Infect with passage 4 virus at a multiplicity of infection (moi) of 0.1 plaque forming units (pfu) per cell. Incubate on a stirring platform at 27°C for 5 days. Clarify the supernatant by centrifuging at 3kpm for 5 minutes (as above) and store the virus at 4°C.

CHAPTER 3

HYBRID Ty-VLPs CONTAINING V3 LOOPS FROM THE HIV ISOLATE IIIB

3.0 INTRODUCTION

As stated in chapter one, a major aim of this project was to investigate the presentation of the major neutralizing epitope of the envelope glycoprotein gp120 (the V3 loop) using the hybrid Ty-VLP system, and to determine whether hybrid HIV V3:Ty-VLPs could induce HIV neutralizing antibodies. As the V3 loop region is hypervariable between different isolates of HIV, initial studies of hybrid VLPs carrying the V3 loop were made using the IIIB isolate that has been the most extensively sudied to date. The main advantages of choosing this isolate were the availability of reagents required for assaying the presentation of the V3 loop sequence by the hybrid Ty-VLPs and the ability to make a direct comparison with other systems reported in the literature. The original IIIB isolate was derived from H9 cells co-cultured with a pool of T cells from several American AIDS patients (Popovic et al., 1984) and is therefore a mixed virus population. Several clones from this isolate have been sequenced and it was decided to produce hybrid VLPs that carried the V3 loop sequence from two of these IIIB clones. The V3 loop sequences from the BH10 clone (Ratner et al., 1985) and the HXB2 clone (Fisher et al., 1985) are shown below. The two sequences differ by only one amino acid, which is underlined:

SNCTRPNNNTRKSIRIQRGPGRAFVTIGKIGNMRQAHCNISG BH10

SNCTRPNNNTRKRIRIQRGPGRAFVTIGKIGNMRQAHCNISG HXB2

The hybrid V3:Ty-VLPs were analysed by Western blot and immunogold electron microscopy. The immunogenicity of the VLPs, including the ability to induce neutralizing antibodies, was studied following immunization of rabbits using different adjuvants.

3.1 CONSTRUCTION OF HIV:Ty-VLPs CONTAINING THE BH10 AND HXB2 V3 LOOPS

In order to construct TYA:HIV V3 loop fusion genes, synthetic oligomers encoding the loop sequences were first cloned into plasmid pSP46 and sequenced. pSP46 is a derivative of plasmid pSP64 (Melton et al., 1984) in which the HindIII site within the polylinker sequence has been replaced with a BglII site. pSP46 therefore contains both BamHI and BglII sites within the polylinker. All of the oligomers synthesized containing the V3 loop sequences had a BglII site at the 5' end of the annealed pair of oligomers and a BamHI site at the 3' end. Cloning of the annealed oligomer pairs into BglII-BamHI digested pSP46 therefore generated a plasmid from which the V3 loop fragment could be easily excised after verification of the sequence (Figure 3.1). If the annealed oligomer pairs were cloned directly into the BamHI site of the VLP expression vectors pMA5620 or pOGS40 (described in chapter 1, section 1.4) the fragment could not be excised again as a BglII-BamHI fragment. The strategy of cloning the oligomers into pSP46 became more important in later constructions where V3 loop fragments from different isolates were linked together in one vector (see chapter 5).

After verification by sequencing, the BglII-BamHI V3 loop fragment was excised from the pSP46 derivative and cloned into the BamHI site of the VLP expression vector pMA5620 (Figure 1.4).

The resulting plasmids were assigned numbers as follows:

Vector	Clone	Plasmid
pSP46	BH10	pOGS508
pSP46	HXB2	pOGS509
pMA5620	BH10	pOGS513
pMA5620	HXB2	pOGS514

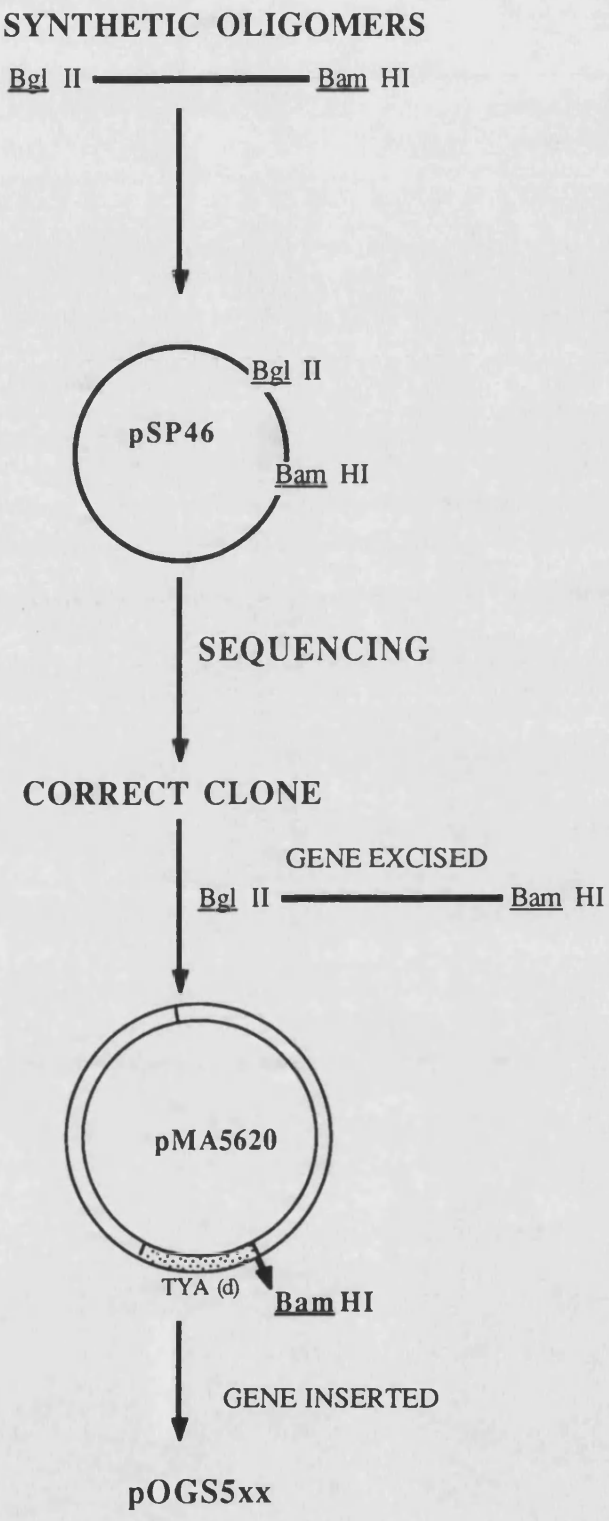


Figure 3.1 Cloning strategy for synthetic oligomers coding for V3 loops

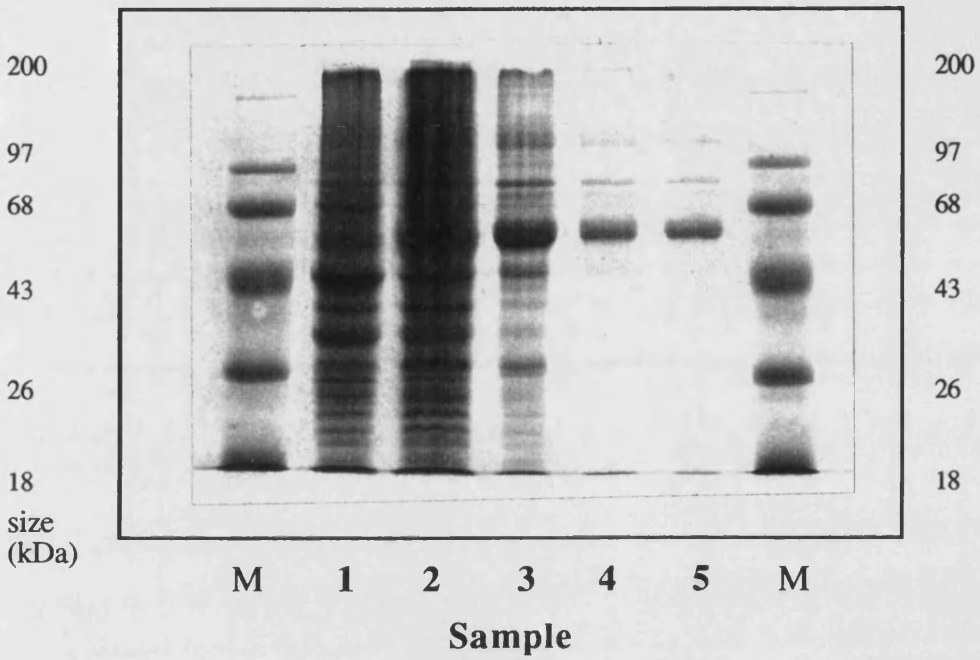
Hybrid Ty-VLPs purified from yeast cells transformed with the VLP expression vectors were given the same name as the plasmid but without the "p" prefix. For example, VLPs purified from pOGS514 transformed cells were OGS514 VLPs.

3.2 PURIFICATION OF HYBRID HIV:Ty-VLPs

The purification of VLPs was facilitated by the fact that they are considerably larger in size than most cellular proteins. Furthermore, as the basic physical properties of each hybrid particle are conferred by the carrier, particle-forming protein, p1, the same purification procedures could be used for each construction.

The protocol used for purifying VLPs has been described in section 2.13. For each preparation, 16l of cells were grown to a density of greater than 2×10^7 cells/ml. The cells were grown under selective conditions in order to maintain the presence of the plasmids. The yeast strain used for the production of VLPs was auxotrophic for leucine, uracil and tryptophan synthesis. Cells transformed with a single plasmid containing the LEU 2 gene (pMA5620 derivatives) were therefore grown in the presence of uracil and tryptophan.

Figures 3.2 and 3.3 illustrate the purification steps involved in the production of VLPs from yeast cells transformed with pOGS514 (isolate HXB2). In figure 3.2, the SDS-PAGE gel shows the particle protein and other yeast proteins at various stages of the purification. The denatured monomeric fusion protein had an estimated molecular weight of 60kD by comparison with the molecular weight markers. However the calculated molecular weight of the truncated p1 protein is 43kD and that of the added HIV protein is approximately 4kD. The fusion protein should therefore have a molecular weight of 47kD. The discrepancy in size is due to the anomalous migration of Ty proteins in SDS-PAGE and has been reported previously (Mellor et al., 1985a).



Sample

1 Broken cells

2 High speed pellet

3 Post-sucrose gradient

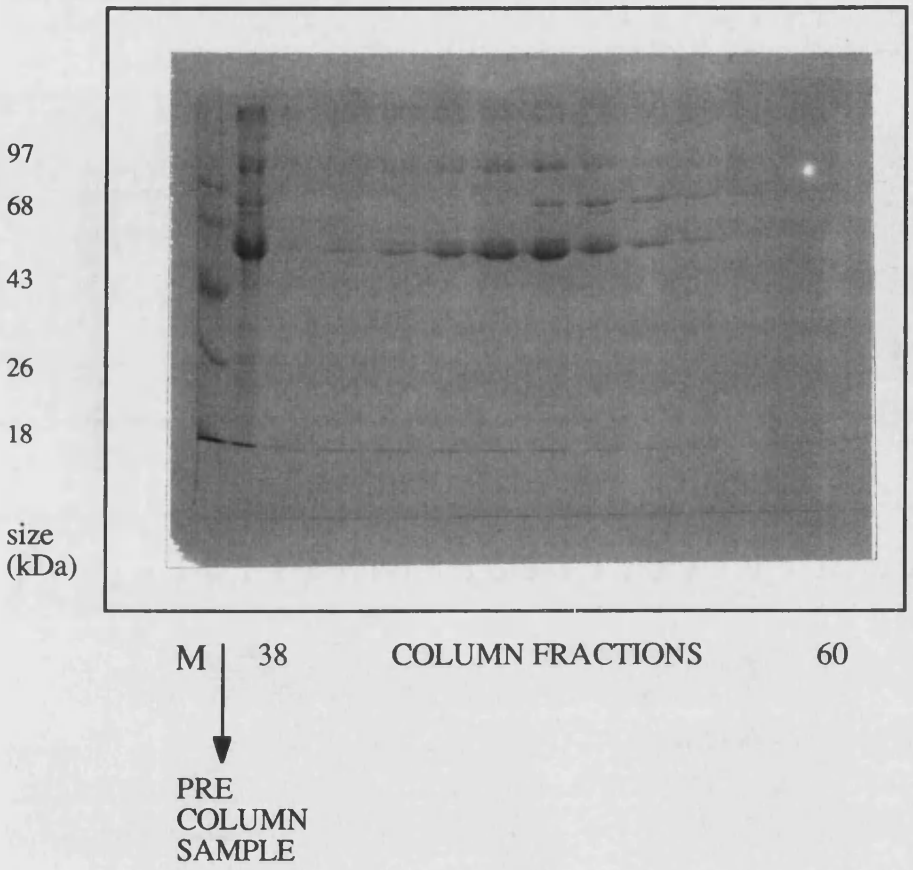
4 Pre-column

5 Post-column

M Molecular weight markers

Figure 3.2 Purification stages of OGS514 VLPs

Samples were run on 10% SDS-PAGE and stained with Coomassie blue.



M Molecular weight markers

Figure 3.3 SDS-PAGE analysis of aliquots of fractions following size exclusion chromatography of OGS514 VLPs

As shown in track 1 of Figure 3.2 it was difficult to distinguish the VLP protein from cellular proteins in the crude cell homogenate. However, the VLP protein became clearly visible after the sucrose gradient step (track 3). The final purification step was to pass the material over a size exclusion chromatography column. Figure 3.3 shows an SDS-PAGE gel of column fractions containing the OGS514 particles. The final stage of the preparation was to concentrate the particles to approximately one mg/ml. The OGS514 purification illustrated here yielded 26 mg of VLPs, i.e., 1.6 mg/l. Preparations of other HIV V3 loop:Ty-VLPs yielded between 10 and 50mg from 16l cultures.

3.3 PRELIMINARY CHARACTERISATION OF HYBRID Ty-VLPs

3.3.1 Western blot analysis

The hybrid VLPs OGS513 (BH10) and OGS514 (HXB2) were analysed by Western blotting. The blot shown in Figure 3.4 (a) was probed with a monoclonal antibody raised against MA5620 control Ty particles. The blot showed the expected increase in size of approximately 4kD for the monomeric proteins of OGS513 and OGS514 as compared to the MA5620 control Ty particles. The blot in Figure 3.4 (b) was probed with a gp120-specific monoclonal antibody 9284 (Du Pont). Monoclonal antibody (Mab) 9284 was raised against isolate IIIB and maps to the left hand side of the V3 loop (Skinner et al., 1988b). Both OGS513 (BH10) and OGS514 (HXB2) VLP proteins reacted positively with Mab 9284, indicating that the V3 loop was present in the fusion proteins comprising these hybrid V3:Ty-VLPs. As mentioned above, the V3 loop sequences from these two clones of the IIIB isolate differ by only one amino acid which is found on the left hand side of the loop. It is therefore interesting to note that this change did not alter the reactivity against the Mab 9284.

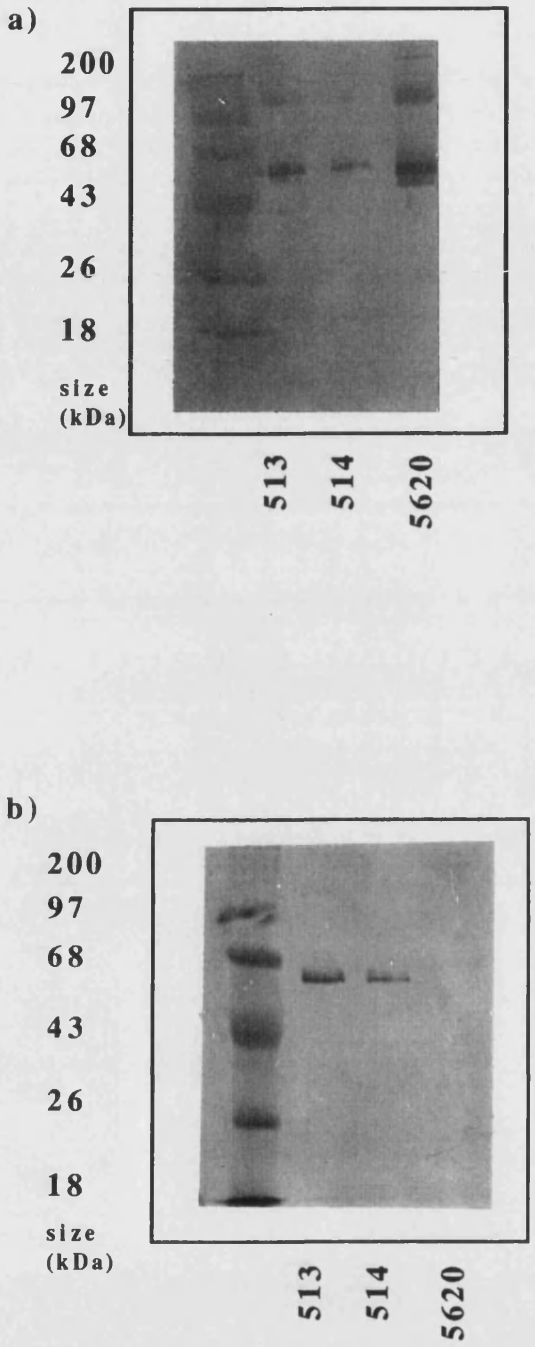


Figure 3.4 Western blot analysis of HIV V3:Ty-VLPs OGS513 (BH10) and OGS514 (HXB2).

Blot (a) was probed with a monoclonal antibody raised against MA5620 control Ty particles. Blot (b) was probed with a gp120 specific monoclonal antibody.

3.3.2 Electron microscopic analysis of OGS 514 (HXB2) Ty-VLPs

Electron micrographs of purified MA5620 (Ty control) and OGS514 VLPs are shown in Figure 1.5. Various conditions were investigated in order to gold-label the OGS514 VLPs (isolate HXB2) with the gp120-specific MAb. The optimal conditions that were determined are summarized in Section 2.16. Using these conditions the OGS514 VLPs were specifically labelled with MAb 9284 (Figure 3.5). As the interior of the particles is unlikely to be accessible to the gold/antibody complexes, these data indicate that at least some of the V3 loop region was exposed on the surface of the particles.

3.4 IMMUNE RESPONSES GENERATED AGAINST THE V3:Ty-VLPs

The major analysis of antigen presentation was by immunization of rabbits with the HIV IIIB V3 loop:Ty-VLPs. The immunizations were carried out at Serotec, Kidlington, Oxfordshire. All of the experiments utilized the same immunization protocol. The dose was 500µg of VLPs which represents approximately 50µg of the V3 loop antigen. Each dose was administered via two intramuscular sites. After a priming immunization, the rabbits received a boost every two weeks for a total of eight boosts. Test bleeds were taken every week in order to allow for close monitoring of the antibody levels. After the eighth boost, rabbits were bled every two weeks so that the longevity of the response could be investigated.

Initially, the immunizations were carried out using Freund's adjuvant. This is a very powerful adjuvant because the antigens are presented as an oil emulsion which acts as a depot, releasing the antigen slowly. However, Freund's adjuvant can cause adverse side effects and is therefore only useful in a primary screen to identify effective antigens in experimental animals. These experiments were then followed by an analysis of the immunogenicity of HIV:Ty-VLPs carrying the V3 loop from isolate HXB2 (OGS514)

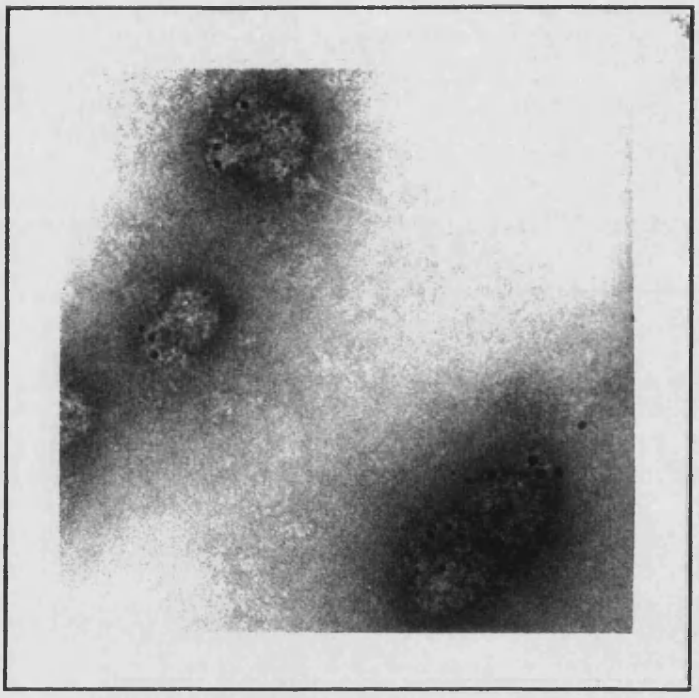


Figure 3.5 Immunogold electron microscopy of OGS514 VLPs

VLPs were probed with the monoclonal antibody 9284. Bound antibody was detected using a gold labelled (5nm) anti-mouse antibody.

in conjunction with aluminium hydroxide. Aluminium hydroxide is currently the only adjuvant approved for use in humans and these experiments were therefore the most relevant in terms of candidate vaccine design.

3.4.1 Serum antibody titres of animals immunized with the V3:Ty-VLPs in conjunction with Freund's adjuvant

Initial immunizations compared the immunogenicity of OGS513 (BH10) and OGS514 (HXB2) V3:Ty-VLPs following administration in conjunction with Freund's adjuvant. The three rabbits which received the OGS513 (BH10) VLPs were designated 513/1/1, 513/1/2 and 513/1/3 and the three rabbits that received the OGS514 (HXB2) VLPS were designated 514/1/1, 514/1/2 and 514/1/3.

The serum antibody titres were determined using an ELISA assay (outlined in Figure 2.1.) First, wells of a microtitre plate were coated with an appropriate antigen. The antigen used in these assays to measure the anti-HIV response was recombinant gp120 (rgp120; isolate IIIB), which was obtained from the Medical Research Council AIDS Directed Programme. To measure the response against the Ty component of the hybrid particle, MA5620 (control Ty) particles were used as the antigen. Secondly, the rabbit antisera raised against the HIV V3 loop:Ty-VLPs were titrated against the antigen, usually beginning at a 1:100 dilution followed by trebling dilutions to 1:72900. After the appropriate washing and detection steps (see figure 2.1), the optical densities (O.D.) recorded were converted to mid-point titres. These were calculated as the dilution which gave rise to half of the maximum O.D. achieved by the lowest dilutions. Mid-point titres were used as they are more comparable between assays than end-point titres.

Sera from the animals immunized with the OGS513 and OGS514 VLPs in conjunction with Freund's adjuvant were assayed against rgp120. Figure 3.6 shows the antibody titres of the bleeds obtained after each boost. In the 513/1 group, animal 513/1/1 showed an anti-gp120 response after the first boost which increased gradually up to post-boost five, declined post-boosts six and seven and then increased again post-boost eight. Animals 513/1/2 and 513/1/3 had relatively low anti-gp120 titres throughout the immunization regime. In the 514/1 group, all three animals showed a strong response against rgp120. The titres of animal 514/1/1 peaked post-boost five at 1:13000 and the titres of animals 514/1/2 and 514/1/3 peaked post-boost four at 1:9000 and 1:12500 respectively. The titres declined after subsequent boosts, which may have been due to anti-V3 loop antibodies present at the time of the boost complexing with the additional V3 antigen presented by the V3:Ty-VLPs. It is possible that this would have had the effect of decreasing the antibody population and/or increasing the rate of clearance of the antigen. As Figure 3.6 demonstrates, the animals in group 514/1 showed a much greater anti-gp120 response as compared to the animals in group 513/1. These data therefore suggested that OGS514 hybrid VLPs were more effective at eliciting an anti-HIV response.

Figure 3.7 shows the antibody titres obtained against the Ty component of hybrid VLPs in the animals in groups 513/1 and 514/1. All the animals in both groups had high anti-Ty titres, although the response was highly variable throughout the immunization schedule. The titres of animals 513/1/1 and 513/1/3 peaked at 1:1000000 post-boosts eight and seven respectively, whereas the maximum response in animal 513/1/2 occurred after the second boost with a titre of 1:650000. In the 514/1 group, the anti-Ty titres of animal 514/1/1 peaked after the third boost at 1:200000 and then declined gradually following subsequent boosts. The peak titre of animal 514/1/2 of 1:600000 occurred after the fifth boost and animal 514/1/3 had maximum titres of

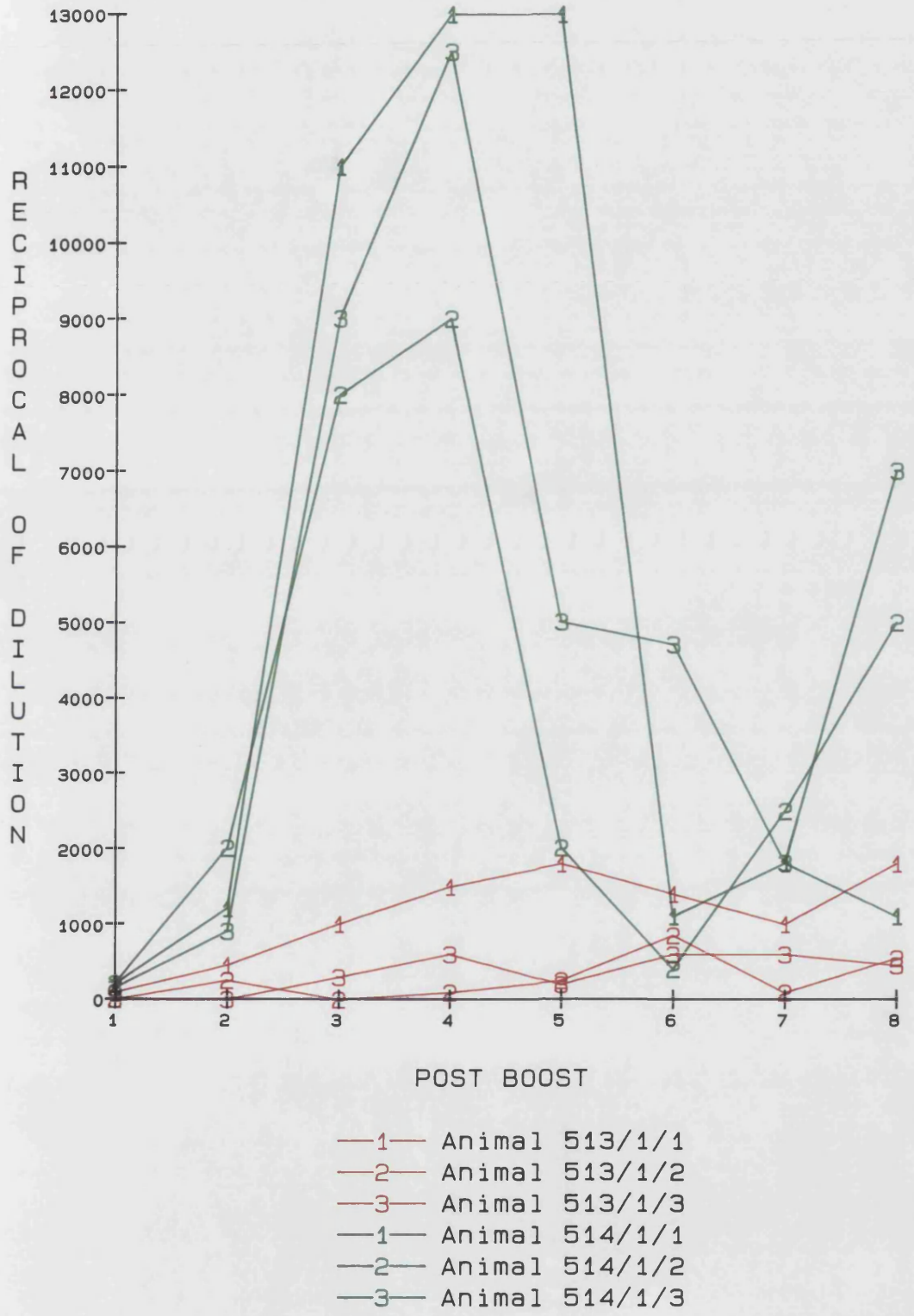


Figure 3.6 ELISA mid-point titres of groups 513/1 and 514/1 assayed against rgp120. The sera were assayed seven days post boost.

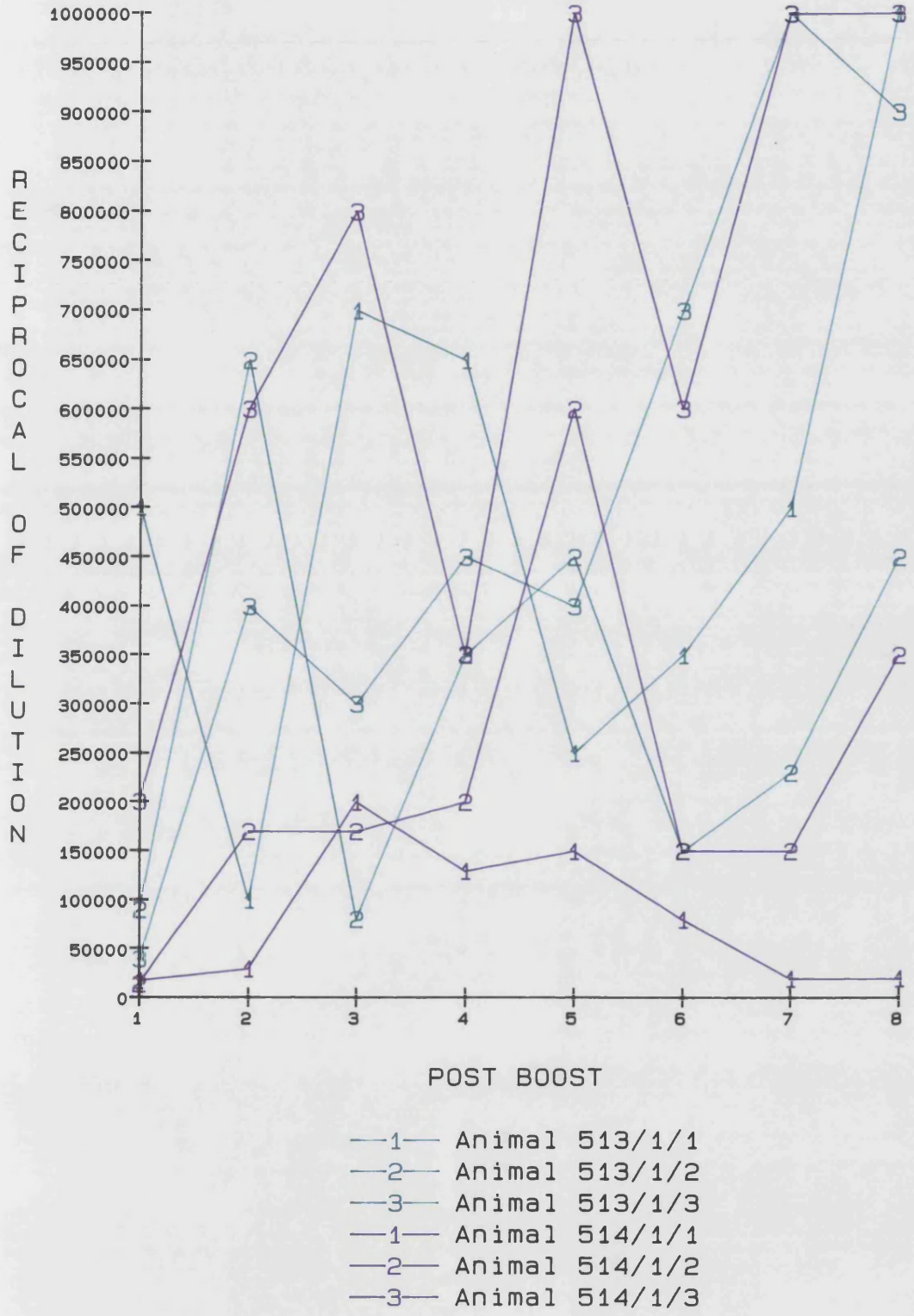


Figure 3.7 Anti-Ty mid-point titres of groups 513/1 and 514/1. The sera were assayed against control Ty particles.

1:1000000 post-boosts five, seven and eight.

Comparison of the anti-gp120 responses with the anti-Ty responses showed that higher titres were generated against Ty. This observation may correspond to the fact that in the hybrid particles the majority of the protein was Ty, and the V3 loop represented only 10% of the particulate protein. There appeared to be little correlation between the responses generated against the two components of the particle, either when comparing the responses in individual animals or comparing the responses between the two different groups.

3.4.2 Induction of anti-HIV neutralizing antibodies following immunizations with V3:Ty-VLPs in conjunction with Freund's adjuvant

Antisera from the animals in groups 513/1 and 514/1 described above in section 3.4.1 were assayed for their ability to neutralize HIV-1 isolate IIIB in vitro. The assay has been described in section 2.22 and is shown diagrammatically in figure 2.2. Virus neutralizing antibody titres were expressed as the reciprocal of the dilution that gave rise to a 90% inhibition of infectivity.

Figure 3.8 compares the neutralization titres of the animals in group 513/1 with the animals in group 514/1. After the first boost, all the animals in group 514/1 had neutralizing antibodies and, in particular, animal 514/1/3 had a substantial titre of 1/128. In contrast, none of the animals in group 513/1 had neutralizing antibodies following the first boost. In general, titres increased in most animals up to post-boost four or five, and titres in group 514/1 were higher than those in group 513/1. The neutralization titres therefore provided further evidence to suggest that a stronger immune response was generated against the HXB2 V3 loop carried by the OGS514 VLPs than to the BH10 V3 loop carried by the OGS513 VLPs.

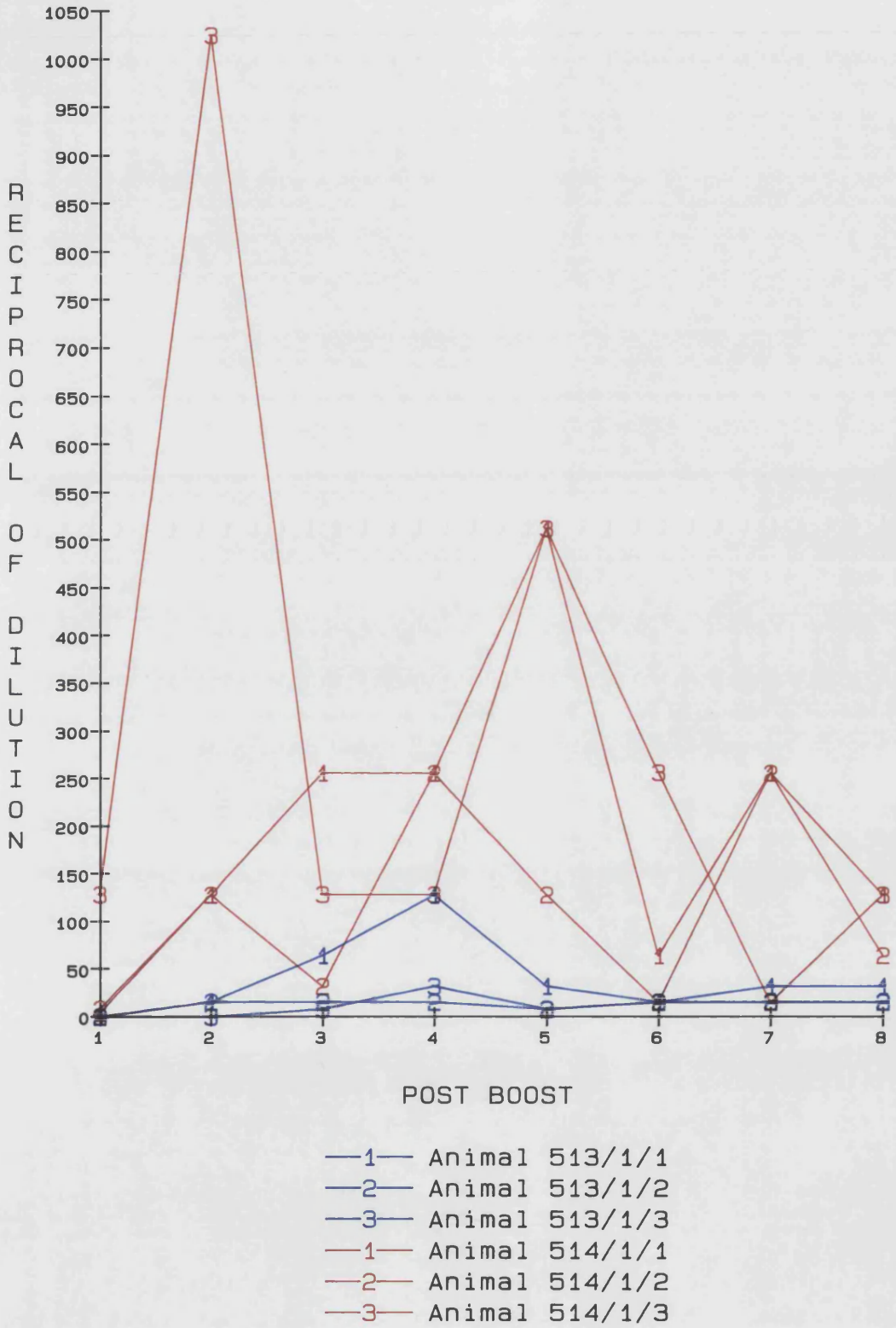


Figure 3.8 Neutralization titres of sera from groups 513/1 and 514/1 assayed against HIVIIIB.

3.4.3 Longevity of the antibody response in animals immunized with V3:Ty-VLPs in conjunction with Freund's adjuvant

The longevity of the antibody response in the animals in groups 513/1 and 514/1 was assessed by measuring the anti-gp120 titres of the sera taken at monthly intervals for six months. Table 3/1 shows that all the animals in both groups still had anti-gp120 antibodies after six months and in some animals the titres increased during this period. This maintenance of antibody levels was probably due to the depot effect of the Freund's adjuvant at the site of injection, slowing releasing antigen for some time after the eighth boost.

3.4.4 Recall of the immune response of animals immunized with V3:Ty-VLPs in conjunction with Freund's adjuvant

In order to determine whether a memory response had been generated against the V3 loops carried by the OGS513 (BH10) and OGS514 (HXB2) VLPs the animals in groups 513/1 and 514/1 were given a further boost seven months after the eighth boost. Table 3/2 gives the HIV neutralization titres before and after the ninth boost. Neutralizing antibodies were still present at the time of boosting and did not significantly increase in titre after the boost. Due to the sustained response in all of the animals prior to boost nine this experiment could not demonstrate whether or not a memory response had been induced.

3.4.5 Serum antibody titres of animals immunized with OGS514 (HXB2) VLPs in conjunction with aluminium hydroxide

As stated above, immunizations carried out in conjunction with Freund's adjuvant can be useful as a primary screen to investigate the immunogenicity of novel antigens. The initial immunizations carried out with OGS513 (BH10) VLPs and OGS514 (HXB2) VLPs administered in conjunction with Freund's adjuvant suggested that

TABLE 3/1. LONGEVITY OF THE ANTI-GP120 RESPONSE IN ANIMALS IN GROUPS 513/1 AND 514/1

Anti-gp120 titres were expressed as the reciprocal of the dilution of the mid-point titre.

	Animal					
	513/1/1	**513/1/2**	**513/1/3**	**514/1/1**	**514/1/2**	**514/1/3**
Weeks after boost 8						
4	1800	600	350	1100	5000	4400
8	550	1400	1200	700	3000	5000
12	650	750	850	1700	1000	2100
16	850	650	600	500	1800	2400
20	950	1500	1500	350	1800	2700
24	350	700	950	350	1800	1500

TABLE 3/2. NEUTRALIZATION TITRES OF ANIMALS IN GROUPS 513/1 AND 514/1 PRE AND POST THE NINTH BOOST

Neutralization titres were expressed as the reciprocal of dilution that gave rise to 90% inihibtion of syncytium formation.

	Animal					
	513/1/1	**513/1/2**	**513/1/3**	**514/1/1**	**514/1/2**	**514/1/3**
pre boost	8	16	8	16	64	128
post boost	32	8	8	32	128	128

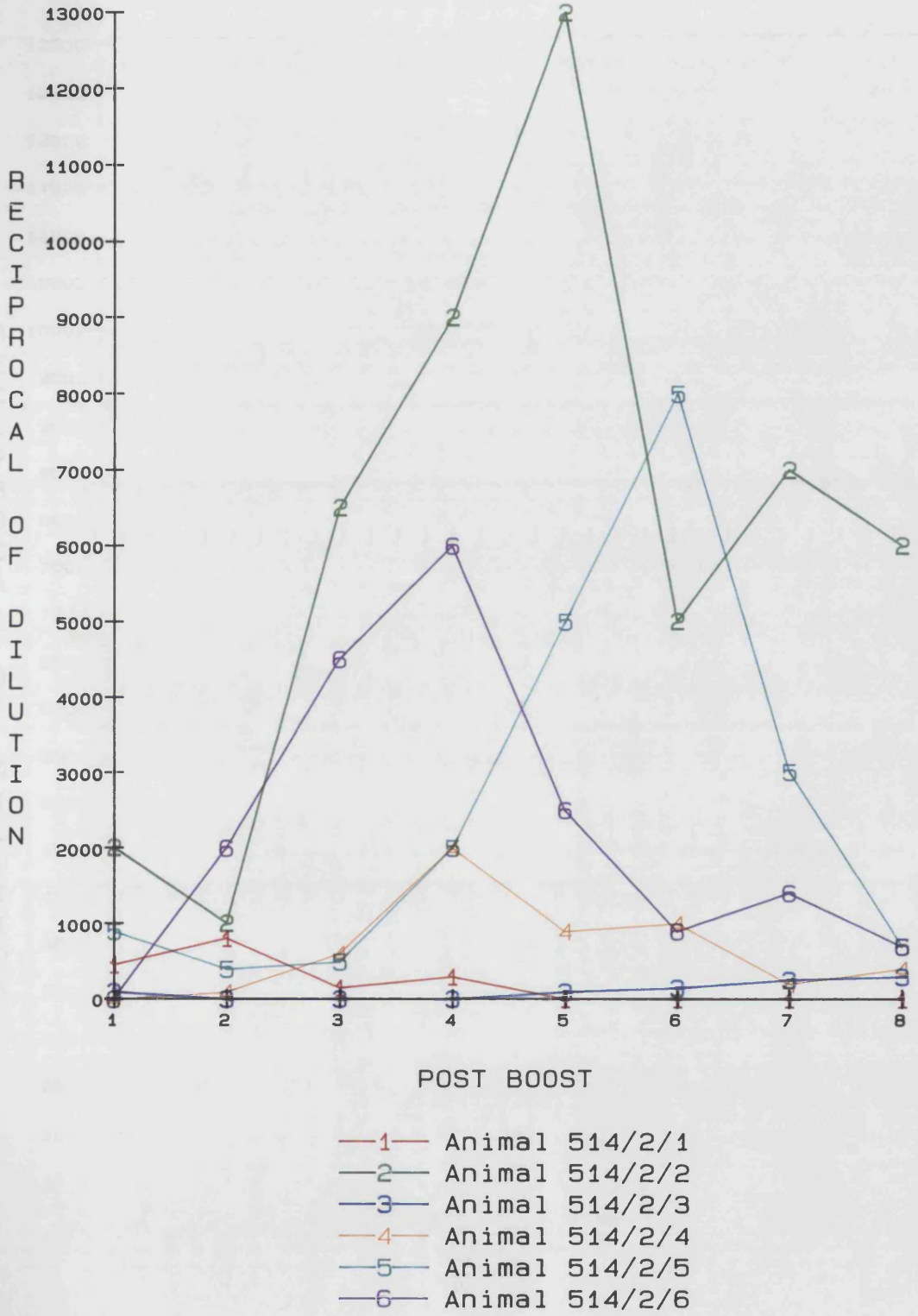


Figure 3.9 ELISA mid-point titres of group 514/2 assayed against rgp120. The sera were assayed seven days post boost.

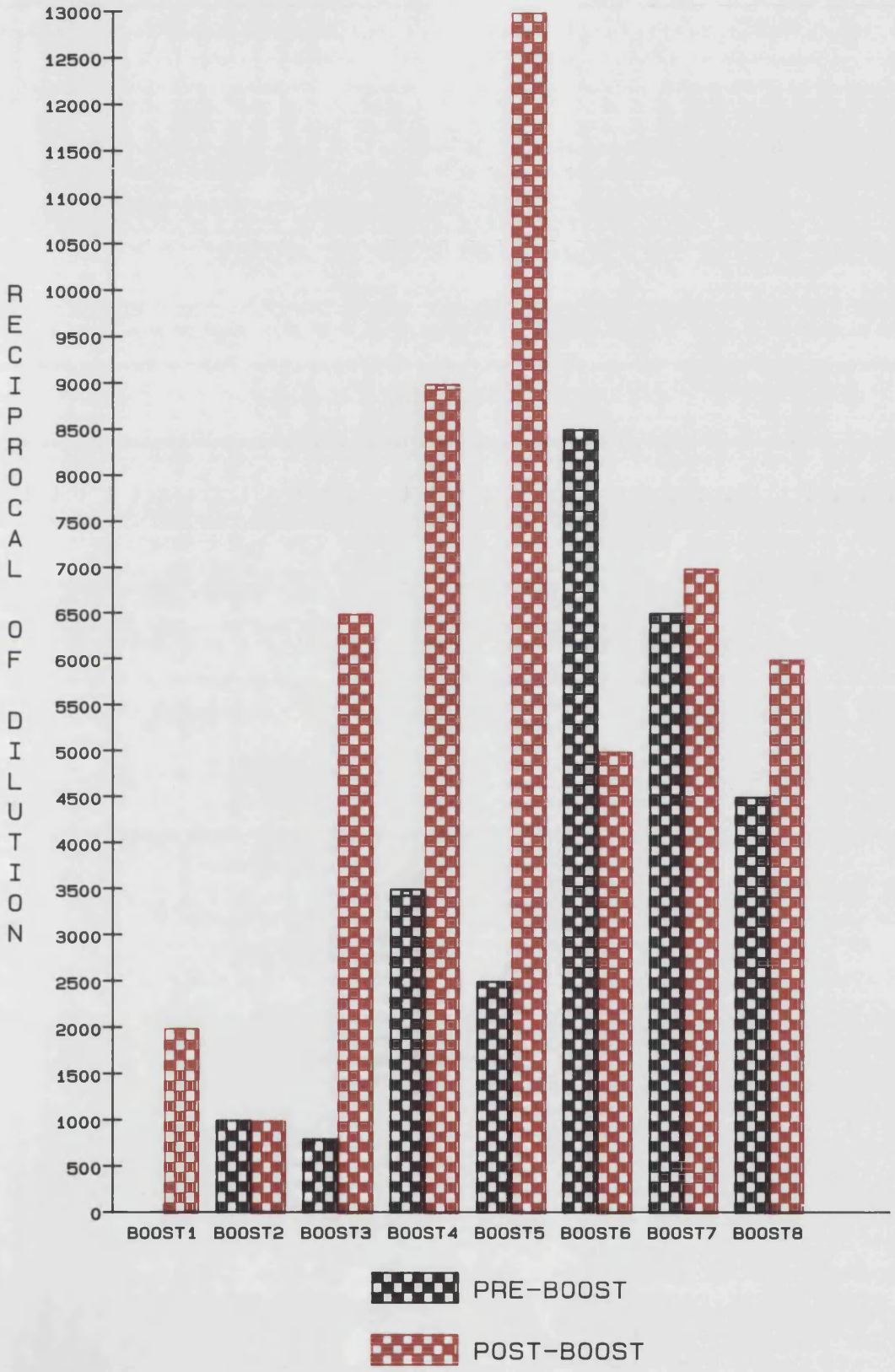


Figure 3.10 ELISA mid-point titres of sera from animal 514/2/2. The sera were assayed against rgp120.

Figure 3.11 shows the Ty responses from the animals in group 514/2. Animals 514/2/1 and 514/2/3 had the highest anti-Ty responses. However these animals also had the lowest anti-gp120 response, which indicated that animals 514/2/1 and 514/2/3 preferentially responded to Ty. In general, in the four animals that had strong anti-gp120 responses, a similar trend in their anti-Ty responses was observed to that of the anti-gp120 responses (Figure 3.9), with peak titres occurring post-boost four or five. These data further indicated that antibodies already present may be complexing with the antigen in subsequent boosts. This therefore suggested that the boosting regime for the experiments, although chosen on the basis of experiments described in the literature, was too frequent. The maximum anti-Ty titres were approximately three to ten fold higher than the titres against gp120. In addition, the anti-Ty titres in the animals immunized with OGS514 VLPs in conjunction with aluminium hydroxide were approximately ten fold lower than the animals immunized with the OGS514 VLPs in Freund's adjuvant.

The sera from animal 514/2/2 were assayed against the control Ty particles before and after each boost. Figure 3.12 shows that although the titres against Ty were higher than the serum antibody titres against gp120 the pattern of response was similar. The decreases in titre between boosts were not as great as those seen with the gp120 antibody titres indicating that the anti-Ty antibodies had a longer half-life than the anti-gp120 antibodies.

3.4.6 Neutralizing antibody titres of animals immunized with OGS 514 (HXB2) VLPs in conjunction with aluminium hydroxide

Sera from the animals in group 514/2 were tested for the presence of HIV neutralizing antibodies in a syncytium inhibition assay. Figure 3.13 shows the neutralization titres obtained. The neutralization titres reflected the anti-gp120 responses with four of the

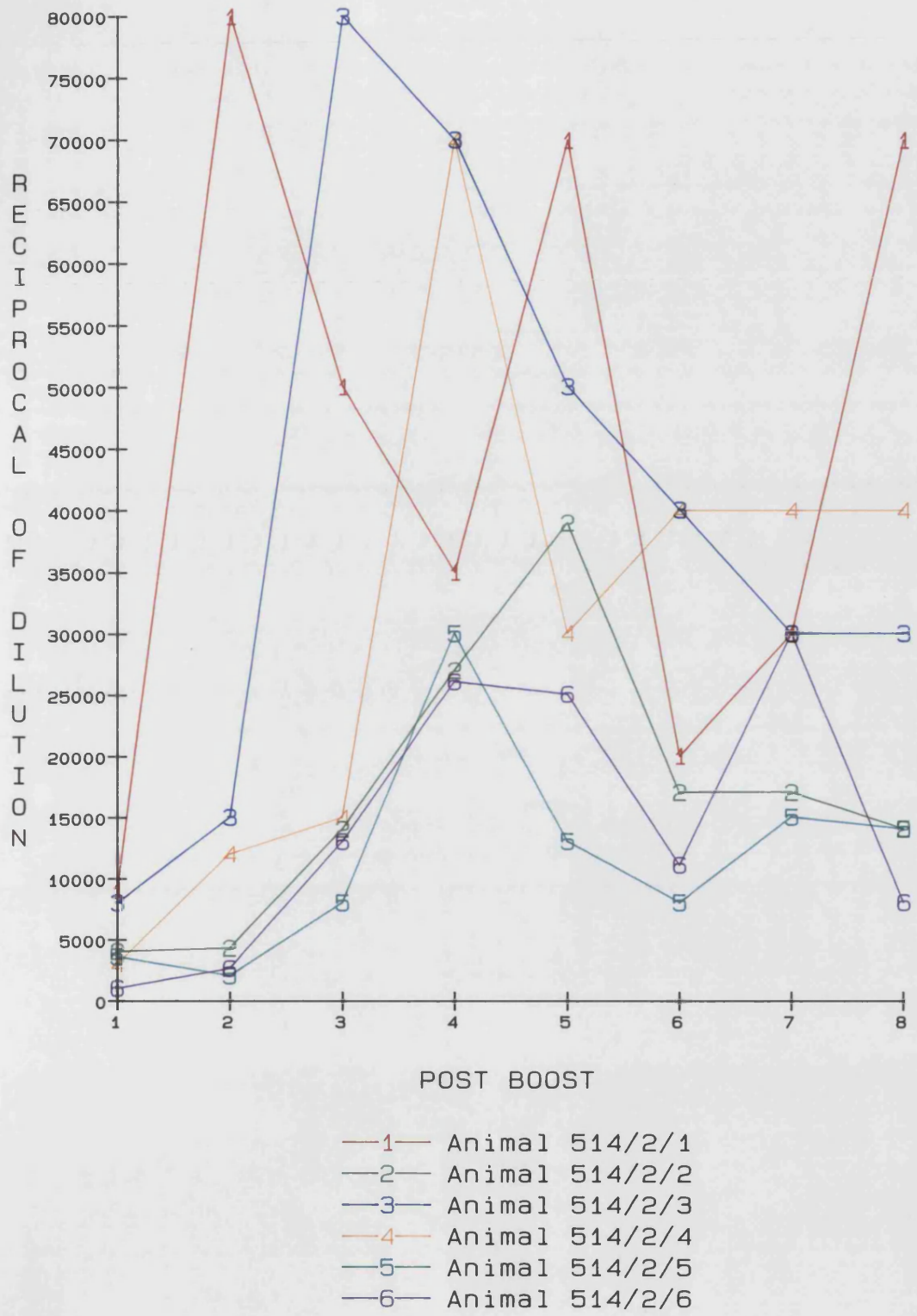


Figure 3.11 Anti-Ty mid-point titres of group 514/2. The sera were assayed against control Ty particles.

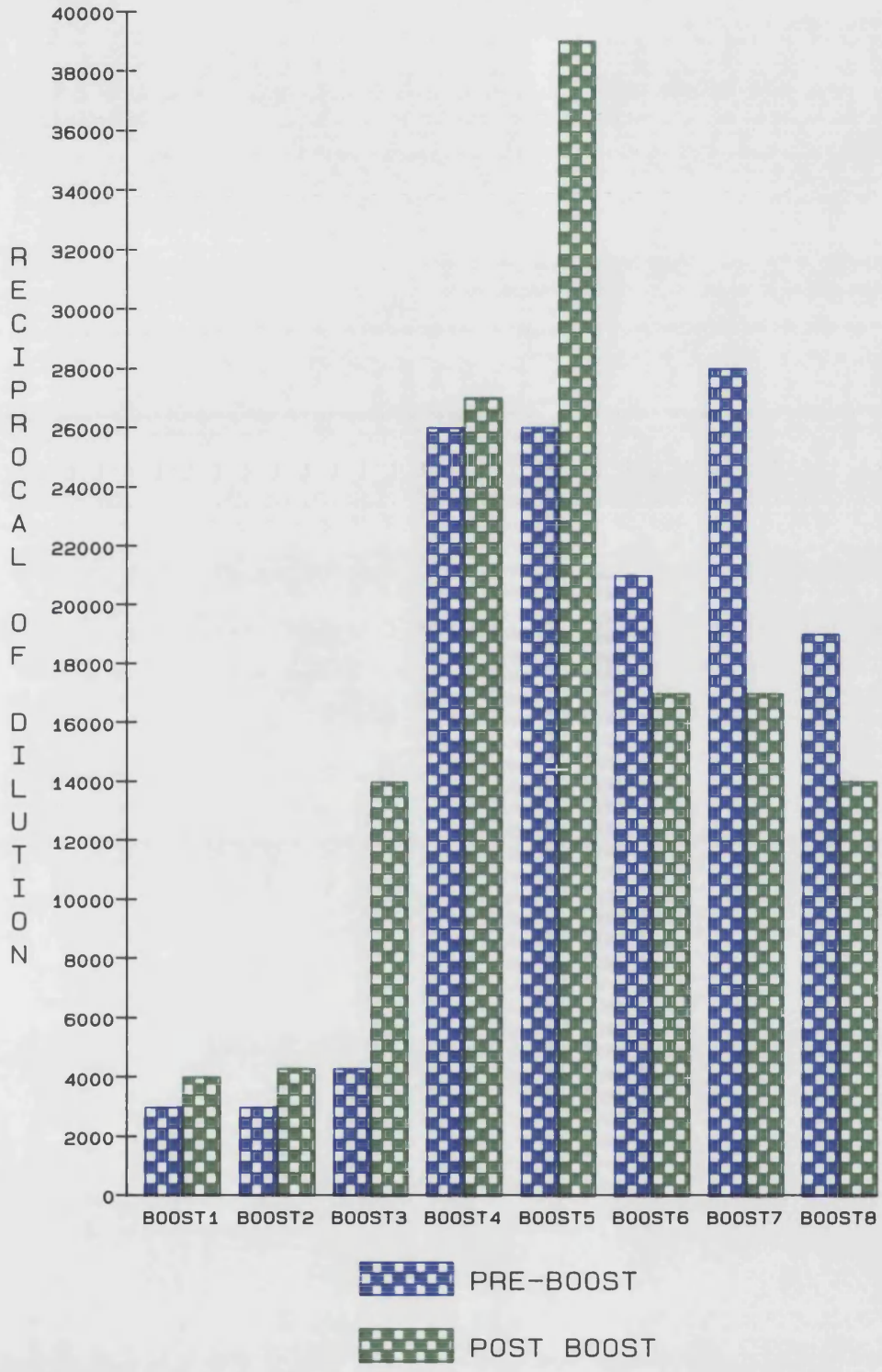


Figure 3.12 Anti-Ty mid-point titres of animal 514/2/2. The sera were assayed against control Ty particles.

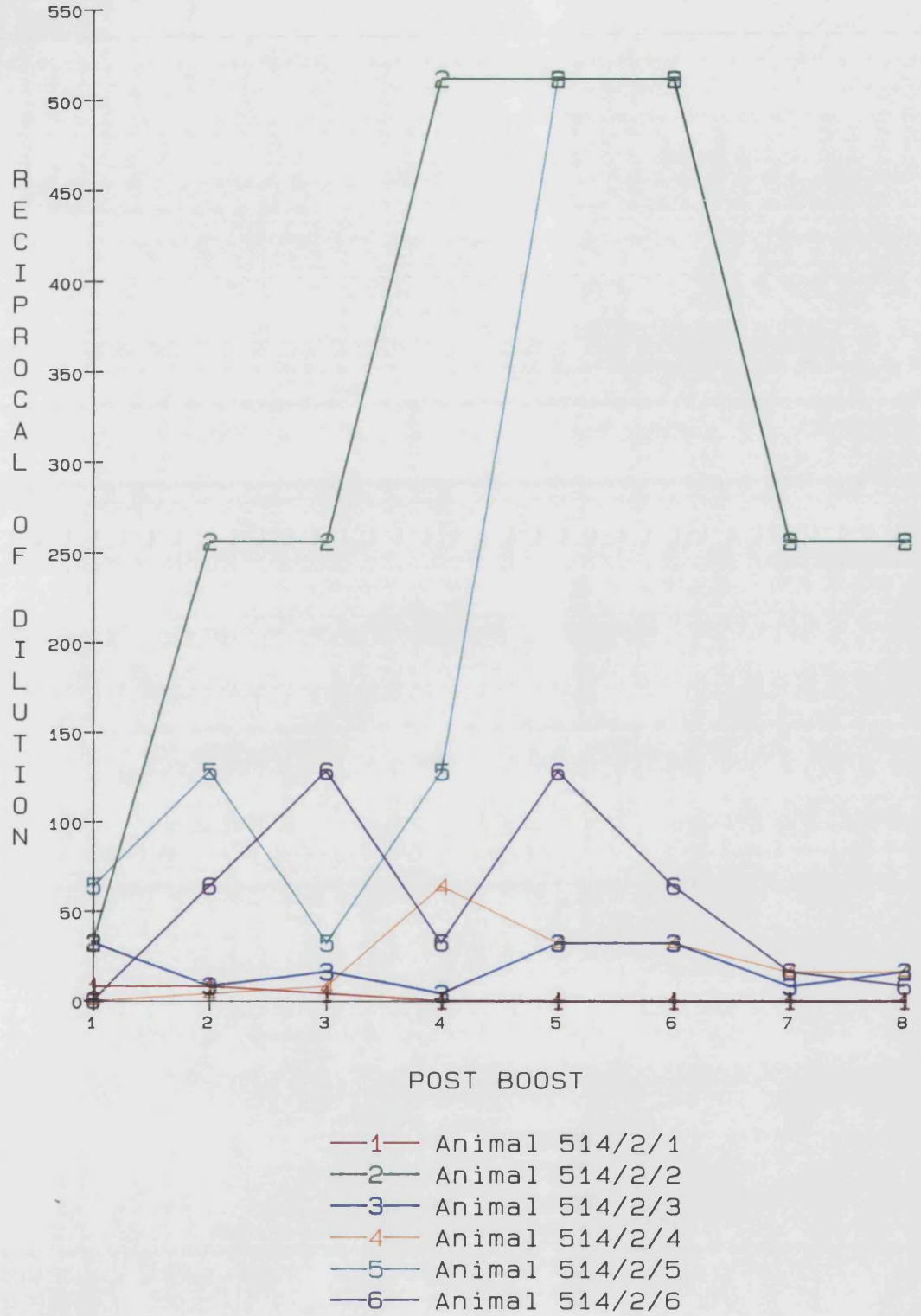


Figure 3.13 Neutralization titres of sera from group 514/2 assayed against HIVIIIB. Titres of 1/512 are actually equal to or greater than 1/512.

animals having high levels of neutralizing antibodies. The pattern of the responses was also similar with peak titres occuring after the fourth or fifth boosts. The neutralization titres of the animals in group 514/2 were comparable with the titres in group 514/1, demonstrating again that similar anti-HIV responses were elicited whether the immunizations were carried out in conjunction with aluminium hydroxide or Freund's adjuvant.

The neutralizing antibody titres from animal 514/2/2 were determined before and after each boost. Figure 3.14 shows that the trend seen in the neutralization titres was similar to that of the anti-gp120 antibody titres shown in Figure 3.10. This suggested that a significant proportion of the antibodies induced against the HXB2 V3 loop presented on OGS514 VLPs were virus neutralizing antibodies. The high titres of neutralizing antibodies observed with a maximum of 1:2048 (animal 514/2/2, post-boost five), demonstrated the efficacy of the OGS514 VLPs following administration in a clinically relevant formulation.

3.4.7 Longevity of the antibody response in animals 514/2/2 and 514/2/6

The longevity of the response in all six animals in group 514/2 could not be monitored due to the loss of four of the animals as a result of an infection contracted in the animal house.

Table 3/3 shows the serum gp120 and neutralizing antibody titres obtained up to 10 weeks after the last boost in animals 514/2/2 and 514/2/6. The neutralizing antibody titres of animal 514/2/2 remained constant at 1/512 for eight weeks following the final boost and then dropped to 1/64, whereas the ability of sera from animal 514/2/6 to neutralize declined gradually throughout the 10 week period. However, in both

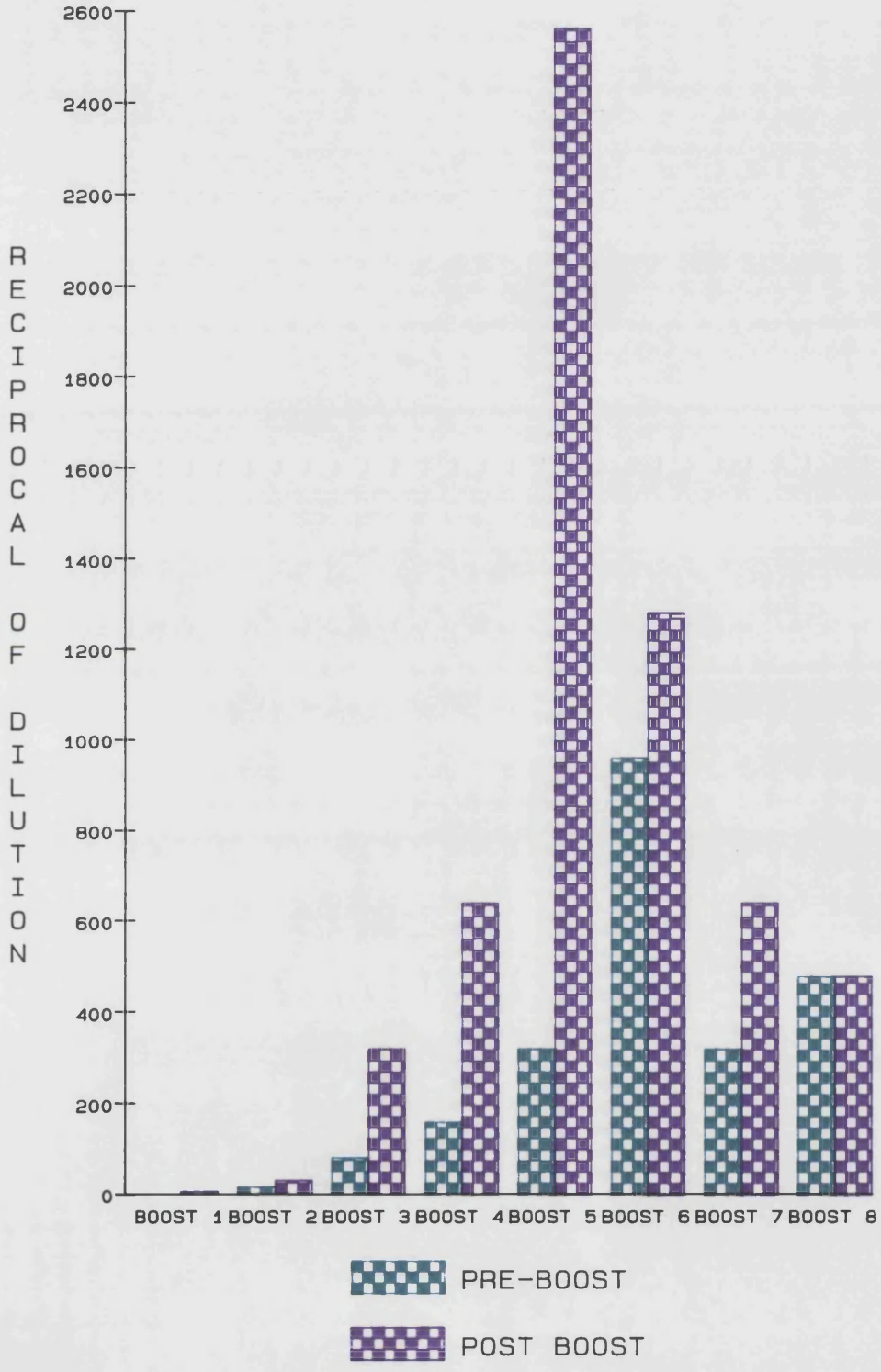


Figure 3.14 Neutralization titres of sera from animal 514/2/2. Sera were assayed against HIVIIIB.

TABLE 3/3. LONGEVITY OF THE ANTIBODY RESPONSE OF ANIMALS 514/2/2 AND 514/2/6

	Weeks following the final boost				
Animal	**2**	**4**	**6**	**8**	**10**
i) Reciprocal gp120 antibody mid-point titre					
514/2/2	1000	4000	800	1100	300
514/2/6	200	300	400	200	300
ii) Reciprocal HIV neutralizing antibody titre					
514/2/2	512	512	512	512	64
514/2/6	64	64	32	16	16

animals, significant levels of gp120 and neutralizing antibodies were still present 10 weeks following boost eight. Sera from animal 514/2/6 still had detectable anti-gp120 titres up to 20 weeks after the eighth boost (data not shown).

3.4.8 Recall of the response in animal 514/2/6

In order to determine whether antigen-specific memory cells had been induced by the OGS514 VLPs, animal 514/2/6 received a further boost after a 6 month rest period. At the time of the boost no gp120 or neutralizing antibodies were detected. However, sera taken 7, 14 and 21 days after the ninth boost had neutralizing antibody titres of 1/16, 1/32 and 1/128 respectively. In the initial immunization regime, neutralizing antibodies were not detected after the first boost and the maximum titre of 1/128 was only achieved after the third boost. Boosting after a six month rest period therefore induced a rapid recall of the response to the HXB2 V3 loop presented on OGS514 VLPs, with the maximum neutralization titre being achieved in three weeks.

3.5 DISCUSSION

Hybrid Ty-VLPs have been constructed that carry the major neutralizing epitope of HIV clones BH10 and HXB2. The VLPs reacted positively by Western blot analysis with a V3 loop monoclonal antibody, confirming the presence of the V3 loop sequence on the hybrid V3:Ty-VLPs. Immunogold labelling experiments with VLPs carrying the HXB2 V3 loop indicated that at least some of the V3 loop sequence was exposed on the surface of the VLPs. However, although the labelling by the anti-gp120 antibody was specific, the antibody did not label the entire surface of the VLPs. One possible explanation was that only a limited amount of antibodies were able to bind due to steric hindrance. Alternatively, as the reactions were not carried out in solution, it is possible that some disruption of the VLP structure occurred as the particles were loaded onto the grid, thereby exposing previously hidden epitopes.

Immunizations of experimental animals have been carried out to test the efficacy of the hybrid V3:Ty-VLPs. Initial immunizations compared the immunogenicity of OGS513 (BH10) and OGS514 (HXB2) VLPs in conjunction with Freund's adjuvant. The data demonstrated that although both the OGS513 (BH10) and OGS514 (HXB2) particles elicited anti-gp120 and virus neutralizing antibodies, a greater response was seen following immunization with OGS514 VLPs. In contrast, similar anti-Ty responses were induced in both groups of animals, indicating that both particle preparations were similarly immunogenic. Three possible explanations for the differences seen in the anti-HIV responses elicited by the OGS513 and OGS514 VLPs are outlined below.

First, higher anti-HIV responses elicited by OGS514 VLPs may be due to the variation in the V3 loop sequences. The V3 loop sequences differ by only one amino acid, with a serine residue in BH10 being substituted by an arginine residue in HXB2. This is not a conservative change; although both residues are polar, serine is uncharged whereas the side chain of arginine is basic. This charge difference may in itself affect antigenicity. In addition, the bulky side chain of the arginine residue may influence the overall structure of the V3 loop, thereby altering the antigenic properties of the sequence.

Secondly, the differences observed in neutralization titres may reflect the relative proportions of the HXB2 and the BH10 clones within the mixed IIIB population used in the assays. If anti-HXB2 and anti-BH10 antibodies can only neutralize HXB2-like and BH10-like clones respectively, differences in titres may simply indicate that the IIIB population contains more HXB2-like than BH10-like viruses. However, there was a good correlation between the anti-gp120 titres and the HIV neutralizing titres suggesting that a significant proportion of the antibodies raised against the V3 loops were virus-neutralizing antibodies. As the recombinant gp120 used in the ELISA assay

had the BH10 V3 loop sequence, it is therefore unlikely that the differences observed were due to the reagents used in the assays.

Thirdly, the observed variation in response may be because only relative small numbers of animals were used in the experiments. As the animals were outbred and therefore likely to have different MHC haplotypes, it is possible that the animals in group 514/1 were fortuitously better responders to the V3 loop sequence.

A second series of immunizations were carried out using the OGS514 VLPs in conjunction with aluminium hydroxide. High-titre anti-gp120 and virus neutralizing antibodies were again generated and these data demonstrated that OGS514 VLPs are efficacious in a clinically relevant formulation. Overall, the titres induced by OGS514 VLPs were similar whether the immunizations were administered in conjunction with aluminium hydroxide or with Freund's adjuvant. These results indicate that the OGS514 VLPs effectively present the V3 loop sequence to the immune system and that this presentation is not dependent on the use of a powerful enhancer of immunogenicity. Differences were observed in the number of animals in the two adjuvant groups that induced strong anti-HIV responses. The animals in the Freund's group all showed similar high titre responses whereas only four out of the six animals in the group that had received OGS514 VLPs in conjunction with aluminium hydroxide had substantial titres. These differences were probably due to the different adjuvants used but could also be attributed to the variations inherent when using small groups of animals. The variations could be addressed by comparing the relative responses induced by OGS514 VLPs following immunization in conjunction with Freund's adjuvant or aluminium hydroxide, using larger groups in which the animals have been MHC haplotyped. A difference was also observed in the longevity of the response induced in the two OGS514 experiments. The more sustained response in the Freund's

group was probably due to the depot effect of this adjuvant.

The results presented in this chapter demonstrate that hybrid Ty-VLPs carrying the HXB2 V3 loop elicit high-titre HIV neutralizing antibodies when administered in a clinically relevant formulation. Immunization also generates a memory response. The OGS514 V3:Ty-VLPs therefore induce responses that are likely to be required of a potential HIV vaccine candidate.

CHAPTER 4

IMMUNE RESPONSES INDUCED BY HYBRID Ty-VLPs CONTAINING THE V3 LOOP FROM THE MN ISOLATE

4.0 INTRODUCTION

In recent years many isolates of HIV from the USA and Europe have been sequenced and this analysis has shown that the isolate IIIB is not prevalent. Isolates from infected individuals have been found to be more similar to the isolate MN that was originally sequenced by Gurgo et al.(1988). Serological studies of HIV infected patients showed that 18% (Devash et al., 1990b) and 14% (LaRosa et al., 1990) had antibodies that recognised the V3 loop from the IIIB isolate. In contrast these studies and a further study by Zwart et al.(1991) demonstrated that a high percentage of patient sera reacted with the V3 loop from the MN isolate. These data suggest that a potential vaccine candidate should include a component that would elicit a neutralizing response against the MN isolate. Hybrid Ty-VLPs were therefore constructed carrying the MN V3 loop whose sequence is shown below:

SNCTRPNYNKRKRIHIGPGRAFYTTKNIIGTIRQAHCNISG

4.1 CONSTRUCTION AND PURIFICATION OF MN V3:Ty-VLPs

A TYA:MN V3 loop fusion gene was constructed by first cloning synthetic oligomers encoding the MN V3 loop sequence into pSP46 in the same way as described in chapter 3, section 3.1. The resulting plasmid was designated pOGS524. The MN V3 loop gene was then cloned into the yeast expression vector pMA5620 to generate pOGS525. However yeast cells that were transformed with pOGS525 grew very slowly and gave a poor yield of hybrid VLPs. This indicated that the p1:MN V3 loop fusion protein in some way had a toxic effect on the yeast cells. The MN V3 loop gene was therefore cloned into the expression vector pOGS40 to generate pOGS530 which expressed the fusion protein from the galactose inducible promoter described in chapter 1, section 1.4.1. Yeast cells were transformed with pOGS530 and then OGS530 (MN) hybrid V3:Ty-VLPs were purified using the method described in chapter 2, section 2.3.

4.2 PRELIMINARY CHARACTERIZATION OF OGS530 (MN) V3:Ty-VLPs

Preliminary characterization of OGS530 (MN) VLPs was carried out by Western blot analysis. The blot in Figure 4.1a was probed with a polyclonal antiserum raised against MA5620 control Ty particles and showed an increase in size of the p1:MN V3 monomeric protein as compared to p1. The blot in Figure 4.1b was probed with a polyclonal antiserum raised against an MN V3 loop peptide and the positive reaction indicated the presence of the MN V3 loop in the OGS530 VLP fusion protein.

Figure 4.1c shows two Western blot filters in which a panel of hybrid Ty-VLPs carrying the V3 loops from a diverse range of isolates were probed with two separate pools of ten HIV-1 positive sera. These included the OGS513 (BH10) and OGS514 (HXB2) VLPs carrying the V3 loops from the IIIB isolate (chapter 3) and also OGS515 VLPs that carry the V3 loop from the Haitian isolate RF. OGS516 (MAL) and OGS519 (ELI) contain the V3 loops from two African isolates. These hybrid V3:Ty-VLPs are discussed in chapter 5. It is interesting to note that both pools of sera gave the same pattern of reactivity. A weak response was observed with OGS513 (BH10) and OGS514 (HXB2) VLPs, a stronger response was seen with OGS516 (MAL) VLPs and a very strong response was seen with the OGS530 (MN) VLPs. These results support the observations outlined in the introduction that MN-like isolates are more common than any others examined to date.

4.3 IMMUNE RESPONSES GENERATED AGAINST THE MN V3:Ty-VLPs

To test the immunogenicity of OGS530 (MN) VLPs, five rabbits were immunized in conjunction with aluminium hydroxide (designated group 530/1). The immunization schedule was as described previously in section 3.4, i.e., each rabbit received a

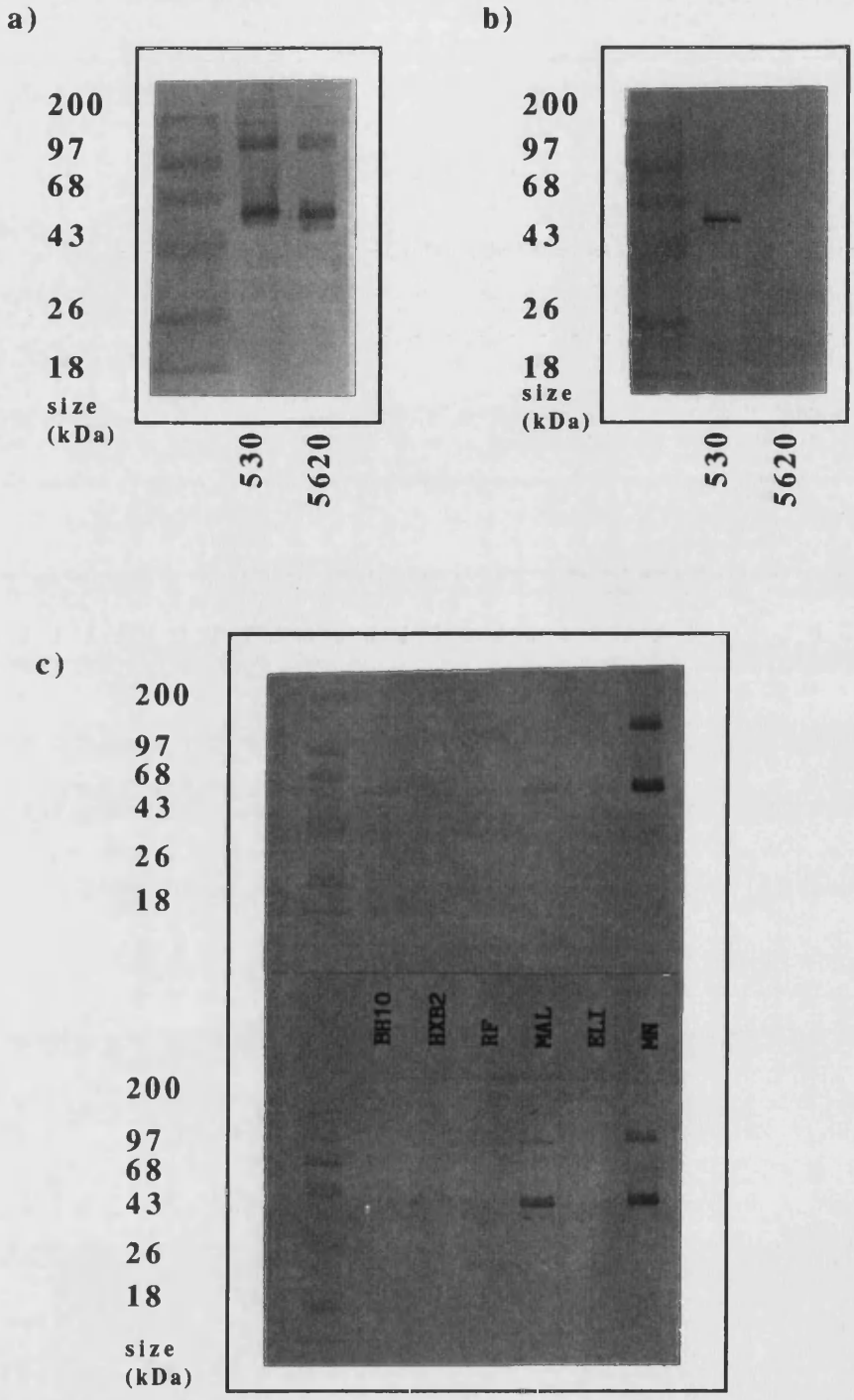


Figure 4.1 Western blot analysis of OGS530 (MN) VLPs

Blot (a) was probed with a monoclonal antibody raised against MA5620 control Ty particles. Blot (b) was probed with a polyclonal serum raised against a MN V3 peptide. Each blot in (c) was probed with a separate pool of HIV-1 positive sera.

priming immunization and a total of eight boosts of 500µg of OGS530 VLPs at two-weekly intervals. Sera from the immunized animals were tested for their ability to neutralize the HIV isolate MN. Only two animals had low levels of neutralizing antibodies against MN. Serum antibody levels measured against a peptide coding for the MN V3 loop were also low. The anti-Ty titres were measured and were found to be approximately ten fold lower as compared to the titres obtained in group 514/2 (chapter 3, section 3.4.5). This indicated that for some unknown reason, the animals in group 530/1 had induced a very poor response against the OGS530 VLPs.

To examine the anti-OGS530 response further, an additional series of immunizations were therefore carried out. One group of five rabbits were immunized with the OGS530 VLPs in conjunction with aluminum hydroxide (designated group 530/2) and another five rabbits were immunized in conjunction with Freund's adjuvant (group 530/3). Antisera from the ten animals up to post-boost five were assayed by ELISA against Ty and the MN peptide. Again a poor response was observed in all ten animals, with similar titres obtained in each group. When the anti-Ty response of animals immunized with OGS514 VLPs had been compared previously, either in conjunction with Freund's adjuvant or aluminium hydroxide, it was found that the titres in the Freund's group were approximately ten fold higher. This enhancement of the anti-Ty response was not observed in the animals that received the OGS530 (MN) VLPs with Freund's adjuvant. The results obtained for the three groups of rabbits immunized with the OGS530 VLPs therefore indicated that a poor immune response was generated against these particles. As the same preparation of OGS530 VLPs was used for the immunizations one possible explanation of the poor responses was that some impurity in this batch had a deleterious effect on the immune system of the rabbits. Another possibility was that the MN V3 loop sequence was in some way immunosuppressive. However, this seemed unlikely as there is significant homology between the MN

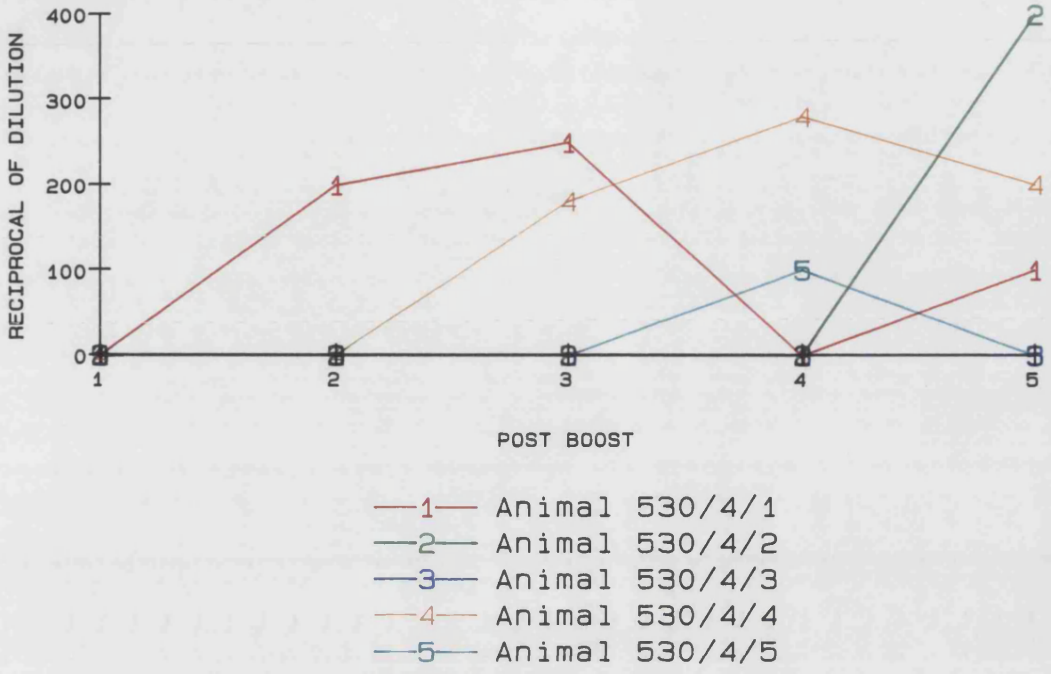
sequence and the HXB2 sequence which had already been shown to be a very effective antigen when presented on the OGS514 VLPs (chapter 3).

To address the possibility of batch-batch variation of the VLPs a further round of immunizations was carried out using a new preparation of OGS530 VLPs. Group 530/4 received the OGS530 VLPs in conjunction with aluminium hydroxide and group 530/5 received the immunizations in conjunction with Freund's adjuvant. There were five animals in each group and each rabbit received a priming immunization and a total of five boosts of 500μg of OGS530 VLPs at two-weekly intervals. The animals were bled at weekly intervals up to the fifth boost and were then bled at two-weekly intervals so that the longevity of the response against the OGS530 VLPs could be measured. The results obtained for these two groups of rabbits are presented below.

4.3.1 Serum antibody titres of animals immunized with OGS530 VLPs in conjunction with aluminium hydroxide or Freund's adjuvant

The serum antibody titres of the animals that received OGS530 VLPs in conjunction with aluminium hydroxide (group 530/4) or Freund's adjuvant (group 530/5) were determined using the ELISA assay described in section 2.18. The anti-MN responses were assayed against a 40 amino acid peptide coding for the MN V3 loop. The anti-Ty responses were measured against the MA5620 (Ty control) particles.

Figure 4.2 compares the anti-MN responses of groups 530/4 (aluminium hydroxide) and 530/5 (Freund's) up to post-boost five. No response was observed in either group of animals after the first boost. Following the second boost animal 530/4/1 and three animals in group 530/5 had generated a response. After the third boost, two animals in group 530/4 had a measurable anti-MN response whereas all five animals in group 530/5 had responded. As Figure 4.2 shows, the responses generated in the 530/4



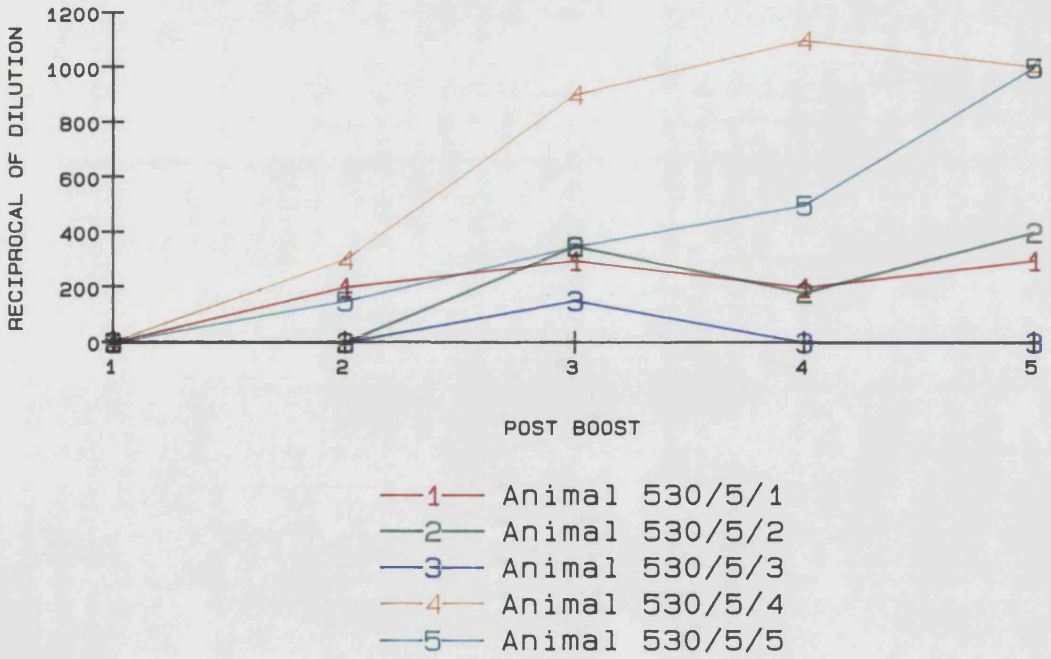
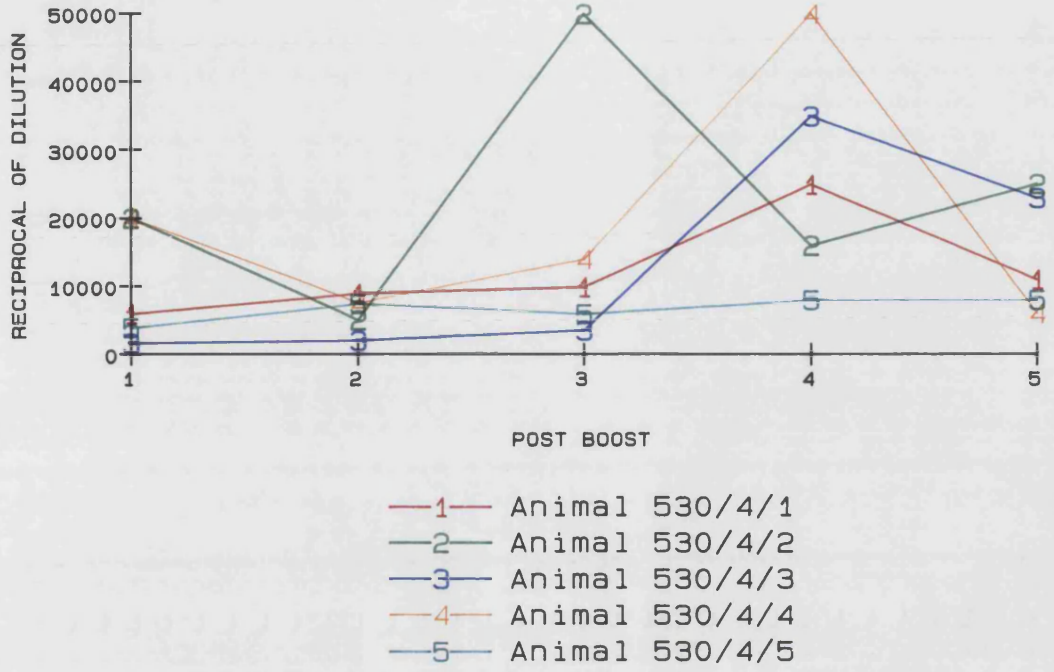


Figure 4.2 ELISA mid-point titres of groups 530/4 and 530/5 assayed against the MN V3 peptide. The sera were assayed seven days post boost.

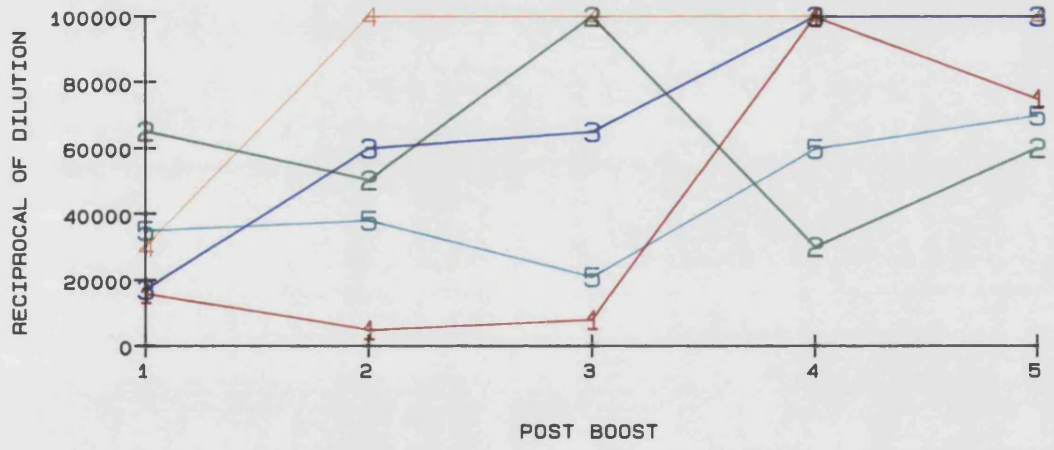
group were variable with maximum responses occurring after boosts three, four or five. Animal 530/4/1 showed a peak response of 1:250 post-boost three, animal 530/4/2 did not respond until after boost five (but showed a significant response of 1:400), animal 530/4/3 failed to respond to the MN V3 loop, animal 530/4/4 showed a peak response of 1:280 post-boost four and animal 530/4/5 only responded after the fourth boost. In contrast, in group 530/5, apart from animal 530/5/3, a general trend in the increase of the response throughout the immunization regime was observed, with peak titres that ranged from 1:300 to 1:1200. It can be seen that higher titres were generated in the group that received the immunizations in conjunction with Freund's adjuvant. These results indicated that to generate a significant response against the MN V3 loop presented on the OGS530 VLPs a potent enhancer of the immune response was required.

The anti-Ty responses of groups 530/4 and 530/5 up to post-boost five are shown in Figure 4.3. In both groups all the animals generated significant anti-Ty responses and it can be seen that higher titres were obtained in group 530/5 where Freund's was used as the adjuvant. In group 530/4 animals 530/4/1, 530/4/3 and 530/4/4 showed peak responses post-boost four of 1:20000, 1:35000 and 1:50000 respectively. The titres of animal 530/4/2 peaked after the third boost at 1:50000 and animal 530/4/5 maintained a relatively low response throughout the immunization schedule. A similar pattern of responses, but with higher titres, was generated in the 530/5 (Freund's) group with peak titres of 1:100000 in three animals occuring after boosts three, four or five. In animal 530/4/4 the peak titre of 1:100000 occurred after the second boost and this titre was maintained up to post-boost five.

In comparing the anti-MN responses with the anti-Ty responses a general trend was observed where peak titres in both groups occurred post-boost three, four or five.



1 Animal 530/4/1
2 Animal 530/4/2
3 Animal 530/4/3
4 Animal 530/4/4
5 Animal 530/4/5



1 Animal 530/5/1
2 Animal 530/5/2
3 Animal 530/5/3
4 Animal 530/5/4
5 Animal 530/5/5

Figure 4.3 Anti-Ty mid-point titres of groups 530/4 and 530/5. The sera were assayed against Ty control particles.

However in individual animals there was little correlation between the maximum responses generated against the V3 loop and the Ty portion of the particle. The peak titres only corresponded in animals 530/4/4 and animal 530/5/5.

4.3.2 Neutralization titres of animals immunized with OGS530 VLPs in conjunction with Freund's adjuvant or aluminium hydroxide

Antisera obtained from the animals in groups 530/4 and 530/5 following boosts four and five were tested in an HIV neutralization assay against the MN isolate. The results are shown in Table 4/1. It can be seen that in the 530/4 group which received the OGS530 VLPs in conjunction with aluminium hydroxide, three of the animals generated low titre neutralizing antibodies. In the 530/5 group which received the immunizations in Freund's adjuvant all five animals generated neutralizing antibodies. Sera from animals 530/5/1 and 530/5/5 had high neutralization titres post-boost five of 1:128. The maximum response of 1:256 elicited by animal 530/5/4 occurred after the fourth boost. These data provided further evidence that a significant response against the MN V3 loop carried by the OGS530 VLPs was only generated in the presence of a powerful enhancer of the immune response, i.e., Freund's adjuvant.

4.3.3 Longevity of the immune response generated against the OGS530 VLPs

The longevity of the immune response generated against the OGS530 VLPs in groups 530/4 and 530/5 was measured using the anti-MN peptide ELISA. Sera taken at monthly intervals after the fifth boost were assayed. In the 530/4 group, anti-MN antibodies were detected in four of the animals four weeks after the final boost. Sera taken up to 24 weeks post-boost five were also assayed, but no antibodies were detected. This indicated that the anti-MN response elicited by the OGS530 VLPs when immunized in conjunction with aluminium hydroxide was not only weak but also

TABLE 4/1. NEUTRALIZATION TITRES OF GROUPS 530/4 AND 530/5 POST BOOSTS 4 AND 5

			Animal		
	530/4/1	**530/4/2**	**530/4/3**	**530/4/4**	**530/4/5**
Post boost					
4	-ve	-ve	-ve	-ve	8
5	16	8	-ve	-ve	-ve

			Animal		
	530/5/1	**530/5/2**	**530/5/3**	**530/5/4**	**530/5/5**
Post boost					
4	32	32	16	256	32
5	128	64	32	128	128

The neutralization titres were expressed as the reciprocal of dilution that gave rise to 90% inhibition of syncytium formation. -ve indicates a neutralization titre of less than or equal to 1:4.

relatively short lived. Table 4/2 gives the anti-MN titres of group 530/5 up to 24 weeks after the fifth boost. In four out of the five animals the anti-peptide response increased up to eight weeks following the fifth boost. This was probably due to the depot effect of the Freund's adjuvant. After 24 weeks, four of the animals had maintained their anti-MN titres which demonstrated that a sustained response against the MN V3 loop was elicited by the OGS530 VLPs when immunizations were carried out in conjunction with Freund's adjuvant.

4.3.4 Recall of the immune response

To test whether a memory response had been induced by the OGS530 VLPs the animals in group 530/4 (aluminium hydroxide) were given a further boost immunization 26 weeks after the fifth boost. At this time only four animals remained in group 530/4. Animal 530/4/2 had died as a result of an accident in the animal house. The remaining animals received 500μg of OGS530 VLPs in conjunction with aluminium hydroxide and were bled weekly for six weeks in order to monitor the response closely. Serum antibody levels were measured against the MN peptide and Ty. A significant increase in the anti-Ty titres was observed. No anti-MN antibodies were detected following the boost which indicated that MN V3 specific-memory cells had not been induced by the OGS530 VLPs. However sera taken three weeks after the sixth boost were tested for the presence of neutralizing antibodies and animal 530/4/1 had a neutralization titre of 1:8. Sera taken after the first boost in the intial regime had not been tested for neutralizing antibodies and it was not therefore possible to determine whether memory B cells had been primed in this animal. However, as only a low titre neutralizing response was detected in just one of the animals post-boost six, this result was not regarded as significant.

TABLE4/2. LONGEVITY OF THE ANTI-MN RESPONSE IN GROUP 530/5

Anti-MN titres were expressed as the reciprocal of the dilution of the mid-point titre.

Animal	**530/5/1**	**530/5/2**	**530/5/3**	**530/5/4**	**530/5/5**
Weeks post boost 5					
4	800	1200	400	2500	150
8	650	1500	500	3000	150
12	500	800	550	1000	150
16	600	600	200	550	100
20	350	350	150	250	0
24	200	400	200	500	0

In order to determine whether a Ty-independent MN-specific memory response had been generated against the V3 loop carried by the OGS530 VLPs, the animals in group 530/5 (Freund's adjuvant) were boosted with a 40 amino acid MN peptide coding for the V3 loop. Each animal received 125μg of the peptide in conjunction with Freund's incomplete adjuvant 26 weeks after the fifth boost with OGS530 VLPs. Sera taken for six weeks following the peptide boost were assayed for the presence of anti-MN and anti-Ty antibodies. Table 4/3 shows the titres obtained pre-boost, one week following the boost and three weeks after the boost when the maximum anti-MN response was observed. As expected, the anti-Ty titres had not increased. Prior to the boost only animal 530/5/4 had a measurable anti-MN response. In four of the animals serum antibodies against the MN peptide were detected one week following the boost and the titres increased up to three weeks after the boost. Sera taken at the time of the peptide boost and three weeks afterwards were tested for the presence of MN neutralizing antibodies. Three of the animals still had neutralizing antibodies present at the time of the boost. However, it can be seen that in four animals a significant increase in the neutralization titres occured following the peptide boost. These results indicated that the immune system had been primed following immunization with OGS530 VLPs in conjunction with Freund's adjuvant and that this anti-MN response was not dependent on the presence of anti-Ty memory cells.

4.4 DISCUSSION

Hybrid Ty-VLPs have been produced that carry the V3 loop from the MN isolate. These OGS530 VLPs were shown to react postitively with a MN V3 specific polyclonal serum and also reacted very strongly with two pools of human HIV positive sera. As mentioned above, this observation supports several reports in the literature that MN-like isolates are the most common sequences identified to date in the population.

TABLE 4/3. ANTIBODY TITRES OF GROUP 530/5 PRE AND POST BOOST SIX

Anti-Ty and anti-MN titres were expressed as the reciprocal of the dilution of the mid-point titre. Neutralization titres were expressed as the reciprocal of dilution that gave rise to 90% inhibition of syncytium formation:

Animal	**530/5/1**	**530/5/2**	**530/5/3**	**530/5/4**	**530/5/5**
Anti-Ty titres					
pre boost	30000	40000	100000	100000	10000
1 week post boost	20000	60000	100000	100000	8000
3 weeks post boost	13000	50000	100000	100000	4500
Anti-MN titres					
Pre boost	0	0	0	100	0
1 week post boost	300	1100	200	600	0
3 weeks post boost	1900	1900	600	3000	0
Neutralization titres					
Pre boost	0	16	0	64	4
3 weeks post boost	256	512	32	512	8

To analyse the immunogenicity of the OGS530 VLPs rabbits have been immunized with the VLPs either in conjunction with aluminium hydroxide or Freund's adjuvant. In contrast to the results obtained with the OGS514 (HXB2) VLPs (chapter 3), where a similar response was generated irrespective of the adjuvant used, different responses were seen in the two groups of rabbits that received the OGS530 VLPs. A comparison of the responses induced by the OGS514 and the OGS530 VLPs is made in chapter 8.

Anti-MN serum and virus neutralizing antibody titres have also been measured. In comparing the levels of anti-MN antibodies between groups 530/4 and 530/5, higher maximum titres were observed in the 530/5 group although several animals in each group had similar titres. However, when the antisera were tested for the presence of neutralizing antibodies a significant difference in the response was observed. All five animals in group 530/5 generated neutralizing antibodies, with high titre neutralizing antibodies present in three of the animals. In contrast, only three of the animals in group 530/4 had neutralizing antibodies and these were of low titre. Comparison of the serum and neutralization titres indicated that there was some discrepancy and suggested that the ELISA assay was not detecting all of the antibodies present. A possible explanation could be that when the MN peptide was coated onto the ELISA plate it was not presented in the correct way to be recognized by the some of the anti-V3 loop antibodies, and in particular the antibody population that neutralized the virus. When antisera raised against the OGS514 (HXB2) VLPs were tested in an ELISA against either a HXB2 V3 peptide or IIIB rgp120 as the coating antigen, much lower titres were recorded against the peptide (data not shown). Taken together, these data indicate that peptides are not particularly effective for detecting V3 loop antibodies. This may well account for the differences observed between the relative serum and neutralizing titres in groups 530/4 and 530/5.

Anaylsis of the longevity of the response in groups 530/4 and 530/5 also showed significant differences between the groups. When the OGS530 VLPs were immunized in conjunction with aluminium hydroxide (group 530/4), the response following the last boost was relatively short lived. In contrast, a sustained response was observed when the OGS530 VLPs were immunized in Freund's adjuvant. This effect was probably due to the depot effect of this adjuvant.

When group 530/4 were tested for a recall response to the OGS530 VLPs it was found that memory B cells had not been primed. This may be because the MN V3 loop on the VLPs is only weakly immunogenic when immunizations are carried out with aluminium hydroxide.

A Ty-independent memory response was observed when the animals in group 530/5 received a boost with MN V3 peptide. Neutralization titres increased significantly which suggested that the immune system had been primed to generate neutralizing antibodies following immunizations with the OGS530 VLPs in conjunction with Freund's adjuvant. However, this result could indicate that the MN peptide itself is strongly immunogenic. This seems unlikely as Javerhian et al. (1989) reported an anti-MN peptide neutralization titre of 1:20. To investigate whether the high neutralizing titres seen following the peptide boost in group 530/5 were entirely due to priming of the immune system by the OGS530 VLPs it would be neccesary to carry out an experiment using the MN V3 peptide as both the priming and boosting immunogen.

The results presented in this chapter indicate that a substantial anti-MN response was only elicited by OGS530 VLPs in the presence of an immune response potentiator. Although the MN V3 loop may be correctly presented on the OGS530 VLPs it is possible that the sequence itself is not intrinsically immunogenic, thereby resulting in

weak responses following immunization with aluminium hydroxide. Ideally, an HIV vaccine candidate should contain a component that elicits the production of anti-MN neutralizing antibodies and it may therefore be necessary to explore alternative methods in order to increase the immunogenicity of the MN V3 loop sequence.

CHAPTER 5

PRESENTATION OF MULTIPLE V3 LOOPS

5.0 INTRODUCTION

The V3 loop is a hypervariable sequence and neutralizing antibodies raised against this region are type-specific. However, an HIV vaccine candidate needs to be able to elicit the production of antibodies that will neutralize a broad range of HIV isolates. A possible way of achieving this would be to present V3 loop sequences from different isolates. The hybrid Ty-VLP system provides several routes for presenting multiple V3 loops to the immune system. One approach would be to produce hybrid Ty-VLPs carrying single V3 loop sequences from different isolates and then make a "cocktail" of the different V3:Ty-VLPs to be used as the immunogen. An alternative would be to produce a hybrid Ty-VLP that carries multiple V3 loop sequences. These two approaches have been investigated and the results are presented below.

5.1 IMMUNE RESPONSES GENERATED AGAINST A COCKTAIL OF V3:Ty-VLPs

Hybrid Ty-VLPs were produced that carried V3 loop sequences from divergent HIV isolates. The isolates used and their origins are:

Isolate	Country of origin	Reference
BH10	USA	Ratner et al., 1985
HXB2	USA	Fisher et al., 1985
RF	HAITI	Starcich et al., 1986
MAL	ZAIRE	Alizon et al., 1986
ELI	ZAIRE	Alizon et al., 1986

The amino acid sequences of the different V3 loops are shown in Table 5/1.

TABLE 5/1. V3 LOOP AMINO ACID SEQUENCES

SNCTRPNNNTRKSIRIQRGPGRAFVTIGKIGNMRQAHCNISG	BH10
SNCTRPNNNTRKRIRIQRGPGRAFVTIGKIGNMRQAHCNISG	HXB2
SNCTRPGNNTRRGIHFGPGQALYTTGIVDEIRRAYCNISG	MAL
SNCTRPGNNTRRGIHFGPGQALYTTGIVDIRRAYCTING	MAL CORRECT
SNCTRPNNNTRKSITKQRGPGRVLYATGQIIGDIRKAHCNSIG	RF
SNCTRPNNNTRKSITKGPGRVIYATGQIIGDIRKAHCNLSGS	RF CORRECT
STCARPYQNTRQRTPIGLGQSLYTTRGRTKIIGQAHCNISG	ELI
STCARPYQNTRQRTPIGLGQSLYTTRSRSIIGQAHCNISG	ELI CORRECT

Synthetic oligomers coding for the different V3 loops were cloned into pSP46 and then into the VLP expression vectors as described in chapter 3, section 3.1. The expression vectors coding for the p1:V3 fusion proteins were designated plasmid numbers as follows:

Isolate	Plasmid
BH10	pOGS513
HXB2	pOGS514
RF	pOGS515
MAL	pOGS516
ELI	pOGS517

Particle preparations were carried out for the five different VLPs using the method described in chapter 2, section 2.13. When pOGS517 (ELI) was transformed into yeast the cells grew very slowly and the yield of hybrid VLPs purified from the cells was extremely low. Therefore the p1:V3 (ELI) fusion gene was cloned into the VLP expression vector pOGS40 containing the PGK:GAL inducible promoter (chapter 1, section 1.4) and the resulting plasmid was designated pOGS519. Hybrid VLPs containing the V3 loop from the ELI isolate were therefore purified from cells transformed with pOGS519.

The sequence information from which the RF, MAL and ELI V3 loop oligomers were originally designed was subsequently found to be incorrect. This finding only came to light after the OGS515, OGS516 and OGS519 VLPs had been made and the immunizations completed. For comparison, the correct sequences are also given in Table 5/1.

OGS515 (RF) VLPs were shown to react positively with an antiserum raised against a RF V3 loop peptide (a gift from Dr. Dani Bolognesi, Duke University). Antisera reactive with the V3 loops from the MAL and ELI isolates was not available to test the OGS516 and the OGS519 VLPs.

Initially the various V3:Ty-VLPs were used to immunize groups of three rabbits as single immunogens in conjunction with Freund's adjuvant. The immunization schedule was as described in chapter 3, section 3.4. Each rabbit received a priming immunization and a total of eight boosts of 500μg of VLPs at two weekly intervals. The immune responses generated by hybrid Ty-VLPs OGS513 and OGS514 carrying the V3 loops from the BH10 and HXB2 clones of the isolate IIIB were described previously in chapter 3. Both VLPs generated IIIB-specific neutralizing antibodies with the highest titres elicited by OGS514 VLPs. Antisera raised against OGS515 (RF) VLPs were tested for the presence of antibodies that neutralized the RF isolate. Neutralizing antibodies were generated against OGS515 VLPs and peak titres of 1/64 in two animals occurred after the fifth boost. The relatively low levels of RF neutralizing antibodies generated by the OGS515 VLPs may be due to differences in V3 sequences between the immunizing antigen and the test virus. Antisera raised against OGS516 (MAL) and OGS519 (ELI) VLPs could not be tested for neutralizing antibodies as the virus isolates were not available. However, the three animals that received OGS516 and OGS519 VLPs had induced antibodies that reacted with MAL and ELI V3 peptides respectively.

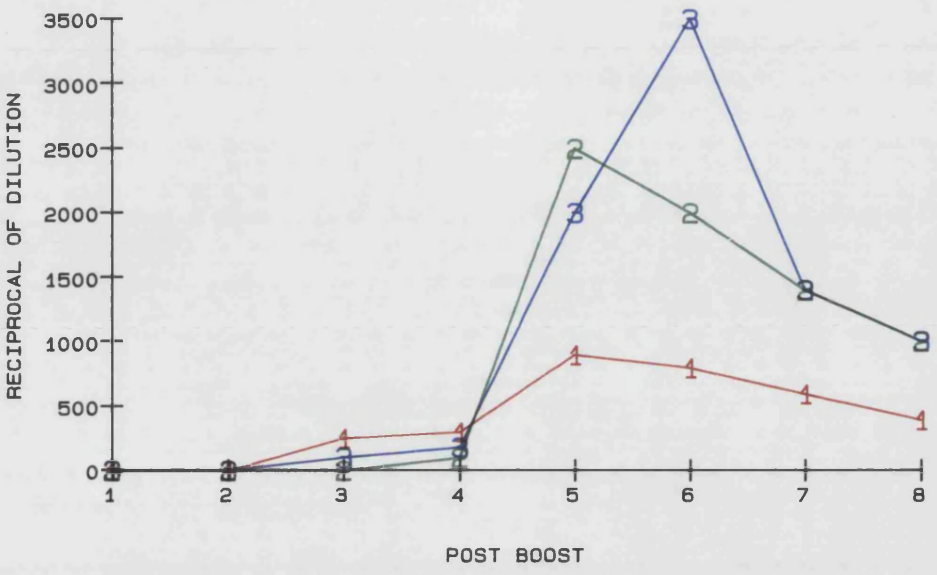
The immunizations carried out seperately with the five hybrid V3:Ty-VLPs demonstrated that each VLP elicited a type-specific response. A further group of three rabbits was then immunized with a "cocktail" of OGS513, OGS514, OGS515, OGS516 and OGS519 VLPs, in conjunction with Freund's adjuvant. The immunization schedule was the same as for the immunizations when the VLPs were

used as single immunogens and each rabbit received 500μg of each VLP per dose. Therefore a total of 2.5mg of VLPs was administered in each immunization. The group was designated MIX5 and the serum and neutralizing antibodies induced are described below.

5.1.1 Serum antibody titres of animals immunized with the cocktail of V3:Ty-VLPs

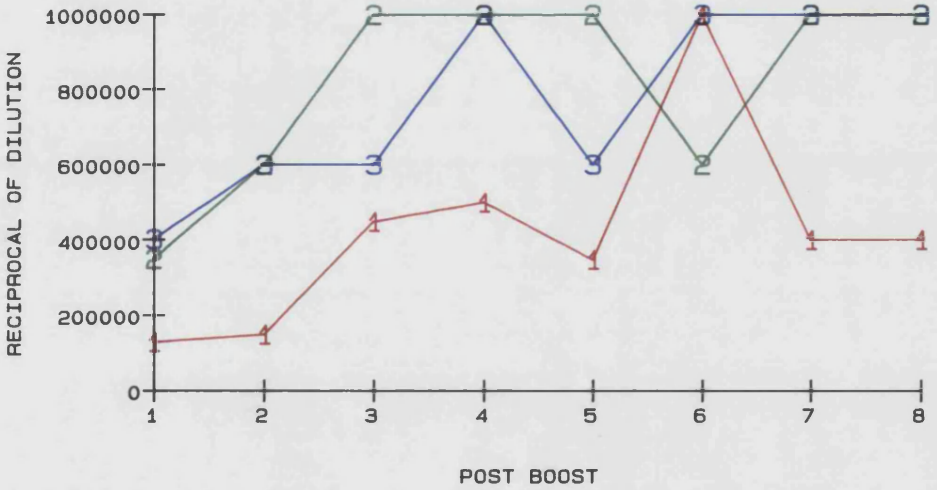
Serum antibody titres were determined against rgp120 (IIIB isolate) using the ELISA assay described in chapter 2, section 2.18. Figure 5.1a shows the titres after each boost. It can be seen that anti-gp120 antibodies were not detected in any of the animals until the third boost had been administered. Animals MIX5/2 and MIX5/3 only produced substantial titres after the fifth boost. Animal MIX5/1 also generated peak titres post-boost five, although only relatively low levels of antibodies were detected in this animal. Sera from the animals in group MIX5 were also assayed against peptides containing V3 loop sequences of isolates RF, MAL and ELI (a gift from Dr. Dani Bolognesi). Animal MIX5/2 had low levels of antibodies against RF and animal MIX5/1 had generated antibodies against ELI. None of the animals had detectable anti-MAL antibodies.

Figure 5.1b shows the anti-Ty titres of group MIX5. The sera were assayed against MA5620 (control Ty) particles. All of the animals generated high levels of anti-Ty antibodies, although the response in animal MIX5/1 was generally lower than in animals MIX5/2 and MIX5/3. This indicated that the intrinsic ability of animal MIX5/1 to respond to the VLPs was lower.



5.1a Anti-gp120 mid-point titres

—1— Animal MIX5/1
—2— Animal MIX5/2
—3— Animal MIX5/3



5.1b Anti-Ty mid-point titres

—1— Animal MIX5/1
—2— Animal MIX5/2
—3— Animal MIX5/3

Figure 5.1 ELISA mid-point titres of group MIX5.

5.1.2 Neutralization titres of animals immunized with the cocktail of V3:Ty-VLPs

As mentioned previously neutralization of MAL and ELI could not be tested as the isolates were not available. Antisera raised against the cocktail of hybrid V3:Ty-VLPs in group MIX5 were tested for the presence of neutralizing antibodies against isolate IIIB. The neutralization titres are shown in Table 5/2. Neutralizing antibodies were not detected until after boost four, with substantial responses observed after the fifth boost in animals MIX5/2 and MIX5/3. Animal MIX5/2 maintained high titres of neutralizing antibodies up to post-boost seven. Animal MIX5/1 generated relatively low levels of neutralizing antibodies after the fifth and seventh boosts. Sera taken following boost five were also tested for anti-RF neutralizing antibodies. Animals MIX5/2 and MIX5/3 had neutralization titres of 1/16 against RF.

A general correlation was observed between anti-rgp120 (IIIB) antibody and anti-IIIB neutralization titres, with substantial responses detected only after the fifth boost was administered. In contrast, animals immunized with OGS514 (HXB2) VLPs alone had both anti-gp120 and neutralizing antibodies after a single boost (see chapter 3). This result indicated that a frequent immunization regime using a mixture of hybrid V3:Ty-VLPs did not result in the rapid induction of V3-specific antibodies, with at least four boosts being required to stimulate a response. In this respect a single VLP immunogen would be a better vaccine candidate where responses need to be elicited after a minimum number of immunizations.

5.2 CONSTRUCTION AND PRODUCTION OF HYBRID HIV:Ty-VLPs CARRYING MULTIPLE V3 LOOPS

In an attempt to generate a combined early and cross-reactive response against hybrid HIV V3:Ty-VLPs, particles were constructed that carry V3 loop sequences from

TABLE 5/2. NEUTRALIZATION TITRES OF ANIMALS IN GROUP MIX5

Animal Post Boost	MIX5/1	MIX5/2	MIX5/3
1	-ve	-ve	-ve
2	-ve	-ve	-ve
3	-ve	-ve	-ve
4	16	16	8
5	32	256	128
6	16	512	128
7	32	512	64
8	4	64	32

The neutralization titres against the HIV isolate IIIB were determined and expressed as the reciprocal of dilution that gave rise to 90% inhibition of syncytium formation. -ve indicates a neutralization titre of less than or equal to 1:4.

isolates HXB2, MN and RF in tandem. The construction of these VLPs is shown schematically in Figure 5.2. Oligomers encoding the V3 loop sequences were first cloned into pSP46 so that the BglII-BamHI fragments could be then be excised for linking together into one vector. The genes encoding the V3 loops were cloned into the VLP expression vector pOGS40 that contains the PGK:GAL inducible promoter (chapter 1, section 1.4) because the MN sequence was toxic to the yeast cells when expressed from the constitutive PGK promoter (chapter 4, section 4.1).

The gene coding for the HXB2 V3 loop was cloned into the BamHI site of pOGS40 and the resulting plasmid designated pOGS531.

The gene encoding the MN V3 loop was inserted into the BamHI site of pOGS531 to generate plasmid pOGS560. pOGS560 therefore contained sequences that coded for the HXB2 and MN V3 loops.

As the original oligomers containing the V3 loop sequence were incorrect new oligomers encoding the correct RF V3 loop sequence were synthesized and cloned into pSP46 to generate plasmid pOGS547. The RF V3 loop fragment from pOGS547 was then inserted into the BamHI site of pOGS560. The resulting plasmid, pOGS561, therefore contained the HXB2:MN:RF V3 loop sequences in a tandem array at the C-terminus of p1.

When particle preparations were carried out using the standard VLP production method described in chapter 2, section 2.13, the yield of OGS561 VLPs was extremely low. Analysis of samples taken throughout the preparation showed that substantial losses of the VLPs occurred at the chromatography stage. A modified purification strategy that produced higher yields of the OGS561 VLPs was therefore developed.

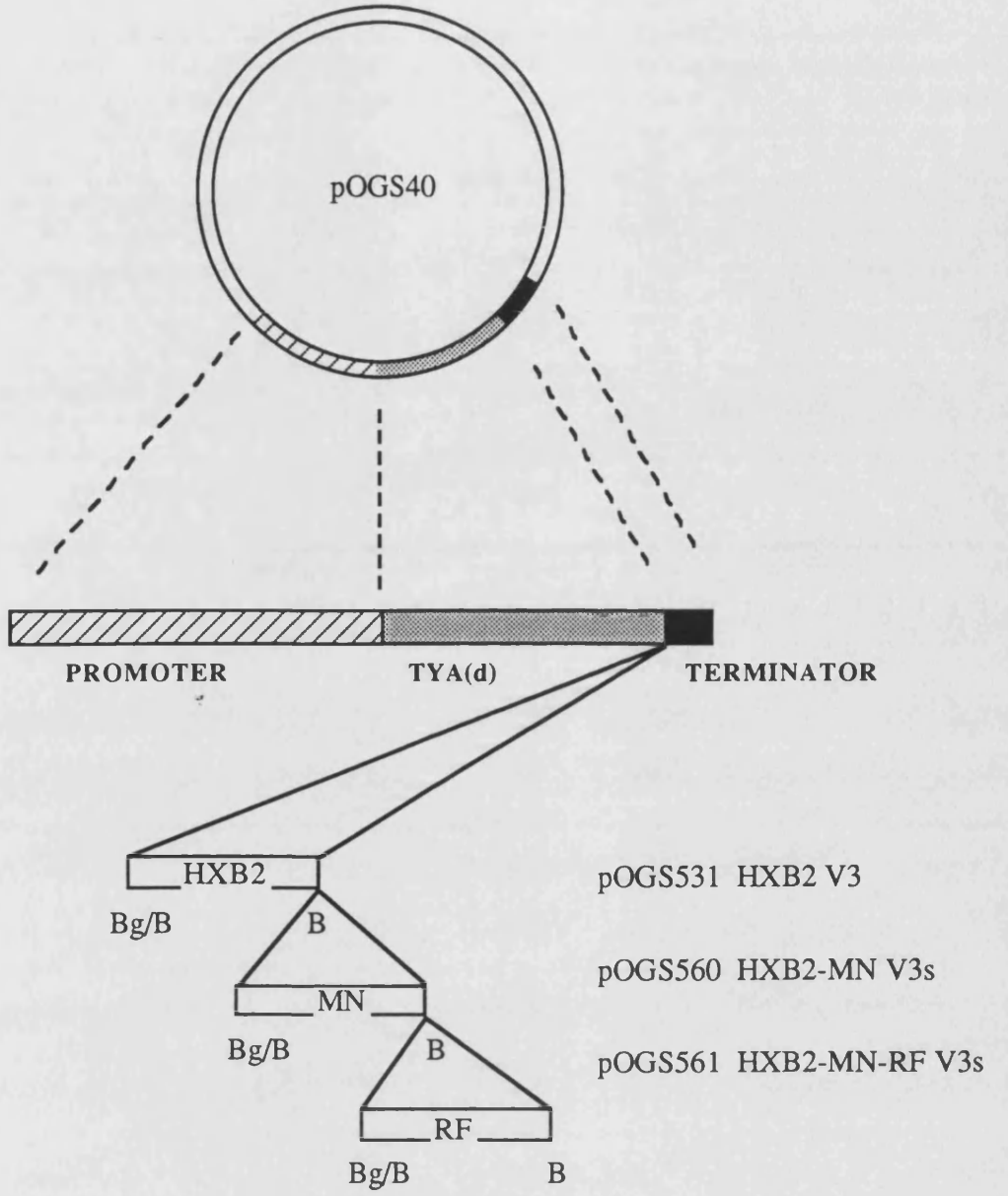
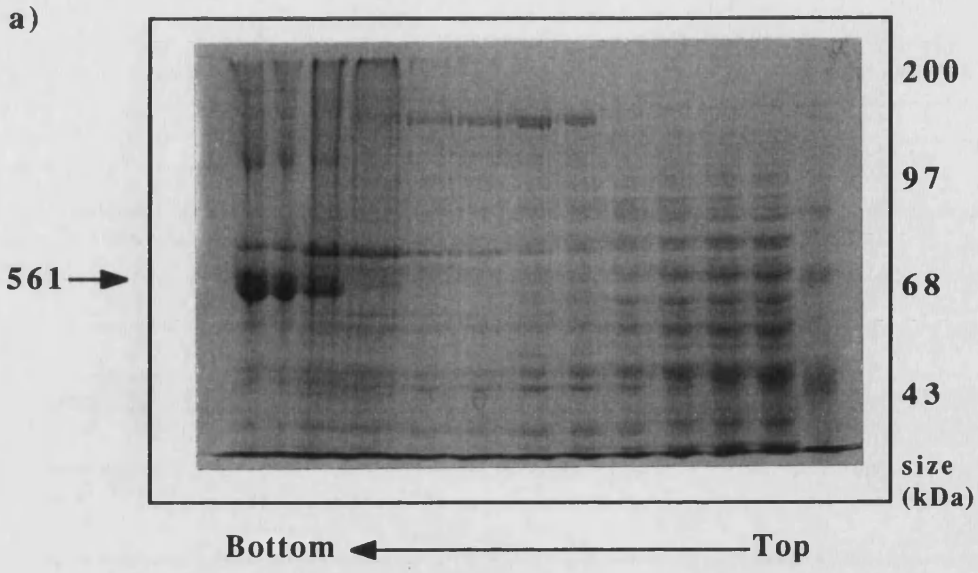


Figure 5.2 Construction of expression vectors for VLPs containing combinations of V3 loop sequences

Yeast cells were broken using a hand bead-beating method. Packed cells (5ml) were resuspended in 10ml of TEN buffer and 5g of glass beads added. The cells were broken by vortexing vigorously for ten minutes, with periods of thirty seconds of vortexing interspersed with thirty seconds on ice. The supernatant was then collected following removal of cell debris by centrifugation at 3krpm (Sorvall RT6000B centrifuge) for five minutes. The procedure was repeated twice more and the resulting supernatants pooled. The supernatant was centrifuged at 9krpm (Sorvall RC5B centrifuge, Sorvall SA600 rotor) for twenty minutes and then centrifuged at 41krpm (Beckman L8-70M ultracentrifuge, SW41 rotor) for one and a half hours onto a 60% sucrose/TEN cushion. The cushion was removed and dialysed overnight against TEN buffer. The dialysate was centrifuged at 9krpm (Sorvall RC5B centifuge, Sorvall SA600 rotor) for twenty minutes and the supernatant was loaded onto a 5-20% sucrose gradient containing a 60% sucrose cushion at the bottom. The gradient was centrifuged at 41krpm (Beckman L8-70M ultracentrifuge, SW41 rotor) for two and a half hours and then fractionated into 1ml fractions. An SDS-PAGE gel of the sucrose gradient fractions is shown in Figure 5.3. The gel shows that the majority of the contaminating yeast proteins remained in the gradient whereas the OGS561 particles were found in the cushion. The cushion was collected and dialysed against PBS buffer overnight, followed by centrifugation of the dialysate at 9krpm (Sorvall RC5B centifuge, Sorvall SA600 rotor) for twenty minutes to remove debris. Figure 5.3 also shows the final material produced from this preparation from which 21mg of OGS561 VLPs were purified from 20ml of packed cells.

When purified OGS561 VLPs were examined by transmission electron microscopy, many of the VLPs appeared to have aggregated into chains (Figure 5.4). This aggregation effect was not observed with VLPs that carried a single V3 loop and possibly accounted for the losses incurred in the standard VLP preparation.



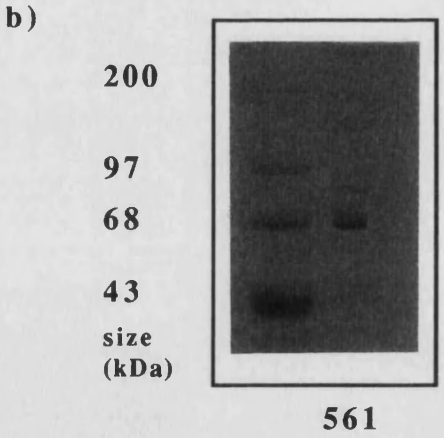


Figure 5.3 SDS-PAGE analysis of an OGS561 particle preparation

a) Sucrose gradient fractions. The gradient was fractionated into 1ml fractions.
b) Purified OGS561 particles. 10μg of OGS561 VLPs were loaded.

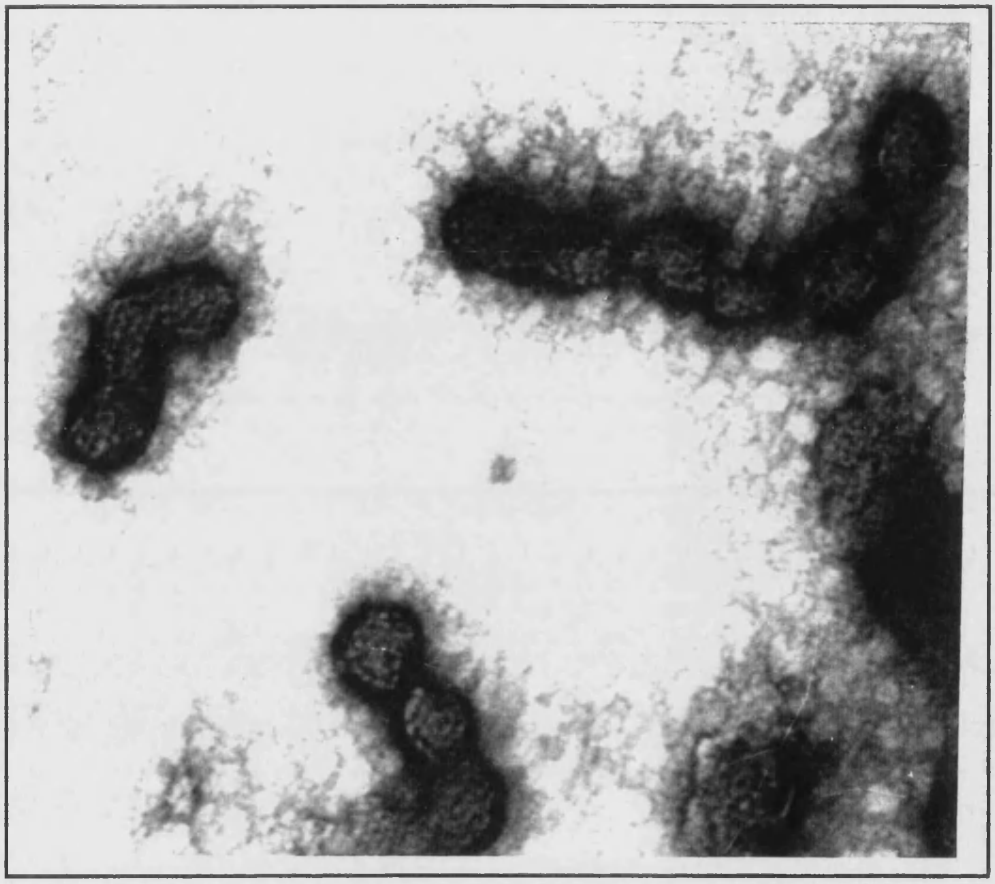


Figure 5.4 Electron micrograph of OGS561 VLPs

The VLPs were stained with uranyl acetate

5.3 WESTERN BLOT ANALYSIS OF OGS561 VLPs

Preliminary characterization of the multiple V3 loop VLPs was carried out by Western blot analysis (Figure 5.5). The OGS560 (HXB2:MN) and OGS561 (HXB2:MN:RF) proteins reacted positively with MAb 9284 (Fig 5.5 (a)), indicating that the HXB2 V3 loop on the multiple loop VLPs was recognised by the IIIB-specific antibody. This Western blot also shows the increase in size of the monomeric proteins of OGS560 and OGS561 as compared to OGS514. When the VLPs were probed with a pool of human HIV positive sera (Fig 5.5 (b)) a strong reaction was observed against both OGS560 and OGS561 proteins. This reactivity was probably due to the presence of the MN V3 loop as previous analyses with human sera had only shown a strong response to OGS530 (MN) VLPs and not to VLPs carrying the HXB2 or RF V3 loops. The OGS561 protein also reacted positively when probed with an antiserum raised against an RF V3 loop peptide (data not shown).

The Western blot analysis therefore indicated that OGS561 VLPs contained regions that were reactive against antisera to the V3 loop sequences HXB2, MN and RF.

5.4 IMMUNE RESPONSES GENERATED BY OGS561 VLPs

A group of five rabbits was immunized in order to determine whether OGS561 VLPs could elicit neutralizing antibodies against the three virus isolates IIIB, MN and RF. The immunizations were administered in conjunction with the clinically relevant adjuvant, aluminium hydroxide. Each rabbit received a priming immunization and eight boosts of 500µg of OGS561 VLPs at two-weekly intervals. The group was designated 561/1, containing animals one to five.

5.4.1 Serum antibody titres of animals immunized with OGS561 VLPs

Serum antibody levels against the three V3 loops carried by OGS561 VLPs were

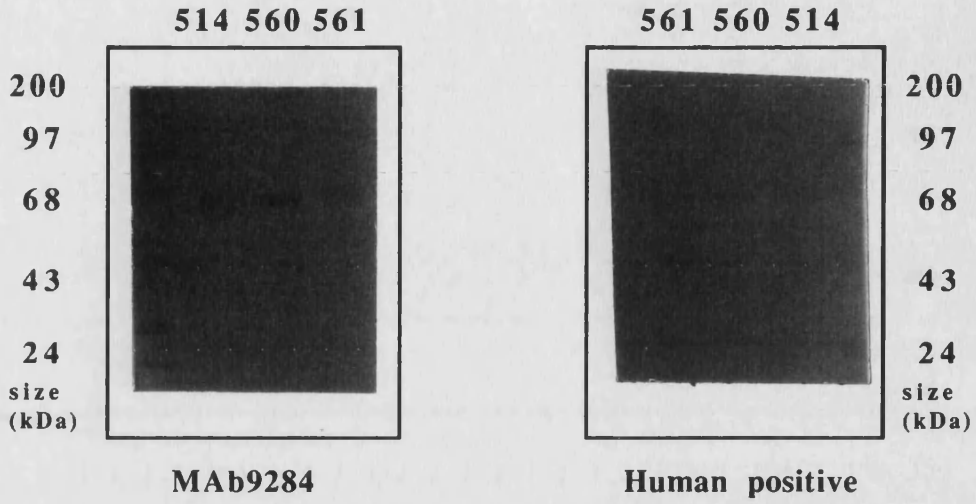


Figure 5.5 Western blot analysis of V3 multiples

OGS514 carried the HXB2 V3 loop, OGS560 carried the HXB2 and the MN V3 loops, and OGS561 carried the HXB2, MN and RF V3 loops

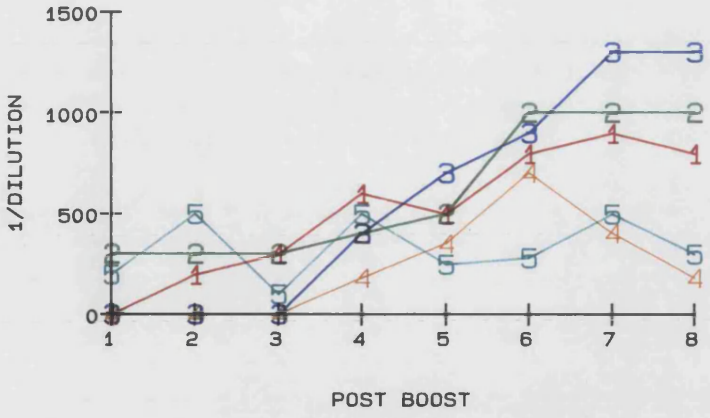
Blot (a) was probed with monoclonal antibody 9284
Blot (b) was probed with human positive sera

measured in an ELISA assay using peptides containing either IIIB, MN or RF V3 loops. Figure 5.6 shows that the five animals generated serum antibodies against all three of the V3 loops. High titres were obtained against the HXB2 and MN peptides, and a lower response was observed against RF. In general, although the antibody levels to each V3 loop differed in individual animals there was a correlation between the pattern of response against each V3 loop throughout the immunization schedule. Responses were not observed in all animals until after the fourth boost was administered, although two animals responded after a single boost immunization.

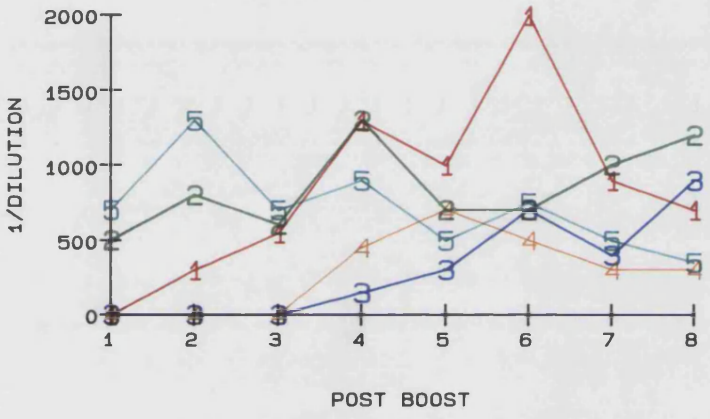
The responses induced against Ty were also measured using MA5620 (control Ty) particles (Figure 5.7). The responses generated in animal 561/1/1 increased gradually after each boost. However, in other animals the anti-Ty titres were variable throughout the immunization schedule. The anti-Ty titres in group 561/1 were comparable to those seen in group 514/2 which received OGS514 (HXB2) VLPs in conjunction with aluminium hydroxide (chapter 3). There appeared to be little correlation between the anti-Ty titres and the anti-V3 titres induced after each boost.

5.4.2 Neutralization titres of animals immunized with OGS561 VLPs

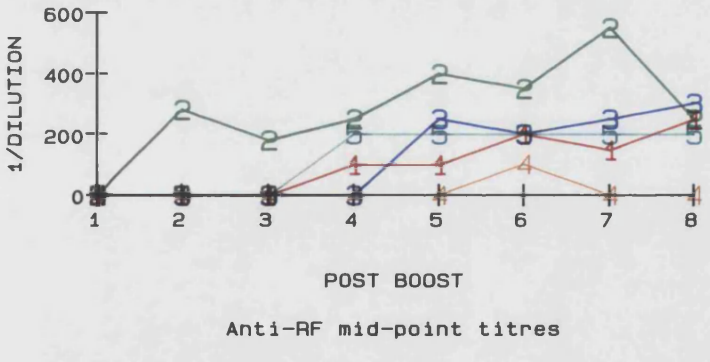
Serum samples taken after boosts three, four, six, seven and eight were tested for neutralizing antibodies against the virus isolates IIIB, MN and RF. Virus neutralizing antibodies were not detected in animal 516/1/1, and animal 561/1/3 only had low levels of neutralizing antibodies against isolate RF following boosts seven and eight. The neutralization titres of animals 561/1/2, 561/1/4 and 561/1/5 are shown in Table 5/3. Only animal 561/1/2 had neutralizing antibodies against all three virus isolates, with a maximum titre of 1/64 against IIIB and MN and low levels of anti-RF antibodies. Animal 561/1/4 generated anti-MN neutralizing antibodies after boost four, although the titre then declined following subsequent boosts. Animal 561/1/5 had a peak anti-MN



Anti-HXB2 mid-point titres



Anti-MN mid-point titres



Anti-RF mid-point titres

1 Animal 561/1/1
2 Animal 561/1/2
3 Animal 561/1/3
4 Animal 561/1/4
5 Animal 561/1/5

Figure 5.6 ELISA mid-point titres of group 561/1

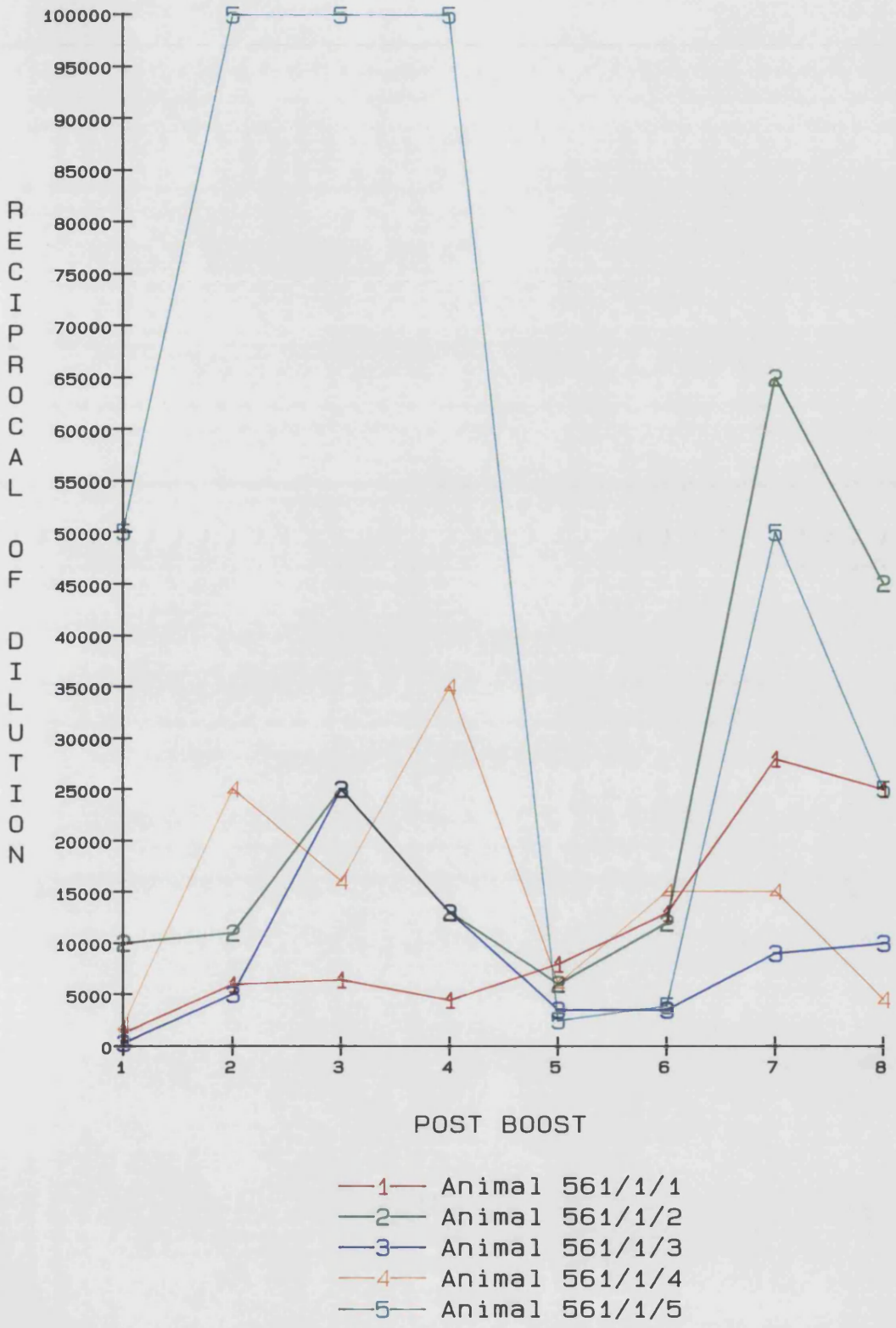


Figure 5.7 Anti-Ty titres of group 561/1. The sera were assayed against control Ty particles.

TABLE 5/3. NEUTRALIZATION TITRES OF ANIMALS 561/1/2, 561/1/4 AND 561/1/5

The neutralization titres against the virus isolates IIIB, MN and RF were determined and the titres expressed as the reciprocal of dilution that gave rise to 90% inhibition of syncytium formation. -ve indicates a neutralization titre of less than or equal to 1:4.

Animal	Virus isolate post boost	IIIB	MN	RF
561/1/2	3	10	-ve	-ve
	4	10	20	-ve
	6	16	32	-ve
	7	32	64	8
	8	64	64	8
561/1/4	3	-ve	-ve	-ve
	4	-ve	20	-ve
	6	-ve	8	-ve
	7	-ve	8	-ve
	8	-ve	-ve	-ve
516/1/5	3	-ve	-ve	-ve
	4	-ve	20	-ve
	6	-ve	32	-ve
	7	-ve	64	-ve
	8	-ve	32	8

neutralization titre after the seventh boost and low levels of anti-RF neutralizing antibodies after boost eight.

No correlation was seen between the anti-V3 antibody titres and neutralization activity. All of the animals had significant anti-HXB2 V3 antibodies whereas only animal 561/1/2 had anti-IIIB neutralizing antibodies. The animals also had high anti-MN serum antibody levels, but only animals 561/1/2 and 561/1/5 had substantial anti-MN neutralization titres. Four animals had anti-RF serum antibodies whereas no animals generated significant levels of anti-RF neutralizing antibodies.

These results demonstrated that antibodies were raised against the V3 loops presented on OGS561 VLPs, but that the majority of the antibodies were not virus-neutralizing antibodies.

5.4.3 Longevity of the immune response

Serum samples taken at four-weekly intervals after the eighth boost were assayed for anti-V3 antibodies. Anti-RF antibodies were only detected in three animals up to four weeks after boost eight. In animal 561/1/1 anti-HXB2 and anti-MN antibodies were detected up to twenty-four and sixteen weeks after the last boost respectively. Animal 561/1/2 had anti-HXB2 and anti-MN antibodies up to eight and sixteen weeks post-boost eight respectively. The responses observed in animal 561/1/3 were similar to those seen in 561/1/2. Animal 516/1/4 had no detectable anti-V3 loop antibodies four weeks after boost eight. Animal 561/1/5 had anti-HXB2 and anti-MN antibodies until four weeks and eight weeks post-boost eight respectively.

In general, responses were detected longer in the animals that had initially generated the highest anti-V3 titres.

5.4.4 Recall of the immune response

To determine whether antigen specific memory cells had been generated following immunizations with OGS561 VLPs in conjunction with aluminium hydroxide, the animals in group 561/1 received a further boost of 500μg of OGS561 VLPs thirty-two weeks after the eighth boost.

Serum samples taken at two-weekly intervals after the ninth boost were assayed for anti-Ty and anti-V3 antibodies. For each animal, similar titres were observed four weeks after boost nine as the maximum response induced during the intial immunization regime. This result therefore indicated that a memory response had been generated against both the Ty and HIV components of OGS561 VLPs.

Sera taken four weeks post-boost nine were also tested for the presence of neutralizing antibodies against virus isolates IIIB, MN and RF. Only two animals showed neutralizing activity. Animal 561/1/2 had a substantial anti-MN neutralization titre and low titres against both IIIB and RF. Animal 561/1/5 had a moderate anti-MN response post-boost nine.

These data therefore provided further evidence that although responses, including a memory response, were generated against the V3 loops on the OGS561 VLPs the sequences were not presented in the optimum configuration to elicit the production of high titre cross-neutralizing antibodies.

5.5 DISCUSSION

Experiments have been carried out to determine whether the presentation of multiple V3 loops using the hybrid Ty-VLP system could induce cross-isolate neutralizing antibodies. Two different approaches were investigated, i.e., presenting different V3

loop sequences as a cocktail of hybrid V3:Ty-VLPs, or presenting three V3 loops on a single hybrid particle.

A cocktail of five different V3:Ty-VLPs was used to immunize rabbits, in conjunction with Freund's adjuvant. After the immunizations had been carried out it became apparent that the amino acid sequence of three of the V3 loops used were incorrect. It was not therefore possible to determine whether this immunization strategy would induce a broad range of cross-isolate neutralizing antibodies. The correct V3 sequences carried by OGS513 and OGS514 VLPs were from clones of the IIIB isolate, therefore analysis was concentrated on the antibody response to this virus. OGS513 (BH10) and OGS514 (HXB2) VLPs had both elicited the production of isolate IIIB neutralizing antibodies when used as single immunogens (see chapter 3). In particular, OGS514 VLPs had induced the production of high titre neutralizing antibodies with reactivity being detected in the OGS514-immunized animals after only one boost. However when OGS514 VLPs were used as part of the cocktail, anti-IIIB neutralizing antibodies were not detected until after the fourth boost. There are several potential explanations for this delayed response. One possibility is that the animals in group MIX5 received five times more Ty immunogen per immunization than the animals that had received OGS514 VLPs alone. The Ty component of the hybrid VLPs is a very effective immunogen (see chapters 3 and 4) which elicits high titre anti-Ty antibodies. It is therefore possible that the 2.5mg total dose of VLPs resulted in the immune system seeing mainly Ty epitopes, thereby reducing the effective dose of V3 antigen. However, the anti-Ty titres in the group which received the VLP cocktail were comparable with the anti-Ty titres of the group that received the OGS514 VLPs alone. This suggests that a maximum Ty response was elicited by a 500μg dose and that the difference observed in the V3 responses was not due to an overload of Ty immunogen.

An alternative explanation is that the V3 loop sequence carried by one of the non-IIIB hybrid VLPs is immunodominant and that antibodies were preferentially raised to this sequence. This hypothesis could not be investigated because the V3 loop sequences carried by the other VLPs were incorrect, therefore reagents were not available to assay the responses against these sequences.

Although the VLP cocktail experiment did not show whether significant cross-neutralizing antibodies could be induced, the results indicated that this would not be the optimum way to present multiple sequences. This is because, where tested, multiple boosts were required to elicit the production of neutralizing antibodies. One requirement of a potential vaccine candidate is that a substantial response needs to be generated after administration of a minimum number of boosts. Therefore, even if cross-isolate antibodies had been induced using this immunization strategy, this requirement would not have been fulfilled.

An alternative strategy for inducing cross-neutralizing responses has been investigated by presenting multiple V3 loop sequences on a single hybrid Ty-VLP. OGS561 VLPs were produced which carried the V3 loop sequences from the HXB2, MN and RF isolates. Western blot analysis of OGS561 VLPs showed that immunoreactive sequences to the three virus isolates were present on the VLPs. Rabbits were then immunized with OGS561 VLPs in conjunction with aluminium hydroxide. The results showed that although antibodies were raised against the three different V3 loop sequences presented on OGS561 VLPs, the V3 loops were not presented in an effective configuration that induced high titre cross-neutralizing antibodies.

The V3 loop sequences were presented in tandem as a C-terminal fusion with the particle-forming protein p1. Electron microscopic analysis of the OGS561 VLPs

indicated that the presence of the three V3 loop sequences in some way caused the VLPs to form chain-like structures. The presence of partly-aggregated material, in which the V3 loop presentation may have been sub-optimal, could be responsible for the poor immune responses. It is not known whether the VLPs remained in this aggregated form following adsorption onto aluminium hydroxide. This could be addressed by electron microscopic analysis of a negatively stained mixture of VLP and aluminium hydroxide.

Neutralization titres against the MN isolate were generated in two of the animals and although multiple boosts were required to elicit this response, the titres were higher than those obtained against the MN V3 loop when it was presented alone as OGS530 VLPs. In contrast, high titre anti-IIIB neutralizing antibodies were generated in the majority of the animals immunized with OGS514 (HXB2) VLPs, whereas only one animal immunized with OGS561 VLPs generated neutralizing antibodies against this isolate. These results may indicate that when the three V3 loop sequences, HXB2-MN-RF, were expressed together, the MN loop was optimally presented relative to the HXB2 and RF loops.

Analysis of the immune response elicited by presenting multiple V3 loop sequences using the hybrid Ty-VLP system in two different ways has shown that neither produced high titre cross-neutralizing antibodies after a minimum number of boosts. Alternative methods will therefore need to be explored to induce the broad response that is required of an HIV vaccine.

CHAPTER 6

PRODUCTION AND ANALYSIS OF HYBRID SIV:Ty-VLPs

6.0 INTRODUCTION

Although non-human primates such as chimpanzees and baboons can be infected with HIV, they do not develop disease symptoms. Therefore, challenge experiments with non-human primates, which are expensive and difficult to perform, can only demonstrate protection against infection. In the absence of a suitable disease model for carrying out challenge experiments of potential vaccine candidates against HIV alternative approaches have to be taken. One such approach may be to develop vaccine candidates against simian immunodeficiency virus (SIV) that could be used as the basis for the design of HIV vaccine candidates. For example, the ability of a hybrid SIV:Ty-VLP carring the major neutralizing epitope of SIV to prevent either infection or disease could be tested in a challenge experiment. If successful, this would lend support to the idea of using an analogous HIV construction in humans.

Considerably less is known about the functional and immunogenic regions of the SIV envelope glycoprotein than is known for HIV and, as yet, the principle neutralization determinant has not been conclusively mapped. Sequence alignment of SIV and HIV-1 sequences does not clearly identify an SIV region with strong homology to the V3 loop. However, by aligning conserved cysteine residues within the gp120 envelope proteins it was possible to identify the most likely SIV "V3" sequence. Unlike HIV sequences, there is no conserved GPGR sequence within the SIV V3 loop, and the SIV V3 sequence is not particularly variable between the SIV envelopes that have been characterized by sequence analysis. The putative SIV V3 sequence is found between amino acids 313 and 347 of the envelope glycoprotein gp120. Hybrid SIV:Ty-VLPs were produced that carried this region of isolate SIV mac251. The hybrid SIV:Ty-VLPs have been analysed by Western blot and by immunogenicity studies carried out in rabbits.

Development of SIV neutralization assays is also somewhat behind the development of assays to determine neutralization of HIV. SIV does not cause a rapid cytopathic effect in cell culture. Therefore an assay which monitors the inhibition of syncytium formation, as described for HIV (section 2.15), is not feasible for testing sera for the presence of anti-SIV neutralizing antibodies. An alternative method is to test for SIV antigen levels in infected cell cultures. A decrease in antigen level following pre-incubation of virus with test sera would show that the virus had been neutralized. An assay to test for SIV neutralization by monitoring the levels of the SIV core protein p27 has been developed in conjunction with Dr. Eleanor Berrie.

6.1 CONSTRUCTION AND PRODUCTION OF HYBRID SIV:Ty-VLPs

The construction of hybrid SIV:Ty-VLPs was similar to the construction of HIV:Ty-VLPs as described previously (chapter 3, section 3.1). The amino acid sequence of the SIV envelope region encoded by the synthetic oligomers is shown below:

CRRPGNKTVLPVTIMSGLVFHSQPLTDRPKQAWC SIVmac251

The synthetic oligomers were first cloned into pSP46 and then into the yeast expression vector pMA5620 (see chapter 1, section 1.4). The plasmids were designated pOGS532 and pOGS533 respectively. When pOGS533 was transformed into yeast, only very low yields of VLPs were obtained, indicating that the SIV sequence was toxic to cells. The gene coding for the SIV V3 sequence was therefore cloned into the expression vector pOGS40 containing the inducible PGK:GAL promoter (chapter 1, section 1.4). The resulting plasmid was designated pOGS534. Hybrid SIV:Ty-VLPs were purified as described in chapter 3, section 3.2.

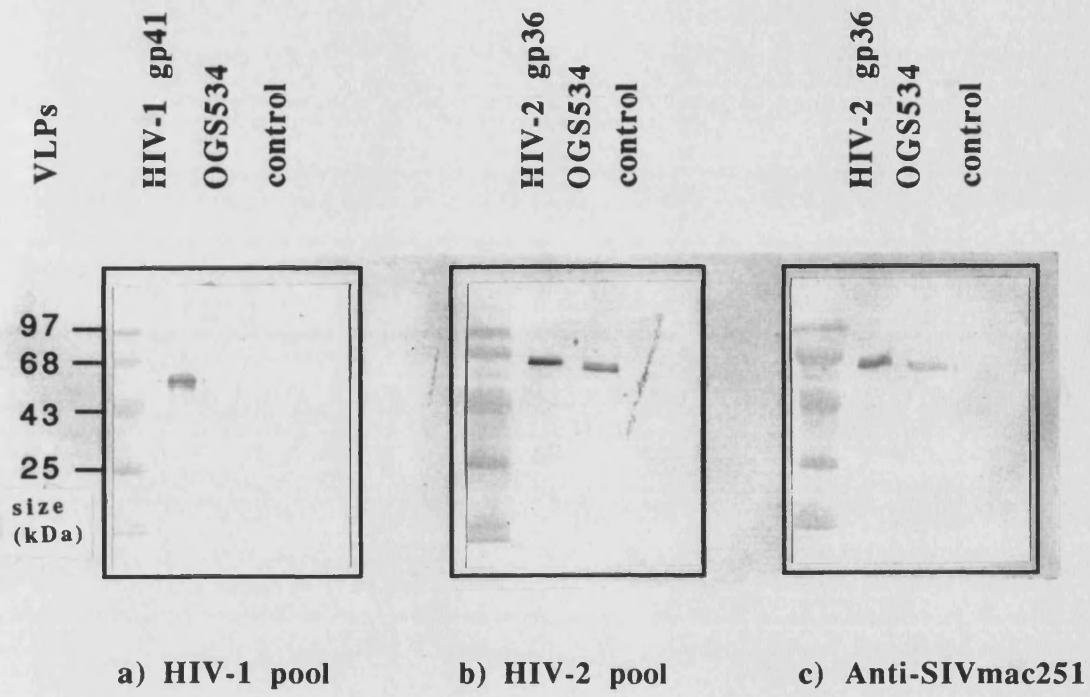
The SIVmac251 V3 sequence contained in the OGS534 VLPs was obtained from the 1988 Los Alamos Data Base. However, in subsequent editions of the data base the sequence given for this region was different. This was only discovered after the immunizations had been carried out. The differences in the amino acid sequences are underlined below:

CRRPGNKTVLPVTIMSGLVFHSQPLTDRPKQAWC SIVmac251 1988
CRRPGNKTVLPVTIMSGLVFHSQPINDRPKQAWC SIVmac251 1991

The peptide used to analyse the reactivity of anti-SIV sera (obtained from the MRC AIDS Directed Progamme) had the sequence from the 1991 Los Alamos database (see section 6.3.1 below). It is not certain which sequences were present in the infectious virus used for neutralization assays because viruses had not been molecularly cloned.

6.2 PRELIMINARY CHARACTERIZATION OF HYBRID SIV:Ty-VLPs

The immunoreactivity of purified hybrid SIV:Ty-VLPs (OGS534) was analysed by Western blot. Figure 6.1 shows the reactivity of VLPs following incubation with HIV and SIV positive sera. VLPs purified from yeast cells transformed with pMA5620 (control Ty) were included as a negative control. The positive control for the blot probed with HIV-1 sera was a VLP construction containing an immunoreactive region of HIV-1 gp41. Similarly, a VLP construction containing an immunoreactive region of HIV-2 gp36 known to react with both HIV-2 or SIV positive sera was used in the blots probed with either HIV-2 and SIV positive sera. OGS534 VLPs gave little reaction when probed with human HIV-1 positive sera (blot (a)). However it can be seen that OGS534 VLPs reacted strongly when probed with either HIV-2 or SIVmac251 positive sera. These Western blots indicated that the region of the SIV envelope carried by the



a) HIV-1 pool b) HIV-2 pool c) Anti-SIVmac251

Figure 6.1 Western blot analysis of hybrid SIV:Ty-VLPs (OGS534)

SIV:Ty-VLPs, control Ty-VLPs and HIV-1 gp41 VLPs or HIV-2 gp36 VLPs were probed with a) a pool of 10 HIV-1 positive sera, b) a pool of 6 HIV-2 positive sera or c) serum from a monkey infected with SIVmac251.

OGS534 VLPs is immunogenic. The cross-reactivity observed with human HIV-2 sera is supported by the significant homology seen between SIV and HIV-2 envelope sequences in this region and indicates that this HIV-2 envelope region could also be immunogenic.

6.3 ANALYSIS OF THE IMMUNE RESPONSE GENERATED AGAINST HYBRID SIV:Ty-VLPs

6.3.1 Serum antibody levels of rabbits immunized with OGS534 VLPs

Initially, five rabbits were immunized with OGS534 VLPs in conjunction with aluminium hydroxide. The group was designated group 534/1. The immunization schedule was the same as that used for immunizations with HIV V3:Ty-VLPs. The animals received 500μg of OGS534 VLPs per dose. Boost immunizations were administered every two weeks until eight boosts had been given. Serum samples were taken every week so that antibody levels could be closely monitored.

The sera taken before and after each boost for each animal in group 534/1 were assayed by ELISA against a 20 amino acid peptide coding for the central region of the SIV V3 sequence. The majority of the samples gave very little reaction against this peptide.

A further ten rabbits were immunized with OGS534 VLPs, five in conjunction with aluminium hydroxide and five in conjunction with Freund's adjuvant. The groups were designated 534/2 and 534/3 respectively. The immunization schedule was the same as for group 534/1. Sera taken from the animals up to the fifth boost were assayed for anti-peptide and anti-Ty responses. The V3 peptide used was the 20 amino acid peptide described above and the Ty responses were measured against control Ty particles, MA5620. The anti-Ty responses were similar to the responses seen against the HIV V3:Ty-VLPs described in chapters 3 and 4, with maximum titres of 1:60000 in group

534/2 and 1:100000 in group 534/3. However, as observed for group 534/1, very little response was detected against the SIV peptide. It was possible that this peptide was not an effective antigen for detecting anti-SIV V3 antibodies. This explanation is suggested by the previous observation that a peptide coding for the central region of the HIV V3 loop (HXB2) did not detect anti-V3 antibodies in anti-OGS514 antisera that had high titre serum antibodies when assayed against rgp120 (data not shown).

Due to concerns about the 20-mer V3 peptide an alternative SIV antigen was investigated in order to detect anti-SIV antibodies in the anti-OGS534 samples. A lysate of cells that had been infected with SIV was obtained from Dr. Karen Kent (NIBSC). A limited amount of this antigen was available, therefore only sera taken after boosts one, five and eight from the three groups of animals immunized with OGS534 VLPs were assayed. Low levels of anti-SIV antibodies were detected and sera giving a positive reaction are shown in Table 6. As the SIV infected cell lysate was known to be effective at detecting anti-gp120 antibodies (Dr. Karen Kent, personal communication), these results suggested that only weak responses had been elicited by OGS534 VLPs.

6.3.2 Development of an SIV neutralization assay

In order to test anti-OGS534 sera for neutralizing antibodies against SIV, and to determine whether the SIV envelope sequence carried by OGS534 VLPs was a neutralization epitope, attempts were made to develop an SIV neutralization assay. During culture of HIV or SIV-infected cells, virus and therefore viral antigens, are released into the culture medium. In particular, core antigens p24 (HIV) and p27 (SIV) are readily detected. The assay was therefore divided into two stages. The first stage was to culture the virus following incubation with anti-OGS534 antisera and the second stage was to assess the levels of SIV core protein p27 present in the cell culture medium. A reduction in p27 levels would indicate that the virus had been neutralized by

TABLE 6. REACTIVITY OF OGS534 ANTISERA AGAINST AN SIV INFECTED CELL LYSATE IN AN ELISA ASSAY

Group	Animal	Post-boost 1	post-boost 5	post-boost 8
534/1	534/1/1	-	-	-
	534/1/2	-	-	-
	534/1/3	-	+	+
	534/1/4	-	-	+
	534/1/5	+	+	+
534/2	534/2/1	-	+	-
	534/2/2	+	+	-
	534/2/3	+	+	-
	534/2/4	+	-	-
	534/2/5	+	-	-
534/3	534/3/1	-	+	+
	534/3/2	-	-	-
	534/3/3	+	+	+
	534/3/4	-	-	-
	534/3/5	-	+	-

Groups 534/1 and 534/2 recieved the immunizations in conjunction with aluminium hydroxide and group 534/3 received the immunizations in conjunction with Freund's adjuvant. + and - indicates an O.D. above or equal to the background O.D. of pre-immune serum respectively.

antibodies in the antisera. The assay is shown diagrammatically in Figure 6.2.

The first stage of the assay was optimised by Dr. Berrie with analysis of various parameters being carried out. For example, different cell lines were examined to determine a cell line to support optimum growth of SIV, and H9 cells were selected for use in the assay. To provide a base line for the assay, the time course of infection was investigated by measuring p27 levels over time. Levels of p27 were measured using the p27 antigen capture assay described below. In general, cells were cultured for 27 days after infection, with viral antigen first being detected between days 16 and 18.

The first step towards developing a sandwich capture p27 ELISA was to identify antibodies that could capture and detect p27 from the cell supernatant fluid of SIV infected cells. Two potential sources of anti-p27 antibodies were available for testing in the ELISA. One source was antisera obtained from a macaque experimentally infected with SIV (a gift from Dr. Kingston Mills, NIBSC). The second source was to raise antisera against VLPs developed by other members of the group which carried the p27 core protein of SIV (OGS249 VLPs). The OGS249 VLPs were constructed as part of another project at British Bio-technology. Five rabbits were immunized with OGS249 VLPs in conjunction with aluminium hydroxide. The immunization schedule was as described above for OGS534 VLPs. Antisera obtained after boost five from each animal was used to probe an SIV infected cell lysate by Western blot. The antisera from all five animals identified a protein band of 27 kD, which indicated that the antisera contained antibodies reactive with p27.

Initially, a rabbit anti-OGS249 antiserum was used to coat ELISA plates to provide capture antibody and the macaque anti-SIV antiserum obtained from NIBSC was used as the detecting antibody. The plates were coated overnight at 4^{o}C with anti-OGS249

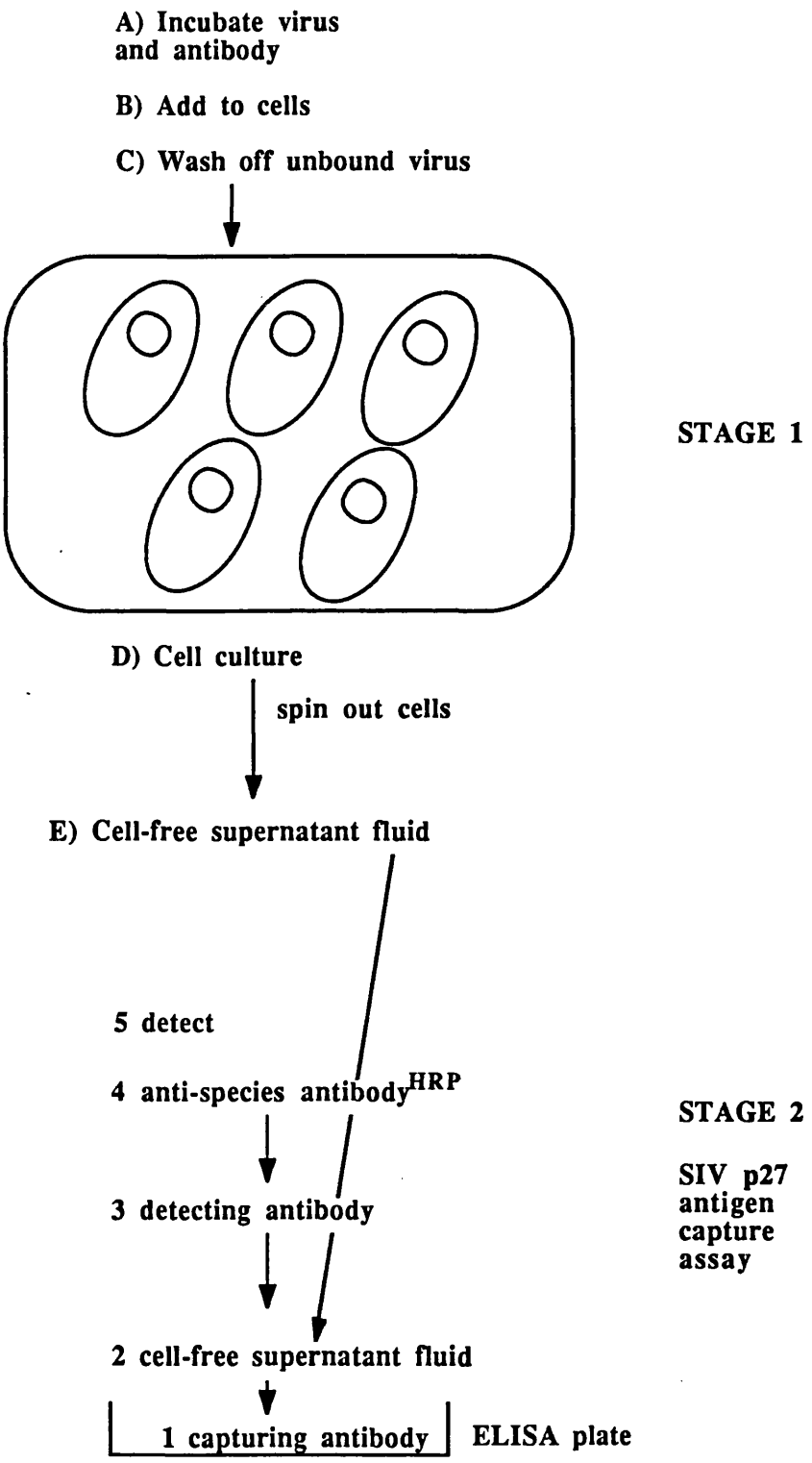


Figure 6.2 Schematic diagram of the SIV neutralization assay

antiserum in a series of dilutions. Plates were also coated with pre-immune sera as a control. All further incubations were carried out for two hours at 4°C and the plates were washed three times in PBS-0.5%Tween between incubations. Following coating, the plates were blocked using PBS-2% casein to prevent non-specific binding. After washing, Triton-X-100-treated supernatant fluid from SIV infected cells was added to the plates. Samples of supernatant fluid from uninfected cells were also included as negative control. After 2 hrs incubation at 4°C the anti-SIV antiserum was added to the plates, again in a series of dilutions. Following incubation of the anti-SIV antiserum and washing, anti-monkey horseradish peroxidase labelled antibodies were added at a single dilution. The plates were developed using hydrogen peroxide in the presence of TMB and the reaction stopped with 2.5M sulphuric acid. The O.D. of the plates were then read at a wavelength of 450nm.

Using this configuration (rabbit anti-OGS249 to capture; macaque anti-SIV to detect) no significant difference was observed between the test (infected cell supernatant) and control (uninfected cell supernatant) samples.

The antigen assay was then carried out in the opposite orientation, using macaque anti-SIV antiserum for capture and rabbit anti-OGS249 antiserum for detection. Figure 6.3 shows the results obtained in this assay. All of the samples were tested in triplicate. The graph shows that differences in optical density (O.D.) readings were observed between test and control samples, although the O.D.s recorded were generally low. Background levels were observed when pre-immune sera was used in the detecting step or when uninfected cell supernatant was used instead of infected cell supernatant. Titration of the detecting antibody (anti-OGS249) against the infected cell supernatant indicated that the reaction was specific. Similar O.D. values were obtained for each of the triplicate samples at each dilution of detecting antibody. Several parameters were

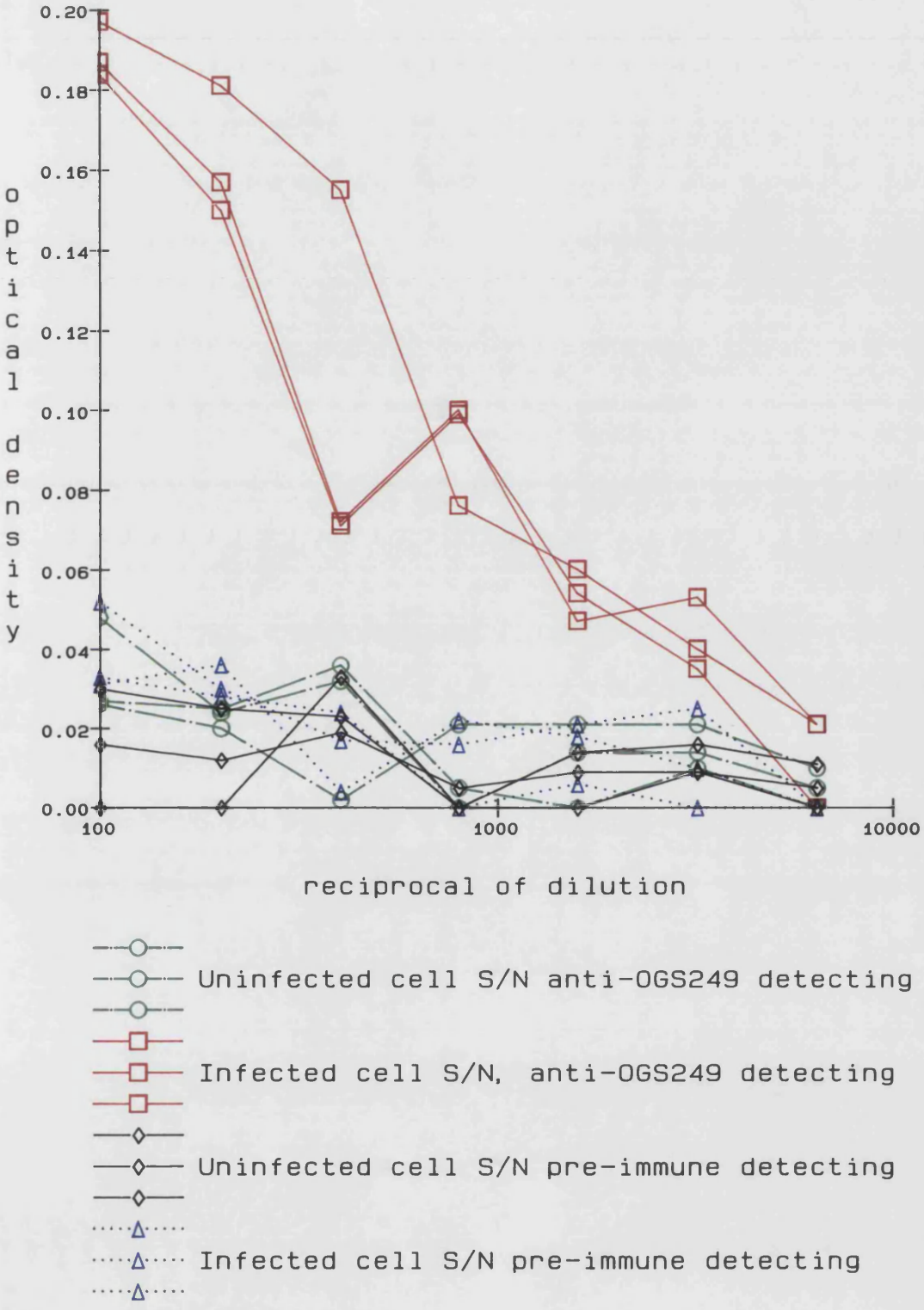


Figure 6.3 p27 antigen capture assay. p27 was captured by the macaque anti-SIV antiserum at 1: 1000.

investigated in order to improve the sensitivity of the assay. These included varying the length and temperature of the incubation times for each of the different stages. However, only incubating the infected cell supernatant at room temperature instead of 4°C resulted in a slight increase in the O.D.s recorded.

Although the assay described above resulted in low O.D.s, the reaction observed was specific and reproducibility was demonstrated between replicates. This assay was therefore used to test serum samples from animals immunized with OGS534 VLPs for the ability to prevent or delay the appearance of p27 antigen in infected cell cultures (see section 6.3.3).

Further studies were conducted to address the sensitvity of the capture assay. The most likely explanation for the low O.D.s recorded was that one or both of the antibodies used was limiting. This would result in either inefficient capture of p27 or inefficient detection of bound p27. Other sources of antibody (HIV-1 human positive sera, mouse anti-OGS249) were also tested but failed to generate higher O.D.s. The next step was to assess a panel of monoclonal antibodies raised against p27 by the Immunology group at British Bio-technology.

The monoclonal antibodies were tested for their ability to capture p27. An example of a test assay to compare two anti-p27 monoclonal antibodies is shown in Figure 6.4. The concentration of each antibody was adjusted so that a 1:10 dilution was equivalent to 50µg/ml. Plates were coated with antibody at doubling dilutions up to a dilution of 1:1280. SIV-infected cell supernatants were tested in duplicate at undiluted, 1:2 and 1:4 concentrations. The rabbit anti-OGS249 antiserum was used as the detecting antibody. The results were expressed as the average of each duplicate supernatant sample. As the graph in Figure 6.4 shows, antibody 12G6 was more effective at capturing p27 than

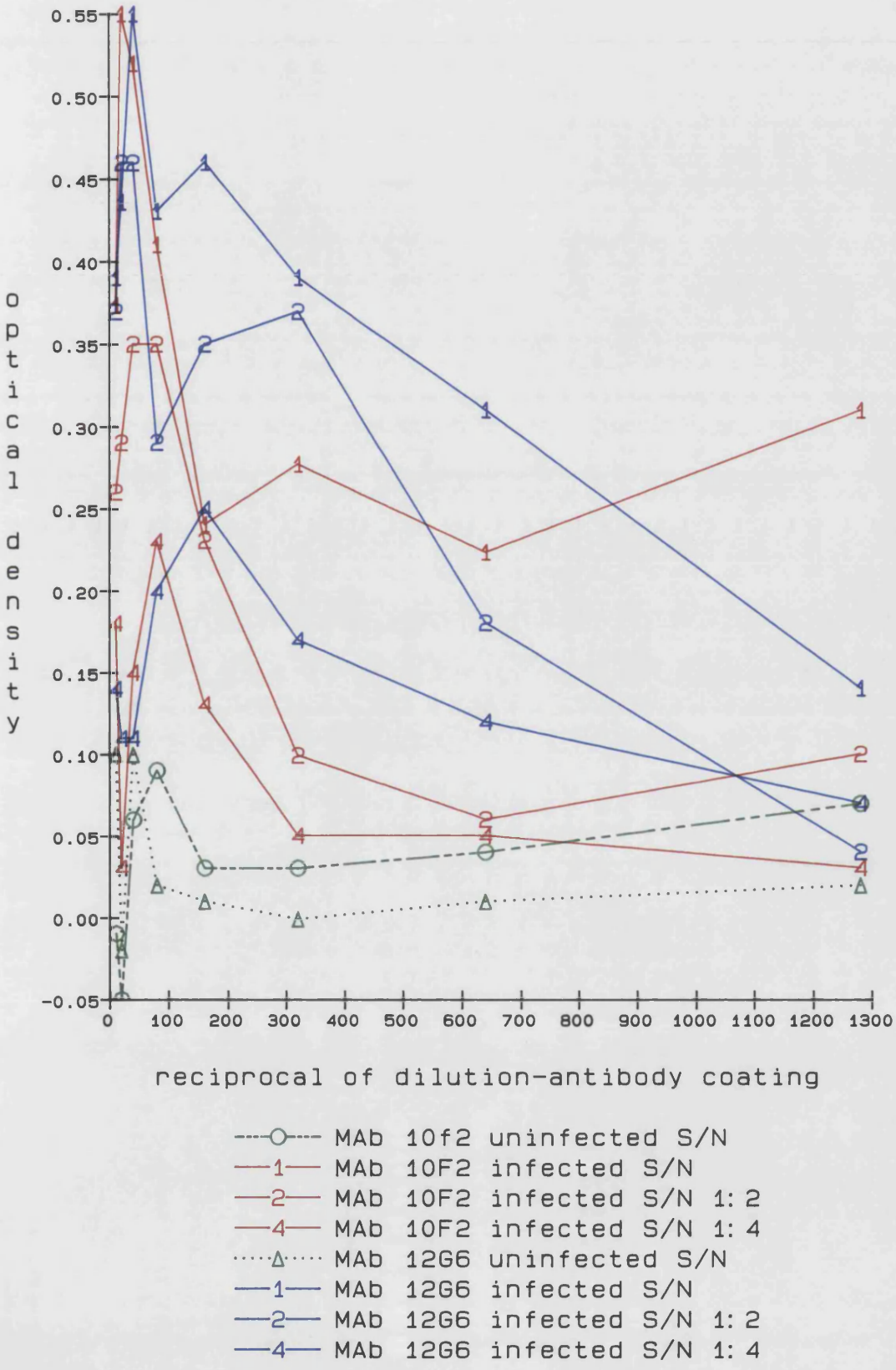


Figure 6.4 An example of a p27 MAb test assay.

antibody 10F2. Antibody 12G6 was found to be the most effective capturing antibody of six monoclonal antibodies tested and this antibody was therefore chosen as the capture antibody for further studies.

Figure 6.5 shows the results obtained with 12G6 as the capture antibody when either the macaque anti-SIV antiserum or the rabbit anti-OGS249 antiserum were used to detect the captured p27. The assay was carried out as described previously. Two different samples of infected cell supernatant fluids were tested in the assay. The results showed that antibodies present in the macaque anti-SIV antiserum were slightly more effective at detecting captured p27 than those in the anti-OGS249 serum. In order to determine the sensitivity of the assay a titration of recombinant p27 (a gift from Dr. Mark Page, NIBSC) was included. The sensitivity of the assay was approximately 50ng/ml and 125ng/ml when the macaque anti-SIV antiserum and rabbit anti-OGS249 antiserum were used as the detecting antibody, respectively.

6.3.3 Induction of SIV neutralizing antibodies by OGS534 VLPs

The cell culture involved in the neutralization assay described in section 6.3.2 has to be carried out under Category III containment conditions and takes 27 days to complete, with samples being taken every two days. Due to the labour intensive nature of this assay, and the limited amount of macaque anti-SIV serum available, only sera from experiment 534/1 (aluminium hydroxide) were tested for the presence of neutralizing antibodies.

First, antisera taken after the fifth boost of animals in group 543/1 were tested. The antisera were diluted 1:10 and then incubated with the virus stock, diluted 1:4, for one hour at 37°C. The virus-antibody mixture was then added to H9 cells. Following a further incubation of two hours at 37°C, unbound virus was washed off and the

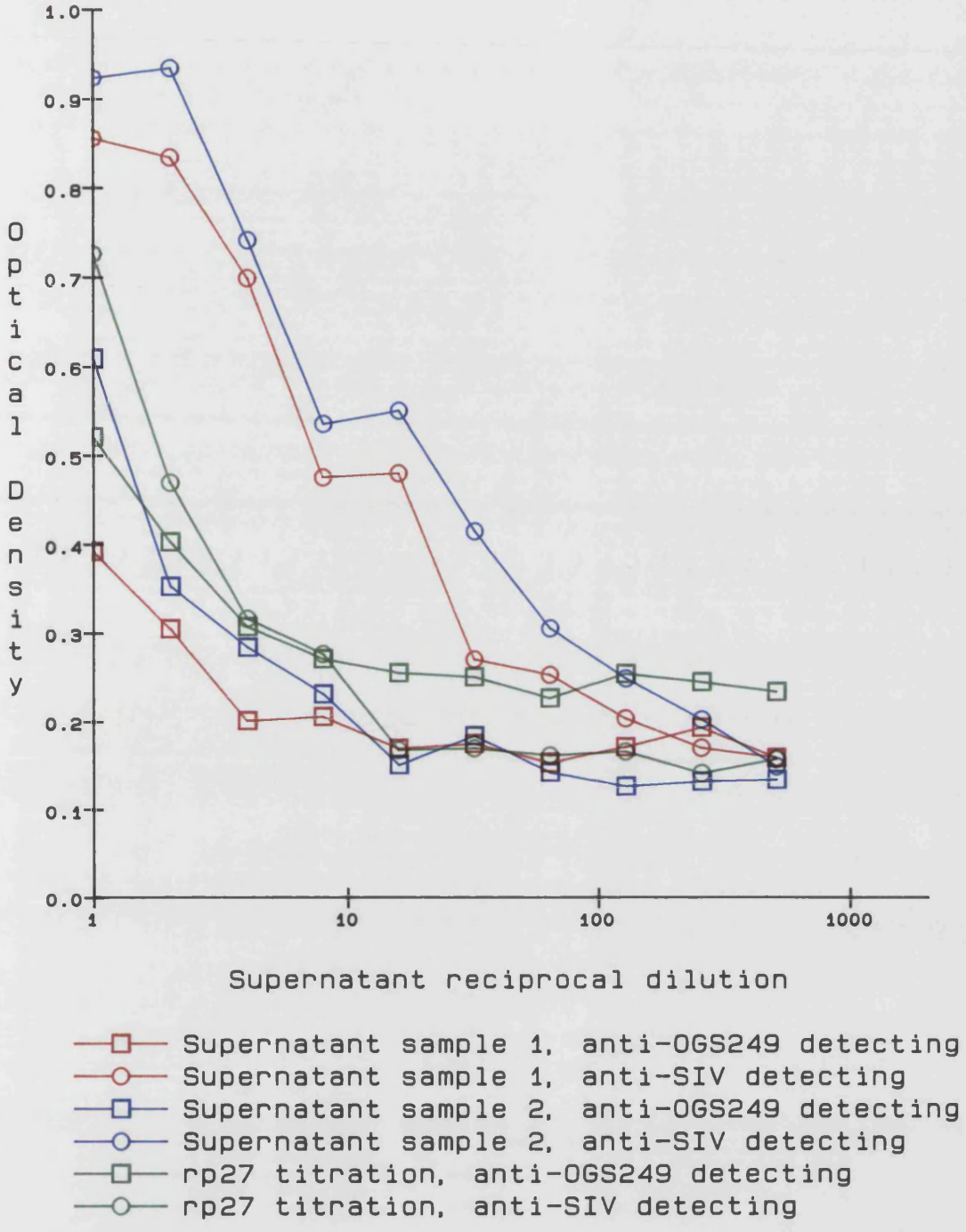
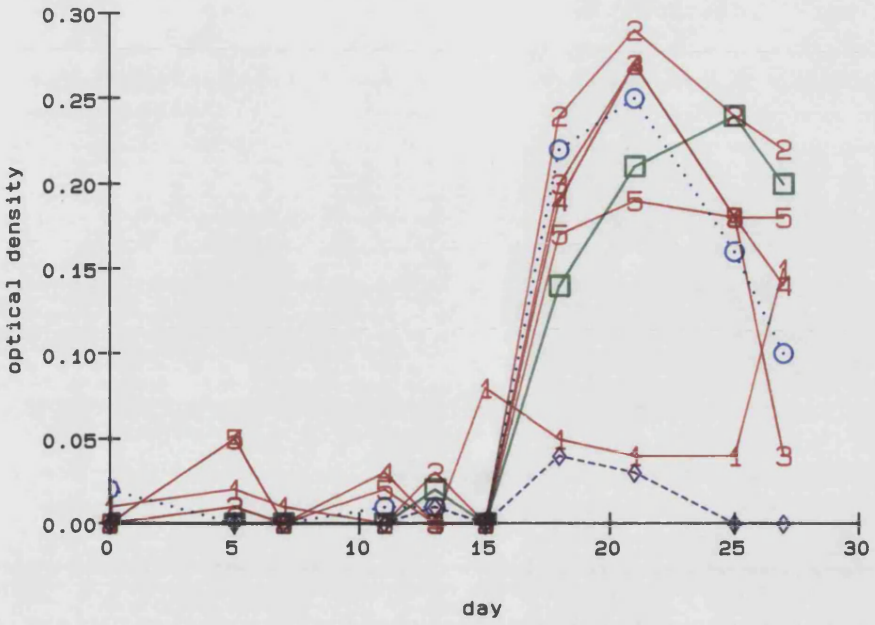


Figure 6.5 SIV antigen capture by MAb12G6 Cell supernatants were titrated in doubling dilutions from neat. Recombinant p27 was titrated in doubling dilutions from 1ug/ml. Anti-OGS249 detecting antibody was used at a dilution of 1:100. Anti-SIV detecting antibody was used at a dilution of 1:1000.

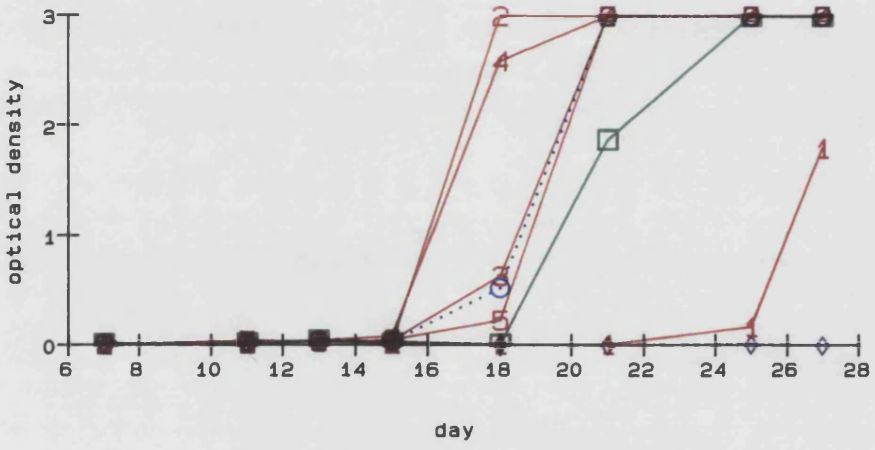
cultures maintained for 27 days. Samples were taken every two days and frozen at -20°C until they could be assayed in the sandwich capture ELISA for p27 levels. The assay used macaque anti-SIV antiserum as the capture antibody and rabbit anti-OGS249 as the detecting antibody (Figure 6.6a). The virus control (no antibody) samples showed that significant p27 levels were not detected until day 17 and that they peaked around day 25. It can be seen that the time course of infection for virus incubated with the anti-OGS534 sera from four of the animals was similar to that of the virus control curve. This indicated that the virus had not been neutralized by sera from animals 543/1/2, 3, 4 or 5. However, antigen levels in the culture medium of cells infected with virus that had been incubated with the serum from animal 534/1/1 remained at basal levels until day 25. This result suggested that neutralizing antibodies against SIV were present.

During the course of these studies a p27 capture ELISA became commercially available from Coulter. The samples tested in the assay described above were therefore also tested in the Coulter assay (Figure 6.6b). Although the O.D.s obtained with this assay were greater than those in the "in-house" assay, the results were generally similar. Pre-immune sera or sera from animals from animals 534/1/2, 3, 4 and 5 did not delay the appearance of p27 in the culture medium, and in some cases antigen was detected before the appearance of p27 in the virus control. However, samples taken after pre-incubation with antiserum from animal 534/1/1 again only showed detectable p27 on day 25.

A second experiment was carried out in which antiserum taken from rabbit 534/1/1 after the fifth boost was diluted 1:10 or 1:40 prior to incubation with the virus. Analysis of the p27 antigen levels through a 27 day period demonstrated a nine day and ten day delay in the detection of p27 in cultures following pre-incubation with 534/1/1 rabbit



6.6a in house p27 assay



6.6b Coulter p27 assay

Uninfected cell control
Virus control
Pre-immune pool
Animal 534/1/1
Animal 534/1/2
Animal 534/1/3
Animal 534/1/4
Animal 534/1/5

Figure 6.6 p27 antigen levels from an SIV neutralization assay.

antiserum at the 1:10 and 1:40 dilutions respectively. This result confirmed that the 534/1/1 antiserum was able to delay the infection of H9 cells with SIVmac251.

6.4 DISCUSSION

Hybrid Ty-VLPs have been produced that carry the putative V3 region from the SIV isolate mac251. Western blot analysis demonstrated that OGS534 VLPs reacted positively with serum from a macaque experimentally infected with SIVmac251, therefore confirming that the sequence contained in the SIV:Ty-VLPs is immunogenic. However, the antibody response elicited by OGS534 VLPs, as determined by ELISA against a V3 peptide or SIV-infected cell lysate, has suggested that this region may only be weakly immunogenic. Even when immunizations were carried out in Freund's adjuvant only low levels antibodies were detected. The results presented in section 6.3.1 therefore suggest that the SIV V3 sequence is weakly immunogenic, in contrast to the HIV V3 loop that is highly immunogenic.

It is possible that the lack of potency of anti-OGS534 sera against the V3 20-mer peptide, SIV-infected lysate or infectious virus was due to differences between the amino acids in the V3 sequence carried by the SIV:Ty-VLPs and the V3 sequence present in the test antigens and infectious virus. The effect of this difference will remain unclear without sequencing the envelope genes of the infectious virus stock or virus derived antigen. Alternatively, a second VLP construction could be evaluated that contained the 1991 Los Alamos database sequence.

In order to determine whether the V3 loop carried by OGS534 VLPs elicited the production of neutralizing antibodies, a two stage SIV neutralization assay has been developed. Difficulties were encountered in both the cell culture and antigen detection stages of the assay. The development of a routine assay is critically dependent on the

availability of reproducible virus stocks. Such stocks were not always available, often resulting in insufficient virus being added to initiate a productive infection within the 27 day period. Productive infection, and possible neutralization, was monitored by measuring the levels of p27 antigen in the cell culture medium. p27 antigen was detected by developing an antigen capture assay. Although optical density readings obtained using polyclonal sera as the capture and detecting antibodies were low, the assay was specific and reproducible. Furthermore, the assay gave results which were comparable with a commercially available assay. Ultimately the use of monoclonal antibodies may result in a more sensitive assay. To this end, anti-p27 monoclonal antibodies were evaluated as capture antibodies and one, 12G6, was shown to be the most effective. The direct labelling of a second antibody, recognising a different epitope to that recognised by 12G6, for p27 detection, would allow further refinement of the assay.

Anti-OGS534 antisera from five animals were tested for the presence of neutralizing antibodies. Only serum from one of the immunized animals was able to delay the time course of SIV infection in vitro. This result can only be regarded as preliminary due to the small numbers of assays carried out and the lack of optimization of the assay itself.

The results presented in this chapter indicate that it may be possible to elicit weakly-neutralizing antibodies against the V3 region of the SIV envelope glycoprotein. However, taken together with more recent data from other laboratories (discussed in chapter 8), it appears that this region is not the principal neutralization determinant and these SIV V3:Ty-VLPs are unlikely to provide a useful model for HIV V3:Ty-VLPs in challenge experiments.

CHAPTER 7

HYBRID HIV GAG VLPs

7.0 INTRODUCTION

HIV infected individuals mount a strong humoral and cellular immune response to the gag encoded proteins, i.e., the core proteins of the virus. Studies of the immune response induced against gag encoded proteins suggest that these proteins should be included in a vaccine candidate against HIV. However, in general, HIV neutralizing antibodies have not been reported against the core proteins, probably because these proteins are internal and therefore anti-GAG antibodies do not block binding or entry of the virus to susceptible cells. It is likely that to achieve successful protection, an HIV vaccine candidate will have to include a component that elicits production of neutralizing antibodies. A preparation that combined the gag encoded proteins and the neutralizing epitope of the envelope glycoprotein gp120, the V3 loop, may therefore be advantageous. Gheysen et al. (1989) have shown that when the GAG precursor polypeptide Pr55 is expressed using the baculovirus system, this protein has the ability to self assemble into virus-like particles. Furthermore, Adams et al. (1987a) have demonstrated that the gag gene of retroviruses is directly analogous to the TYA gene of Ty, i.e., the gene that encodes the p1 carrier protein of the hybrid Ty-VLP system. Therefore, an attractive way forward may be to engineer GAG as a carrier protein for the V3 loop. A GAG:V3 hybrid particle would combine the neutralizing epitope of gp120 with the T helper cell and CTL epitopes of GAG to make a potential HIV vaccine candidate.

Two expression systems have been used to investigate expression of GAG:V3 fusion proteins. The baculovirus system was chosen because, as mentioned above, expression of the GAG precursor polypeptide in insect cells resulted in the production of GAG particles (Gheysen et al., 1989). Expression in yeast was also investigated because of the analogy between gag and TYA and also work with the Ty-VLP system had previously demonstrated that high levels of fusion proteins could be achieved using this

expression configuration. In addition, using two expression systems gave the advantage of generating reagents from different cell backrounds for testing immune responses to the whole of GAG. For example, GAG particles produced in yeast could be used to assay the anti-GAG responses generated against hybrid GAG:V3 particles produced in insect cells.

The baculovirus system exploits the Autographica californica nuclear polyhedrosis virus (AcNPV) (reviewed by Miller, 1988). Progeny virus is produced in two forms; extracellular virus particles and occluded virus particles. Following infection extracellular virus particles are produced, then later in the life cycle virus particles are occluded into a paracrystalline matrix composed of a 29kD protein called polyhedrin. In this form the virus can infect new hosts by horizontal transmission. As cell to cell infection is mediated by extracellular virus, the synthesis of polyhedrin is a non-essential function in cell culture. Therefore the gene coding for polyhedrin can be replaced by a foreign gene of interest. Expression of the foreign protein is driven by the polyhedrin promoter. The expression strategy is outlined in Figure 7.1. Production of a recombinant baculovirus is dependent on homologous recombination between sequences present in the expression vector and the Autographica califronica nuclear polyhedrosis virus (AcNPV) DNA. Recombination occurs at a frequency of about 1%. Following co-transfection of insect cells with AcNPV DNA and the transfer vector encoding the gene of interest, selection of a putative recombinant virus is by microscopic examination of viral plaques. A recombinant baculovirus does not express polyhedrin, therefore polyhedrin-negative plaques are picked. The putative recombinant baculoviruses then undergo several rounds of plaque purification and a virus is selected by analysis of expression of the foreign protein.

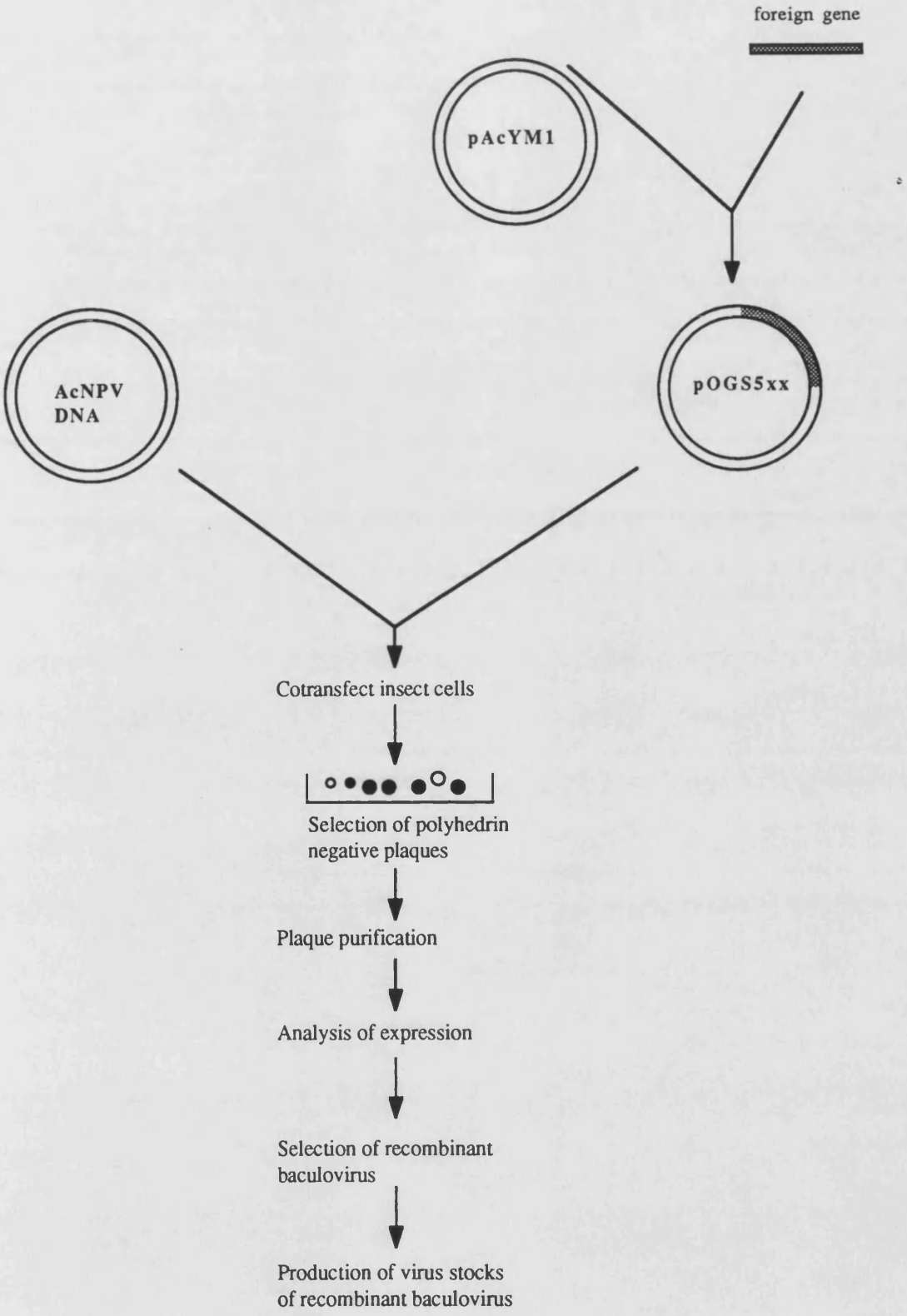


Figure 7.1 Baculovirus expression system

The yeast expression system utilizes a modified galactose, PGK-based inducible promoter (Kingsman et al., 1991). The promoter is similar to that of the yeast expression vector pOGS40 described in chapter 1, section 1.4, but is more tightly regulated in the presence of glucose. The expression vector containing this promoter was modified by Mr. David Krige to allow insertion of genes with BamH1 sites at their 5' and 3' ends. The resulting expression vector was designated pOGS700.

A range of GAG and GAG:V3 proteins have been expressed and analysed for their ability to form particles in both systems. Characterization of the fusion proteins has been carried out by Western blot analysis, immunofluorescence and electron microscopy, and immunogenicity studies have been carried out in rats.

7.1 ANALYSIS OF EXPRESSION OF FULL LENGTH GAG AND GAG:V3 FUSION PROTEINS

7.1.1 Constructions

Figure 7.2 illustrates the constructions made to facilitate expression in yeast and in the baculovirus system. The GAG protein of HIV-1 (isolate BH10) is encoded by a 1.6 kb gene. The gag gene encodes a precursor protein, Pr55, which is subsequently cleaved to produce the core proteins p17, p24 and p15 which is further cleaved to p7 and p6. This gene was engineered to have BamHI restriction sites at its 5' and 3' ends and cloned into pSP46 so that it could be easily inserted into the expression vectors. This tailored gag gene in pSP46 was constructed by Dr. Sally Adams and was designated pOGS15. A gag:V3 fusion gene was made by inserting the HXB2 V3 loop gene (described in chapter 3, section 3.1) at a BglII site located 300 base pairs from the 3' end of the gag gene.The first stage of making the gag:V3 fusion gene was to insert synthetic oligomers in the BglII site to maintain the gag reading frame following insertion of the V3 loop. Insertion of the oligomers was confirmed by sequencing and

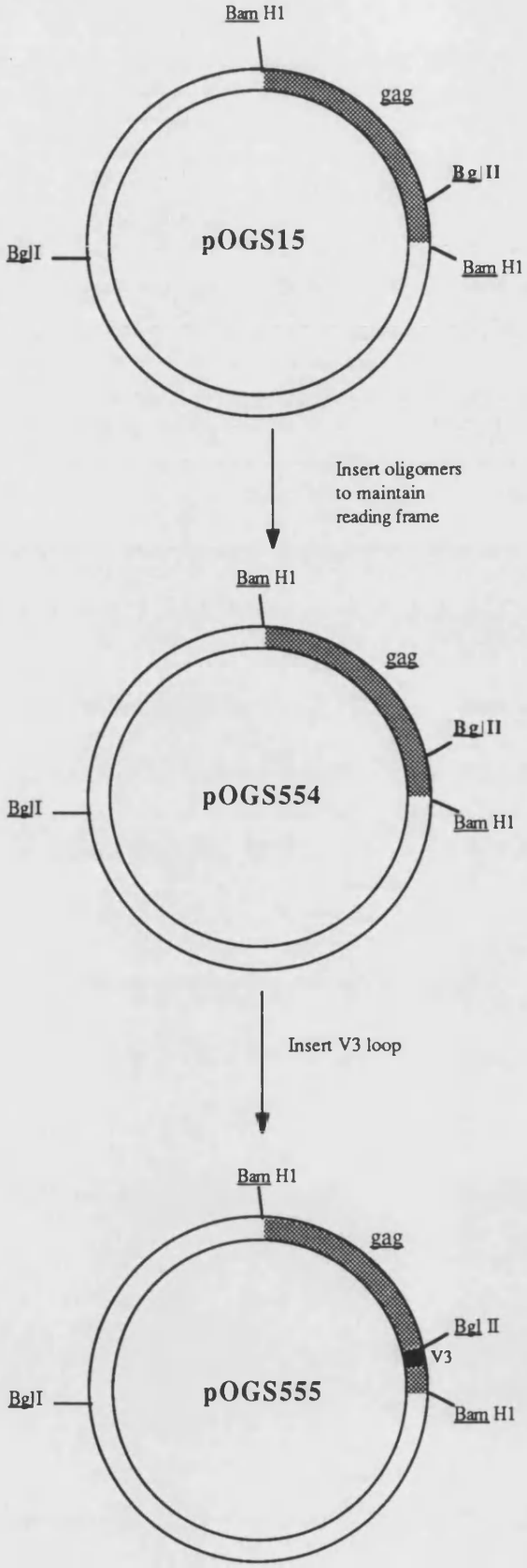


Figure 7.2 Construction of the gene coding for the GAG:V3 fusion protein

the resulting plasmid was designated pOGS554. The V3 loop gene was then inserted into the BglII site to generate pOGS555. Cloning into this site positioned the V3 loop gene in the gag gene between the regions encoding the p7 and p6 proteins.

The gag and the gag:V3 fusion genes were excised from pOGS15 and pOGS555 as BamH1 fragments and cloned into the yeast expression vector pOGS700 containing the galactose inducible promoter. The resulting plasmids were designated pOGS557 (gag) and OGS559 (gag:V3) respectively. The gag and the gag:V3 fusion genes were also cloned into the baculovirus transfer vector pAcYM1 and the plasmids designated pOGS553 and pOGS556 respectively.

7.1.2 Expression in yeast

Yeast strain BJ2168 was transformed with plasmids pOGS557 (full length GAG) and pOGS559 (full length GAG:V3) and cultures grown as described for the production of hybrid Ty-VLPs (chapter 2, section 2.13). After induction by galactose, samples of cells were taken and disrupted by vortexing in the presence of glass beads. The resulting cell lysates were analysed by Western blot using a monoclonal antibody (DuPont, antibody 9283) that is reactive with the p24 core protein of HIV. The antibody was reactive with a band of 55kD in the lysate of cells transformed with pOGS557 that contained the gag gene encoding the GAG precursor protein Pr55. The antibody was also reactive with a band of 59kD in the lysate of cells transformed with pOGS559 which contained the gag:V3 fusion gene. The increase in the size of the GAG:V3 fusion protein was as expected as the V3 loop is approximately 4kD. The p24 immunoreactive protein observed in the OGS559 cell lysate also reacted positively with the V3 loop monoclonal antibody 9284. These results confirmed that the 59kD protein contained both GAG and gp120 components.

Cells transformed with OGS557 and OGS559 were examined by transmission electron microscopy to determine whether the expression of GAG and GAG:V3 fusion protein resulted in particle formation. Electron micrographs of a pOGS557 transformed cell (Figure 7.3a) showed that the GAG protein was associated with the cell membrane as an electron dense double banded layer around the membrane. This layer was not present in control BJ2168 cells. Large protrusions were also seen at the cell surface, suggesting that budding of the GAG protein had been prevented by the cell wall. Figure 7.3b shows an electron micrograph of a yeast cell transformed with pOGS559. The GAG:V3 fusion protein accumulated as aggregated material in the nucleus of the cell. The nuclear localization of this GAG:V3 fusion protein is not easily explained but will be discussed in section 7.5.

The targeting of other retroviral gag encoded precursors at the cell membrane has been shown to be dependent on myristilation (Schultz and Oroszkin, 1983). Myristilation is a post-translational modification which involves removal of the N-terminal methionine and addition of a myristic acid moiety at the next amino acid which is always a glycine residue. The signal for myristilation therefore includes an M-G sequence at the N-terminus, although the presence of a serine residue at amino acid position six is also thought to be important. Studies with the HIV GAG precursor protein expressed using the baculovirus system have confirmed that myristilation is required for association with the cell membrane (Gheysen et al, 1989) and that removal of this sequence resulted in intracellular accumulation of the GAG protein in insect cells.

To test whether removal of the GAG myristilation signal would result in intracellular particle formation in yeast, a further construction was made, shown in Figure 7.4. The myristilation signal was removed by inserting synthetic oligomers at the BamHI site at the 5' end of the gag gene in pOGS14. This insertion created an ATG with no

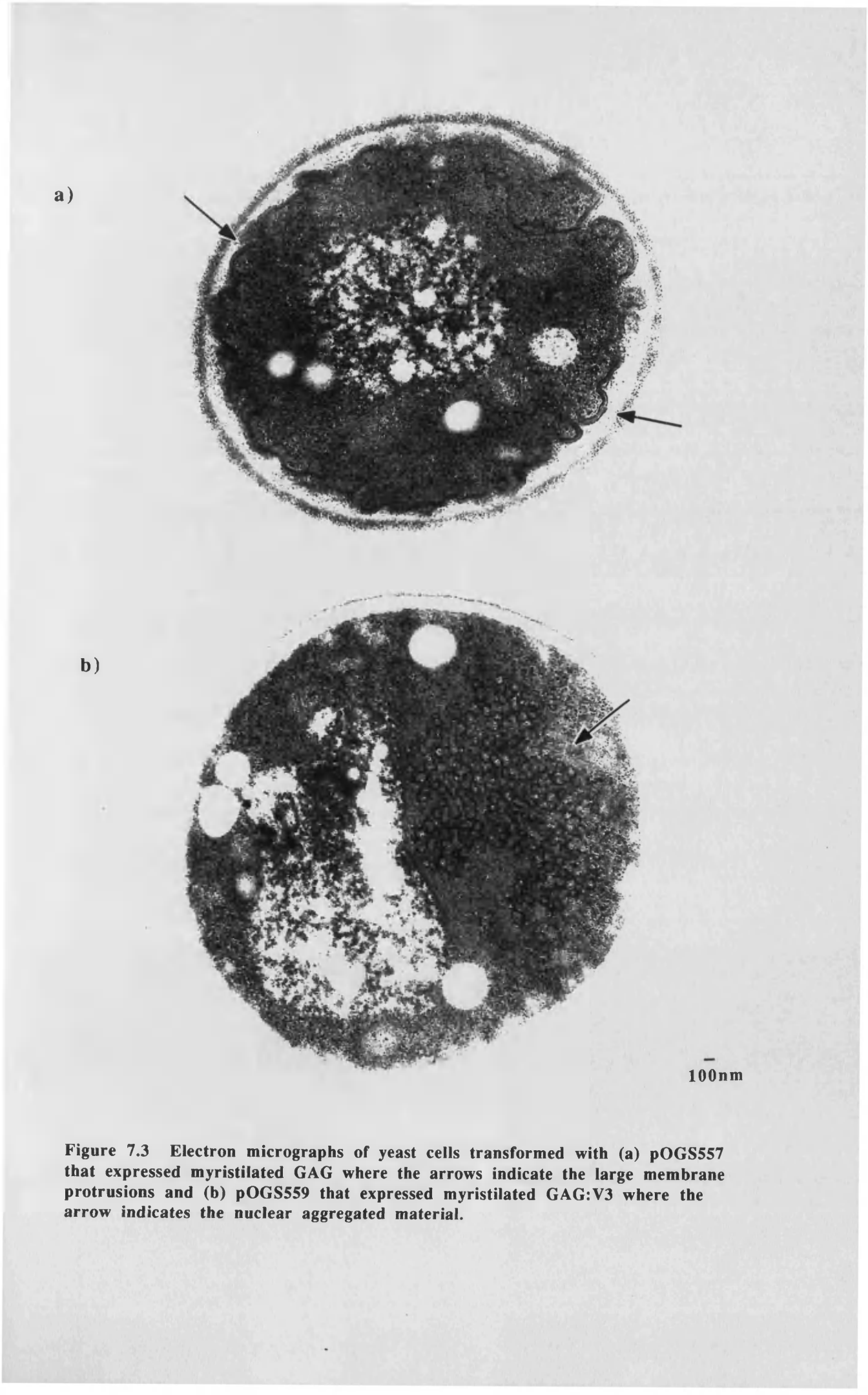


Figure 7.3 Electron micrographs of yeast cells transformed with (a) pOGS557 that expressed myristilated GAG where the arrows indicate the large membrane protrusions and (b) pOGS559 that expressed myristilated GAG:V3 where the arrow indicates the nuclear aggregated material.

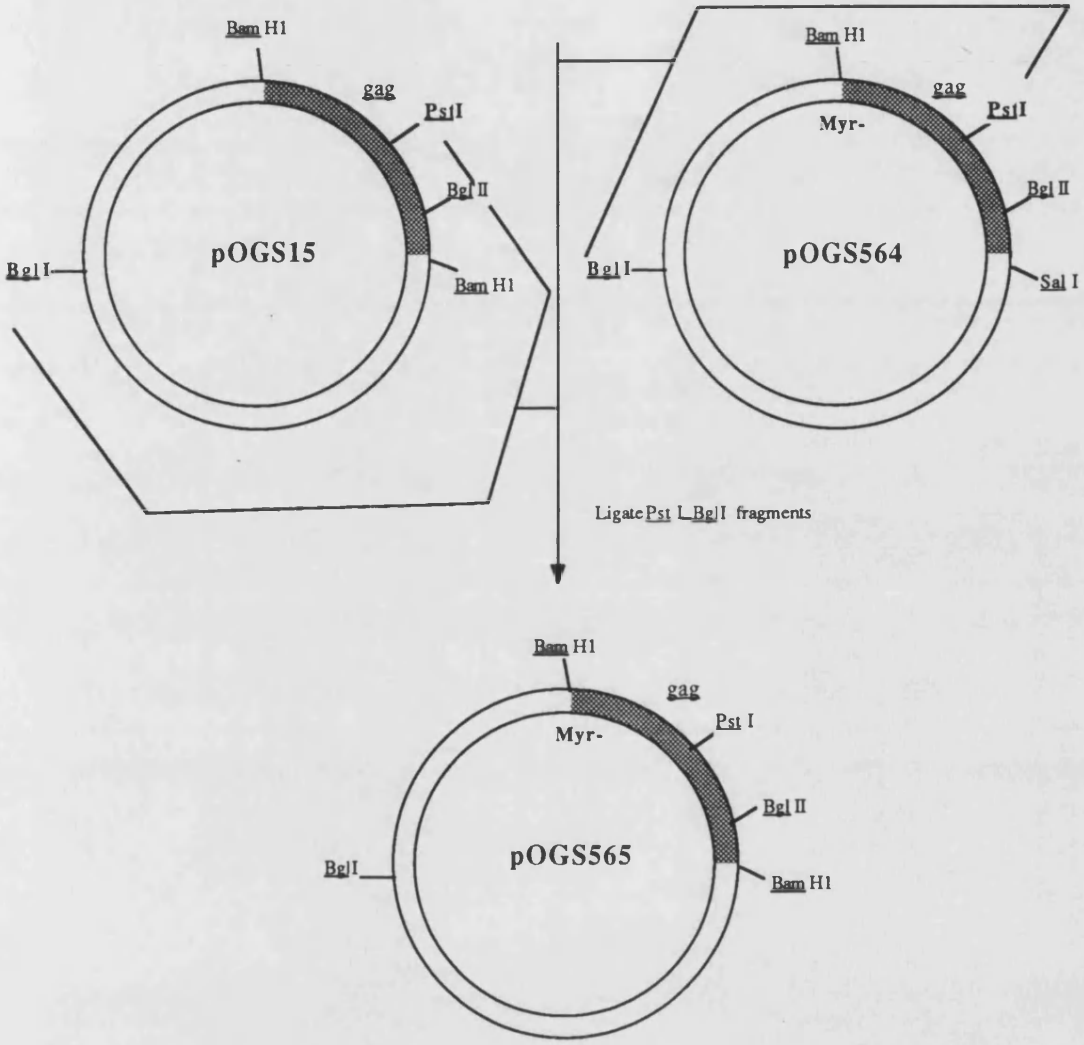


Figure 7.4 Construction of the gene coding for non-myristilated GAG

myristilation signal downstream of the a re-created BamHI site. The resulting plasmid was designated pOGS564. The 5' end of the gag gene in pOGS15 was replaced with a PstI-BglI fragment from pOGS564. The resulting plasmid pOGS565 therefore contained a BamHI cassette coding for full-length non-myristilated GAG. The BamHI fragment was excised from pOGS565 and inserted into the yeast expression vector pOGS700 to generate pOGS566. A gene encoding a non-myristilated GAG:V3 fusion protein was also made by a PstI-BglI replacement of the 5' end of the gag gene in pOGS555 with that in pOGS564 and the resulting plasmid designated pOGS568. The BamHI fragment from pOGS568 was inserted into pOGS700 to generate pOGS569.

Figure 7.5 shows an electron micrograph of a yeast cell transformed with pOGS566 encoding the non-myristilated GAG protein. Although the protein that accumulated in the cytoplasm was particulate, the majority was found to be aggregated rather than present as discrete particles. However, this observation confirmed that the myristilation signal on GAG was essential for cell membrane targeting. The non-myristilated GAG:V3 fusion protein was also expressed and was found to accumulate in the nucleus in the same way as the myristilated GAG:V3 fusion protein. This observation indicated that the myristilation signal responsible for the cell membrane targeting of GAG was in some way overidden when the protein was expressed as a GAG:V3 fusion.

These data demonsrated that expression of full length GAG and GAG:V3 fusion proteins in yeast, either myristilated or non-myristilated, did not result in the fomation of discrete particles.

7.1.3 Expression using the baculovirus system

The myristilated, full length GAG and GAG:V3 proteins were expressed in insect cells using the baculovirus system. Recombinant baculoviruses expressing these proteins

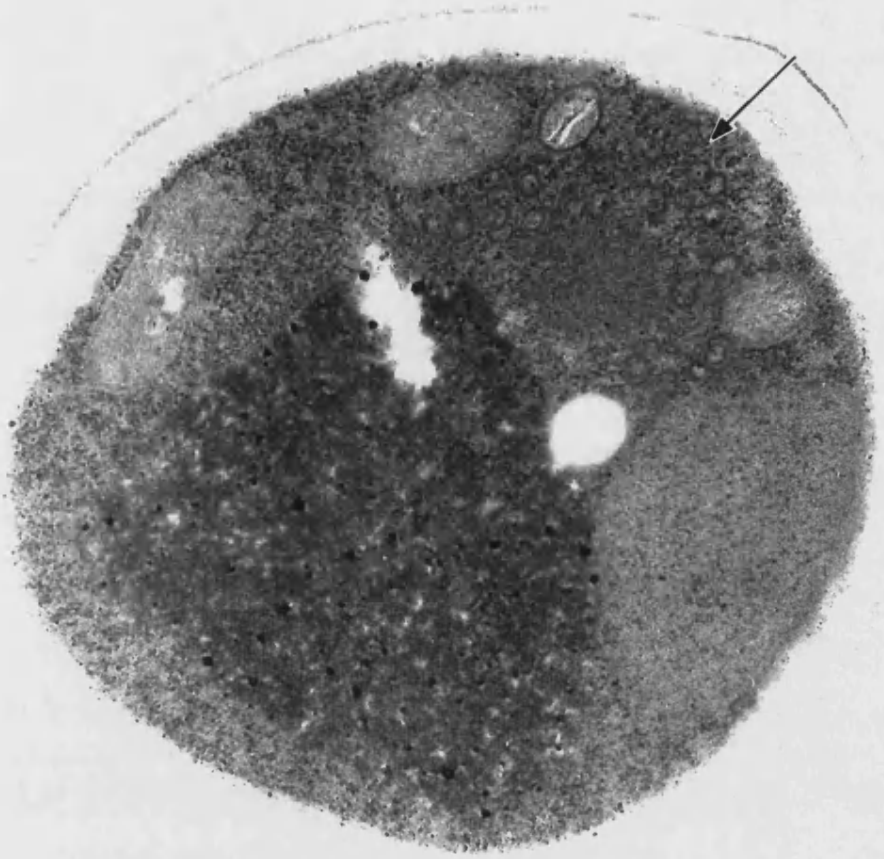


Figure 7.5 Electron micrograph of a yeast cell transformed with pOGS566 that expressed non-myristilated GAG. The arrow indicates the particulate aggregated material in the cytoplasm.

were made by Dr. Katy Gearing at British Bio-technology and infected insect cells were examined by electron microscopy by Dr. Keith Gull at Manchester University. The results are shown here to provide background information. Figure 7.6a shows an insect cell infected with the recombinant baculovirus expressing GAG and confirmed the work of Gheysen et al. (1989) where virus-like particles of approximately 100nm diameter budded from the cell membrane. Figure 7.6b shows an insect cell infected with the recombinant baculovirus expressing the GAG:V3 fusion protein. As seen in yeast cells (Figure 7.3b), an aggregated mass of the protein was found in the nucleus. This indicated that the nuclear localization observed in yeast was not simply a cell type/expression system phenomenon.

Taken with the results described in section 7.1.2 these data demonstrated that hybrid GAG:V3 particles did not assemble when full length GAG:V3 fusion proteins, which contained the V3 loop within GAG, were expressed using either the yeast or the baculovirus systems. Alternative constructions were therefore examined in order to re-establish particle formation and budding.

7.2 ANALYSIS OF EXPRESSION OF TRUNCATED GAG

As mentioned previously the GAG precursor Pr55 is proteolytically cleaved to yield p17, p24, p7 and p6. p17 is the matrix protein, p24 is the major core protein and p7 is involved in nucleic acid binding. However, the role of p6 in infectious virions is as yet unclear. Particle formation by a truncated form of GAG that had p6 removed from the C-terminus was therefore analysed as a step towards producing a GAG:V3 C-terminal fusion protein where the V3 loop effectively replaced p6.

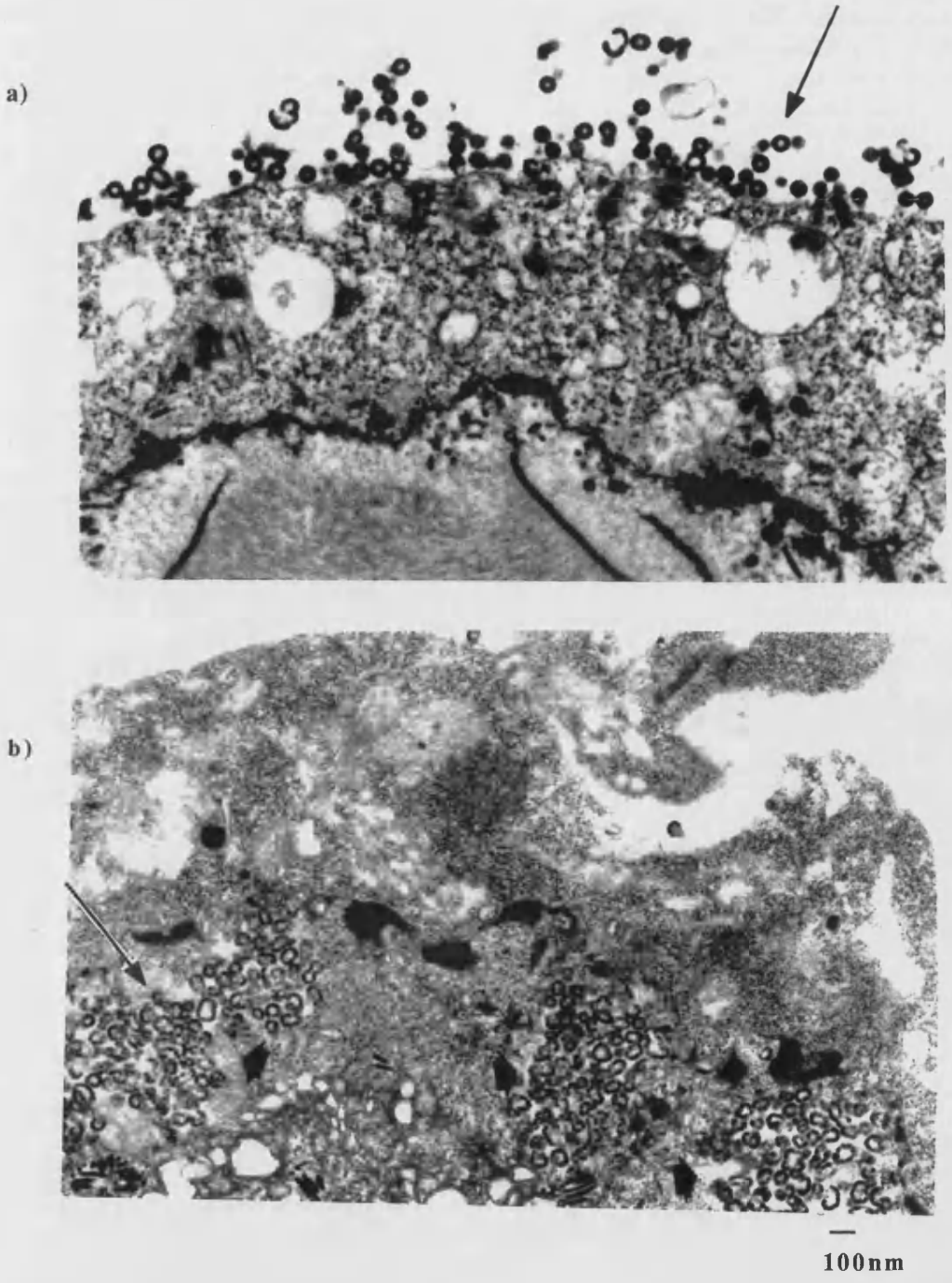


Figure 7.6 Electron micrographs of insect cells infected with recombinant baculoviruses that (a) expressed GAG where the arrow indicates budded particles and (b) expressed GAG:V3 fusion protein where the arrow indicates the nuclear aggregated material.

7.2.1 Constructions

To construct a gene coding for a p6-deleted GAG protein, the gag gene was truncated to 1.3kb by insertion of oligomers coding for a stop codon at the BglII site in pOGS554 (Figure 7.7). The oligomers also contained a BamHI site that enabled excision of the truncated gag gene as a 1.3kb BamHI fragment for insertion into the expression vectors. The plasmid containing this cassette coding for truncated GAG was designated pOGS562. The 1.3kb BamHI fragment from pOGS562 encoding myristilated p6-deleted GAG was cloned into the baculovirus transfer vector pAcYM1 and the resulting plasmid designated pOGS572.

A gene coding for non-myristilated truncated GAG was also constructed by replacing the 3'end of the gag gene in pOGS564 with a PstI-BglI fragment from pOGS562 to generate pOGS571.The BamHI fragment from pOGS571 was inserted into the yeast expression vector pOGS700 to yield pOGS575.

7.2.2 Expression in yeast

Yeast cells were transformed with pOGS575 (non-myristilated truncated GAG) and cultures grown as described for production of hybrid Ty-VLPs (chapter 2, section 2.13). Initial evaluation of the expression of the truncated GAG protein was by Western blot and sucrose gradient analysis. A 4ml packed-cell volume was resuspended in 4ml TEN buffer and cells were broken by vortexing with glass beads. The resulting cell lysate was analysed by Western blot. A band corresponding to the predicted size of 47kD reacted positively with the anti-p24 MAb 9283. Cellular debris was removed from the lysate by centrifugation at 3krpm for five minutes (Beckman RT6000B centrifuge) and 1ml of the resulting supernatant was loaded onto each of two 10ml 10 to 60% sucrose/TEN gradients. The gradients were spun at 40krpm (Beckman L8-70 ultracentrifuge, SW41 rotor) for either 45 minutes or for two hours and 45

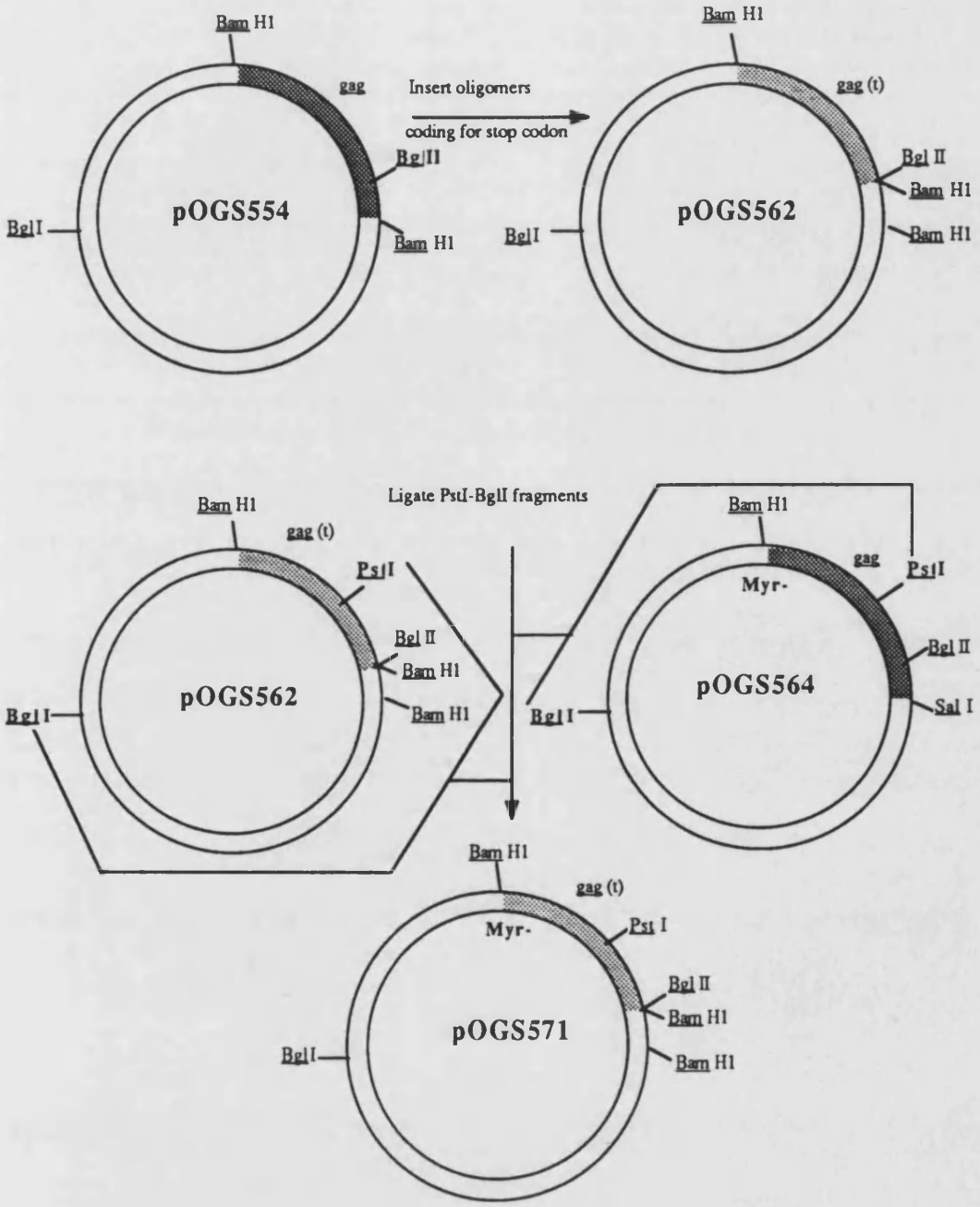


Figure 7.7 Construction of myristilated and non-myristilated truncated gag gene

minutes. The gradients were fractionated to 1ml fractions. Figure 7.8 shows Western blot analysis of the gradient fractions of the two different spin times. After 45 minutes some of the 47kD GAG protein still remained at the top of the gradient. After longer centrifugation all of the 47kD GAG protein had moved into the gradient, suggesting that the protein was particulate in nature. The spread of GAG protein through the gradient indicated that the particles were heterogeneous. A lower molecular weight protein was also observed that remained at the top of the gradient which indicated that some of the GAG protein had been proteolytically cleaved and that this cleaved protein did not form particles.

Yeast cells transformed with OGS575 were examined by electron microscopy (Figure 7.9). In this case GAG particles were observed in the cytoplasm and were approximately 100nm in diameter. Removal of the myristilation signal and p6 therefore established particle formation by the GAG protein in yeast cells.

7.2.3 Expression using the baculovirus system

To produce a recombinant baculovirus expressing a myristilated truncated GAG protein, insect cells were cotransfected with AcNPV viral DNA and the transfer vector pOGS572 (myristilated, truncated GAG) using the method described in chapter 2, section 2.25. Recombinant baculovirus formation was screened in a Brown-Faulkner plaque assay (chapter 2, section 2.26) and ten polyhedrin negative plaques were selected. The putative recombinant baculoviruses underwent three further rounds of plaque purification. Expression of the truncated GAG protein was analysed by Western blot analysis of cell lysates infected with the recombinant baculoviruses using the anti-p24 MAb 9283. A single recombinant baculovirus was then selected and designated AcOGS572. Virus stocks of AcOGS572 were prepared as described in chapter 2, section 2.27.

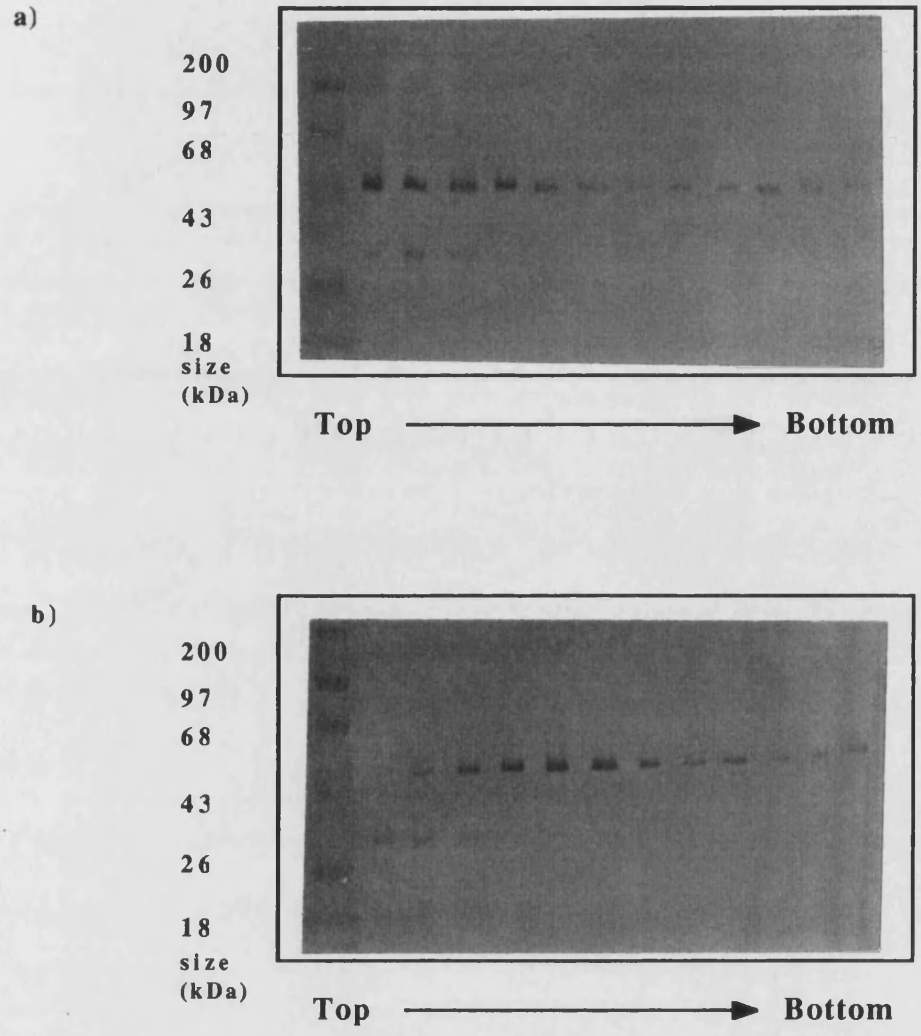
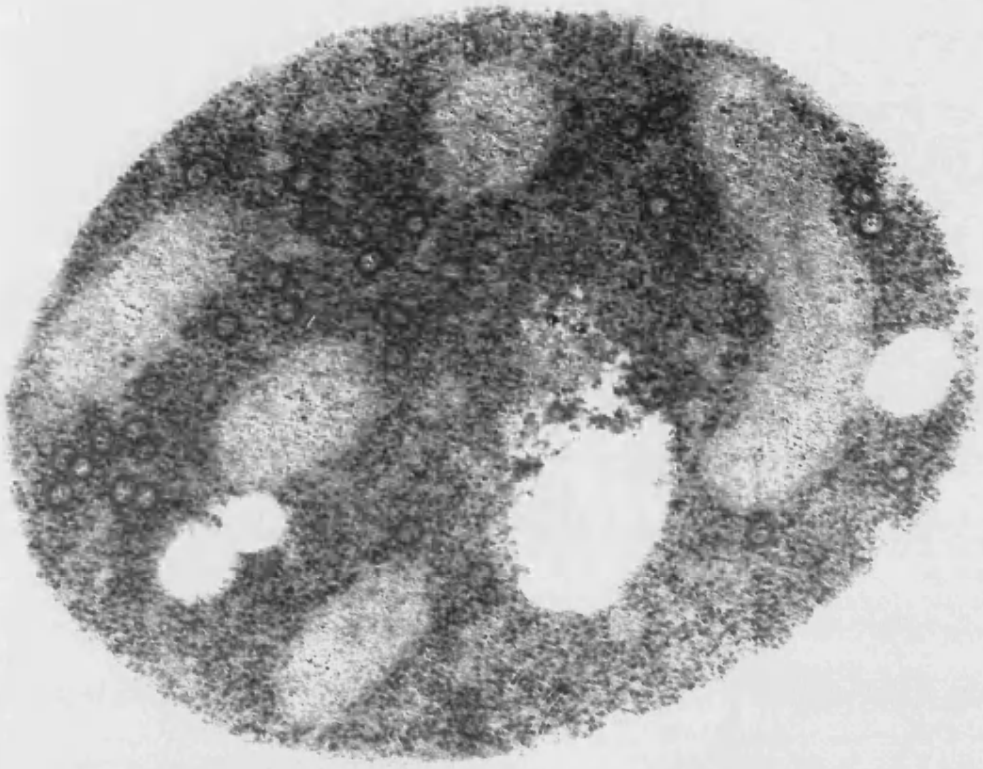


Figure 7.8 Western blots of sucrose gradient analysis of truncated GAG. The gradient shown in (a) as spun for 45 minutes and the gradient in (b) was spun for 2 hours 45 minutes.



100nm

Figure 7.9 Electron micrograph of a yeast cell transformed with pOGS575 that expressed non-myristilated truncated GAG

Insect cells infected with the recombinant baculovirus AcOGS572 were examined by electron microscopy (Figure 7.10). Myristilated, truncated GAG protein assembled into particles that budded from the cell membrane. This observation indicated that p6 was not required for the formation and release of GAG particles in the baculovirus system. The truncated GAG particles were a similar size to those produced from the full length GAG protein (approximately 100nm in diameter) but were perhaps more regular in appearance.

The results presented in section 7.2 therefore demonstrated that particles were produced by expression of non-myristilated, truncated GAG in yeast and myristilated, truncated GAG in insect cells.

7.3 ANALYSIS OF EXPRESSION OF TRUNCATED GAG:V3 FUSION PROTEINS

Having demonstrated that removal of p6 did not prevent particle assembly, the next step was to determine whether expression of a truncated GAG:V3 protein, with the V3 loop fused at the C terminus, could produce hybrid GAG:V3 particles.

7.3.1 Constructions

For expression in yeast the HXB2 V3 loop gene was cloned into the BglII site of the expression vector pOGS575 to generate pOGS579. Plasmid pOGS575 was described in section 7.2.1 and contains a gene encoding non-myristilated, truncated GAG. Plasmid pOGS579 therefore contained a non-myristilated gag:V3 fusion gene (Figure 7.11).

The V3 loop gene was also cloned into the baculovirus vector pOGS572 at the BglII site located at the 3' end of the myristilated truncated gag gene. Plasmid pOGS573

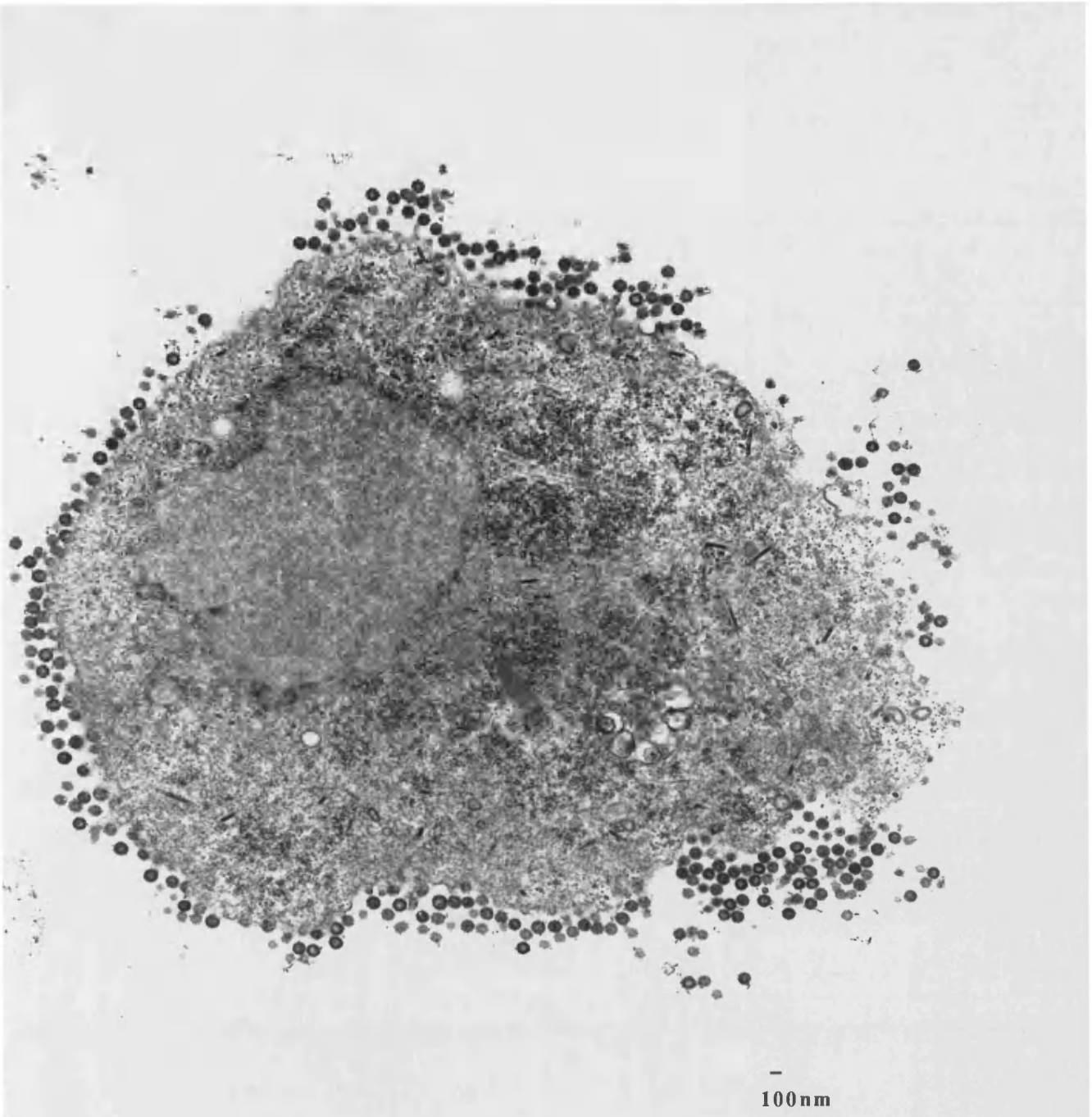


Figure 7.10 Electron micrograph of an insect cell infected with baculovirus AcOGS572 that expressed truncated GAG

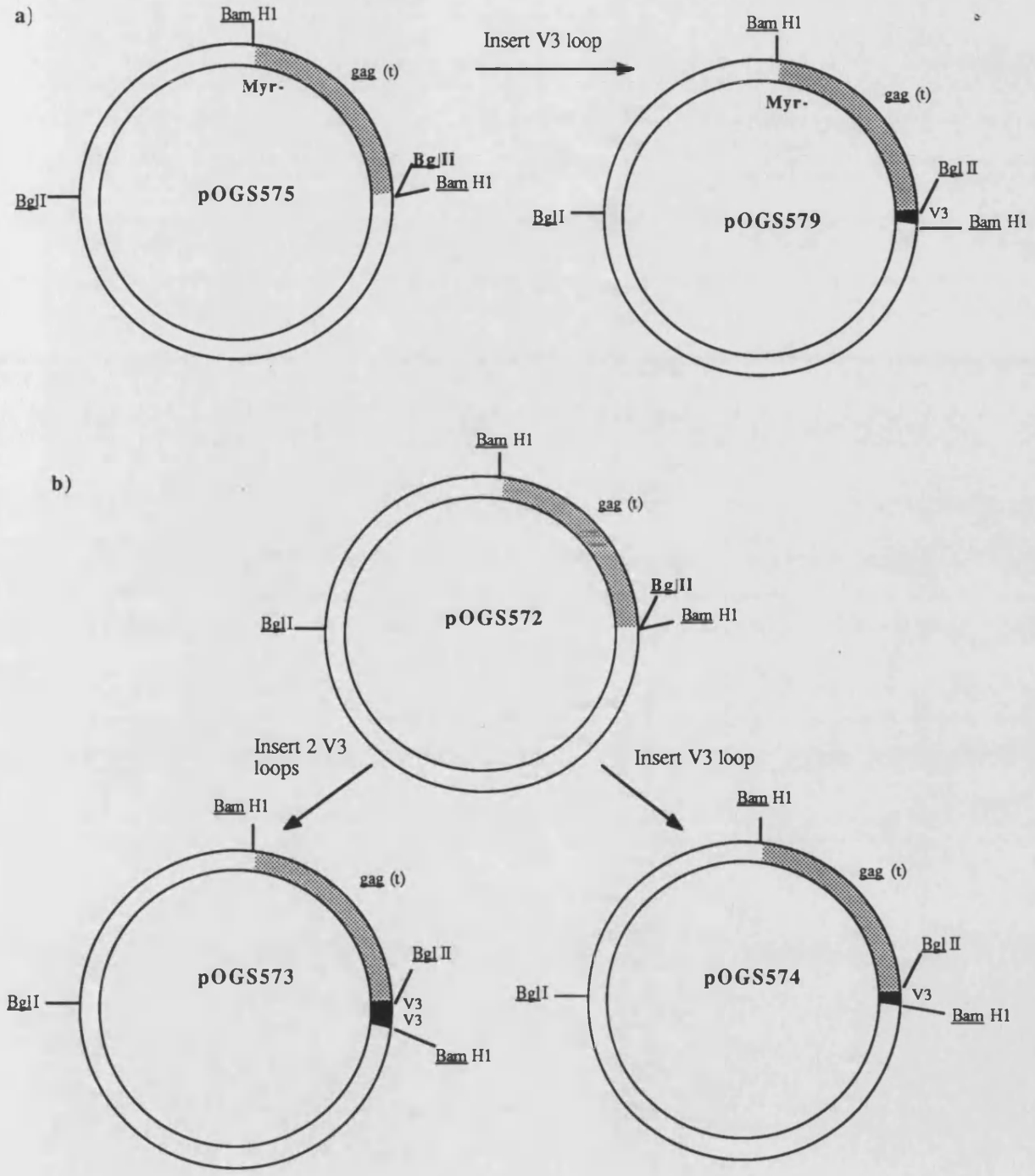


Figure 7.11 Construction of vectors for expression of GAG:V3 fusion protein in (a) yeast and (b) insect cells

contained two copies of the V3 loop gene whereas plasmid pOGS574 contained a single copy (Figure 7.11). Expression of both the gag:V3 and the gag:V3:V3 fusion genes in insect cells was investigated.

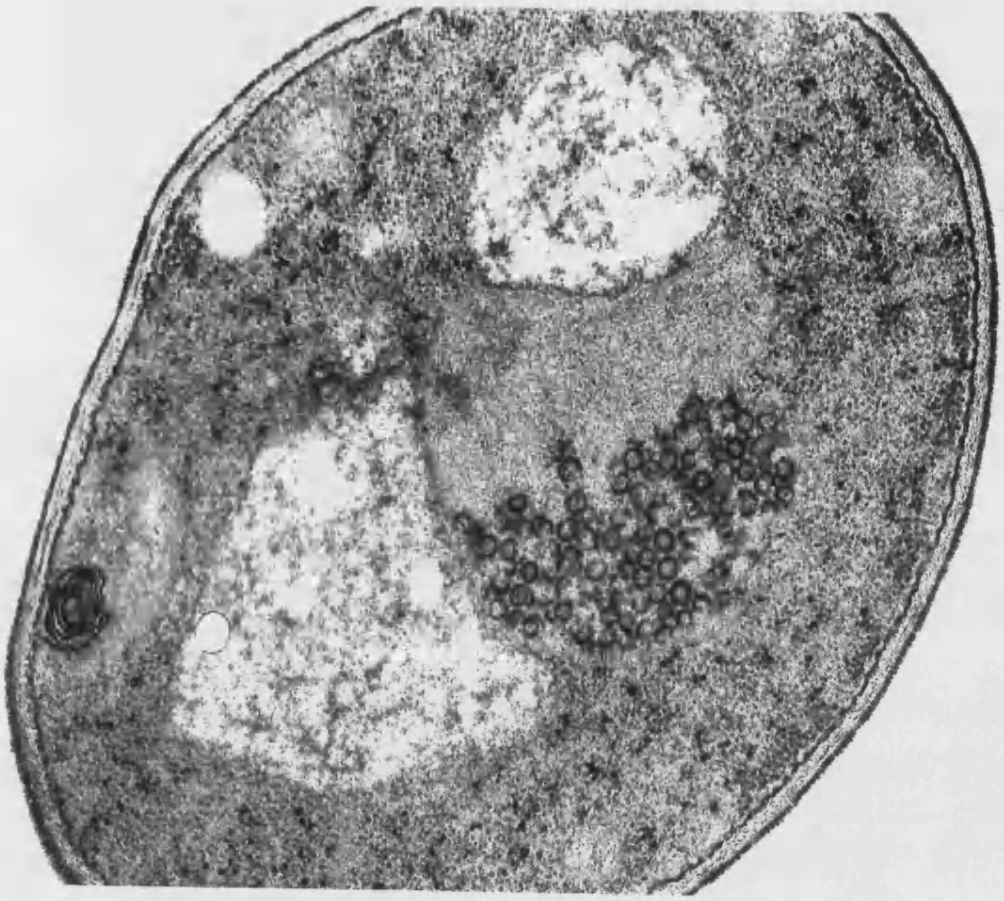
7.3.2 Expression in yeast

Yeast cells transformed with pOGS579 (non-myristilated truncated GAG:V3) were analysed by Western blot and sucrose gradients as described in section 7.2.2. Western blots of cell lysates confirmed that a protein was expressed of the expected molecular weight of 51kD. This protein reacted positively with both the anti-p24 MAb 9283 and the anti-V3 loop MAb 9284. Sucrose gradient analysis revealed a similar profile as that seen with truncated GAG particles (see Figure 7.8). Although the GAG:V3 fusion protein moved into the gradient, it was spread throughout the fractions. This suggested that the particulate material was heterogeneous. Non-particulate low molecular weight degradation products were also observed.

The electron micrograph in Figure 7.12 shows particles present in the nucleus of OGS579 transformed yeast cells. These data demonstrate that hybrid GAG:V3 particles can self-assemble in yeast cells. The nuclear location of this non-myristilated, truncated GAG:V3 fusion protein was consistent with that of the other GAG:V3 fusion proteins described above.

7.3.3 Expression using the baculovirus system

Recombinant baculoviruses were produced that expressed the GAG:V3 and the GAG:V3:V3 fusion proteins as described in section 7.2.3. The baculoviruses were designated AcOGS574 (GAG:V3) and AcOGS573 (GAG:V3:V3) respectively. Infected insect cell lysates were examined by Western blot analysis. The blot shown in Figure 7.13a was probed with the anti-p24 MAb9283 and demonstrated the increase in



100nm

Figure 7.12 Electron micrograph of a yeast cell transformed with pOGS579 that expressed non-myristilated truncated GAG:V3

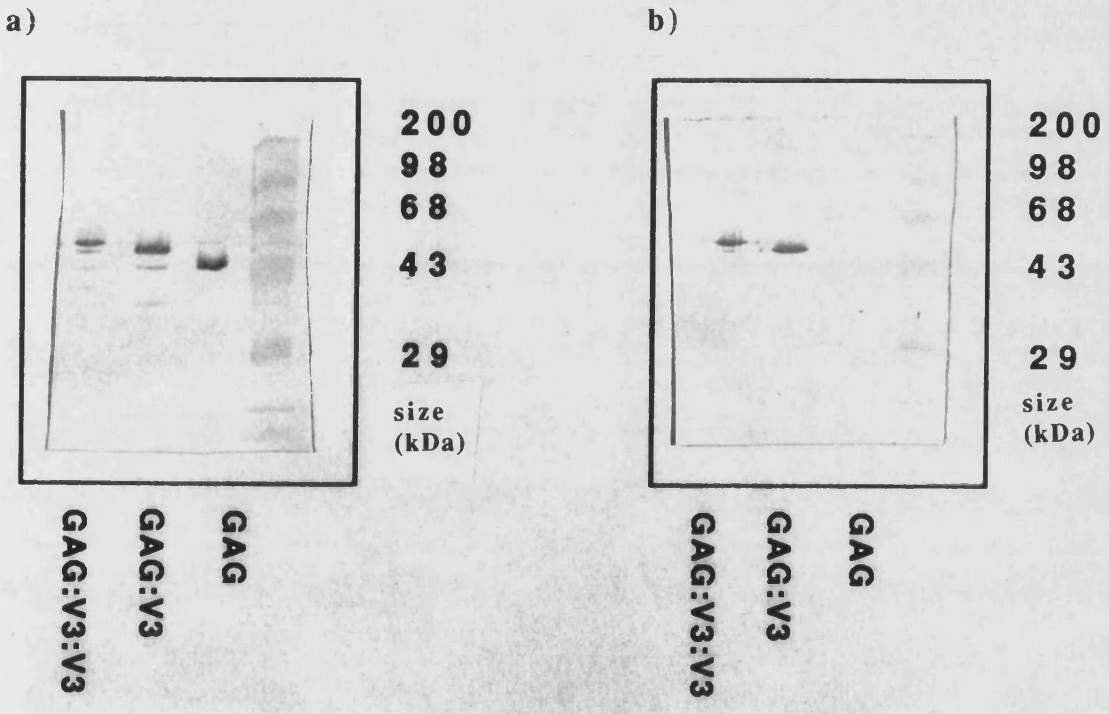


Figure 7.13 Western blot analysis of infected insect cell lysates. The blot in (a) was probed with an anti-p24 MAb and the blot in (b) was probed with an anti-V3 MAb.

size of the GAG:V3 and the GAG:V3:V3 fusion proteins as compared to GAG alone. The blot seen in Figure 7.13b showed a positive reaction of the anti-V3 loop MAb9284 to both the GAG:V3 and the GAG:V3:V3 fusion proteins. As expected, the anti-V3 loop MAb9284 did not react with GAG.

The presence of both GAG and V3 components in the infected insect cells was analysed by fixed cell fluorescence described in chapter 2, section 2.17. Figure 7.14a shows the negative control of AcNPV infected cells incubated with the anti-p24 MAb9283 where no staining was observed. Figure 7.14b shows anti-p24 staining of insect cells infected with the recombinant baculovirus AcOGS572 (truncated GAG). The staining pattern around the edge of the cells suggested that the GAG protein was on the surface of the cells. In contrast anti-p24 staining of insect cells infected with recombinant baculovirus AcOGS574 (truncated GAG:V3) suggested that the protein was present both on the surface and the interior of the cells as indicated by the punctate pattern, with some cells showing brightness at the edges. (Figure 7.14c). Cells infected with the recombinant baculovirus AcOGS573 (truncated GAG:V3:V3) and stained with the anti-p24 MAb indicated that the majority of the protein was inside the cells (Figure 7.14d).

Immunofluoresence of the infected insect cells was also carried out with the anti-V3 loop MAb9284. Cells infected with the recombinant baculovirus AcOGS572 (truncated GAG) did not stain with MAb9284 (Figure 7.15a). Figure 7.15b and c shows that both the AcOGS574 (GAG:V3) and AcOGS573 (GAG:V3:V3) infected cells were specifically stained with the MAb9284, but that the intensity of staining was lower than with the anti-p24 antibody. This could indicate that the anti-V3 loop monoclonal antibody had a lower affinity than the anti-p24 monoclonal antibody, or that the V3 sequence was less accessible.

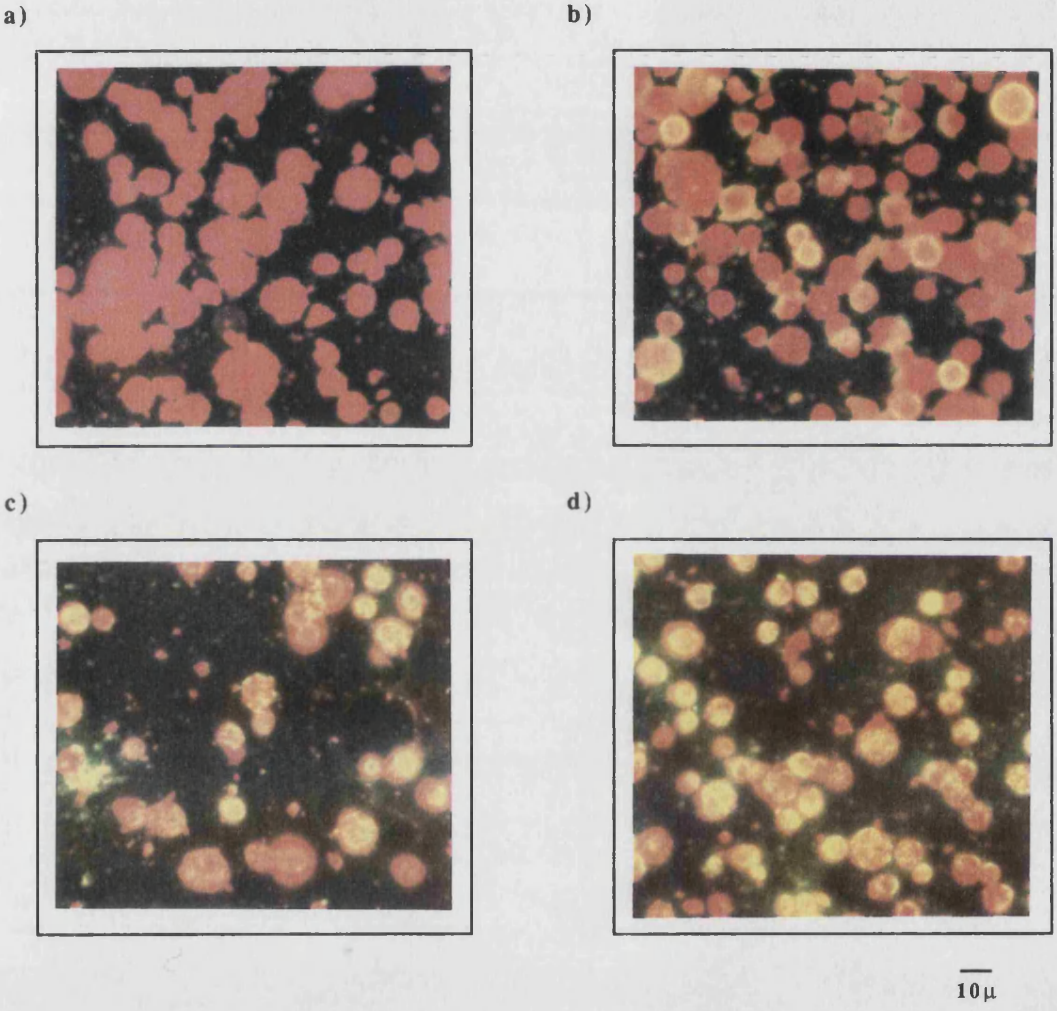


Figure 7.14 Immunofluorescence of baculovirus infected insect cells. The cells were stained with an anti-p24 MAb. The cells in (a) were infected with AcNPV. The cells in (b) were infected with AcOGS572 that expressed truncated GAG. The cells in (c) were infected with AcOGS574 that expressed truncated GAG:V3. The cells in (d) were infected with AcOGS573 that expressed truncated GAG:V3:V3.

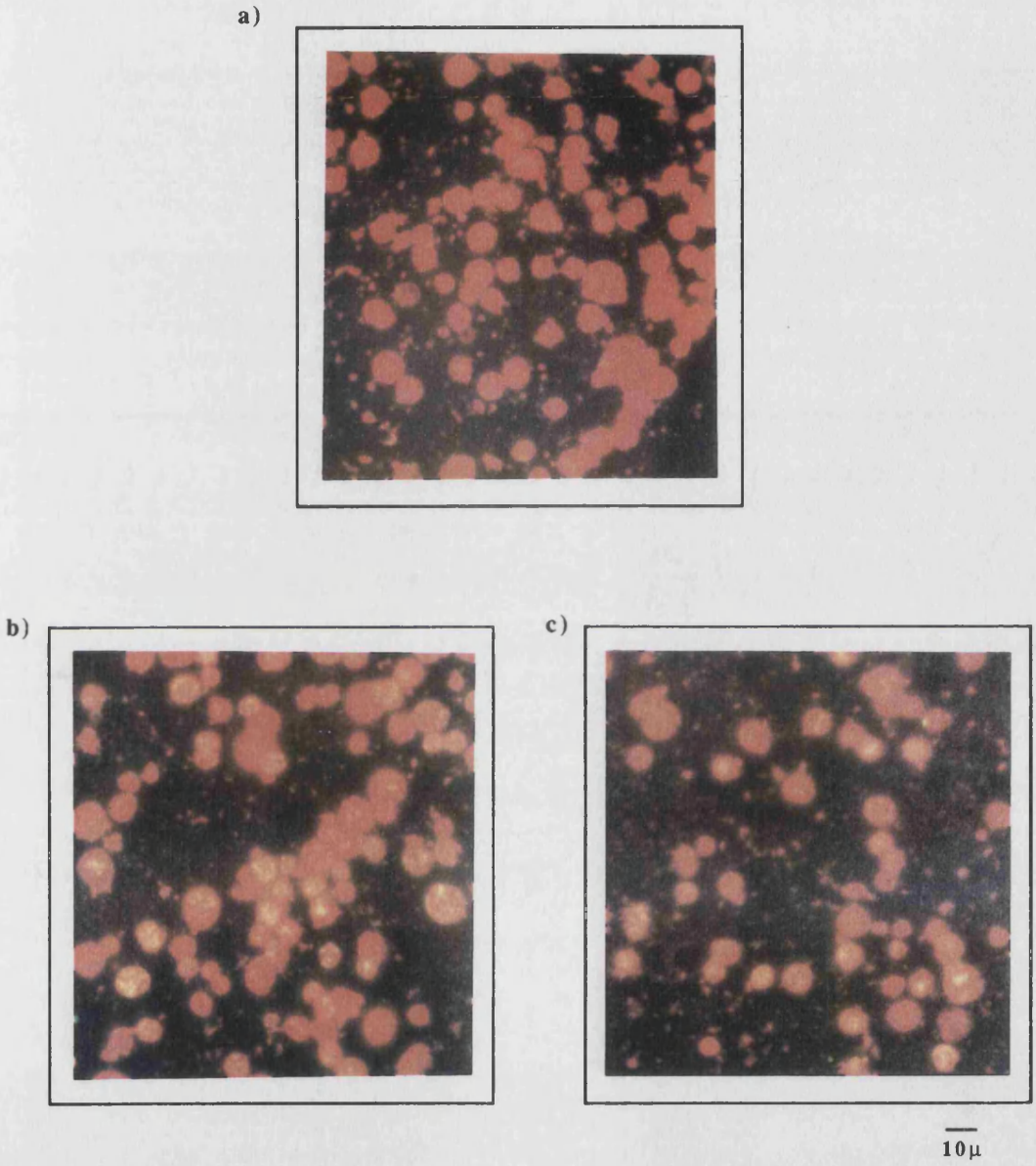


Figure 7.15 Immunofluorescence of (a) OGS572, (b) OGS574 and (c) OGS573 infected cells. The cells were stained with anti-V3 MAb9284.

The results obtained by immunofluorescence were confirmed by examining AcOGS574 (GAG:V3) and AcOGS573 (GAG:V3:V3) infected cells by electron microscopy. Figure 7.16 shows an electron micrograph of an insect cell infected with AcOGS574 and indicates that the myristilated truncated fusion protein had retained the ability to assemble into particles. Approximately half the particles were budding from the cell, whereas the remainder were located in the nucleus. Furthermore the electron micrograph of an AcOGS573 infected cell (Figure 7.17) showed that the GAG:V3:V3 fusion protein formed particles that were all located in the nucleus. Again, this result suggested that nuclear localization of the fusion protein was influenced by the V3 loop sequence.

In summary, hybrid GAG:V3 particles were produced following expression of a non-myristilated, truncated GAG:V3 fusion protein in yeast and after expression of myristilated truncated GAG:V3 and GAG:V3:V3 fusion proteins in insect cells. An unexpected change in the location of the hybrid GAG:V3 particles as compared to the location of GAG particles was observed.

7.3.4 Purification of GAG:V3 and GAG:V3:V3 particles from the nuclei of insect cells

To produce particles for immunization studies, a purification strategy for the hybrid OGS574 (GAG:V3) and the OGS573 (GAG:V3:V3) particles from insect cells was developed. These particles were selected for evaluation so that a comparison could be made of the immune responses induced by the single and double V3 components.

Initially, the optimum parameters of infection for expression were determined. Cultures were infected at different multiplicities of infection (m.o.i) and expression examined over time by Western blot. Optimum expression of both GAG:V3 and GAG:V3:V3

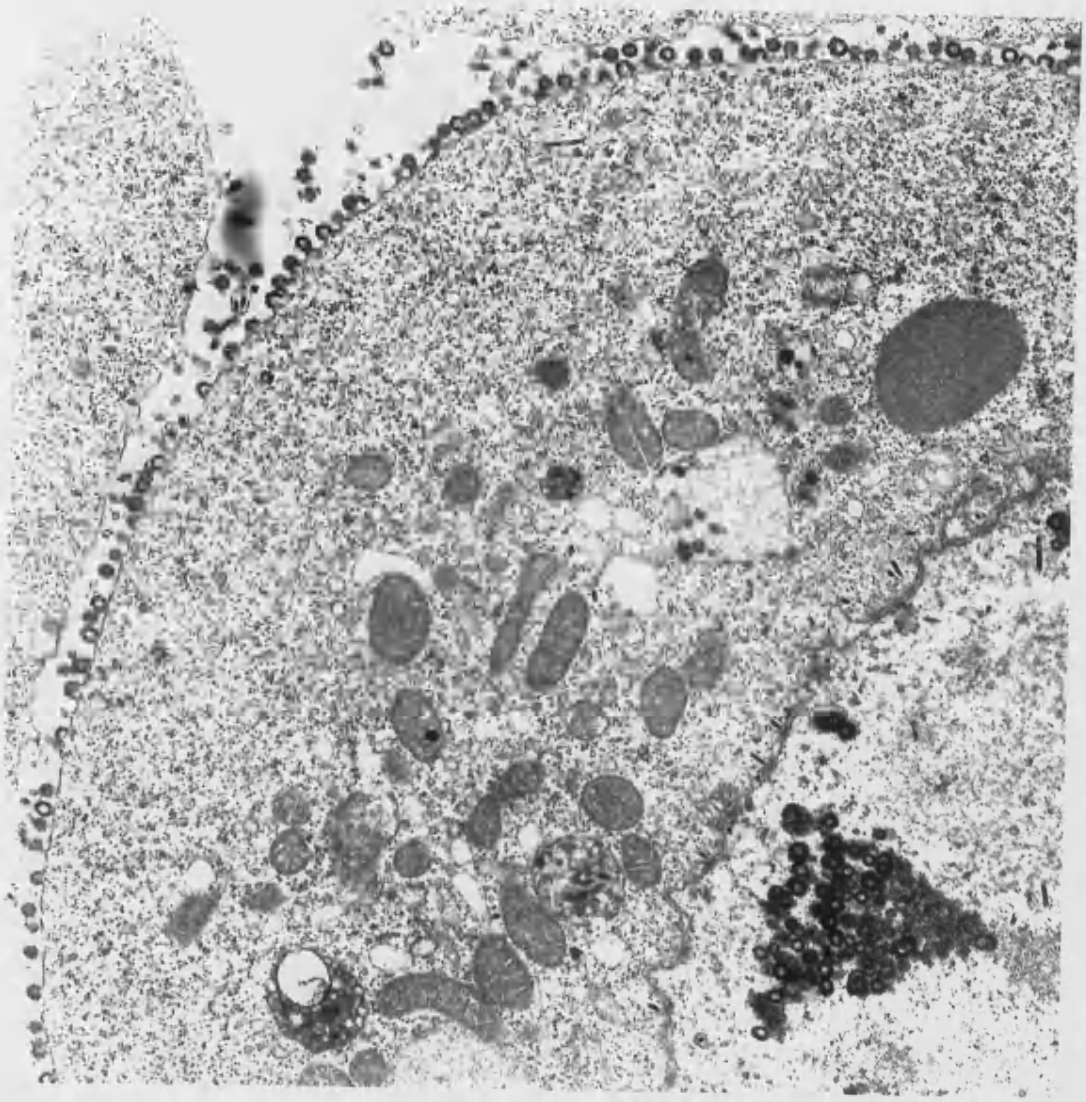


Figure 7.16 Electron micrograph of an insect cell infected with recombinant baculovirus AcOGS574 that expressed myristilated truncated GAG:V3

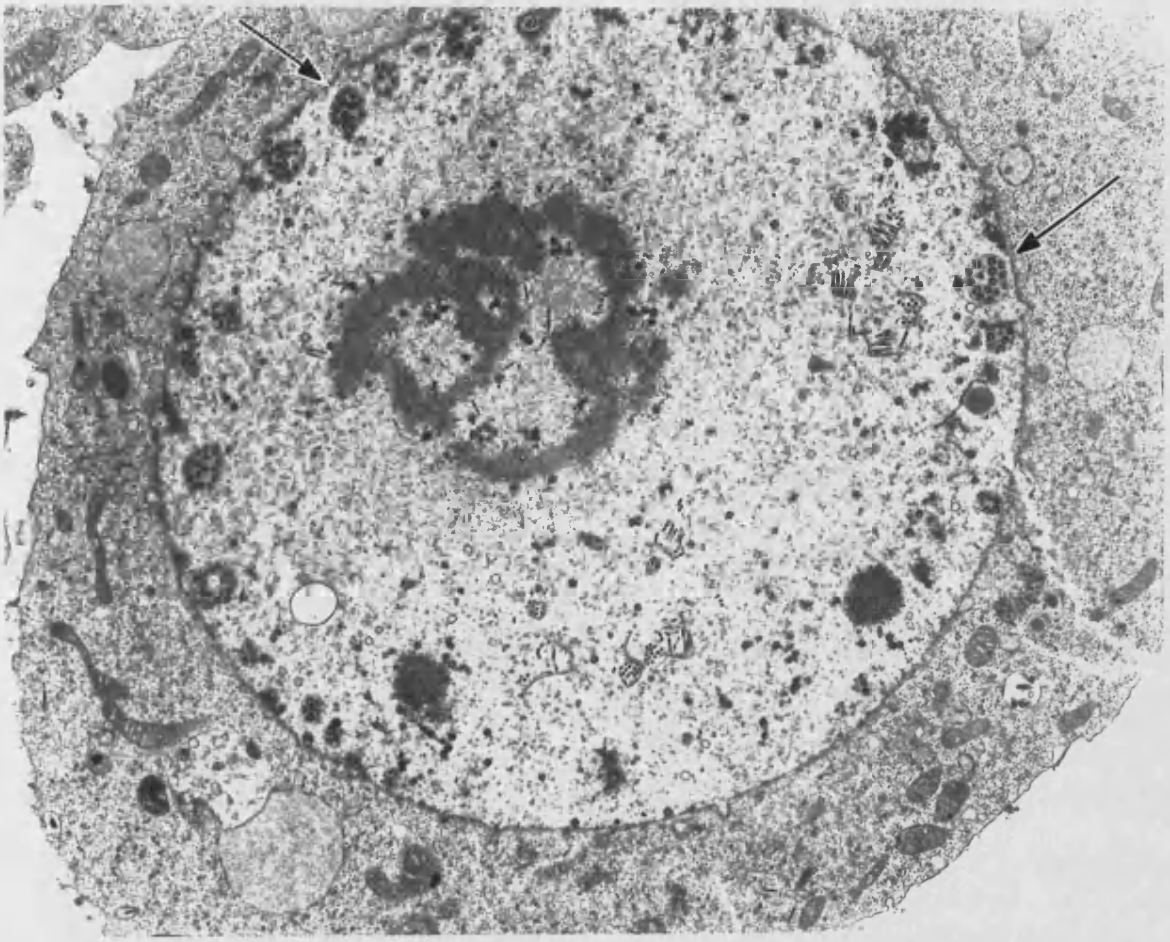


Figure 7.17 Electron micrograph of an insect cell infected with recombinant baculovirus AcOGS573 that expressed myristilated truncated GAG:V3:V3. The arrows indicate the nuclear particles.

fusion proteins occurred after infection with two plaque forming units (pfu)/cell and 48 hours incubation.

The presence of both the GAG:V3 and the GAG:V3:V3 particles in the nuclei of infected cells was exploited as part of the purification process. Various methods and conditions were investigated to prepare nuclei (detergent treatment, centrifugation), to release the particles from purified nuclei (DNAse treatment, sonication) and to purify further the released particles (centrifugation parameters). The purification procedure developed and an example of OGS574 GAG:V3 particle preparation is described below.

A 400ml insect cell culture at $1x10^6$cells/ml was infected with the recombinant baculovirus AcOGS574 at 2pfu/cell. The 400mls of culture was split into 2x200ml aliquots in 500ml spinner flasks. The cultures were incubated at 27°C for 48 hours. Cells were harvested into 12 tubes by centrifugation at 3krpm for five minutes (Beckman RT6000B centrifuge). Each tube was resuspended in 4ml of TEN buffer containing 0.5%NP40 and incubated on ice for 10 minutes. The nuclear and cytoplasmic fractions were separated by centrifugation at 3krpm for 5 minutes (Beckman RT6000B centrifuge). The nuclear pellets were washed three times in PBS buffer and resuspended in a total of 36mls of PBS. The nuclei were sonicated for 6 cycles, 15 seconds on, 10 seconds off (MSE Soniprep 150), and the sonicate was then loaded onto a step gradient. The gradients were prepared in SW41 tubes (Beckman) by pipetting 1ml 60% sucrose/TEN followed by 3ml 30% sucrose/TEN. The sonicate (6ml) was loaded onto each gradient and the gradients were centrifuged at 10krpm for 10 minutes (Beckman L8-70 ultracentrifuge, SW41 rotor). The gradients were harvested into 1ml fractions and aliquots were analysed by SDS-PAGE (Figure 7.18a). The GAG:V3 particles were mainly located in the 60% sucrose/TEN cushion which

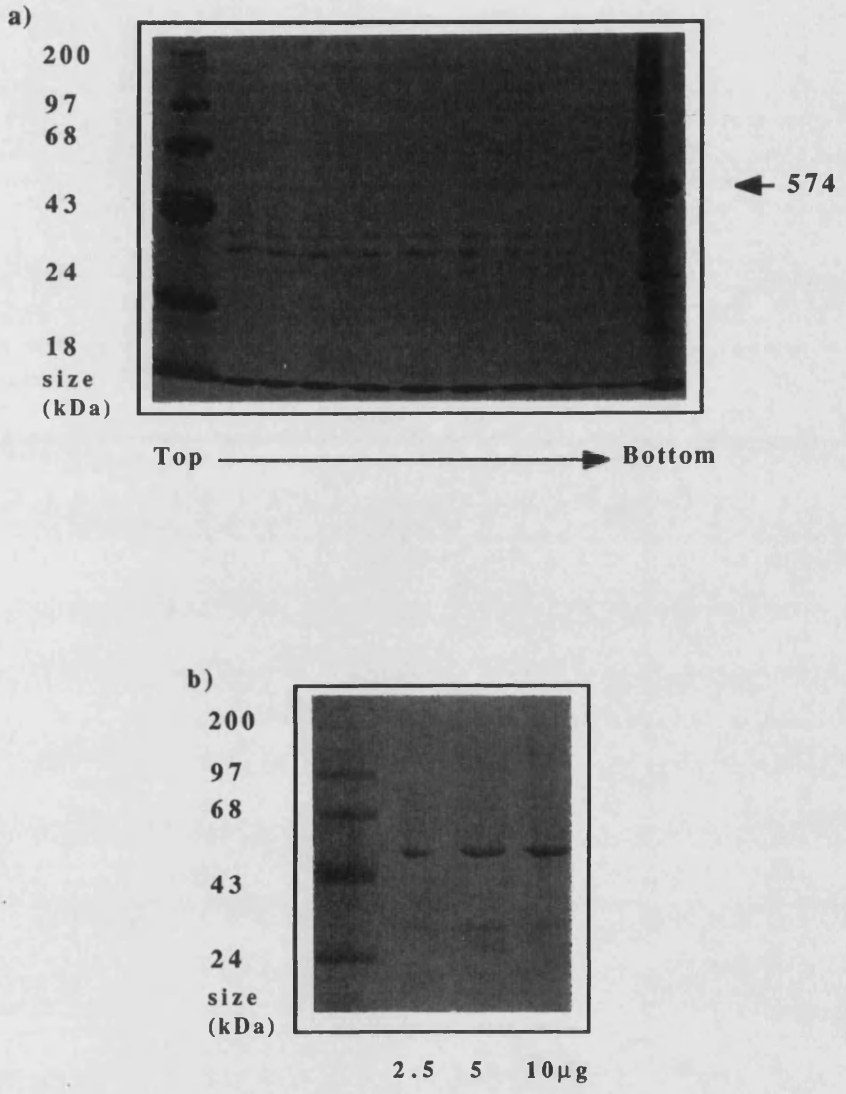


Figure 7.18 Purification of OGS574 GAG:V3 particles
The SDS-PAGE gel in (a) shows a fractionated sucrose gradient
The SDS-PAGE gel in (b) shows 2.5, 5 and 10 µg of the final material

also included some contaminating proteins. The 60% sucrose cushions were pooled and dialysed against 500ml of TEN buffer overnight at 4°C. The protein concentration of the dialysate was estimated by Biorad assay and adjusted to 1mg/ml. The SDS-PAGE gel in figure 7.18b shows loadings of 2.5, 5 and 10µg of the final material. This preparation yielded 17mg of OGS574 GAG:V3 particles.

The rapid pelleting of the GAG:V3 particles suggested that the material was aggregated to some extent. This possibility was further examined by negative stain electron microscopy of the particle preparation as described in chapter 2, section 2.16. The electron micrograph in Figure 7.19a shows that the OGS574 GAG:V3 particles were heterogenous in size. The particles appeared to be aggregated in filamentous material that may have been DNA. OGS573 GAG:V3:V3 particles appeared similar to OGS574 GAG:V3 particles (Figure 7.19b).

7.4 IMMUNE RESPONSES GENERATED AGAINST HYBRID GAG:V3 PARTICLES

Rats were immunized in order to determine the immune responses generated against OGS574 GAG:V3 and OGS573 GAG:V3:V3 particles. Six groups of five rats received 100µg doses of each of the immunogens. Groups 573/1 and 574/1 received the priming immunization in complete Freund's adjuvant and boost immunizations in incomplete Freund's adjuvant. Groups 573/2 and 574/2 received the OGS573 and OGS574 particles in conjunction with aluminium hydroxide and groups 573/3 and 573/4 were immunized with particles alone. The animals were given a priming immunization and two boosts at three weekly intervals. Following a ten week period after the second boost the animals received three further boosts at three weekly intervals. Serum samples were taken before and one week following each boost. Serum antibody levels against the V3 loop were measured against the HXB2 40-mer peptide described in

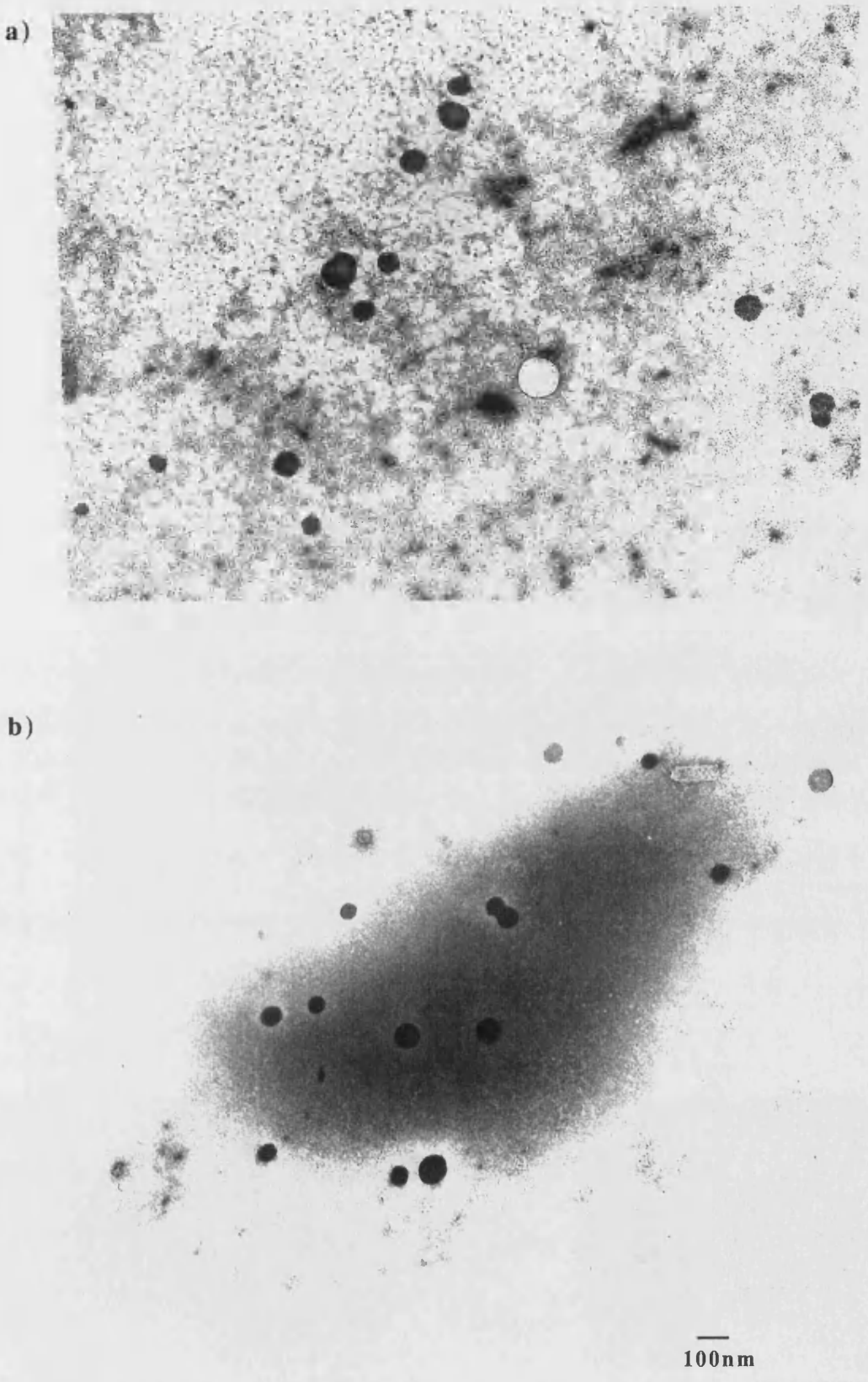


Figure 7.19 Negative stain electron microscopy of (a) OGS574 GAG:V3 and (b) OGS573 GAG:V3:V3 particles

chapter 5. Sera were also tested for the presence of virus neutralizing antibodies in the syncytium inhibition assay described in chapter 2, section 2.22. To measure the anti-GAG response an ELISA was developed using GAG protein expressed in yeast cells (see section 7.4.1).

7.4.1 Development of an ELISA to measure the anti-GAG response

As described in section 7.3.4, OGS573 and OGS574 particles were purified from the nuclei of baculovirus insect cells. In order to measure the GAG-specific responses induced by immunization with this material, the GAG antigen used in the assay needed to be derived from an alternative cell-type. Expression of GAG in yeast cells provided such material for the development of an ELISA assay.

The anti-GAG ELISA was developed using partially purified OGS575 (non-myristilated truncated GAG) particles (see section 7.3.2). pOGS575 transformed yeast cells were disrupted in the presence of glass beads and cellular debris was removed by centrifugation at 3krpm for five minutes (Beckman RT6000B centrifuge). The concentration of the GAG component was estimated by SDS-PAGE and Biorad analysis and adjusted to approximately 1mg/ml. A microtitre plate was coated at concentrations of 1µg, 5µg,10µg and 20µg/ml. The ability of OGS575 particles to detect anti-GAG responses was determined by titration of the anti-p24 monoclonal antibody 9283 and an anti-p17 monoclonal antibody 9282 (DuPont). The anti-V3 loop monoclonal antibody 9284 was included as a negative control. The three monoclonal antibodies were also tested against an untransformed yeast lysate. No reactivity was observed against this lysate for any of the antibodies. Figure 7.20 shows the titration curves obtained at the different GAG coating concentrations. As expected, no reactivity was seen with the anti-V3 antibody. At a coating concentration of 1µg/ml lower reactivities were observed than at the higher concentrations. Mid-point titres of the

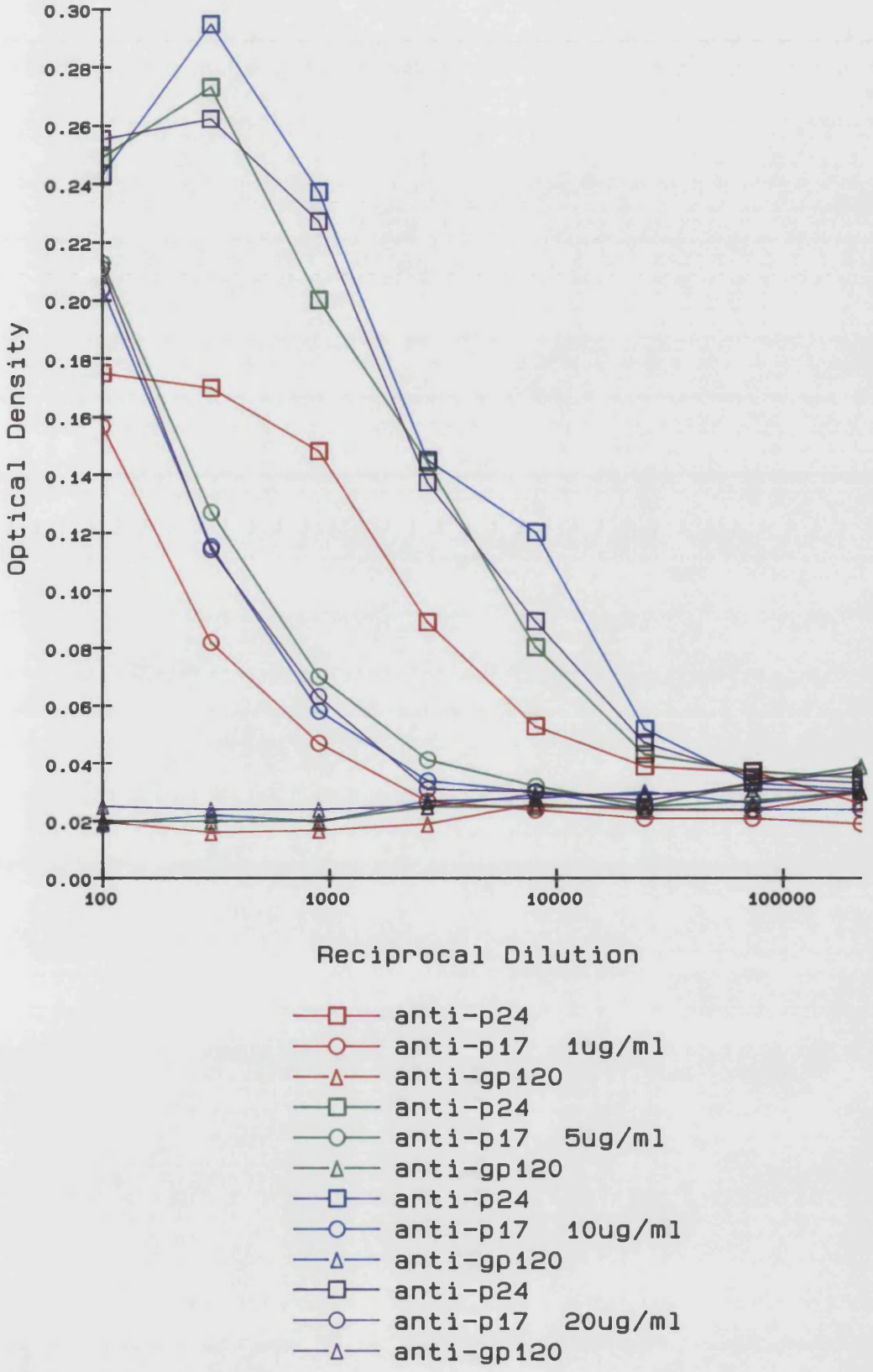


Figure 7.20 Monoclonal antibody titration by GAG ELISA

anti-p17 and anti-p24 monoclonal antibodies were 1:400 and 1:4000 respectively. The differences in titre were probably due to either a difference in binding affinity of the antibodies or in accessibilty of the recognised epitopes. The anti-p24 mid-point titre was comparable with the titre obtained for the 9283 antibody in a DuPont anti-p24 assay (Mr. John Senior, personal communication) and therefore demonstrated that the GAG ELISA was effective at detecting anti-GAG responses. In subsequent tests, OGS575 particles were used at a coating concentration of 5μg/ml and the assay was carried out as described in chapter 2, section 2.18.

7.4.2 Antibody titres of animals immunized with hybrid GAG:V3 particles

7.4.2.1 Anti-GAG responses

Anti-GAG serum antibody titres were determined using the assay described above and are detailed in Table 7. In group 573/1 animals received the OGS573 (GAG:V3:V3) particles in conjunction with Freund's adjuvant and all elicited high titre anti-GAG antibodies. Four of the animals had maximum responses after the second boost, with titres ranging from 1:6000 to 1:15000. Animal 573/1/4 had a maximum response of 1:12000 after the first boost. Sera taken 10 weeks following the second boost (pre-boost three) showed that the anti-GAG response had declined then increased again following the third boost. Titres remained high throughout the rest of the immunization regime. Similar anti-GAG responses were observed in group 574/1, in which rats received OGS574 (GAG:V3) particles in conjunction with Freund's adjuvant. High titres were induced after the first and second boosts in all animals. Animals 574/1/3, 4 and 5 had maximum titres of 1:13000, 1:13000 and 1:8000 respectively after the second boost. Animals 574/1/1 and 574/1/2 had maximum responses post-boost four and three respectively.

TABLE 7. ANTI-GAG RESPONSES OF OGS573 GAG:V3:V3 AND OGS574 GAG:V3 IMMUNIZED ANIMALS

	Reciprocal mid-point titres					
Animal	**Post boost 1**	**Post boost 2**	**Pre boost 3**	**Post boost 3**	**Post boost 4**	**Post boost 5**
573/1/1	1500	6000	700	3500	3000	4000
573/1/2	5000	7000	2000	NT	4000	3000
573/1/3	1200	9000	300	3500	4000	8000
573/1/4	12000	5000	1400	7500	NT	NT
573/1/5	14000	15000	750	7000	11000	13000
574/1/1	8000	9000	2500	9000	11000	9000
574/1/2	8000	9000	600	11000	11000	9000
574/1/3	1000	13000	600	1500	2000	2500
574/1/4	1800	13000	3500	7000	11000	6000
574/1/5	5000	8000	1800	2500	4500	3500

Groups 573/1 and 574/1 received the immunizations in conjunction with Freund's adjuvant.

Table 7 continued

	Reciprocal mid-point titres					
Animal	**Post boost 1**	**Post boost 2**	**Pre boost 3**	**Post boost 3**	**Post boost 4**	**Post boost 5**
573/2/1	7000	8000	1400	2000	6000	12000
573/2/2	1700	1000	100	2000	2000	4000
573/2/3	1800	1800	150	1500	1400	1400
573/2/4	2000	1800	100	3000	12000	15000
573/2/5	2800	4000	300	1800	NT	3500
574/2/1	9000	10000	1500	4000	5000	4000
574/2/2	3500	5500	600	2500	4500	4000
574/2/3	13000	15000	800	5000	3500	5500
574/2/4	8000	14000	1700	4000	14000	NT
574/2/5	2500	1800	700	1500	2000	2000

Groups 573/2 and 574/2 received the immunizations in conjunction with aluminium hydroxide.

Table 7 continued

	Reciprocal mid-point titres					
Animal	**Post boost 1**	**Post boost 2**	**Pre boost 3**	**Post boost 3**	**Post boost 4**	**Post boost 5**
573/3/1	<100	1400	1000	4000	3000	1300
573/3/2	<100	250	150	300	600	500
573/3/3	200	450	100	200	300	250
573/3/4	<100	750	<100	2500	3500	900
573/3/5	<100	350	<100	NT	250	250
574/3/1	800	1000	100	500	750	1000
574/3/2	<100	1000	100	300	500	650
574/3/3	900	600	<100	300	350	350
574/3/4	<100	5000	700	1500	2800	4500
574/3/5	600	NT	100	500	650	800

Groups 573/3 and 574/3 received the immunizations in the absence of adjuvant.

High titre anti-GAG responses were also observed in the groups that received the particles in conjunction with aluminium hydroxide (573/2 and 574/2). All of the animals elicited a substantial response after the first boost. Maximum responses ranging from 1:5500 to 1:15000 were generated after the second boost in four of the animals in group 574/2. In group 573/2, animals 573/2/3 and 573/2/5 had maximum titres of 1:1800 and 1:4000 post-boost two and the highest titres in the other three animals occurred post-boost five.

The responses elicited by OGS573 and OGS574 in the presence of adjuvants were comparable to the anti-GAG titres of human positive sera tested in the same assay (data not shown). Interestingly, the data shown in Table 7 demonstrate that the anti-GAG response was similar whether the immunizations were carried out in Freund's adjuvant or aluminium hydroxide. Furthermore, anti-GAG antibodies were also generated in the absence of adjuvant (groups 573/3 and 574/3; see Table 7). In particular, animals 573/3/1, 573/3/4 and 574/3/4 elicited comparable responses to those immunized with particles formulated with adjuvant. However, in the majority of the animals the overall response was lower in the absence of adjuvant.

7.4.2.2 Mapping of linear epitopes of GAG recognised by anti-OGS573 and anti-OGS574 antisera

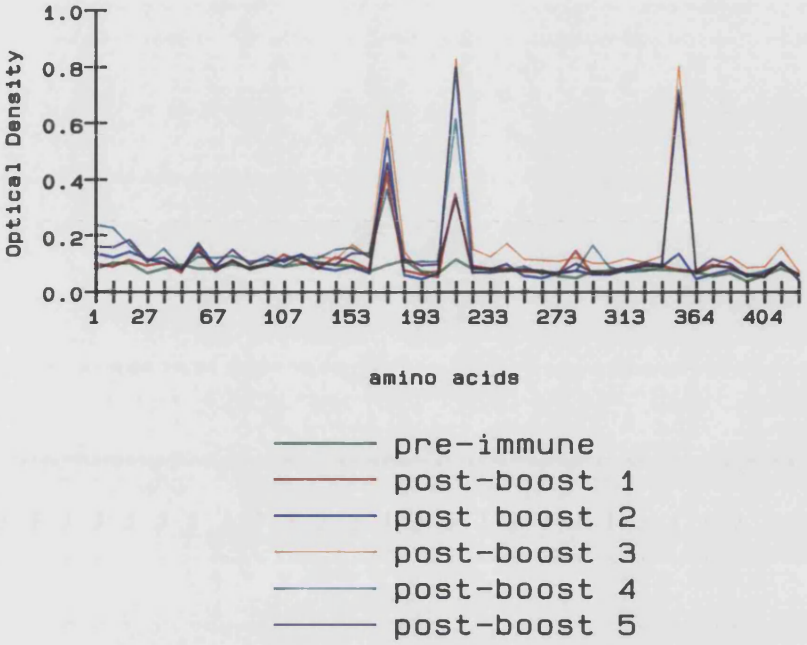
To further analyse the anti-GAG response elicited by the hybrid GAG particles, ELISA's were carried out to determine the reactivity of anti-OGS573 and OGS574 antisera against a series of overlapping GAG peptides obtained from the MRC AIDS Directed Programme. The peptides used covered p17 (amino acids 1-132), p24 amino acids 133-362) and p7 (amino acids 363-438). Peptides covering the p17 and p7 sequences were 15-mers overlapping by five and peptides covering p24 were 20-mers overlapping by ten. Microtitre plates were coated with the individual peptides at a

concentration of 5μg/ml and the anti-OGS573 and OGS574 antisera were tested at a dilution of 1:100. The assays were carried out as described in chapter 2, section 2.18.

Sera taken after each of the five boosts from one animal in each of the six groups were assayed. Figure 7.21a shows the peptide reactivity of antisera taken from animal 573/1/5 that had received the immunizations in Freund's adjuvant. Antibodies recognised three sequences from p24 corresponding to amino acids 173-192, 213-232 and 343-362. Antisera from animal 574/1/2 (Figure 7.21b) also contained antibodies that recognised the amino acid sequence 343-362 and antibodies that recognised p17 and p7 peptides. Antisera from animal 573/2/1 (aluminium hydroxide) reacted against three peptides towards the C-terminus of p24 and one peptide sequence in p7 (Figure 7.21c). Antisera from animal 574/2/2 gave a positive reaction against the N-terminus of p24, a sequence located towards the C-terminus of p24 and also contained antibodies that recognised the same peptide sequence in p7 as antisera from animal 573/2/1(Figure 7.21d). Antisera from animal 573/3/1 that had received the OGS573 particles in the absence of adjuvant reacted against the N-terminus of p24 post-boosts one and two, although sera taken after subsequent boosts did not appear to react with this sequence. Antibodies were also present that reacted against a p7 peptide (Figure 7.21e). Little reaction was observed against any of the peptides with antisera taken from animal 574/3/4 (Figure 7.21f).

Sera taken from all animals after the second boost were also assayed for antibodies that recognised the linear peptide sequences of GAG. The results are shown in Figure 7.22. Sera from the animals in group 573/1 (GAG:V3:V3; Freund's) reacted with a C-terminal peptide of p17, and several peptides within the p24 sequence. In group 574/1 (GAG:V3; Freund's) peptides at the N and C termini of p17 were recognised, as were p24 peptides and an N terminal p7 peptide. Sera from group 573/2 (aluminium

a) Animal 573/1/5



b) Animal 574/1/2

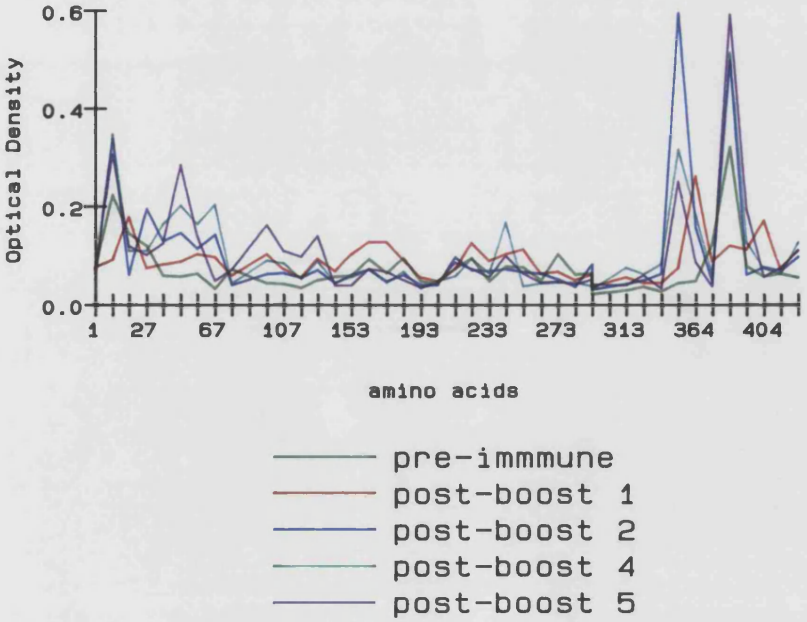
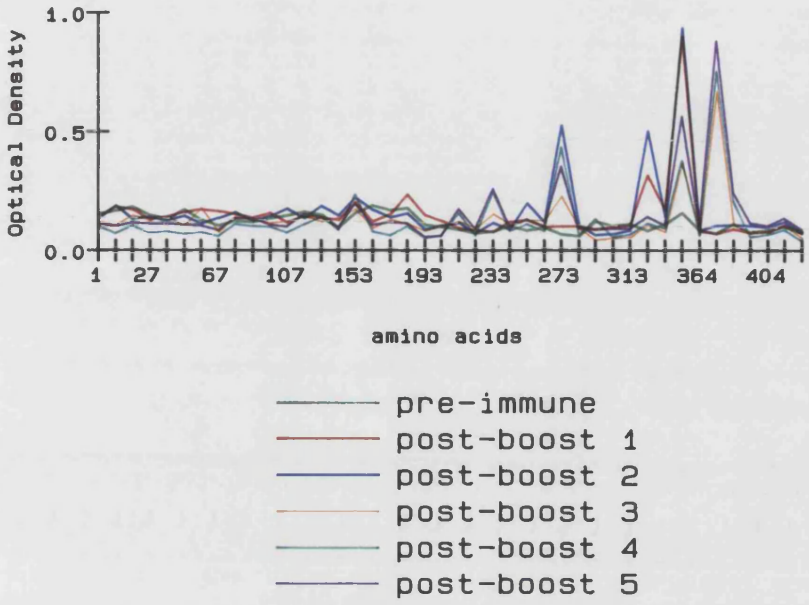


Figure 7.21 GAG linear epitope mapping
p17=1 to 132, p24=133 to 362, p7=363 to 438

c) Animal 573/2/1



d) Animal 574/2/2

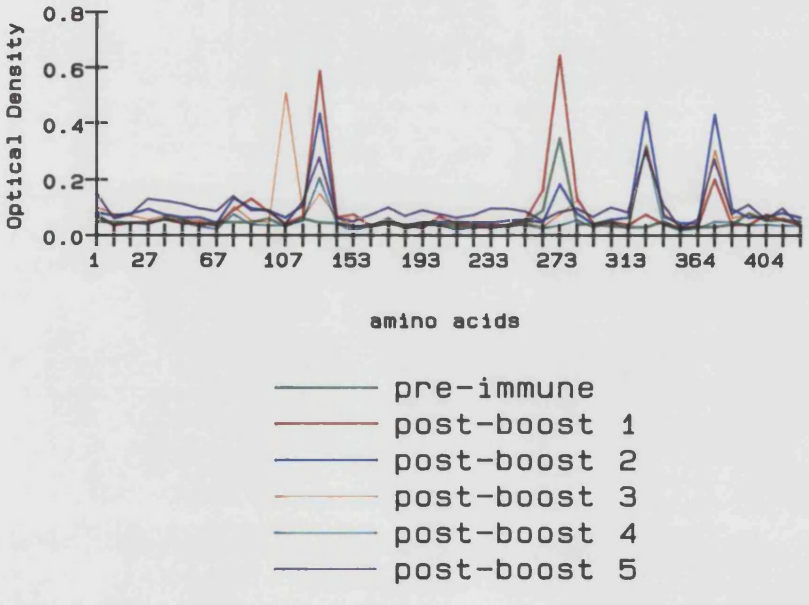
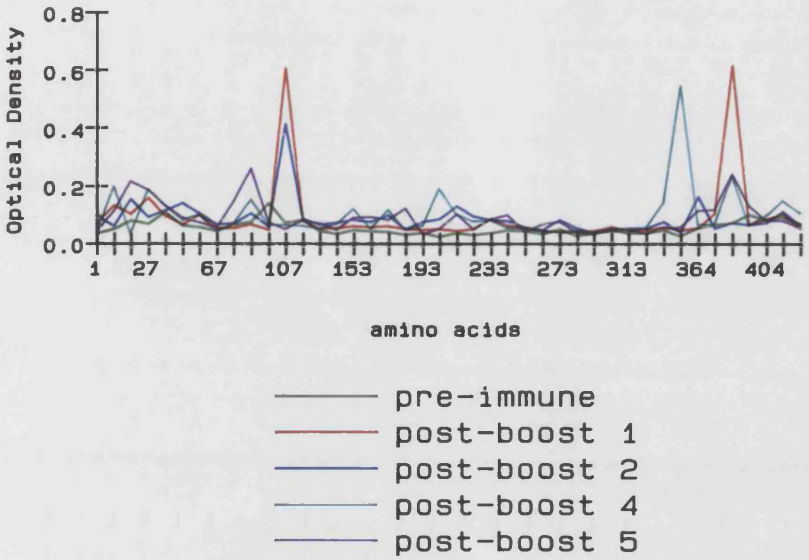


Figure 7.21 GAG linear epitope mapping
p17=1 to 132, p24=133 to 362, p7=363 to 438

e) Animal 573/3/1



f) Animal 574/3/4

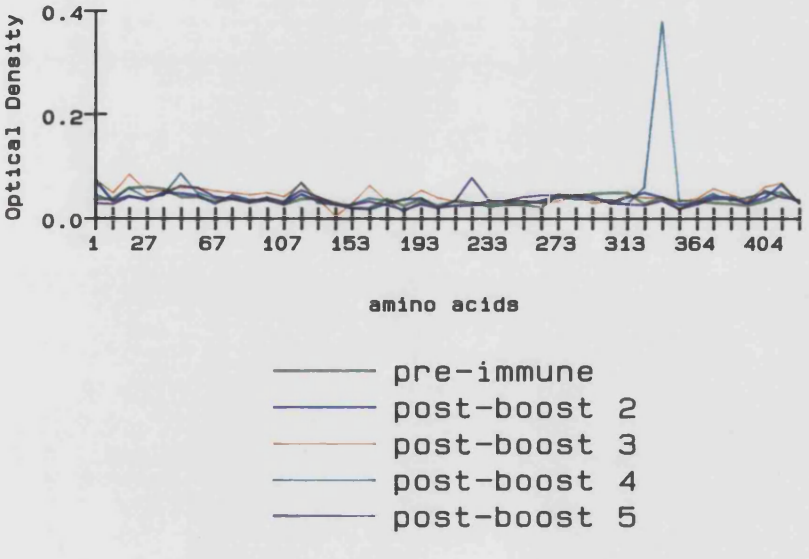
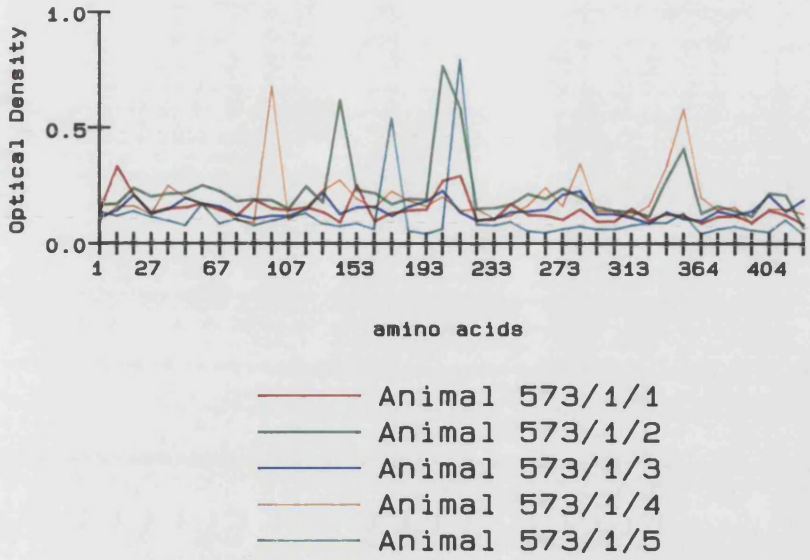


Figure 7.21 GAG linear epitope mapping
p17=1 to 132, p24=133 to 362, p7=363 to 438

a) Group 573/1



b) Group 574/1

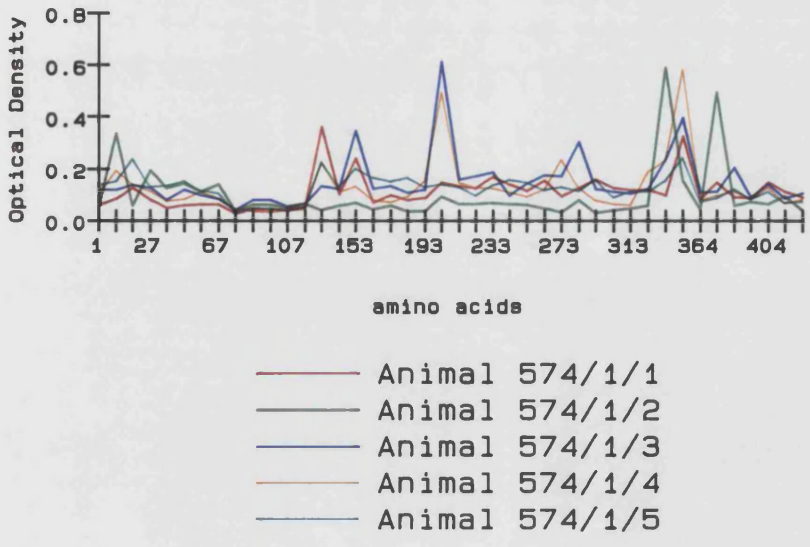
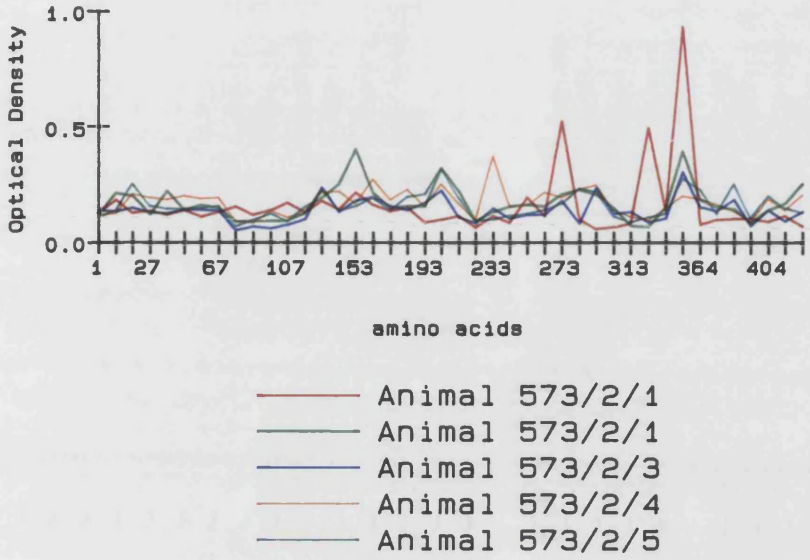


Figure 7.22 GAG linear epitope mapping
p17=1 to 132, p24=133 to 362, p7=363 to 438

c) Group 573/2



d) Group 574/2

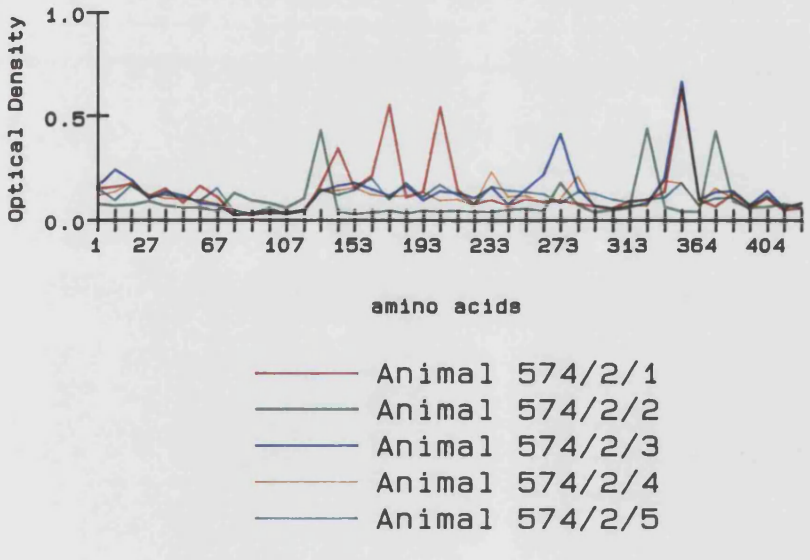
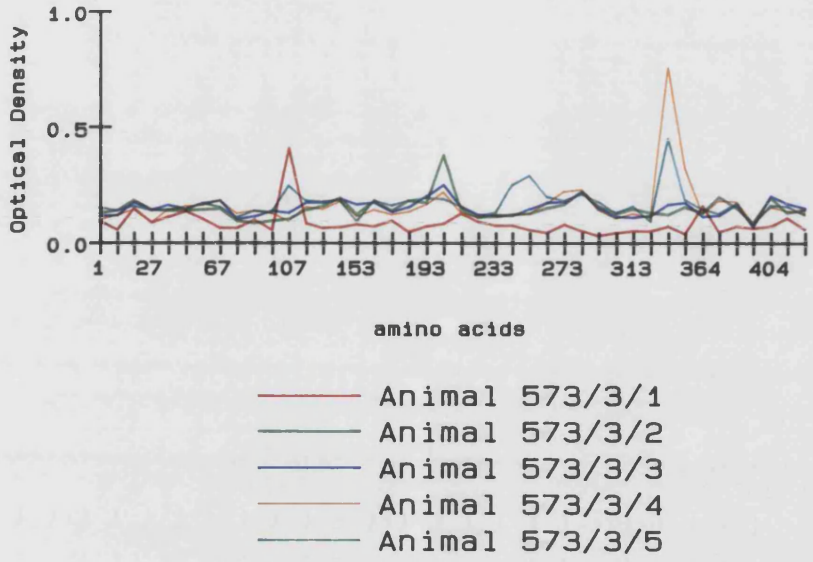


Figure 7.22 GAG linear epitope mapping
p17=1 to 132, p24=133 to 362, p7=363 to 438

e) Group 573/2



f) Group 574/3

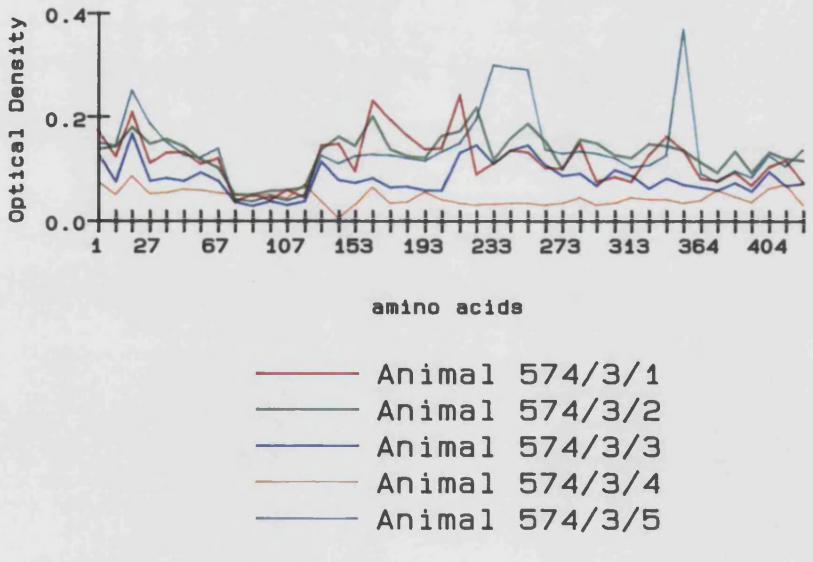


Figure 7.22 GAG linear epitope mapping
p17=1 to 132, p24=133 to 362, p7=363 to 438

hydroxide) showed most reactivity with a C-terminal p24 peptide, with weaker reactions with other p24 regions. In group 574/2 (aluminium hydroxide), reactivity was again seen with the C-terminal p24 region, with positive reactions throughout the rest of p24 and an N-terminal p7 peptide. The reactivities of the sera from groups 573/3 and 574/3 (no adjuvant) were generally weaker, with reactions detected mainly in the p24 peptides.

Antisera raised against the OGS573 (GAG:V3:V3) and OGS574 (GAG:V3) particles contained antibodies that recognised linear sequences throughout the GAG proteins. However, there was a tendency towards a greater number of reactivities toward the N- and C-terminal regions of the complete GAG sequence. In particular, most reactivity was observed at the C-terminus of p24.

7.4.2.3 Anti-V3 responses

Anti-OGS573 and OGS574 sera were assayed for the presence of anti-V3 antibodies against the HXB2 40-mer peptide. However, none of the sera taken after each boost had detectable anti-V3 antibodies in this assay. The sera were also tested for virus neutralizing antibodies using the syncytium inhibition assay. Sera taken post-boosts one and two failed to neutralize the virus. In contrast, sera taken post-boosts three, four and five from some of the animals reduced the number of syncytia present, but none gave rise to a 90% level of inhibition at any dilution. These data therefore suggested that the V3 loop was not presented in a suitable configuration on hybrid GAG:V3 particles to induce virus neutralizing antibodies. Alternatively, the GAG epitopes may have been immunodominant over the V3 epitope, resulting in strong responses against GAG and only weak responses against V3.

7.5 DISCUSSION

Expression of a number of GAG and GAG:V3 fusion proteins using both yeast and baculovirus systems have been investigated. The results are summarized in Figure 7.23. As previously described by Gheysen et al. (1989), full-length myristilated GAG formed budded particles when expressed in the baculovirus system. In contrast, full length, myristilated GAG accumulated at the cell membrane in yeast. In this case, budding of particles was probably prevented by the yeast cell wall. It may be possible to produce budding GAG particles in yeast if expression was induced after removal of the cell wall to produce sphaeroplasts. Expression of non-myristilated full length GAG in yeast resulted in the formation of large inclusion bodies in the cytoplasm. This observation demonstrated that myristilation is essential for accumulation of the protein at the cell membrane and that modification of the GAG protein is required for intracellular particle formation in yeast.

Initially, the V3 loop was inserted between the p7 and p6 domains of the GAG precursor protein, Pr55. This location was chosen because it was believed that insertion between these domains would be less disruptive than insertion within any of the GAG proteins and because a convenient restriction enzyme site was available. However, this insertion not only disrupted particle formation but also altered the cellular location of the GAG:V3 protein in both yeast and insect cells. As full length GAG alone did not accumulate in the nucleus in either yeast or insect cells this suggested that the V3 loop either directly or indirectly caused this change in cellular localization.

Expression of p6-deleted GAG has also been analysed. This approach was justified for two reasons. Firstly, the role of p6 in HIV has yet to be fully elucidated and similar proteins are not found in other retroviral GAG proteins. It is therefore possible that p6 is not involved in or required for particle formation and that hybrid particles could be

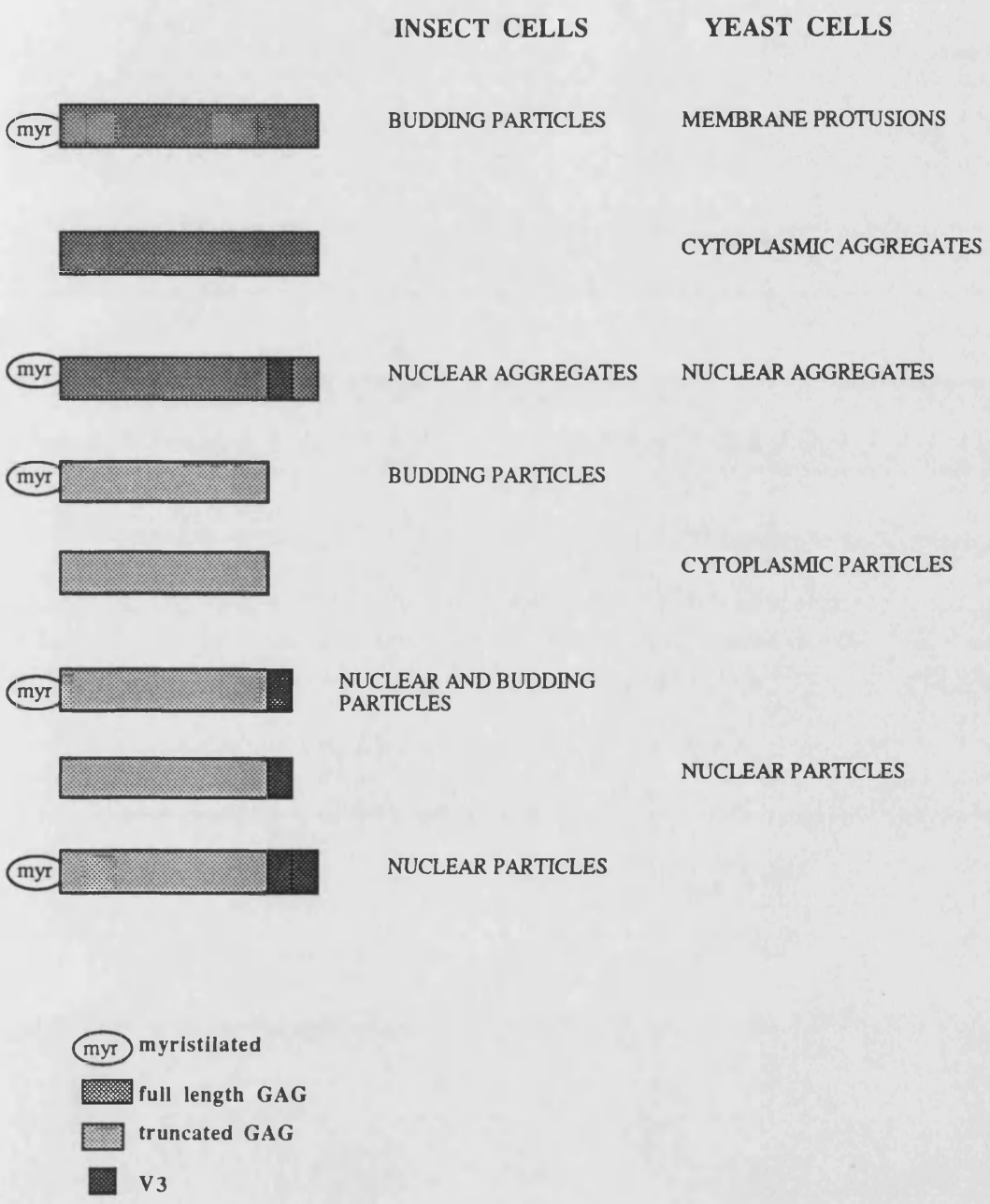


Figure 7.23 Summary of GAG and GAG:V3 expression

formed by replacing p6 with a heterologous antigen. Secondly, previous studies with Ty (Dr. Sally Adams, personal communication) had demonstrated that truncation of the p1 protein resulted in the formation of more stable particles.

Myristilated, truncated GAG formed budded particles that were similar to the particles produced from full length GAG when expressed in insect cells. This result showed that p6 was not required for particle assembly and release in this system. Furthermore, the particles appeared less heterogenous, perhaps supporting the idea that removal of p6 might result in more stable or regular particle formation. The removal of p6 from non-myristilated GAG protein expressed in yeast resulted in the formation of cytoplasmic particles. In contrast, full length non-myristilated GAG did not form discrete particles in yeast. These data suggest that, in yeast, the p6 sequence is deleterious to particle formation.

A non-myristilated truncated GAG:V3 C-terminal fusion protein produced hybrid GAG:V3 nuclear particles in yeast. Similarly, a myristilated, GAG:V3 fusion protein also assembled into particles in insect cells. However, in this case both budded and nuclear particles were observed. A GAG:V3:V3 fusion protein produced only nuclear particles in insect cells. These data demonstrated that genetic modification of the gag gene of HIV can produce a carrier protein that can be used for the presentation of heterologous antigens. All of the constructions containing the V3 loop sequence, whether myristilated or non-myristilated, in either insect or yeast cells, resulted in nuclear localization of the fusion protein. The most obvious explanation for this phenomenon would be that V3 itself contains a nuclear localization signal. However, the V3 loop is not known to contain a conventional nuclear localization signal, although it does contain a series of basic residues as discussed below. The V3 loop forms part of the envelope protein of HIV that is believed to be stripped from the virion following

infection. Even if the V3 loop contained a nuclear targeting signal it therefore seems unlikely that it would function as such in the infection cycle of the virus. A possible explanation for nuclear targeting of GAG:V3 particles is that the V3 loop in the GAG:V3 fusions causes a conformational change in GAG that reveals a previously hidden nuclear localization signal. In all of the constructions described above the V3 loop was introduced at a viral protease cleavage site. In the systems used this site would not be cleaved as the viral protease was not present. It is however possible that the V3 loop sequence could itself be cleaved in these systems as it has previously been reported that the V3 loop contains a non-viral protease cleavage site. When the envelope glycoprotein gp120 is expressed in mammalian cells, an unknown cellular protease specifically cleaves gp120 within the V3 loop (Stephens et al., 1990). It is not known whether this cleavage occurs when gp120 is expressed in yeast or insect cells. However, if cleavage did occur, the V3 loop could be acting as a replacement cleavage site for that normally cleaved in GAG. Protease cleavage of GAG in the virus occurs post budding (Kohl et al., 1988) and therefore the possible conformational change caused by cleavage would happen after the virus had left the cell. If the GAG:V3 constructions were cleaved intracellularly the putative, cryptic nuclear localization signal could have been activated and the particles would then locate to the nucleus. A GAG fusion protein containing a sequence that is not cleaved by cellular proteases could be made in order to investigate this possibility.

Another possible explanation is that the combined GAG:V3 sequence itself creates a nuclear localization signal. A review by Dingwall and Laskey (1991) has suggested a nuclear targeting consensus sequence comprising a bipartite motif defined as two basic residues, a spacer region of any ten amino acids, followed by a second basic cluster in which at least three of the next five amino acids is basic. The HXB2 V3 loop contains the amino acid sequence RKRIR which could be the second basic cluster of the

bipartite motif. Two basic amino acids, **KK**, are located in the p7 sequence of GAG. The spacing between these two basic clusters in the GAG:V3 fusion protein is 34 amino acids. Bipartite signals have been described with spacer lengths other than ten. For example, Morin et al. (1989) showed that the spacing between the two basic clusters necessary for nuclear targeting of the adenovirus DNA-binding protein is 38 amino acids. Therefore the two basic clusters present in the p7 sequence and the V3 loop sequence could be responsible for the nuclear localization of the GAG:V3 fusion proteins.

A third possibility, and perhaps most likely, is that the V3 sequence alone is responsible for the nuclear targeting of the fusion proteins. A review by Roberts (1989) suggested a consensus sequence for a single basic cluster targeting signal. This consensus sequence is **XXKKXX** or **XXKRXR**. The sequence present in the V3 loop described above conforms to this consensus. Also discussed in this review (Roberts 1989) was experimental evidence that efficiency of nuclear targeting is increased with increased copies of the signal sequence. This phenomenon was observed with the expression of the GAG:V3 and GAG:V3:V3 fusions in insect cells where the addition of two V3 loop sequences was required for the protein to locate entirely to the nucleus. The additional V3 loop sequence could also act to increase the efficiency of the putative bipartite motif discussed above.

The possibilties that the nuclear targeting of GAG:V3 particles is caused by either a bipartite motif or by the basic cluster in the V3 loop could be investigated by mutational analysis.

In order to produce hybrid GAG:V3 particles for analysis of the immune response a strategy has been developed for purifying OGS574 (GAG:V3) and OGS573

(GAG:V3:V3) particles from the nuclei of insect cells. The nuclear location of the particles facilitated purification to some extent. However, during the purification it became apparent that the hybrid particles were aggregating and, due to incorporation of cellular components into the aggregates the final material was only approximately 80% pure. Negative stain electron microscopy revealed that discrete particles had been purified in a mesh of contaminating material derived from either nuclear proteins or DNA.

Immunogenicity studies of OGS574 and OGS573 particles have been carried out in rats. The animals received the immunizations in conjunction with Freund's adjuvant, aluminium hydroxide or without adjuvant. All of the animals generated anti-GAG responses and, in particular, high titres were induced after one or two boosts in the animals that received the particles in an adjuvant formulation. Titres were comparable between groups that had received the immunizations in conjunction with either Freund's adjuvant or aluminium hydroxide, demonstrating that the responses were not dependent on a powerful enhancer of the immune response and that they could be generated in a clinically relevant formulation. Anti-GAG titres obtained against the GAG hybrid particles were similar to anti-GAG titres of human HIV positive sera. Decline of anti-core protein antibodies has been correlated with the onset of disease (Schubach et al., 1985; Lange et al., 1986; Weber et al., 1987). If hybrid GAG:V3 particles induced a similar high titre response in humans they could therefore have a potential immunotherapeutic use.

Further analysis of the anti-GAG response induced by OGS574 and OGS573 hybrid particles has been carried out by evaluating the recognition of linear GAG sequences. There was some variation in the sequences reactive with the anti-OGS574 and OGS573 antisera which has also been reported to be the case when examining linear sequences

recognised by HIV positive patients (Broliden et al., 1989). However, in general, the majority of sequences that were recognised corresponded to the N and C-termini of the individual GAG proteins p17, p24 and p7, and in particular the C-terminus of p24. As the termini are at the junctions of viral protease cleavage sites it may be expected that these sequences would be exposed on the surface of the GAG polyprotein and therefore more accessible for antibody recognition. However, the location of the viral protease within the virion remains to be determined. One of the p24 peptide sequences, amino acids 208 to 227, that was recognised by antisera from five of the OGS573 and OGS574 immunized animals, has also been shown to give a positive reaction with antibodies from 35% of a sample of 20 HIV positive patients (Broliden et al., 1989). However, the peptide scan used in the OGS573/OGS574 study was not exhaustive due to the relatively large overlap between the peptides. Also this type of analysis does not identify conformational epitopes and is therefore limited in its usefulness in dissecting the anti-GAG immune response.

The anti-V3 response induced by OGS574 and OGS573 particles has also been analysed. In contrast to the anti-GAG response, the anti-V3 response was negligible, with only weak neutralizing antibodies being induced after a number of boosts. There was no difference in the response generated against one (OGS574) or two (OGS573) copies of the V3 loop which may suggest that the loop was not presented in a configuration that could induce neutralizing antibodies. An alternative explanation could be that a region of the GAG sequence is immunodominant over the V3 sequence. This would seem unlikely as V3 specific antibodies are present in human HIV positive sera (Goudsmit et al., 1988; Goudsmit et al., 1989). However, the sequence at the C-terminus of p24 identified as the most reactive region when tested against anti-OGS573 and OGS574 antisera has not been reported to be reactive with HIV positive sera. This may be due to the sequence being presented in a different

configuration, i.e., as part of the GAG polypeptide rather than as a cleaved viral product. The C-terminal sequence of p24 may not be a "natural" viral epitope and in this alternative configuration it may show immunodominance over the V3 loop in eliciting antibody production when hybrid GAG:V3 particles are used as immunogens. A possible way of testing this would be to insert the V3 loop at the C-terminus of p24 rather than at the C-terminus of p7. This could also be an effective way for improving the anti-V3 response without disrupting an important, clinically relevant epitope. It is possible that insertion of the C-terminus of p24 within GAG may disrupt particle formation. However, the sequence of p24 in this region contains the amino acid sequence GPGHKA. As Javerhian et al. (1989) have shown, virus neutralizing antibodies can be induced against the six amino acid sequence, GPGRAF, found at the tip of the V3 loop. Therefore, an alternative way forward may be to replace the next three amino acids C-terminal to the GPG sequence in p24 to create the GPGRAF sequence by site directed mutagenesis of the GPGHKA p24 sequence. Presentation of part of the V3 loop in this way may result in an effective conformation for the induction of neutralizing antibodies.

Although presentation of the V3 loop on OGS574 and OGS573 particles did not lead to the production of virus neutralizing antibodies, the particles have subsequently been shown to be highly effective at inducing V3-specific cytotoxic T-cell responses (CTL) (Mr. Stephen Harris, personal communication). Therefore, despite the lack of induction of neutralizing antibodies, the presence of high-titre GAG antibodies and V3-specific CTL may still make these particles attractive for immunotherapeutic treatment of HIV-positive individuals. In addition, with further modification of these hybrid particles, it may be possible to elicit virus neutralizing antibodies thereby producing a prophylactic vaccine candidate containing both humoral and cellular components believed to be required for protection against establishment of infection.

The results presented in this chapter have described the production of a novel particulate antigen presentation system, using the HIV GAG polyprotein as a carrier for the heterologous antigen, the V3 loop. The particles have been found to be very effective at eliciting anti-GAG B-cell responses and in generating the production of V3 specific CTLs.

CHAPTER 8

DISCUSSION

8.1 HYBRID HIV:TY-VLPs

Hybrid Ty-VLPs have been constructed that carry the major neutralizing epitope of the envelope protein gp120 from different isolates of HIV-1. Analysis of antigen presentation by hybrid HIV:Ty-VLPs concentrated on VLPs that carry the sequences from clones BH10 and HXB2 of isolate IIIB and also the sequence from the MN isolate. VLPs carrying the IIIB sequences gave a weak reaction with human positive sera, whereas VLPs carrying the V3 loop from the MN isolate gave a very strong reaction. This observation supports reports that the most commonly occuring isolates have sequence homology within this region to isolate MN (Devash et al., 1990; LaRosa et al., 1990; Zwart et al., 1991). Neutralizing antibodies to the V3 loop are type-specific whereas a potential vaccine candidate should elicit broad cross isolate neutralizing antibodies. Presentation of multiple V3 loop sequences has been investigated to determine whether a broad response could be generated.

The HIV V3:Ty-VLPs were used to immunize experimental animals and elicited anti-gp120 and virus-neutralizing responses. However, the results showed that these responses were variable. Analysis of the responses generated against VLPs carrying V3 loops from the clones of isolate IIIB demonstrated that higher titre antibodies were generated against HXB2 V3:Ty-VLPs as compared to BH10 V3:Ty-VLPs. A similar observation has been reported by Neurath and Strick (1990) where rabbits immunized with HXB2 V3 peptide generated higher levels of IIIB neutralizing antibodies than rabbits immunized with BH10 V3 peptide. These data further suggest that the differences in response are due to the immunogenicity of the sequence rather than to animal variation.

High titre neutralizing antibodies were generated against HXB2 V3:Ty-VLPs when the immunizations were carried out in either Freund's adjuvant or aluminium hydroxide.

Aluminium hydroxide is currently the only adjuvant approved for use in humans, and these results therefore demonstrate the efficacy of a hybrid VLP in a clinically relevant formulation. A memory response was also demonstrated. The HXB2 V3:Ty-VLPs therefore fulfilled two of the requirements of a potential vaccine candidate. However the immunogenicity of these VLPs was only tested in small groups of animals. Further experiments would be required, including dose responses and primate immunizations, before HXB2 V3:Ty-VLPs could be considered for evaluation in a clinical trial. Preferably, a challenge experiment would be carried out in chimpanzees.

Although other HIV envelope immunogens that have been used in clinical trials (Zagury et al., 1988; Cooney et al., 1991) are derived from IIIB sequences, the clinical relevance of this isolate is questionable. Sequence analysis of virus isolates and serological analysis of infected individuals have shown that the IIIB isolate is rare, whereas MN-like isolates are common (Devash et al., 1990b; LaRosa et al., 1990; Zwart et al., 1991). This suggests that potential vaccine candidates should include the MN sequence.

Immunogenicity studies of MN V3:Ty-VLPs showed that induction of high titre neutralizing antibodies was dependent on the presence of an immune response potentiator. High levels of MN neutralizing antibodies were generated when the VLPs were used to immunize animals with Freund's adjuvant, although peak titres were lower than the IIIB titres generated by the HXB2 V3:Ty-VLPs, whereas a poor response was generated when the particles were administered in aluminium hydroxide. As the anti-Ty titres were comparable in the groups that received HXB2 and MN V3:Ty-VLPs in aluminium hydroxide, these results suggest that the MN V3 loop presented on Ty-VLPs is less immunogenic than the HXB2 V3 loop, rather than differences being attributable to the VLP preparation. Although the amino acid

sequences are similar at the C- and N-termini and the GPGRAF tip of the loop sequence is the same, non-conservative changes occur in the amino acids immediately to the left and right of the tip. This could alter the antigenicity of the principal neutralizing determinant in the MN loop. Javerhian et al., (1989) have mapped the principal neutralization determinant to eight amino acids at the tip of the loop and have also shown that the location of the neutralization epitope varies between virus isolates with respect to the GPG sequence. The Javerhian study mapped the type specific neutralization epitope of IIIB to include the amino acids to the left of the tip. Immediately to the left of the tip are two amino acids QR in the IIIB V3 loop that are absent in the MN V3 loop. As discussed in chapter 3, the amino acid arginine has properties that could influence structure and therefore antigenicity. However, the MN neutralization epitope has not been specifically mapped and amino acids to the right of the tip could also be influential. As MN like isolates are the most common, it could be speculated that the virus has evolved in such a way as to render this sequence less immunogenic with the result that neutralizing antibodies raised to this epitope are less effective.

Immunization with multiple V3 loop VLPs also generated variable responses. A cocktail of five single V3 loop:Ty-VLPs, including VLPs carrying the sequences from the IIIB isolate, resulted in a delayed response in the generation of IIIB neutralizing antibodies. The use of such a cocktail is not therefore the optimum way of presenting multiple V3 sequences for generating cross neutralizing antibodies. Immunizations with a Ty-VLP carrying V3 loops from three different isolates, HXB2, MN and RF, also failed to induce high titre cross-neutralizing antibodies. However, the majority of animals generated relatively high levels of serum antibodies to the V3 loops. This suggested that presentation of V3 loops in this way resulted in sub-optimal configuration of neutralization epitopes and favoured the presentation of epitopes to

which antibodies with no neutralization activity were raised. Higher neutralization titres were generated against MN in this configuration compared to when the MN loop was presented as a single V3 loop immunogen whereas the converse was found for the HXB2 loop. Palker et al. (1989) reported a similar observation with immunizations of a cocktail of peptides coding for the IIIB, MN and RF loops. Low titre neutralizing antibodies were generated, but with higher titres against MN. Immunogenicity was improved and higher cross-neutralizing titres induced by coupling each of the V3 loop peptides to a peptide coding for an additional T-cell helper epitope of gp120. One report in the literature shows that it is possible to raise high titre neutralizing antibodies by presentation of multiple V3 loop sequences. Wang et al. (1991) presented an octameric peptide of the BH10 V3 loop that was 30 to 50 times more potent than a single peptide conjugated to BSA. Presentation of a cocktail of octamer peptides from six different isolates generated significant cross-neutralization titres. Possible alternative V3:Ty-VLP constructions that may increase the potential to induce high titre cross-neutralizing antibodies are outlined below.

One route to improve the response might be to incorporate additional HIV epitopes in the hybrid V3:Ty-VLPs that could provide "help" in the production of cross-neutralizing antibodies. As discussed above, Palker et al. (1989) demonstrated this principal by coupling an additional T-helper epitope, known as T1, of gp120 to the V3 sequence. The sequence encoding this epitope could be inserted between multiple V3 sequences on a hybrid VLP, for example a p1 C-terminal fusion of T1:HXB2:T1:MN:T1:RF could be made. This may provide an additional advantage by spacing the V3 loop sequences which could enhance the virus-neutralizing capacity as fusing the V3 sequences directly together appears to be deleterious for presentation.

Presentation of sequences coding for the relatively conserved tip of the V3 loop could also be investigated. Javerhian et al. (1990) induced cross-isolate neutralizing antibodies by presenting a peptide coding for three copies of the GPGRAF sequence. As a hybrid Ty-VLP contains approximately 300 copies of the fusion protein it may be possible to induce high-titre cross-isolate neutralizing antibodies using this sequence or multiples of it.

Alternative sites could be explored in p1 to improve the responses against a less immunogenic sequence. The protein p1 has been found to a powerful immunogen and immunodominant sites are likely to be found on the exposed surface of the particle. Such a site could be identified by immunogold labelling of particles using a panel of monoclonal antibodies raised against p1. The immunoreactive sequence that had been surface labelled by a monclonal antibody could then be mapped by Western blot analysis of p1 that had been cleaved by, for example, cyanogen bromide and subsequent N-terminal sequencing of the products. Attempts could then be made to replace this sequence with the V3 loop. This may result in more efficient presentation and would also have the advantage of reducing the immunogenicity of p1.

It is difficult to compare the neutralization titres obtained in this study with those reported in the literature as experiments are carried out in different animal species and many different assays are used. Analysis of immunogenicity has concentrated on immunogens which contain IIIB sequences, including synthetic peptides coding for the V3 loop and recombinant forms of gp120 and gp160.

The most direct comparison is probably with the data from Neurath and Strick (1990), where anti-V3 loop peptide antisera were tested for IIIB neutralizing antibodies. Rabbits were immunized with 200µg/dose of peptide in Freund's adjuvant with a

similar schedule to that used for the V3:Ty-VLP immunogenicity studies. Neutralization titres of 1:8 and 1:100 were recorded for anti-BH10 and anti-HXB2 peptide antisera respectively. A cocktail preparation of 21 V3 loop peptides, which included BH10 and HXB2 peptides, generated neutralization titres of 1:24. Goudsmit et al. (1988) immunized rabbits with a IIIB (BH10) V3 loop peptide conjugated to keyhole limpet haemocyanin (KLH), although a shorter sequence coding for amino acids 305-321 was used. The immunizations were carried out in conjunction with Freund's adjuvant and generated neutralization titres of 1:10. Palker et al. (1988) immunized goats with 28mg of synthetic IIIB or RF V3 peptides coupled to tetanus toxoid in conjunction with Freund's adjuvant and reported maximum neutralization titres of 1:160 after five boosts. In the study outlined above, Javerhian et al. (1989) immunized goats and guinea pigs with peptides encoding the IIIB, MN and RF V3 loops coupled to KLH and reported fusion inhibition titres of 1:40, 1:20 and 1:320 respectively. Using an assay based on reduction of reverse transcriptase activity higher titres of 1:1500, 1:280 and 1:2560 were recorded. This highlights the variations that are seen in the literature depending on the assay used. It is also interesting to note that lower titres were recorded against MN. In a further study Javerhian et al. (1990) reported maximum fusion inhibition titres of 1:240 and 1:80 in sera raised against IIIB and MN peptides respectively. These studies further suggest that the MN V3 sequence is intrinsically less immunogenic than the IIIB sequence.

Only limited data are available for the presentation of V3 loop sequences in conjunction with aluminium hydroxide. Hart et al. (1990) carried out immunogenicity studies with V3 loop peptides in rhesus monkeys using different adjuvant formulations. The monkeys were given a cocktail of peptides containing the sequences from three clones of the IIIB isolate, each coupled to a T cell epitope peptide (amino acids 428-443 of gp120). Immunizations in conjunction with Freund's incomplete adjuvant generated

substantial neutralization titres after three injections as measured by reverse transcriptase assays and after five injections using syncytial inhibition assays. However, in one of the animals immunized in conjunction with aluminium hydroxide low neutralization titres were detected using the reverse transcriptase assay after the fourth, fifth and sixth immunizations and no neutralizing activity was detected using the syncytia inhibition assay.

Other immunogens that have been tested in conjunction with aluminium hydroxide are recombinant preparations of gp120 or gp160. Barrett et al. (1989) reported that when a vaccinia-derived recombinant gp160 was used as an immunogen in goats the response was very poor when the material was adjuvanted with aluminium hydroxide compared to a good response when it was administered in Freund's adjuvant. In conclusion, where studies have been carried out with aluminium hydroxide, the titres achieved are generally of low titre. This is in contrast to the V3:Ty-VLP studies described here, where titres induced by HXB2 V3:Ty-VLPs were equivalent, irrespective of the adjuvant used.

An exception is the work of Berman et al. (1990) who have reported protection of chimpanzees against infection by HIV after immunization with recombinant IIIB gp120 in aluminium hydroxide. Protection of the animals was correlated with levels of antibodies generated against the V3 loop. The animals had neutralization titres of 1:320-1:640. Emini et al. (1992) provided direct evidence that V3 antibodies protect against virus challenge where a chimpanzee was successfully protected by passive immunization with a V3 mouse-human chimaeric monoclonal antibody. The neutralization titre at the time of challenge was 1:320.

It is difficult to compare these results with those obtained using hybrid VLPs carrying the HXB2 V3 loop as the experiments were carried out in different species. However, in rabbits immunized with hybrid HIV HXB2 V3:Ty-VLPs in conjunction with aluminium hydroxide, neutralizing antibodies were detected with titres in a similar range to that reported in the protected chimpanzees. It is therefore possible that immunization with hybrid V3:Ty-VLPs can elicit neutralizing antibody levels above a threshold titre needed for protection.

8.2 Hybrid SIV:Ty-VLPs

Hybrid Ty-VLPs have been produced that carry the putative V3 region from SIVmac251 to investigate whether such a hybrid SIV:Ty-VLP would have use as a model vaccine candidate that could be tested in a challenge experiment. Western blot analysis of the SIV:Ty-VLPs with sera from an experimentally infected macaque demonstrated that this region is immunogenic. However, immunogenicity studies on the SIV:Ty-VLPs have suggested that this region is only weakly immunogenic as low levels of anti-SIV envelope antibodies were detected even when immunizations were carried out in Freund's adjuvant. Preliminary data suggest that it may be possible to elicit weakly-neutralizing antibodies against the V3 region of the SIV envelope. However, taken together with data from other laboratories it appears that this region is not the principal neutralization epitope of SIV.

Putney et al. (1991) have shown that high titre neutralizing antibodies against SIV are generated using native gp120, but not against non-native envelope or peptides. This study concluded that the V3 loop is not, at least independently, a neutralizing determinant because V3 synthetic peptides do not raise neutralizing antibodies. The conformationally dependent principal neutralizing determinant was mapped to the C-terminal third of gp120 using a 45kD native fragment. Recent studies by Kent et al.

(1991b) have shown that monoclonal antibodies raised against the V3 region do not neutralize in vitro. Moreover, these monoclonal antibody studies indicate that the V2 region of the envelope glycoprotein gp120 contains a neutralization epitope of SIV and confirmed that conformational epitopes exist.

As outlined in chapter 1, with the exception of one study, protection of macaques from SIV challenge experiments has been by vaccination with inactivated SIV or infected cell preparations. Lack of correlation with high-titre neutralizing antibodies and protection has been a major concern in these experiments. However recent findings have shown that uninfected cell preparations can induce protection against SIV challenge and that protection of these animals, and the animals vaccinated with SIV preparations, appeared to correlate with anti-cell antibody titres (Stott, 1991). These studies have therefore not yet demonstrated the relevance of neutralizing antibodies in successful challenge experiments. However, the SIV challenge model will remain valid for testing vaccine preparations designed to elicit production of neutralizing antibodies directed towards conformational epitopes, although it would appear that the extrapolation of results with the V3 region of SIV to the HIV model and vice versa, is not applicable.

8.3 Hybrid HIV:GAG VLPs

Investigations have been carried out to determine whether the GAG precursor protein of HIV could act as a carrier for the V3 loop. This approach was taken as Gheysen et al. (1989) demonstrated that GAG expressed in insect cells self-assembled into budded particles and Adams et al. (1987a) have shown that the gag gene of HIV is directly analogous to the TYA gene which encodes the p1 carrier protein of the hybrid Ty-VLP system.

Expression of a number of GAG and GAG:V3 fusions in yeast and insect cells has been investigated and has led to the production of hybrid GAG:V3 particles. In both systems this was achieved by replacing sequences coding for the p6 protein at the C-terminus of GAG with the V3 loop. Electron microscopic analysis revealed an unexpected change in the cellular location of the hybrid particles. Formation of GAG particles in yeast required removal of the membrane targeting signal, i.e., the myristilation signal from the N-terminus of GAG and the deletion of p6. These particles were located in the cytoplasm. Expression of a non-myristilated, p6-deleted GAG:V3 fusion protein resulted in the formation of nuclear particles. In insect cells, a p6-deleted GAG protein formed budded particles similar to particles produced from full length GAG, whereas expression of a GAG:V3 fusion protein resulted in the production of both nuclear and budded particles. Expression of a fusion protein containing two V3 loop sequences resulted in the formation of particles located entirely in the nucleus. Possible explanations for nuclear targeting of the GAG:V3 fusions has been discussed in detail in chapter 7 and it seems possible that a cluster of basic residues present in the V3 loop sequence is responsible for this localisation.

The development of hybrid GAG:V3 particles required modification of the GAG protein which in turn led to some interesting observations in both yeast and insect cells. Expression of GAG in yeast has confirmed the work of others where a protein of the correct molecular weight of 55kD, that was immunoreactive with a p24 antibody, was produced (Jacobs et al.,1989; Vlasuk et al., 1989). As in this study, Jacobs et al. (1989) showed that myristilated GAG targeted to the plasma membrane and non-myristilated GAG was located in the cytoplasm. However, comparison of electron microscopic analysis of transformed cells revealed differences in the nature of the GAG protein produced. The large protusions seen at the cell membrane (Figure 7.3a) were not found by Jacobs et al. (1989) and cytoplasmic particulate aggregates of

non-myristilated GAG (Figure 7.5) were not reported. This may be due to a difference in the promoters used for expression, where higher levels of GAG expression were probably achieved by using the PGK/GAL system, resulting in sufficient accumulation of the protein to form more obvious structures.

In contrast to expression of full-length non-myristilated GAG, the p6-deleted GAG protein formed discrete particles in yeast. This demonstrated that in yeast the presence of p6 appeared to be deleterious for discrete particle formation. However expression of non-myristilated, full-length GAG in insect cells resulted in production of intracellular particles that located in the cytoplasm and nucleus (Gheysen et al., 1989; Royer et al., 1991). These observations suggest that different expression systems may have some influence on both the form and location of non-myristilated GAG because in yeast, GAG was only found in the cytoplasm. In insect cells nuclear localization of recombinant protein may be due to the baculovirus system used as baculovirus locates to the nucleus as part of the infection cycle.

Royer et al. (1991) have expressed a number of non-myristilated and myristilated GAG proteins in insect cells. Expression of myristilated and non-myristilated p15-deleted GAG was investigated and it was reported that the non-myristilated form did not produce particles whereas the myristilated form produced budded particles. In contrast Gheysen et al. (1989) showed that myristilated p15 deleted GAG did not form particles. Expression of a non-myristilated, p6-deleted GAG protein resulted in a reduction of the number of particles found in the nucleus as compared to the full-length, non-myristilated GAG protein and it was proposed that p6 is involved in nuclear localization of GAG (Royer et al., 1991). Expression of myristilated, p6-deleted GAG resulted in budded particles as found in this project. Gottlinger et al. (1991) constructed and expressed mutated proviruses that had all or part of p6 deleted. Virus particles

assembled at the cell membrane and, although budding of p6-deleted virus particles occurred, they did not appear to be released. The particles also had an immature morphology and it was proposed that p6 is involved in final assembly and release of viral particles.

The discrepancies discussed above regarding the expression of modified GAG proteins by different laboratories indicate that the conclusions drawn by the authors in relation to the possible functions of GAG proteins in the virus should be regarded with caution. In an extensive electron microscopic examination of mammalian cells that had been shown by biological assays to be actively producing HIV, it was extremely rare to encounter cells with many budding or budded particles (personal observations). It is, however, common to find cells expressing recombinant forms of GAG producing large amounts of particles. This suggests that, in comparison to viral GAG production, recombinant GAG proteins are often expressed at very high levels, due to the promoters used or because of the absence of other viral proteins that may influence the level of particle production. The discrepancies found between this and other studies expressing the same modified GAG proteins suggest that these different observations may be due to the differences in levels of expression from the different promoters used. In addition, involvement of host cell factors may influence the form and function of GAG proteins. Taken together, these observations highlight the need to test any suggested functions of GAG proteins arising from work on recombinant proteins in HIV infected cells.

Immunogenicity studies have been carried out using hybrid GAG:V3 and GAG:V3:V3 particles purified from the nuclei of insect cells. High-titre anti-GAG antibodies were generated in conjunction with either Freund's adjuvant or aluminium hydroxide. These responses were comparable to the anti-GAG responses seen in sera from HIV infected individuals tested in the same assay. In contrast, a weak humoral response to the V3

component of the particles was observed and the data suggest that the V3 loop was not presented in a favourable configuration to induce the production of virus-neutralizing antibodies. However, work carried out by other members of the group has demonstrated that GAG:V3 particles are effective at inducing cytotoxic T-cell responses. Suggested ways to improve presentation of neutralization epitopes by GAG have been discussed in chapter 7. These include insertion of the V3 loop at an alternative identified site in GAG and modification of a sequence in p24 to create a GPGRAF sequence. This in effect would create a GAG:V3 tip hybrid by minimal disruption of the sequence.

Limited data are available in the literature for comparison with this immunogenicity study, although Haffar et al. (1991) have studied the immune responses generated against recombinant GAG-ENV particles produced from coinfection of mammalian cells with vaccinia viruses carrying gag and env genes. Positive anti-GAG responses were recorded by Western blot analysis and low titre virus-neutralizing antibodies were detected.

The production of hybrid GAG:V3 particles has demonstrated that a modified GAG protein can be used as a carrier for a heterologous antigen. Although only weakly neutralizing antibodies were induced following immunization, high titre anti-GAG antibodies and V3 specific cytotoxic T-cell responses were generated.

8.4 Summary

Analysis of antigen presentation of the V3 loop by two particulate carrier systems has been investigated. Hybrid particles have been produced using the system developed at British Bio-technology based on p1, a protein encoded by the yeast retrotransposon, Ty, and a novel particulate carrier system has been developed by exploiting the

self-assembly properties of the GAG polypeptide precursor protein of the HIV core proteins.

Hybrid V3:Ty-VLPs carrying the V3 loop sequence from the HXB2 clone of isolate IIIB elicited the production of high titre neutralizing antibodies, and a memory response, in a clinically relevant formulation. The titres were comparable to those in two protection studies reported to date (Berman et al., 1990; Emini et al., 1992). However, the hybrid Ty-VLP system may offer advantages over the immunogens used in these studies. In the Berman study the protected chimpanzees had been immunized with recombinant gp120 and in the Emini study protection was achieved by passive immunization with an anti-V3 loop neutralizing monoclonal antibody. Presentation of the V3 loop as a hybrid Ty-VLP rather than as part of gp120 eliminates concerns of possible deleterious side effects of the gp120 interaction with the T-cell receptor CD4. Priming of the immune system and subsequent memory response offers an advantage over passive immunization with antibody.

The results presented in this project demonstrate the potential of using hybrid Ty-VLPs to induce HIV neutralizing antibodies and provides a baseline on which to improve the system to induce high titre cross-isolate neutralizing antibodies that would be required of a potential vaccine candidate.

Hybrid GAG:V3 particles have been produced in yeast and insect cells using a modified GAG protein as the carrier protein. The results have shown that a strong humoral immune response is generated against the GAG component and, as decline in anti-GAG antibodies has been correlated with onset of disease in HIV infected individuals, the particles could have a potential immunotherapeutic use. Although the results have demonstrated that GAG can act as a particulate carrier system for a heterologous

antigen, the V3 loop, only weak humoral responses were observed against this component. If further modification of these hybrid particles can be carried out to elicit the production of virus neutralizing antibodies, they would represent an attractive HIV vaccine candidate containing both the core and envelope components that may be required for protection in humans.

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Induction of High-Titer Neutralizing Antibodies, Using Hybrid Human Immunodeficiency Virus V3-Ty Viruslike Particles in a Clinically Relevant Adjuvant

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The localization of neutralization determinants within the envelope glycoproteins of human immunodeficiency virus (HIV) has been largely achieved by immunizing small animals in conjunction with Freund's adjuvant. However, for eventual use in humans, candidate HIV vaccine components must also be efficacious in a nontoxic formulation. We describe here the production of hybrid Ty viruslike particles carrying the major neutralizing domain of HIV and demonstrate the induction of high-titer virus-neutralizing antibodies and an HIV-specific T-cell proliferative response after immunization in conjunction with aluminum hydroxide. As aluminum hydroxide and aluminum phosphate are the only adjuvants currently licensed for use in humans, these observations have implications for the development of an effective vaccine against HIV.

The humoral immune response can be an important component of protective immunity against infectious agents, and one strategy for immunoprophylaxis against AIDS may be the administration of an immunogen designed to elicit virus-neutralizing antibodies. Although many laboratories have demonstrated that all or parts of the human immunodeficiency virus (HIV) envelope glycoproteins induce neutralizing antibodies when administered in Freund's adjuvant (9, 18, 22, 33, 34, 36), very few studies have been done using clinically approved adjuvants. Aluminum hydroxide has generally only been used in studies involving primates, and in most cases in which neutralization has been observed, the titers have been low (3, 4, 6, 33). However, significant progress toward the development of an HIV vaccine has been made recently with the demonstration that recombinant gp120 formulated in aluminum hydroxide can elicit protective immunity in chimpanzees against a homologous strain of HIV type 1 (HIV-1) (5). The protection was correlated with the induction of high-titer neutralizing antibodies and also with the presence of antibodies targeted against the principal neutralizing determinant of HIV (19) contained within the disulfide-cross-linked third variable domain (V3 loop) of gp120. Although no adverse side effects were observed in the immunized chimpanzees, the immunosuppressive effects that may result from gp120 binding to CD4 and inhibiting T-cell function in humans should still be taken into consideration (10, 13, 24, 31). The use of immunogens containing neutralization determinants but deficient in CD4 binding may therefore be advantageous. Surprisingly, despite considerable interest in the ability of V3 loop peptides to elicit neutralizing antibodies (19, 34, 35, 38) and the in vivo evidence that anti-V3 antibodies may prevent infection by HIV (5, 11), there have been no reports of the efficacy of V3-based immunogens when administered in adjuvants approved for use in humans.

The approach that we took was to analyze the immunogenicity of a V3 loop sequence presented as a particulate fusion protein. We have shown previously that a protein encoded by the *Saccharomyces cerevisiae* retrotransposon Ty can be used as a carrier for producing recombinant HIV antigens that induce both B-cell and T-cell responses in experimental animals (1, 30). The Ty particle itself can tolerate a wide range of additional protein sequence without disruption, allowing small peptide-sized fragments, protein domains, and full-length proteins to be accommodated (1, 15, 30). In addition, any hybrid Ty viruslike particle (Ty-VLP) can be purified by using a generic protocol based on the physical properties of the particle. To produce hybrid HIV V3-Ty-VLPs containing a V3 loop, synthetic oligomers encoding gp120 amino acids 295 to 333 from isolate HXB2 (14) were inserted into the *Bam*HI site of plasmid pMA5620 (1) (Fig. 1a). The resulting plasmid (pOGS514) therefore contained a Ty-HIV fusion gene under the control of the efficient yeast phosphoglycerate kinase promoter (Fig. 1a). After transformation of plasmid pOGS514 into yeast cells, the fusion protein was expressed at high levels and assembled into 50-nm particles (Fig. 1b). These hybrid HIV V3-Ty-VLPs were purified by a standard procedure that has been described previously (15), and the presence of the V3 sequence was confirmed by Western immunoblotting (data not shown). The location of the HIV component was analyzed by immunogold electron microscopy with a V3 loop-specific monoclonal antibody (MAb) (Fig. 1c). When control

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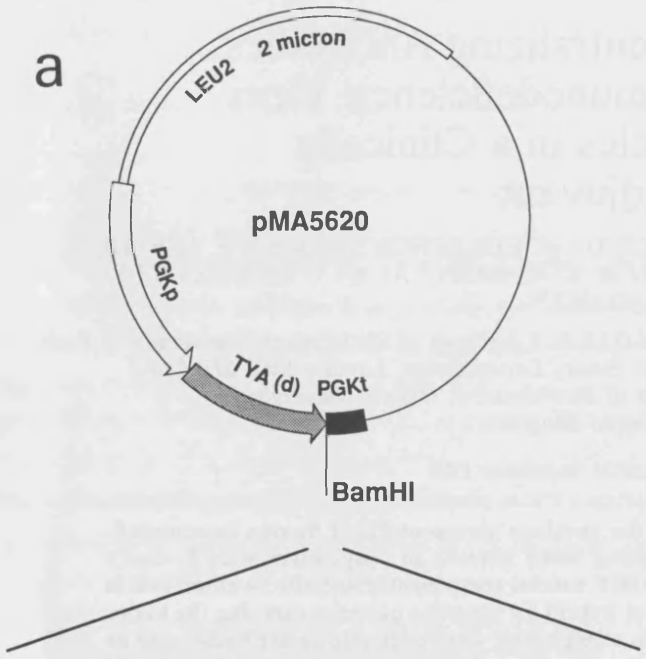


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        N   C   T   R   P   N   N   N   T
GA TCT AAT TGC ACC CGT CCT AAC AAT AAC ACT
     A TTA ACG TGG GCA GGA TTG TTA TTG TCA

 R   K   R   I   R   I   Q   R   G   P   G
AGA AAG AGA ATT AGA ATC CAA AGA GGT CCA GGT
TCT TTC TCT TAA TCT TAG GTT ACA CCA GGT CCA

 R   A   F   V   T   I   G   K   I   G   N
AGA GCT TTC GTT ACC ATT GGT AAA ATC GGT AAT
TCT CGA AAG CAA TGG TAA CCA TTT TAG CCA TTA

 M   R   Q   A   H   C   N   I   S
ATG AGA CAA GCT CAC TGT AAC ATT TCT G
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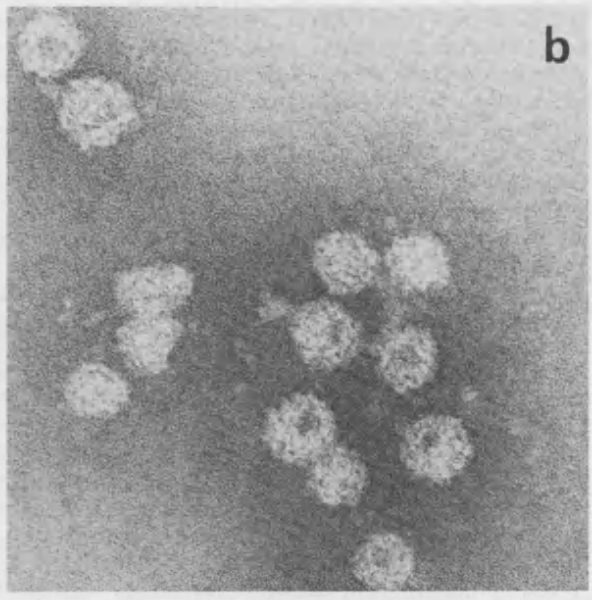


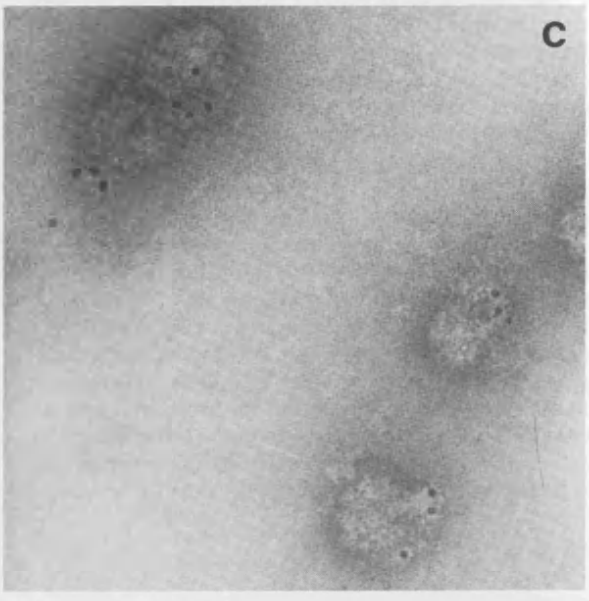
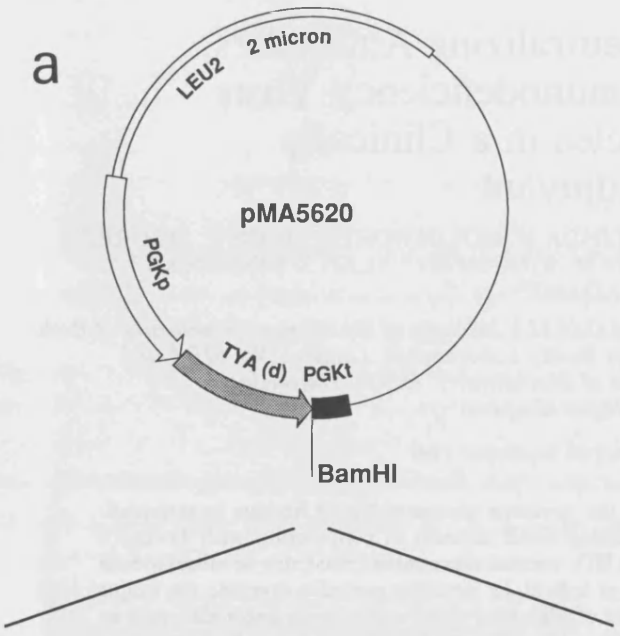


FIG. 1. Construction of HIV V3-Ty-VLPs. (a) Structure of the expression vector pMA5620 and the nucleic acid and predicted amino acid sequence of oligomers encoding the V3 loop of HIV isolate HXB2. Plasmid pMA5620 has been described previously (1) and contains the first 381 codons of the *TYA* gene from the yeast retrotransposon Ty1-15 (20) [TYA(d); shaded arrow], promoter sequences from the yeast *PGK* gene (open arrow), a *LEU2*-2μ selection and replication module (open box), a replication and ampicillin selection module from pBR322 (thin line), and a *PGK* terminator fragment containing stop codons in all three reading frames and a transcriptional stop signal (black box). The codons of the synthesized oligomers were assigned according to yeast codon bias, using published tables of codon usage (16). Annealed oligomers were inserted into the *Bam*HI site of pMA5620 to generate plasmid pOGS514. (b) Electron micrograph of HIV V3-Ty-VLPs, prepared for electron microscopy by procedures described previously (28). (c) Electron micrograph of HIV V3-Ty-VLPs probed with a gold-labeled anti-gp120 MAb. Immunogold labeling was done by incubating HIV V3-Ty-VLPs with a gp120-specific MAb, 9284 (36) (Du Pont; raised against isolate IIIB). Bound antibody was detected after incubation with anti-mouse antibody conjugated to 5-nm gold particles (Sigma) and staining with 3% phosphotungstic acid.

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     A TTA ACG TGG GCA GGA TTG TTA TTG TCA

 R   K   R   I   R   I   Q   R   G   P   G
AGA AAG AGA ATT AGA ATC CAA AGA GGT CCA GGT
TCT TTC TCT TAA TCT TAG GTT ACA CCA GGT CCA

 R   A   F   V   T   I   G   K   I   G   N
AGA GCT TTC GTT ACC ATT GGT AAA ATC GGT AAT
TCT CGA AAG CAA TGG TAA CCA TTT TAG CCA TTA

 M   R   Q   A   H   C   N   I   S
ATG AGA CAA GCT CAC TGT AAC ATT TCT G
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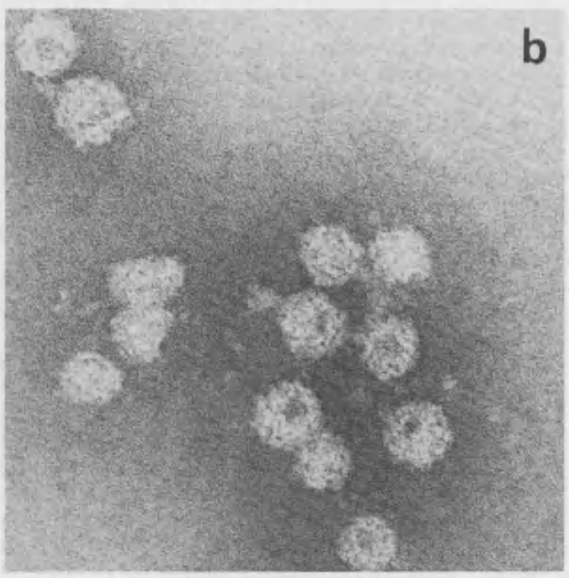
FIG. 1. Construction of HIV V3-Ty-VLPs. (a) Structure of the expression vector pMA5620 and the nucleic acid and predicted amino acid sequence of oligomers encoding the V3 loop of HIV isolate HXB2. Plasmid pMA5620 has been described previously (1) and contains the first 381 codons of the *TYA* gene from the yeast retrotransposon Ty1-15 (20) [TYA(d); shaded arrow], promoter sequences from the yeast *PGK* gene (open arrow), a *LEU2*:2μ selection and replication module (open box), a replication and ampicillin selection module from pBR322 (thin line), and a *PGK* terminator fragment containing stop codons in all three reading frames and a transcriptional stop signal (black box). The codons of the synthesized oligomers were assigned according to yeast codon bias, using published tables of codon usage (16). Annealed oligomers were inserted into the *Bam*HI site of pMA5620 to generate plasmid pOGS514. (b) Electron micrograph of HIV V3-Ty-VLPs, prepared for electron microscopy by procedures described previously (28). (c) Electron micrograph of HIV V3-Ty-VLPs probed with a gold-labeled anti-gp120 MAb. Immunogold labeling was done by incubating HIV V3-Ty-VLPs with a gp120-specific MAb, 9284 (36) (Du Pont; raised against isolate IIIB). Bound antibody was detected after incubation with anti-mouse antibody conjugated to 5-nm gold particles (Sigma) and staining with 3% phosphotungstic acid.



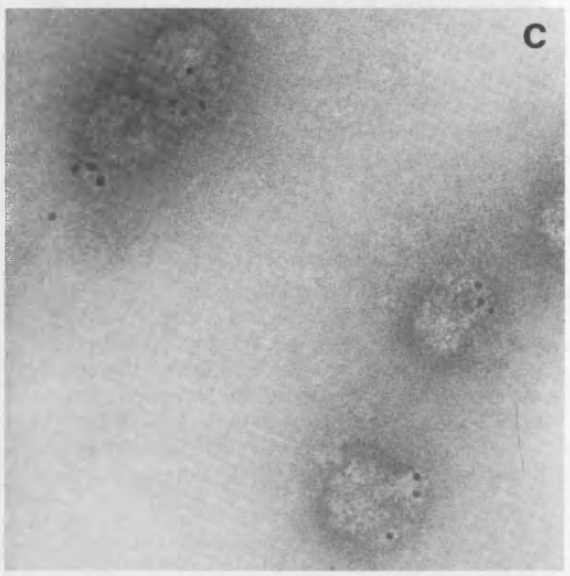


TABLE 1. Serum antibody and neutralizing antibody titers of rabbits immunized with HIV V3-Ty-VLPs[a]

Adjuvant and animal no.	Reciprocal serum antibody midpoint titer of serum sample:						Reciprocal HIV neutralizing antibody titer of serum sample:					
	PI	PB1	PB2	PB3	PB4	PB5	PI	PB1	PB2	PB3	PB4	PB5
Freund's adjuvant												
F1	<100	200	1,200	11,000	13,000	13,000	<4	4	16	256	256	512
F2	<100	200	2,000	8,000	9,000	2,000	<4	8	32	32	256	128
F3	<100	150	900	9,000	12,500	5,000	<4	256	64	128	128	512
Aluminum hydroxide												
A1	<100	2,000	1,000	6,500	9,000	13,000	<4	32	256	256	512	2,048
A2	<100	900	400	500	2,000	5,000	<4	64	128	32	128	512
A3	<100	<100	2,000	4,500	6,000	2,500	<4	<4	64	128	32	128

[a] Animals F1 to F3 received a priming immunization in Freund's complete adjuvant and were then boosted with HIV V3-Ty-VLPs in Freund's incomplete adjuvant. Animals A1 to A3 received priming and booster immunizations of HIV V3-Ty-VLPs as an aluminum hydroxide precipitate. The levels of serum antibodies and virus-neutralizing antibodies were assessed in preimmune sera (PI) and in sera taken 7 days after each booster immunization (PB1 to PB5). Serum antibodies against gp120 were measured by ELISA, and midpoint titers were calculated as the dilution of serum which gave rise to one-half the maximum optical density at 450 nm. Neutralizing antibody titers are expressed as the dilution of serum that resulted in 90% inhibition of syncytium formation. 90% inhibition of syncytium formation was defined as the degree of cytopathic effect observed in control wells infected with 50 $TCID_{50}$ units of untreated virus. Six human immune sera (QC1 to QC6) from anonymous British HIV-positive blood donors (27) gave reciprocal neutralization titers of 80, 80 to 320, 40, 10, 320, and 320, respectively, in this assay.

Ty-VLPs were probed with the same MAb, no specific labeling was observed. As the interior of the particles is unlikely to be accessible to the gold-antibody complexes, the specific labeling of the HIV V3-Ty-VLPs indicates that at least part of the V3 loop region is exposed on the surface of the particles.

To establish that the V3 loop region was immunogenic when presented in this conformation, we initially used purified particles to immunize rabbits in conjunction with Freund's adjuvant. Rabbits were immunized intramuscularly with 500 μg of purified HIV V3-Ty-VLPs at 2-week intervals. A 500-μg sample of HIV V3-Ty-VLPs contains 50 μg of the HIV V3 loop component. The animals received a priming immunization in Freund's complete adjuvant and were then boosted with HIV V3-Ty-VLPs in Freund's incomplete adjuvant. Serum antibody titers were determined against recombinant gp120 (isolate IIIB [37]) secreted from Chinese hamster ovary (CHO) cells (Celltech Ltd.; obtained from the MRC AIDS Directed Programme) in an enzyme-linked immunosorbent assay (ELISA). Neutralization titers were measured by an infection inhibition assay based on the method described by Kinney-Thomas et al. (21). Briefly, heat-inactivated serum was incubated with 500 50% tissue culture infectious dose ($TCID_{50}$) units of HIV-1 isolate IIIB for 1 h at 37°C and then added to 10^5 C8166 cells. After 48 h at 37°C, the cultures were examined for the presence of syncytia. Titers were expressed as the dilution of serum that resulted in 90% inhibition of syncytium formation. Table 1 (top) demonstrates that both serum antibodies and virus-neutralizing antibodies were detected after a single boost and increased to high titers after subsequent boosts. These data therefore demonstrated that HIV V3-Ty-VLPs could be used to elicit significant levels of anti-HIV neutralizing antibodies when administered with a potent adjuvant.

To evaluate the immunogenicity of the HIV V3-Ty-VLPs in a formulation that has been previously approved for use in humans, we administered the particles to rabbits as an aluminum hydroxide precipitate (Table 1, bottom). Surprisingly, both the anti-gp120 serum antibody titers and virus-neutralizing titers obtained against HIV isolate IIIB were at least equivalent to, and in some cases higher than, the responses obtained with Freund's adjuvant. The specificity of the neutralization response was assessed with the divergent HIV isolates RF (40) and MN (17). Sera taken from animals A1, A2, and A3 after the fifth booster immunization showed no significant neutralization of either of these isolates. The longevity of the anti-IIIB response was evaluated by analyzing sera taken from two of the immunized animals every 2 weeks after the final boost for a period of 10 weeks (Table 2). The neutralizing antibody titers of animal A1 remained constant at 1/512 for 8 weeks after the final boost and then dropped to 1/64, whereas the ability of sera from animal A3 to neutralize HIV isolate IIIB declined gradually throughout the 10-week period, with a half-life of 20 to 30 days. However, in both animals, significant levels of serum and neutralizing antibodies were still present 10 weeks after the last immunization.

Javaherian et al. (19) and Meloen et al. (29) have recently demonstrated that the major neutralizing epitope of HIV-1 isolate IIIB is defined by eight amino acids (QRGPGRAF) at the tip of the V3 loop (Fig. 2A). We therefore investigated whether the antibodies raised against the HIV V3-Ty-VLPs

TABLE 2. Longevity of the antibody response in animals immunized in conjunction with aluminum hydroxide[a]

Animal no.	Reciprocal serum antibody midpoint titer at wk:					Reciprocal HIV neutralizing antibody titer at wk:				
	2	4	6	8	10	2	4	6	8	10
A1	1,000	4,000	800	1,100	300	512	512	512	512	64
A3	200	300	400	200	300	64	64	32	16	16

[a] The levels of serum antibodies and neutralizing antibodies were assayed in sera taken from animals at 2-week intervals after the final immunization with HIV V3-Ty-VLPs.

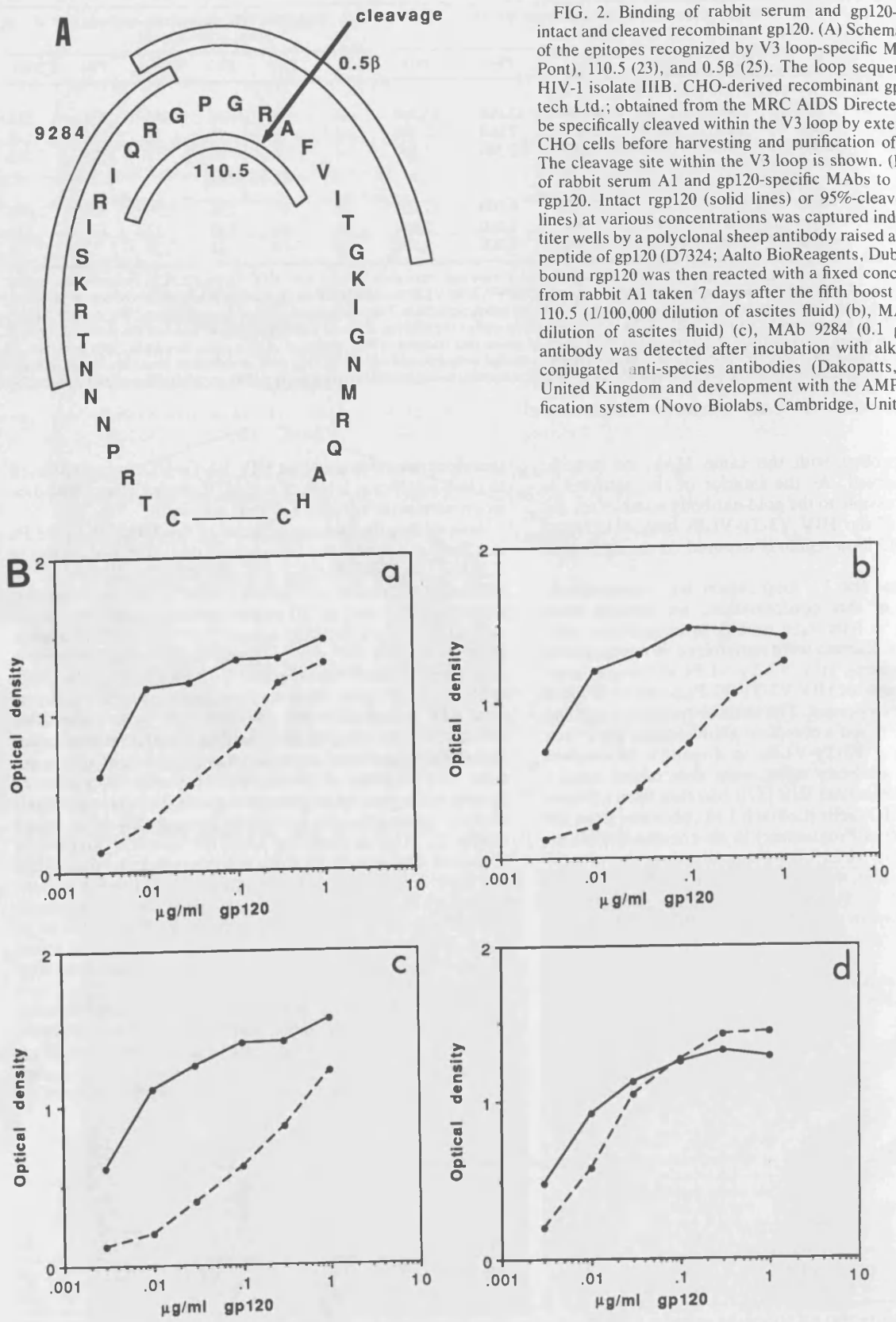


FIG. 2. Binding of rabbit serum and gp120-specific MAbs to intact and cleaved recombinant gp120. (A) Schematic representation of the epitopes recognized by V3 loop-specific MAbs 9284 (39) (Du Pont), 110.5 (23), and 0.5β (25). The loop sequence shown is from HIV-1 isolate IIIB. CHO-derived recombinant gp120 (rgp120; Celltech Ltd.; obtained from the MRC AIDS Directed Programme) can be specifically cleaved within the V3 loop by extended culture of the CHO cells before harvesting and purification of the material (41). The cleavage site within the V3 loop is shown. (B) Binding profiles of rabbit serum A1 and gp120-specific MAbs to intact and cleaved rgp120. Intact rgp120 (solid lines) or 95%-cleaved rgp120 (broken lines) at various concentrations was captured indirectly onto microtiter wells by a polyclonal sheep antibody raised against a C-terminal peptide of gp120 (D7324; Aalto BioReagents, Dublin, Eire) (32). The bound rgp120 was then reacted with a fixed concentration of serum from rabbit A1 taken 7 days after the fifth boost (1/3,000) (a), MAb 110.5 (1/100,000 dilution of ascites fluid) (b), MAb 0.5β (1/100,000 dilution of ascites fluid) (c), MAb 9284 (0.1 μg/ml) (d). Bound antibody was detected after incubation with alkaline phosphatase-conjugated anti-species antibodies (Dakopatts, High Wycombe, United Kingdom and development with the AMPAK ELISA amplification system (Novo Biolabs, Cambridge, United Kingdom).

Clapham (Chester Beatty Laboratories) for human sera; Robert Gallo (National Cancer Institute, Bethesda, Md.) for HIV isolates IIIB, RF, and MN; Nigel Burns (British Bio-technology) for advice on VLP purifications; and Kate Powell (Bath University) for assistance with electron microscopy.

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