ANTIBODY MEDIATED NEUTRALIZATION AND ENHANCEMENT OF HIV-1 INFECTION

(ANTILICHAAM GEMEDIEERDE NEUTRALISATIE EN VERHOGING VAN HIV-1 INFECTIE)

PROEFSCHRIFT

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CHAPTER 1

General introduction

TAXONOMY, GENOMIC ORGANIZATION AND STRUCTURE OF HIV-1

Human immunodeficiency virus type 1 (HIV-1) was identified in 1983 as the causative agent of the acquired immunodeficiency syndrome (AIDS)[1-3]. HIV-1 has been included in the genus *Lentiviruses* of the Retroviridae family (subfamily Lentivirinae). Several related lentiviruses have been identified,which in some cases induce a pathogenesis in their natural host, similar to that induced by HIV-1(*). These include:

* HIV-2 in humans[4]
* simian immunodeficiency virus (SIV) in non-human primates[5-11]
* feline immunodeficiency virus (FIV) in cats, and closely related lentiviruses in other Felidae[12-14]

  bovine immunodeficiency virus (BIV) in cows[15]

  caprine arthritis-encephalitis (CAEV) in goats[16]

  equine infectious anaemia virus (EIAV) in horses[17]

  Maedi-Visna virus in sheep[17]

The genomic organisation of these lentiviruses shows many similarities although differences exist (Figure 1)[18]. On basis of these similarities and their phylogenetic relationship (Figure 2) it has been postulated that the immunodeficiency viruses (HIV, SIV and FIV) have diverged from a common ancestor[11,19]. Phylogenetically HIV-1 may be divide into different subtypes[20]. Results of analysis of a large panel of V3 domain sequences of HIV-1 strains from each phylogenetic subtype seems to indicate that these subtypes represent introductions of cladal founder viruses into different human subpopulations[21]. Similarities in structure, genomic organization and pathogenesis have prompted investigators to use infection of animals with lentiviruses related to HIV-1, as model systems for HIV-1 infection in humans. However, differences in the pathogeneses of lentivirus infections and in the mechanisms of envelope glycoprotein mediated entry, indicate that caution should be taken in extrapolating findings in these systems to HIV-1 infection of humans.

The mature HIV-1 particle is sphericle with a diameter of approximately 100nm[22]. The two positive stranded RNA molecules encoding the virus proteins bind several small structural proteins and the reverse transcriptase, thereby forming the ribonucleoprotein. During the budding process several other regulatory proteins are also encapsulated within the virion. The cone shaped core consisting of the p24 protein holds the ribonucleoprotein. This ribonucleoprotein is surrounded by the myristilated p17 which is attached to the virion lipid bilayer via an interaction with the transmembrane glycoprotein gp41. The highly glycosylated surface protein gp120 is non covalently attached to the gp41 molecule. Electron microscopical studies have shown that 72 spikes, which each consist of tri- or tetrameric complexed gp41-gp120 molecules, protrude from the HIV-1 particle[23,24]. Also proteins of the cell are incorporated in the virion membrane, during the budding process[25].

HIV-1 INFECTION IN HUMANS: TRANSMISSION AND PATHOGENESIS

Infectious HIV-1 can be recovered from several human organs and tissues including peripheral blood and semen[1]. As a consequence HIV-1 may be transmitted during hetero- and homosexual contacts, by needle sharing, by needle stick accidents, by blood transfusions and vertically from mother to infant[26-28]. HIV-1 infection at the cellular level is initiated by the high affinity interaction of the gp120 molecule with it's primary cellular receptor CD4[29-31]. The main targets of HIV-1 infection are therefore CD4 expressing cells. These include T lymphocytes of the helper

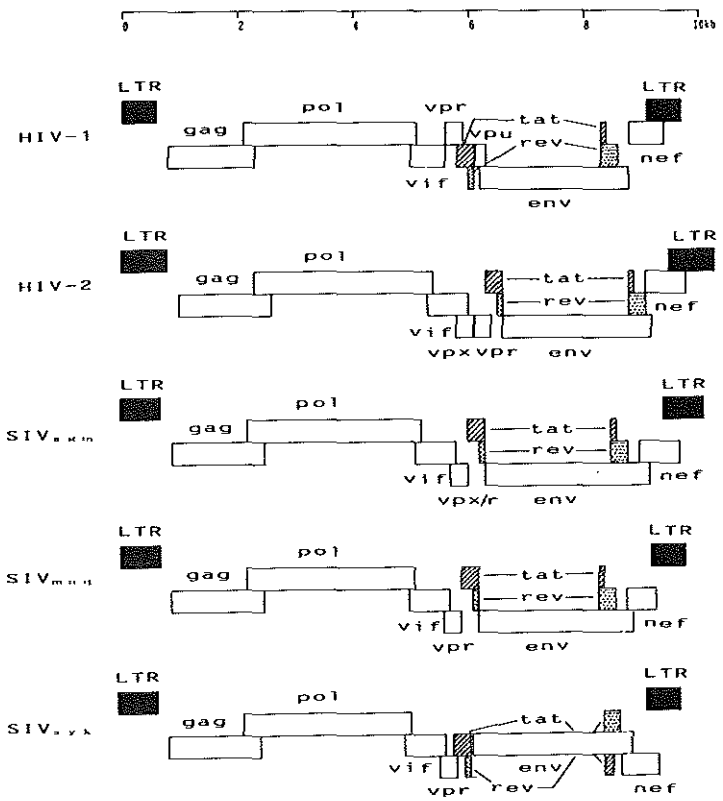

Fig. 1. Genomic organization of the five major groups of primate lentiviruses. The HIV-1 group includes HIV-1 and SIVcpz; the HIV-2 group includes HIV-2, SIVsmm, SIVmne and SIVmac. Adapted with permission from Hayami, M., Ido, E. and Miura, T. Survey of SIV among nonhuman primate populations. in Current Topics in Microbiology and Immunology, vol. 188, pages 1-20.

phenotype[1], cells of the monocyte/macrophage lineage in lymphoid organs[32], brain[33], alveolar fluid[34] and bone marrow[35]. Furthermore, follicular dendritic cells (FDC's), Langerhans cells and peripheral blood dendritic cells may become infected[36-38]. Only recently it was shown that dendritic cells in secondary lymphoid organs are a primary reservoir of HIV-1 producing cells *in vivo* during the asymptomatic phase of the infection[36]. Both HIV-1 and HIV-2 may also infect $CD4^-$ cells *in vitro*, but the significance of this finding for the *in vivo* situation has not been demonstrated[39-41].

Three different stages of HIV-1 induced pathogenesis in humans may be distinguished:

The acute phase:

The acute phase of the HIV-1 infection runs a subclinical course in 30-50% of the cases[42]. Mild and transient symptoms may occur similar to an influenza- or mononucleosis like illness. More sever clinical symptoms during the acute stage of the HIV-1 infection are associated with a rapid progression towards AIDS[43]. Initially high levels of HIV-1 p24 core antigen can be detected in the peripheral blood, a concurrent sharp decline in $CD4^+$ T cells suggests a high replication rate and

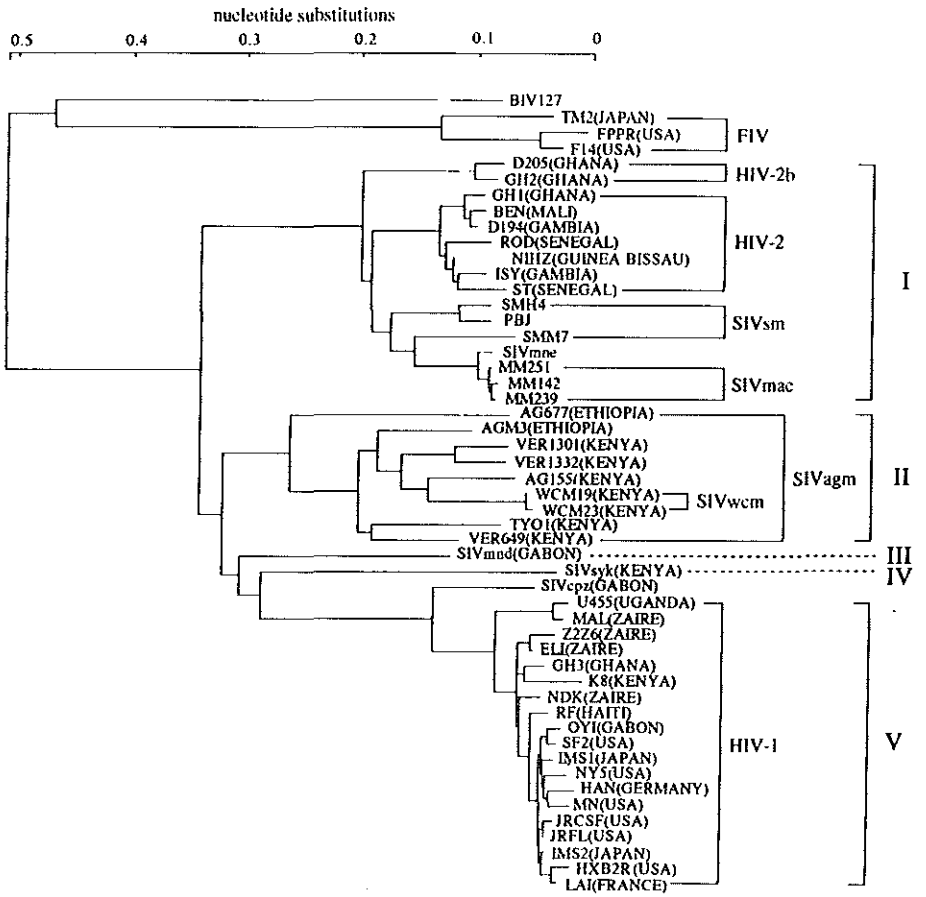

Fig. 2. Phylogenetic tree of AIDS-related viruses. The tree was constructed after allignment of the nucleotide sequence of a part of the *pol* gene (integrase). The number of estimated nucleotide substitutions per site is given at the top of the figure; horizontal branch length indicates the genetic distance. Adapted with permission from Hayami, M., Ido, E. and Miura, T. Survey of SIV among nonhuman primate populations. in Current Topics in Microbiology and Immunology, vol. 188, pages 1-20.

a direct cytopathic effect of the virus in these cells[44]. An immune mediated cell killing of HIV-1 infected $CD4^+$ cells can't be excluded however despite the fact that the first HIV-1 specific $CD8^+$ cytotoxic T lymphocytes (CTL) in the circulation are generally detected after initiation of the $CD4^+$ cell decline[45,46]. Furthermore redistribution of $CD4^+$ cells may contribute to the decline in $CD4^+$ cells in the peripheral blood. The subsequent decrease in free HIV-1 p24 core antigen levels and the concurrent rise of $CD4^+$ cells to nearly normal levels has been attributed to the development of an HIV-1 specific immune response and a self limiting nature of the primary HIV-1 infection[47]. HIV-1 specific $CD8^+$ CTL and HIV-1 specific antibodies are detected around the time that the HIV-1 p24 core antigen levels in circulation start to decline[46,48]. Despite the fact that *in vitro* virus neutralizing (VN) antibodies are detected much later, *in vivo* locally produced VN antibodies,

antibody dependent cellular cytotoxicity and formation of antigen-antibody complexes by HIV-1 specific antibodies may contribute to the decline in the free HIV-1 p24 core antigen levels found soon after infection.

The asymptomatic phase:

The period between initial infection and the time of AIDS diagnosis may vary from one to more than fifteen years[49]. A recent study has shown that the viral load in an infected individual is highly predictive for the length of the asymptomatic period[50]. Studies on asymptomatic individuals, who differ significantly in their disease progression rates, suggested that the length of the asymptomatic period may depend on the biological phenotype of the initial virus inoculum and/or on the quantity and quality of the HIV-1 specific immune response[51-54]. Only recently host genetic factors have been shown to contribute to the susceptibility of the host to HIV infection and the rate of disease progression[55]. During the asymptomatic phase CD4 čell numbers and the ratio between $CD4^+$ and $CD8^+$ cells usually decline gradually[56]. Only low to undetectable levels of free HIV-1 p24 core antigen levels are found in the peripheral blood[50,56]. Antibodies against all major HIV-1 proteins are found in HIV-1 seropositive individuals during this phase whereas the $CD8^+$ CTL response seems to be more restricted[57,58]. Most of the latter data were however generated with antigens from heterologues virus strains. During this phase only low numbers of HIV-1 infected cells are detected in the peripheral blood. In the primary lymphoid organs (lymph nodes and spleen) high numbers of HIV-1 particles are sequestered on the cell surface of dendritic cells and a high percentage of $CD4^+$ T cells are non productively infected[32,59]. The FDC network and the lymph node architecture are both relatively intact although follicular hyperplasia is observed in most cases[60]. As mentioned above HIV-1 *in situ* hybridization studies of adenoids have shown that cells expressing DC markers are productively infected and may form syncytia in the absence of *in vitro* syncytium inducing (SI) virus variants in the peripheral blood lymphocytes[36].

The symptomatic phase:

The symptomatic phase of HIV-1 infection is preceded by a sharp decline in $CD4^+$ cell numbers[56]. The diagnosis AIDS is made on basis of AIDS defining illness and/or $CD4^+$ cell numbers below 200/ml of blood[61]. During the later stages of the HIV-1 infection, the FDC network and it's HIV-1 trapping ability are destroyed and germinal centres start to involute[60]. Furthermore the percentage of productively infected $CD4^+$ T cells increases[32]. Several different manifestations of the immunological disorder resulting from HIV-1 infection have been described[61]. Many of the opportunistic infections observed are caused by intracellular pathogens, which indicates a loss of cellular immunity in the final stages of the disease.

GENETIC AND PHENOTYPIC VARIATION OF HIV-1

One of the hallmarks of HIV-1 infection in the human host is the existence of genotypic and phenotypic variation within one individual[62]. The variation observed is caused by the incorporation of other nucleic acid residues than the originally encoded, during reverse transcription of viral RNA strands into DNA. Due to the lack of a proof reading system, up to 10 nucleic acid changes are introduced in the HIV-1 genome during each replication cycle *in vitro*[63,64]. The *in vivo* nucleotide substitution rate has been estimated to be between $0.4x10^{-3}$ and $15.8x10^{-3}$ /site/year depending on the gene studied[62]. The *in vivo* population diversity is limited by at least two mechanisms. First not each nucleic acid change results in a "viable" mutant or in a mutant that differs in amino acid

composition (silent mutations), and second both humoral and cellular immune responses may limit the replication of certain mutants. The variation introduced in the envelope glycoprotein clusters in five discrete regions called hypervariable regions one to five (V1 to V5)[65]. Naturally occurring changes introduced in several of these regions have been shown to change the sensitivity of the virus to neutralizing antibodies and the *in vitro* biological phenotype of the virus[66-72]. These findings suggest that both properties are involved in adaptation to, and subsequent persistence of the virus in the host. Alternatively these properties may be linked, resulting in simultaneous changes of which only one is important for persistence of the virus in the host.

In vitro biological properties, used to classify viruses include: the ability of the virus to induce formation of multinucleated cells (the so called SI capacity)[73,74], the replication rate of the virus[75,76], the ability to persistently infect either monocyte derived macrophages (MDM's) or immortalized T cell lines[73,74,77] and the ability to induce single cell lysis[78-80]. These biological properties proved to be highly interrelated[81,82]: non syncytium inducing (NSI) HIV-1 strains are little cytopathic, have a low replication rate and usually have the ability to infect MDM's. SI HIV-1 strains on the other hand are usually highly cytopathic, replicate to high titers and are able to infect immortalized T cell lines. It should be realized however, that a whole range of intermediate phenotypes may exist simultaneously in the host[81,83]. It has furthermore been shown that the *in vitro* biological characteristics of HIV-1 strains, like SI capacity and overall pathogenicity do not always correlate with their *in vivo* behaviour[84-88]. The most commonly used system to classify HIV-1 strains according to their *in vitro* biological phenotype was described by Cheng-Mayer *et al.*[73] and Tersmette *et al.*[74,89]. This system uses the difference in SI capacity and cellular host range to classify viruses into SI and NSI strains. While introducing the system it was recognized that the presence of viruses of the respective phenotypes correlates with the stage of the disease and the rate of disease progression in the host. Many studies described thereafter have confirmed that SI variants are isolated from about 50% of the individuals that progress towards AIDS with a severe clinical disease manifestation[56,62]. Furthermore, several studies have shown that SI variants may be isolated around seroconversion from patients with more severe clinical manifestation of primary infection[51,52,90]. These individuals usually progress more rapidly towards AIDS. After primary infection in most cases NSI variants persist in the host despite the fact that SI variants may be isolated *in vitro* from the initial inoculum[90-92]. Whether this is a reflection of a low prevalence of SI variants in the inoculum that have a selective advantage *in vitro*, of a preferential outgrowth *in vivo* of NSI variants, or of a reflection of the route of infection still remains elusive.

Several of the other biological characteristics may also be used to classify HIV-1 strains[75,79,81]. It has been suggested that these features are more relevant for the *in vivo* pathogenicity of the virus[86-88]. The virus strains used in the studies presented in this thesis are predominantly classified according to their SI capacity.

HIV-1 ENTRY

In vitro studies have shown that HIV-1 entry is a result of pH-independent fusion of viral and cell membrane[93-95]. It can however not be excluded that *in vivo* opsonization of virus particles may lead to endocytosis by cells of the monocytic cell lineage and subsequent fusion in the early endosome at low pH as observed with e.g. influenza virus[96,97]. It has recently been shown that opsonized virus particles may be rendered infectious by binding to FDC's[98]. The mechanism by which these cells specialized in antigen presentation, render seemingly inactivated virus particles

infectious and the resulting mechanism of entry is unclear. It has been postulated that FDC's present antigen in the presence of antigen specific antibodies by disrupting the antigen-antibody complexes. In the case of HIV-1 this would allow the initial interaction of gp120 with CD4 on the target cell to take place.

Three subsequent phases may be postulated in the process of HIV-1 glycoprotein mediated cell entry:

1) Binding of the virion to the cell membrane,
2) Induction of conformational changes within the CD4-gp120 complex resulting in a fusogenic state of the complex in close proximity to the cell membrane,
3) Induction of conformational changes within the gp41 molecule resulting in insertion of the gp41 N-terminal stretch of hydrophobic amino acids into the opposing cell membrane thereby destabilizing the cell membrane and allowing fusion of the virion and cell membranes.

Binding of the virion to the cell membrane

The V3 loop on HIV-1 gp120 of T cell line adapted (TCLA) HIV-1 strains and supposedly also other positively charged loops protruding from the gp120 molecule, has been shown to bind to negatively charged cellular counterparts, like sugar residues and the CDR3 domain on CD4[99-101]. It has been postulated that this initial low affinity binding enables the virus to scan the cell membrane for CD4, after which the entry process is initiated by the high affinity interaction of gp120 with CD4. The V3 loop on primary HIV-1 strains does not seem to be readily accessible on the glycoprotein complex however for such an interaction[100,102]. Furthermore V3 loop specific antibodies block HIV-1 entry at a stage following HIV-1 binding[103,104]. It therefore seems likely that HIV-1 binding is initiated by the high affinity interaction between gp120 and CD4 or by the interaction between cell encoded proteins incorporated in the HIV-1 particle and a cellular counterpart[105].

Induction of conformational changes within the CD4-gp120 complex

A whole array of conformational changes have been described in both gp120 and CD4 after binding of gp120 to sCD4 and cell membrane associated CD4[68,106-108]. The exact role of these changes in the process of HIV-1 envelope glycoprotein mediated entry is not clear yet. After initial binding of the virion to the cell a considerable distance must be bridged by the N-terminal part of the gp41 molecule in order to insert itself into the cell membrane. Furthermore both the virion and cell membrane are negatively charged entities which results in a electrostatic repulsion of virion and cell membranes. Conformational changes in the HIV-1 glycoprotein-CD4 complex therefore seem to be necessary to bring the virion membrane in closer proximity to the cell membrane. At least two changes are believed to contribute to this phenomenon. First CD4 has been postulated to bend at a hinge region between D2 and D3[109] and second, shedding of gp120 after binding to CD4 may also contribute to this phenomenon[110]. Since primary HIV-1 strains do not readily shed gp120 after CD4 binding[108,111,112], alternative changes within the glycoprotein complex may lead to bending gp120 outside the gap needed for the insertion of the gp41 fusion domain into the cell membrane. Only little is known about the changes in the envelope glycoprotein complex of primary HIV-1 strains after it's binding to CD4. It seems likely however that the changes parallel those described for HIV-2 and SIVagm during receptor induced activation of membrane fusion since these viruses also do not readily shed their SU after binding to CD4[106,113,114].

Induction of conformational changes within the gp41 molecule

Virus receptor mediated membrane fusion has been most thoroughly studied in influenza HA mediated fusion. Analysis of the crystal structure of HA2 in the fusogenic state indicates that the association of HA2 leucine zipper regions into a coiled coil structure is essential for the formation of the fusion attack complex. The bending of the HA2 molecule results in a stretch of alpha helices forming the central coiled coil core of the HA2 fusion attack complex[97,115]. This triple stranded central core seems to be required to bridge the distance between virion and cell membrane. Studies on the conformational changes within the HIV-1 glycoprotein complex induced by CD4 binding, are seriously hampered by the fact that crystal structures of the envelope glycoprotein in it's native conformation and in the fusion attack complex, have sofar not been produced. The present knowledge about the conformation of the glycoprotein complex is largely limited to the TM part of the glycoprotein complex. Based on peptide inhibition experiments, and in line with the model proposed for the HA2 part of the influenza virus heamagglutinin in it's native form and as a fusion attack complex, Matthews *et al.* proposed a model for the formation of a HIV-1 TM fusion attack complex[116]. In this model the formation of a central coiled coil core structure by the leucine zipper regions of the TM glycoprotein, is an essential step in the fusion process[117]. Formation of this central core in the native state is inhibited by the putative distal alpha helix[118]. In parallel with the HA2 model, this alpha helix may be part of the central core in the fusion attack complex by placing itself on top of the central core, thereby bridging the space between viral and cellular membranes[97]. Insertion of the fusion related sequence into the adjacent membrane is thought to destabilize the cell membrane, which eventually allows fusion of the membranes to take place[119].

The interaction of the HIV-1 glycoprotein complex with membrane associated CD4 is required but not sufficient for HIV-1 entry[120]. Recently it was shown that expression of proteins belonging to the C-X-C and C-C chemokine receptor subfamilies of the G-protein binding receptor superfamily, is required for HIV-1 entry[121,122]. HIV-1 strains proved to have different requirements for these second receptors. Infection of TCLA HIV-1 strains proved to depend on expression of the β chemokine receptor LESTR also called fusin, whereas primary macrophage-tropic HIV-1 strains required expression of the CC CKR5 chemokine receptor. It was furthermore shown that genetic variation in the CC CKR5 chemokine receptor may have a pronounced influence on the susceptibility to HIV-1 infection and on the rate of disease progression after HIV-1 infection[55].

RELATIONSHIP BETWEEN ANTIGENIC STRUCTURE OF THE HIV-1 ENVELOPE GLYCOPROTEIN COMPLEX AND BIOLOGICAL ACTIVITY OF ANTIBODIES

Presently the database of antibodies directed against linear epitopes on the gp41 and gp120 molecules, constructed by the Los Alamos Laboratories at the world wide web site http://hiv-web.lanl.gov/immuno/ab, contains antibodies which recognize virtually the complete amino acid sequence of the gp160 molecule. On the folded monomeric gp120/gp160 a limited set of these antibodies bind to their epitope[67]. Besides these antibodies, a range of antibodies have been described of which the binding is dependent on either the tertiary or quaternary structure of the envelope glycoproteins[67,123]. The linear and tertiary epitopes on gp120 all cluster within discrete and sometimes overlapping antigenic sites on monomeric gp120[68]. The antigenic sites that are able to induce VN antibodies all cluster on one face of the gp120 molecule suggesting that this face is pointed towards the surface of the multimeric glycoprotein complex, whereas non VN

antibody inducing antigenic sites seem occluded within the complex (Figure 3).

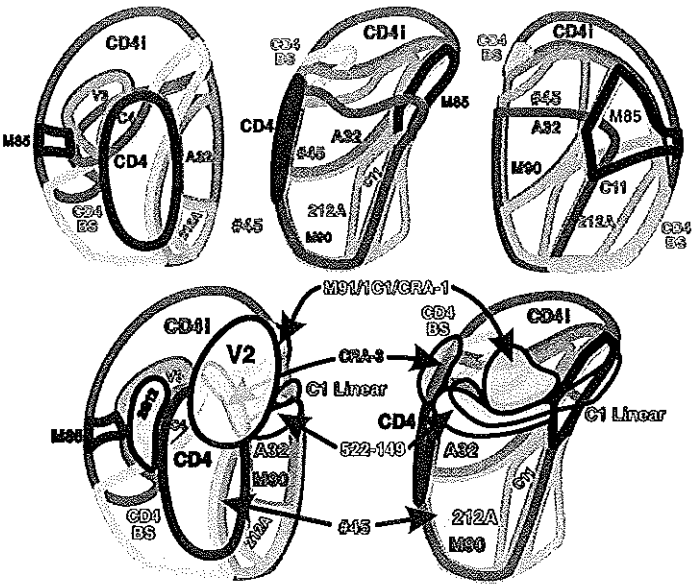

Fig. 3. Antibody cross-competition map of the surface of the HIV-1 gp120 glycoprotein. The top three drawings illustrate the core of the competition map of gp120 from three different perspectives, with the surface seen in the upper left-hand illustration presumably exposed on the assembled envelope glycoprotein oligomer. The binding sites for CD4 and groups of monoclonal antibodies are depicted. Overlap of eptiopes indicates reciprocal inhibition of monoclonal antibody binding in ELISA, whereas adjacent epitopes are used to model unidirectional inhibitory or enhancing effects. The lower two drawings include epitopes that were displayed as surface projections. In the lower right-hand figure, the V2 epitopes were removed for clarity. Adapted with permission from J.P. Moore and J. Sodroski, J. Virol. 70,1863-1872.

Of the linear and tertiary epitopes able to induce VN antibodies the V2, V3 and CD4 binding domain (CD4 bd) on HIV-1 gp120 and amino acid 679-685 on gp41 (ELDKWAS) are described in most detail (for review see Poignard *et al.*[124]). TCLA HIV-1 strains, may be readily neutralized by antibodies directed against these antigenic sites. The VN capacity of these antibodies is both dependent on the relative affinity of the antibody for the HIV-1 strain tested and on the exact location of the epitope within the antigenic site[68,107,125,126]. Several mechanisms by which HIV-1 glycoprotein specific antibodies neutralize TCLA HIV-1 strains may be considered, including:

1) inhibition of CD4 binding
2) induction or inhibition of conformational changes leaving the envelope glycoprotein complex unable to exert further function
3) interference with HIV-1 glycoprotein/second receptor binding
4) interference with post entry events

Inhibition of CD4 binding

A vast amount of MAb's directed against the C4 domain and the CD4bs that interfere with monomeric gp120/sCD4 binding has been described. It does not seem likely however, that the

mechanism of neutralization of these MAb's is the direct result of inhibition of virion binding to it's cellular receptor[127].

Induction or inhibition of conformational changes

It has recently been shown that V3 specific MAb's may induce shedding of gp120, which results in the irreversible inactivation of the virion[128]. TCLA HIV-1 strains may also be neutralized under conditions where shedding of gp120 does not take place[129]. This observation indicates that additional mechanisms of neutralization may contribute to the neutralizing capacity of these MAb's. VN antibodies directed against other antigenic sites like V1/V2 , CD4i, C4 and the CD4bs, which do not induce shedding of gp120[128], have been shown to induce conformational changes in monomeric gp120 which increase or decrease the affinity of sCD4 and MAb's directed against other antigenic sites[68]. Whether these changes are also induced in the multimeric envelope glycoprotein complex and whether these changes reduce the fusogenic or CD4 binding capacity of the HIV-1 glycoprotein complex remains elusive. The binding of the human monoclonal antibody (HuMAb) IAM-2F5 to it's epitope is reduced in the presence of sCD4[130]. This implies that the epitope of IAM-2F5 is obscured within the fusogenic state of the HIV-1 envelope glycoprotein complex. It may be postulated that IAM-2F5 blocks the induction of conformational changes required to get the HIV-1 envelope glycoprotein complex into it's fusogenic state. Alternatively IAM 2F5 may either directly or sterically hinder the binding of gp41 to a cell membrane protein.

Interference with HIV-1 glycoprotein/second receptor binding

Secondary to the high affinity interaction of gp120 with CD4, a low affinity interaction is required to induce the fusion attack complex[120]. Parts of the V1/V2, V3 and C4 domains seem likely candidates for taking part in such an interaction, since these domains have been shown to interact as a complex in receptor mediated membrane fusion[131,132]. Antibodies directed against these antigenic sites may neutralize HIV-1 by inhibiting this secondary interaction. The Ca^{2+} binding site within gp41 has been postulated to bind to a cellular second receptor[133]. VN antibodies binding to epitopes in the vicinity of this second receptor contact site, like IAM-2F5 may sterically hinder this interaction.

Interference with post entry events

It has been speculated that Fab fragments of HuMAb IgG1-b12 may exert their function after entry of HIV-1 into the cell by inhibiting the uncoating of the ribonucleoprotein (N. Dimmock, personal communication).

ANTIBODY MEDIATED ENHANCEMENT OF HIV-1

Besides mediating virus neutralization, antibodies directed against the envelope glycoprotein complex may also mediate enhancement of infectivity *in vitro*. Until recently two mechanisms by which HIV-1 specific antibodies may facilitate HIV-1 entry have been described[134]. These include: Fc receptor and complement receptor mediated antibody dependent enhancement of HIV-1 infection (Fc-ADE an C'-ADE). Several cell types express receptors for the Fc portion of antibodies and for factors of the complement system that bind to antibody-antigen complexes[135,136]. These

receptors mediate endocytosis of foreign antigens, thereby facilitating inactivation of the pathogen, break down of the antigen in the lysosomal compartment and subsequent antigen processing and presentation. *In vitro* studies indicate that HIV-1 may have found ways to take advantage of this system[137-140]. The mechanism by which HIV-1 specific antibodies facilitate entry of HIV-1 into these cell types is not fully understood. Fc-ADE and C'-ADE are however thought to facilitate initial binding of the virion to the cell after which the gp120-CD4 interaction may initiate fusion[141-143]. Whether the resulting mechanism of HIV-1 glycoprotein mediated entry is indeed similar to HIV-1 envelope glycoprotein mediated entry into T cell lines still remains to be shown. Alternatively opsonisation of virus particles has been shown to enable HIV-1 to enter these cells independent of membrane associated CD4[139]. How CD4 independent entry in the presence of antibodies and complement is facilitated remains unclear. It may be hypothesized that opsonization of the HIV-1 particle may facilitate endocytosis of the virion and subsequent pH induced viral fusion within the early endosome, by a mechanism that would parallel entry of influenza virus[97,115]. Alternatively, binding of the virion to the cell via Fc- or C' receptors may allow the HIV-1 transmembrane molecule to bind to a cellular receptor, eventually allowing pH independent fusion of virion and cell membrane to take place.

The soluble form of the CD4 receptor may also enhance entry of specific HIV-2 strains and SIVagm into $CD4^-$ and $CD4^+$ cell lines respectively by a mechanism called receptor activated membrane fusion[41,144]. We and others have recently shown that in HIV-1 infection such a mechanism is also operational[145,146]. Furthermore HIV-1 specific antibodies also enhanced entry of HIV-1 strains susceptible to sCD4 mediated enhancement.

The role of antibody mediated enhancement in *in vivo* HIV-1 infection has been subject of debate. Differences in properties of HIV-1 strains isolated *in vitro* in the absence of HIV-1 specific antibodies and cell free HIV-1 strains found *in vivo* however suggest that antibody mediated enhancement of HIV-1 does play a role in *in vivo* HIV-1 infection[85,147]. Furthermore, enhancement of EIAV and FIV infection has been observed *in vivo* after vaccination and subsequent challenge of ponies and cats respectively[148,149]. These observations indicate that indeed enhancement of lentivirus infection may become a serious threat for lentivirus vaccine development.

AIM OF THIS STUDY

The induction of HIV-1 specific antibodies that prevent HIV-1 infection or limit the spread of the virus during the initial stages of the infection is thought to be an essential feature of a successful future vaccine. HIV-1, SIV and FIV candidate vaccines that elicit whole envelope glycoprotein specific antibody responses have sofar had limited success in preventing infection[148,150]. These observations indicate that increasing our knowledge of mechanisms that underlay primary HIV-1 glycoprotein mediated entry and of the way in which HIV-1 glycoprotein specific antibodies modulate this entry, may be crucial for successful HIV-1 vaccine development. The studies presented in this thesis were initiated to increase our understanding of the way in which HIV-1 envelope specific antibodies may interfere with, or modulate the sequence of events that lead to viral entry.

The generation and characterization of materials and *in vitro* and *in vivo* systems, used to study these phenomena are described in chapter 2, 3, 4 (HIV-1 envelope specific HuMAb's), 5 (envelope trans-complementation assay) and 7 (xeno-GvHD mouse model). In chapters 5 and 6 *in vitro* studies into the mechanism of primary HIV-1 entry and the modulation of primary HIV-1 glycoprotein mediated entry by human antibodies and sCD4 are described. *In vitro* studies on the

protective capacity of HIV-1 specific antibodies have several obvious shortcomings like the absence of HIV-1 susceptible monocytes and macrophages and the absence of tissue distribution of HIV-1 infected and susceptible cells. These aspects may all contribute to the outcome of HIV-1 infection in humans. In chapter 7 the protective capacity of *in vitro* virus neutralizing human antibodies is studied in a human to mouse chimeric model, in which these aspects are taken into account. In the general discussion (chapter 8), the data presented, are discussed in the light of the current knowledge in this field and of their relevance for HIV-1 vaccine development.

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CHAPTER 2

Further characterization of an antigenic site of HIV-1 gp120 recognized by virus neutralizing human monoclonal antibodies.

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Further characterization of an antigenic site of HIV-1 gp120 recognized by virus neutralizing human monoclonal antibodies

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Objective: The aim of this study is to characterize antigenic sites on HIV-1 gp120 which may be important for the development of active and passive immunization strategies against HIV-1 infection.

Design: Two HIV-1-seropositive individuals were selected from the Amsterdam cohort and Epstein–Barr virus (EBV)-transformed B cells were generated from their peripheral blood mononuclear cells, which produce HIV-1-specific human monoclonal antibodies (HuMAb).

Methods: HuMAb were generated and selected based on their reactivities with native gp120. Reactivity with HIV-1 strains from phylogenetically different subfamilies was determined by immunostaining and virus neutralization assays. Specificity for the CD4-binding site was tested by an inhibition enzyme-linked immunosorbent assay and amino acids (aa) involved in the binding of the HuMAb were identified with a set of gp120 molecules with single aa substitutions.

Results: Three HuMAb (GP13, GP44, GP68) were generated, all recognizing a conserved conformation dependent epitope within, or topographically near, the CD4-binding site of gp120. HuMAb GP13 and GP68 neutralized a broad range of HIV-1 strains from phylogenetically different subfamilies, whereas HuMAb GP44 exhibited a more restricted pattern of neutralizing activity. The patterns of gp120 aa involved in their binding were unique for each of these HuMAb.

Conclusions: The pattern of reactivities of these three HIV-1-neutralizing HuMAb developed in these studies is similar to, but distinct from other human and rodent MAb that recognize this antigenic site of HIV-1 gp120.

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Keywords: HIV-1, neutralizing antibodies, human monoclonal antibody, gp120, CD4-binding site, virus neutralization, passive immunization.

Introduction

Several studies in which virus-neutralizing (VN) antibodies were passively transferred, indicated that antiviral antibodies play a major role in the protection from lentivirus infections [1–4]. Sera from HIV-1-infected individuals have been shown to neutralize a wide range of HIV-1 strains originating from Europe,

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the United States of America and Africa [5]. Antibodies recognizing a conformation dependent antigenic site, overlapping with the CD4-binding site on gp120 are responsible for a major part of the VN cross-reactivity in human sera [6,7]. To investigate the nature of this antigenic site in more detail, monoclonal antibodies (MAb) of this specificity are of great value. To date, a limited number of human MAb/(HuMAb) specific for this site have been derived from North American HIV-1-seropositive individuals. Furthermore, rodent MAb, which inhibit binding of gp120 to CD4, have been generated against HIV-1 IIIB gp120 [8–12]. Here, we describe the generation of VN HuMAb produced by Epstein–Barr virus (EBV) transformation of peripheral blood mononuclear cells (PBMC) from two Dutch HIV-1-seropositive individuals. The characterization of these antibodies and the corresponding epitopes are presented.

Materials and methods

Cell lines and virus strains

Human HeLa CD4+ cells and the human lymphoid cell lines C8166 and H9 were maintained in Dulbecco's modified Egale's medium (DMEM) or RPMI-1640 suplemented with fetal calf serum (FCS) [13]. The following virus strains, with the subtype indicated according to Meyers *et al.* [14], were used for immunostaining and virus neutralization: IIIB (LAI isolate, subtype B), RF and MN (subtype B) [15,16], SF2 (subtype B)[17], NY-5/LAV-1 (subtype B), Z34, Z84 (subtype D) and Z129 [5], GL-1 and GL-3 [9], U455 (subtype A)[5] and CBL-4 [5]. The HIV-2 strain CBL-22 [18] and ROD [19] were also used.

Generation of human B cell clones and HuMAb

PBMC were isolated from 40 ml blood of four HIV-1-seropositive asymptomatic adult males of the Amsterdam cohort, and generation of EBV-transformed B-cell lines with these PBMC was performed as described previously [20]. For the identification and characterization of antibodies in culture supernatants, an enzyme-linked immunosorbent assay (ELISA) system described by Robinson [11] was used with recombinant HIV-1 IIIB gp120, recombinant HIV-1 IIIB gp160 and recombinant HIV-2 gp105 produced in a baculovirus expression system (American Biotechnologies Inc., Cambridge, Massachusetts, USA), feline immunodeficiency virus (FIV) glycoprotein [21] and simian immunodeficiency virus (SIV_{mac251}) cell lysate [22] as antigens.

Immunostaining assay

The degree of conservation of the epitopes recognized by the HuMAb generated in these studies was tested in a previously described immunostaining assay with the above mentioned virus strains [23].

ELISA systems

The HuMAb GP13 and GP68 generated in the course of this study were biotinylated with NHS-d-succinimidobiotin as recommended by the manufacturer (Sigma Chemie, Axel, The Netherlands). Recombinant gp120 (see above, 75 ng/ml) was coated to the ELISA plate with affinity-purified polyclonal antibody raised in sheep against the HIV-1 gp120 carboxy terminus [24,25]. gp120-coated plates were incubated for 1 h at 37°C with serial dilutions of biotinylated HuMAb and after washing incubated for 1 h at 37°C with 1:20 diluted StreptABComplex HRP (DAKO A/S, Glostrup, Denmark). The dilution resulting in the half maximum optical density (OD) 450 nm (mid-point titre) of the biotinylated HuMAb was determined. To determine the inhibition of the biotinylated HuMAb by the different HuMAb and soluble CD4 (sCD4), the gp120 coated plates were preincubated for 1 h with 80 µl serially diluted HuMAb or sCD4. As a negative control, a broadly reactive HuMAb (K14, immunoglobulin G1 (IgG1) specific for HIV-1 gp41 was used [20], and 20 µl of the biotinylated HuMAb (mid-point titre) was added. The ELISA was further developed as described above. These assays were performed in triplicate; the mean of the results of the three independent assays is given.

The ELISA used for the determination of the specificity of the HuMAb for a described set of mutant gp120 glycoproteins [26] was performed with a previously described assay developed by Dr J. McKeating (Chester Beatty Laboratories, London, UK) [25,27]. The reduction in binding is given as the mean percentage of the binding by the wild-type glycoproteins, and calculated from the results of two independent assays according to the formula:

$$\% = 100 \times \left(\frac{\text{OD450mutant} - \text{OD450background}}{\text{OD450WT} - \text{OD450background}} \right)$$

VN assay

The microtiter VN assay used in these studies has been described previously [13]. Briefly, 1000 median tissue culture infective doses ($TCID_{50}$) of virus stock in 40 µl was incubated with 10 µl of serial dilutions of protein A purified HuMAb for 1 h at 37°C, then incubated with 100 µl of 2×10^5/ml C8166 or H9 cells for 1 h at 37°C. After 5 days of culture, the wells were microscopically scored for the presence of syncytia. Data presented are taken from representative experiments. Several control experiments were performed, including multiple testing of different virus stocks with different HuMAb preparations. Similar results were obtained in all of these cases.

Results

General properties of newly generated gp120-specific HuMAb

Screening of cloned EBV-transformed PBMC from two out of four donors (numbers 1171 and 658) yielded one B-cell line from the first donor (EBV GP13) and two from the second (EBV GP44, EBV GP68), all three produced HuMAb of the IgG1 subclass reactive in an ELISA with recombinant HIV-1 IIIB gp120 and gp160. These cell lines were subcloned until > 99.9% chance of clonality was obtained [20]. These HuMAb failed to recognize envelope glycoproteins of HIV-2, SIV_{mac}, and FIV in ELISA (data not shown), indicating that these three HuMAb react specifically with HIV-1 gp120. To delineate the antigenic sites recognized by these three HuMAb, we tested their capacity and that of sCD4 to inhibit the binding of biotinconjugated HuMAb GP13 (Fig. 1a) and GP68 (Fig. 1b) at their midpoint titer (18.8 and 96 ng/ml, respectivelly) to HIV-1 IIIB gp120 in the inhibition ELISA. The binding of GP13 and GP68 to gp120 was not inhibited by the gp41 specific HuMAb. GP13 and GP68 mutually inhibited each other to approximately the same extent whereas nearly 100 times more GP44 was needed for the same inhibition of the binding of both antibodies. The binding of GP13 and GP68 to gp120 could also be inhibited by pre-incubation of the wells with approximately 10 or 100 ng sCD4 per ml respectively. These data indicate that the HuMAb GP13, GP44 and GP68 recognize an antigenic site located within or topographically near the CD4-binding site of gp120.

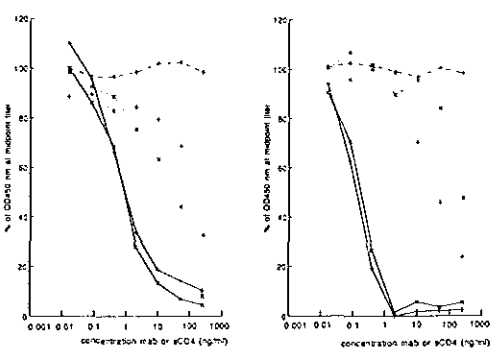

Fig. 1. Mean reduction percentages of OD450 nm values obtained by preincubation of gp120 coated enzyme-liked immunosorbent assay plates with non-labelled human monoclonal antibodies (HuMAb) or soluble(s) CD4, and subsequent addition of biotin labelled HuMAb GP13 (a) and GP68 (b) at midpoint titres (18.8 and 98 ng/ml respectivelly). Concentrations of non-labelled HuMAb are given in ng/ml (horizontal axis). All assays were carried out in triplicate and s.d. were less then 2% in all cases. GP13 (— + —); GP44 (– + –); GP68 (— × —); K14 (- - + - -); sCD4 (×).

Specificity of HuMAb GP13, GP44 and GP68

To determine the degree of conservation of the epitope recognized by these three HuMAb, they were tested for their ability to stain cells infected with HIV isolates from three out of five phylogenetic subfamilies of HIV-1 [14] in the immunostaining assay. All HIV-1 strains tested (n = 12) were recognized by these HuMAb, whereas the two HIV-2 strains tested were not recognized in this assay (data not shown).

Table 1. HIV-1 neutralizing activity of human monoclonal antibodies.

Virus	Strain	GP13	GP44	GP68
HIV-1	SF2	0.1*	60	0.7
HIV-1	MN	30	> 240	11
HIV-1	IIIB	45	> 240	30
HIV-1	RF	90	> 240	> 250
HIV-1	CBL4	12	> 240	11
HIV-1	Z34	8	> 240	11
HIV-2	ROD	> 250	> 240	> 250
HIV-2	CBL-22	> 250	> 240	> 250

*In vitro virus neurtalizing activity presented as the lowest concentration of human monoclonal activity in µg/ml, giving 90–95% reduction of the numbers of syncytia in a syncytium inhibition assay using 1000 median tissue culture infective doses ($TCID_{50}$).

The broadest HIV-1-neutralizing activity was observed for HuMAb GP13 which neutralized all the HIV-1 strains tested. The concentrations of antibody needed to neutralize 1000 $TCID_{50}$ of the different virus strains, ranged from 0.1 to 90 µg/ml for $HIV\text{-}1_{SF2}$ and for $HIV\text{-}1_{RF}$, respectively. Also, HuMAb GP68 exhibited a relatively broad VN activity. It only failed to neutralize HIV-1 RF at the antibody concentrations tested (< 250 µg/ml). HuMAb GP44 only neutralized HIV-1 SF2 under these conditions at levels ≥ 60 µg/ml (Table 1).

Recognition of mutant HIV-1 gp120 molecules by HuMAb GP13, GP44 and GP68

The reactivities in ELISA of these three HuMAb with a panel of mutant HIV-1 gp120, containing single amino-acid (aa) substitutions [26], are presented as percentages of their reactivities with the wild-type (HXBc2) gp120 in Fig. 2. For comparison, the reactivities of these mutant glycoproteins with sCD4 and HuMAb F105, a previously described HuMAb of similar specificity, are also shown [10,28]. Changes in threonine 257, aspartic acid 368 or glutamic acid 370 abrogated recognition by the GP13, GP44 and GP68 antibodies. Changes in recognition by these antibodies were also seen for the 256 S/Y and 262 N/T mutants, which exhibit a certain degree of confomational alteration relative to the wild-type envelop glycoproteins [26]. Recognition of the gp120 glycoprotein by the GP68 MAb was uniquely affected by changes in lysine 117 and tyrosine 435.

Discussion

In this study we have described the generation of three EBV-transformed B-cell lines from the PBMC of

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CHAPTER 3

Characterization of a V3 domain-specific neutralizing human monoclonal antibody that preferentially recognizes non syncytium inducing human immunodeficiency virus type 1 strains.

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Characterization of a V3 domain-specific neutralizing human monoclonal antibody that preferentially recognizes non-syncytium-inducing human immunodeficiency virus type 1 strains

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A type-specific human immunodeficiency virus type 1 (HIV-1)-neutralizing human monoclonal antibody (HuMAb MN215) is described that reacts with the V3 domain of a number of subtype B virus strains. Pepscan analysis indicated that amino acids at both sides of the tip of the V3 loop were involved in the binding of HuMAb MN215. The minimum epitope in a V3 sequence, obtained from the donor from whom the cell line originated, was 9 amino acids long and proved to be located at the C-terminal side of the tip of the loop. In a replacement Pepscan analysis, individual amino acids of the V3 loop important for binding of HuMAb MN215 were identified. Amino acids at positions 15 (H), 16 (I), 17 (G) and 18 (P) were found to be essential for binding of the antibody, whereas changes at positions 19 of G to N, 20 of R to K and 23 of F to L, as well as the addition of a negative charge at the C terminus, improved binding. Thus, amino acids involved in the binding of HuMAb MN215 are primarily located within highly variable regions of the V3 loop. HuMAb MN215 showed a higher affinity for the V3 domain sequences and recombinant envelope glycoproteins derived from non-syncytium-inducing strains than for those derived from syncytium-inducing strains.

Introduction

Virus neutralizing (VN) antibodies are believed to play a major role in the pathogenesis of human immunodeficiency virus type 1 (HIV-1) infection. Suppression of virus replication during the relatively long asymptomatic period is believed to be mediated at least in part by the humoral immune response (Page *et al.*, 1992; Watkins *et al.*, 1993). The identification and characterization of antigenic sites on the glycoproteins of HIV-1 that elicit VN antibodies therefore seems to be a prerequisite for the rational development of passive and active immunization strategies. The third variable domain (V3 domain) of the outer membrane glycoprotein (gp120) of HIV-1 has been shown to elicit VN antibodies (Goudsmit *et al.*, 1988; Palker *et al.*, 1988). Despite the variability within this antigenic site, certain antibodies directed against this domain may neutralize a wide range of virus strains (Langedijk *et al.*, 1991; Ohno *et al.*, 1991; Gorny *et al.*, 1992). Certain human monoclonal antibodies (HuMAbs) directed against the V3 domain are therefore likely candidates for passive immunization therapies. A mouse monoclonal antibody directed against the V3 domain of the HIV-1 IIIB strain has been shown to protect a chimpanzee against a challenge with the homologous virus strain when the antibody was administered prior to challenge (Emini *et al.*, 1992).

The V3 domain has been shown to be an important denominator for the phenotype of the virus: it directly influences the syncytium-inducing (SI) capacity and cell tropism of the virus. Mutations in the V3 domain of HIV-1 strains or the exchange of this region between highly related but phenotypically distinct glycoproteins may change their biological phenotypes (Freed & Risser, 1991; de Jong *et al.*, 1992; Andeweg *et al.*, 1993). It has also been shown that the V3 domain consensus sequence of non-syncytium-inducing (NSI) HIV-1 strains differs in several aspects from the V3 consensus sequence of SI HIV-1 strains (Fouchier *et al.*, 1992). A similar phenomenon has been observed when the V3 domain consensus sequence of macrophage-tropic and T cell-tropic HIV-1

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strains were compared (Chesebro *et al.*, 1992). Therefore, it may be expected that certain antibodies directed against this site display different reactivities with HIV-1 strains of different phenotypes. HIV-1 strains isolated during the asymptomatic period of HIV-1 infection are predominantly of the NSI phenotype and are therefore considered important targets for early passive immunization therapies (Roos *et al.*, 1992; Zhang *et al.*, 1992; Zhu *et al.*, 1993). Studies with V3 domain-specific HuMAbs have primarily focused on their reactivities with HIV-1 strains of the SI phenotype, since these HIV-1 strains are most commonly used in assay systems. Here we describe the generation and characterization of a HIV-1 V3 domain-specific HuMAb that reacts predominantly with HIV-1 strains of the NSI phenotype.

Methods

Generation of a human B cell clone producing V3 domain-specific HuMAb. Peripheral blood mononuclear cells (PBMC) were isolated from 40 ml heparinized blood of an HIV-1-seropositive asymptomatic adult male of the Amsterdam cohort (donor #658) four years after seroconversion; generation of Epstein–Barr virus (EBV)-transformed B cell lines was performed essentially as previously described (Teeuwsen *et al.*, 1990; Schutten *et al.*, 1993). The B cell line described in the present paper was fused with the mouse–human heterohybridoma F3B6 (Foung *et al.*, 1984) and subcloned several times, after which it was expanded into flasks.

For the identification of antibodies in culture supernatant, ELISA plates (Costar) were coated with a 23 amino acid peptide, purchased from American BioTechnologies, spanning the tip of the V3 loop of HIV-1 MN gp120. This peptide was coated overnight at 4 °C in PBS pH 7·5 at 750 ng/ml. The ELISA was developed as previously described (Schutten *et al.*, 1993). Twelve peptides representing the V3 region of HIV-1 subtype B [Universal (subtype B consensus; Meyers *et al.*, 1992), MN, SC, SF2, CDC4, HXB2, WM52, RF and NY/5 strains] and subtype D (ELI, Z2 and Z6 strains) (Meyers *et al.*, 1992) were purchased from American BioTechnologies and coated under the same conditions for determination of the cross-reactivity. IgG subclass determination was carried out in ELISA with subclass-specific mouse monoclonal antibodies (Seralab). The HIV-1 gp120 V3 loop sequence from virus in the serum of donor #658 was determined as described previously (Wolfs *et al.*, 1991).

Peptide inhibition ELISA. HuMAb MN215 was incubated with serial dilutions of the HIV-1 Universal, MN, SF2 and SC V3 peptides at a concentration at which half of the maximum binding was achieved in a solid phase V3 loop peptide ELISA using the HIV-1 MN V3 peptide. Furthermore, peptides generated by Dr C. Sia (Connaught Laboratories, Canada) were used in this assay. These peptides were generated on the basis of consensus SI and NSI sequences obtained from a previously described set of eight recombinant envelop proteins derived from two donors (#16 and #320) from the Amsterdam cohort (Fig. 1; Andeweg *et al.*, 1992). This set consisted of four recombinant glycoproteins per donor, of which two were derived from viruses with the SI phenotype and two from viruses with the NSI phenotype. ELISA plates coated with the HIV-1 MN V3 peptide were incubated for 1 h at 37 °C with these HuMAb MN215–peptide mixtures, which were preincubated overnight at 4 °C. The ELISA was completed as described above.

Pepscan analysis. Pepscan analysis was performed as originally described by Geysen *et al.* (1984) and modified by Langedijk *et al.* (1991). In short, peptides were synthesized on polyethylene rods and tested with HuMAb MN215 in an ELISA according to established procedures (Langedijk *et al.*, 1991). A set of peptides of between 8 and 16 amino acids in length, with all but 1 amino acid overlapping, were synthesized with sequences derived from the HIV-1 MN gp120 V3 loop

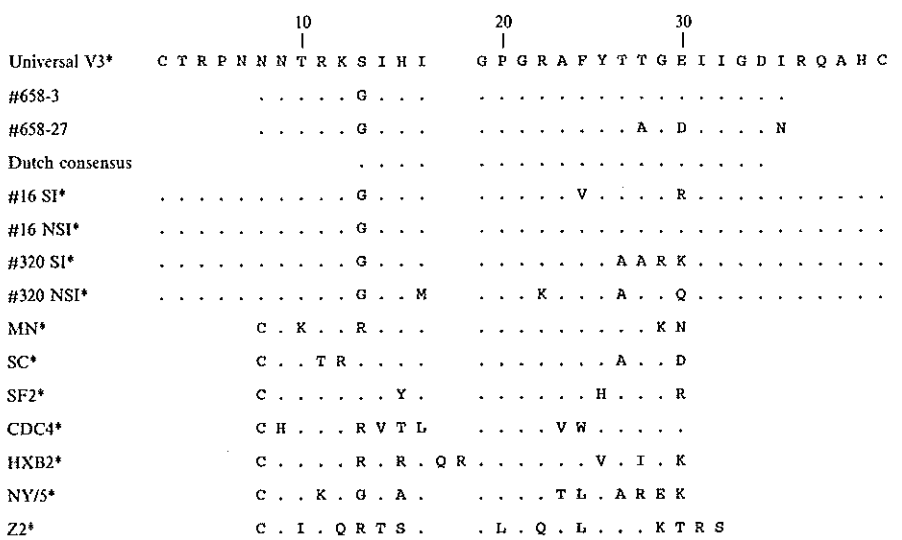


Fig. 1. Comparison of HIV-1 V3 domain amino acid sequences. The asterisk indicates V3 sequences from different HIV-1 strains from which peptides were used in solid phase peptide and peptide inhibition ELISA. The Dutch consensus, #658–3 and #658–27 V3 sequences were used in Pepscan.

(Fig. 1; Meyers *et al.*, 1992) and one sequence more related to the Dutch consensus V3 sequence (Fig. 1; Holley *et al.*, 1991). To determine the individual amino acid which was important for binding of HuMAb MN215 to its epitope, peptide analogues were made from the Dutch V3 consensus sequence (from position 12 to 27) by substituting single amino acids. The amino acids within this sequence were substituted for amino acids which have been proven to be prevalent at each position in Dutch HIV-1 isolates (Holley *et al.*, 1991) or by amino acids with different biochemical properties. Numbering of the amino acids in the HIV-1 gp120 V3 loop was according to LaRosa *et al.* (1990).

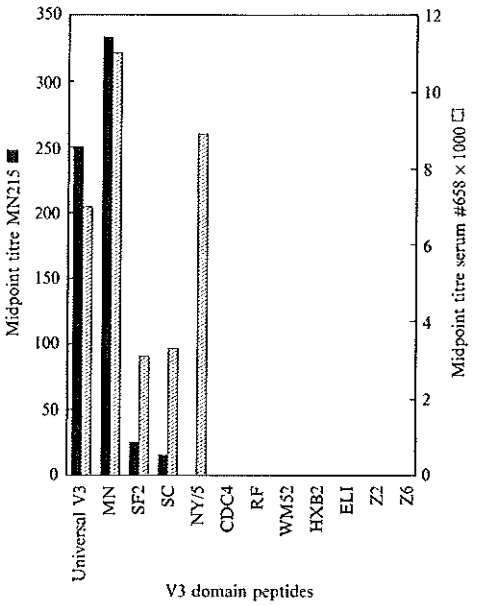
Relative affinity of monoclonal antibodies for HIV-1 gp160. Recombinant HIV-1 envelope glycoproteins, which we have described recently (Andeweg *et al.*, 1992), were expressed in HeLa cells by a recombinant vaccinia virus (rVV) expression system. The rVVs used were 320-2a.5 and 320-2a.7 (donor #320; phenotype SI), 320-2a.3 and 320-2a.6 (donor #320; phenotype NSI), 16.1 and 16.2 (donor #16; phenotype SI), and 16.3 and 16.4 (donor 16; phenotype NSI) (Andeweg *et al.*, 1992). At 24 h after rVV infection, HeLa cells were lysed with 1% empigen in PBS, in the presence of proteinase inhibitors (10 μg/ml aprotinin, 1 mM-PMSF; Boehringer Mannheim). For HIV-1 MN glycoproteins, persistently infected SupT1 cells were cocultured with uninfected SupT1 cells at a ratio of 1:4. After 3 days cells were centrifuged and lysed as described above. Cell lysates were divided into aliquots and the amount of gp120/160 was determined in a catching antibody ELISA as previously described (Moore, 1990). Equal amounts of gp120/160 were coated to ELISA plates and the concentration where 50% of the maximum A_{450} was reached with the HuMAbs was taken as a relative measure for affinity. The HuMAbs directed against more conserved sites on gp160 used in this ELISA have been previously described (Tecuwsen *et al.*, 1990; Gorny *et al.*, 1993; Schutten *et al.*, 1993).

Virus entry inhibition assay. The *env* gene encoding the recombinant 16.2 SI glycoprotein was cloned into an expression vector as previously described (Andeweg *et al.*, 1993) and subsequently used to generate chimeric infectious virus using a *trans* complementation assay. In short, an *env*-defective HIV-1 provirus, encoding the bacterial CAT reporter gene, was complemented by the 16.2 SI *env* gene for a single round of replication by co-transfection into the $CD4^-$ COS cell line. This resulted in the production of virions carrying heterologous envelope glycoproteins which could infect $CD4^+$ cells like the SupT1 cells used in this study (Helseth *et al.*, 1990). After 3 days cell-free supernatant was collected by centrifugation and frozen at −135 °C. For infection the amount of p24 antigen, as determined with a p24 ELISA kit (V5; Organon Teknika), was calibrated and the level of envelope expression was checked with the capture antibody ELISA (see above). Infection was performed overnight at 37 °C in 1 ml medium. After 24 h the cells were washed and cultured for 72 h. CAT activity in these cells was measured in a previously described CAT assay using a two-phase extraction system (Seed & Sheen, 1988). CAT activities of the cells were considered a direct measure of virus infectivity in these assays.

Results

Generation of HuMAb MN215

Screening of the supernatants of more than 2000 B cell lines obtained by EBV transformation of PBMC collected from donor #658 and subcloning of positive cell cultures yielded one B cell line (MN215) producing an IgG1 antibody that recognized a peptide with the V3 domain sequence of the HIV-1 MN strain. This B cell line continued to produce these V3 domain-specific antibodies at levels of 4 μg/ml after fusion with the mouse–human heterohybridoma F3B6 and further subcloning. The cell line was considered clonal on the basis of the limiting dilution procedure used (Schutten *et al.*, 1993).



MN215	250	333	25	15	0	0	0	0	0	0	0	0
Serum 658	7	11	3.1	3.3	8.9	0	0	0	0	0	0	0

Fig. 2. Reactivities of HuMAb MN215 and serum from donor 658 with different HIV-1 V3 peptides. Midpoint titres of HuMAb MN215 (filled bars) and serum taken from donor 658 4 years after seroconversion (hatched bars), at which 50% of maximum A_{450} was reached in an ELISA with V3 domain peptides from different subtype B (Universal, MN, SF2, SC, NY/5, CDC4, RF, WM52, HXB2) and subtype D (ELI, Z2, Z6) HIV-1 strains (see Fig. 1).

Cross reactivity of HuMAb MN215 with different HIV-1 V3 domain peptides

V3 loop sequences of HIV-1 derived from donor #658 at 3 and 60 months after seroconversion (#658–3 and #658–27, respectively) proved to be closely related to the subtype B HIV-1 V3 loop consensus sequence (Fig. 1). When tested in a solid phase peptide ELISA with V3 domain peptides representing subtype B and D HIV-1 strains, the serum from donor #658 taken 4 years after seroconversion showed specificity for peptides derived from subtype B HIV-1 strains (Fig. 2). We subsequently tested HuMAb MN215 for its reactivity in a solid phase

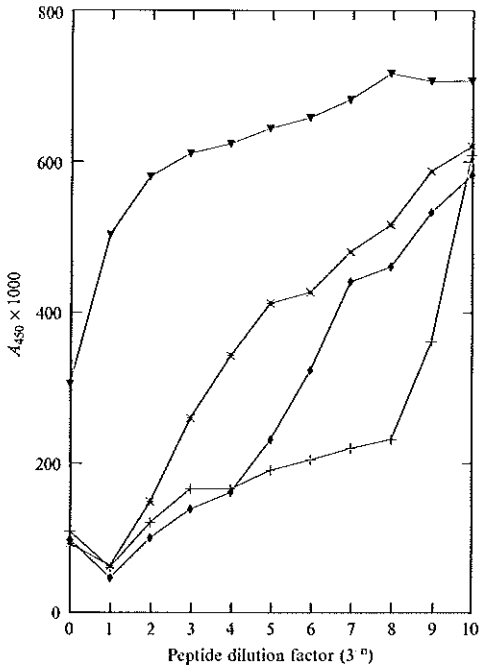


Fig. 3. Peptide inhibition ELISA of HuMAb MN215 with V3 loop peptides. A_{450} values obtained in a solid phase HIV-1 MN V3 peptide ELISA with a standard concentration HuMAb MN215, incubated with serial threefold dilutions of the respective peptides [HIV-1 Universal (◆), MN (+), SC (*), SF2 (▼); see Fig. 1] using a starting peptide concentration of 3×10^{-11} mol.

peptide ELISA with peptides derived from different subtype B HIV-1 strains and with peptides derived from subtype D HIV-1 strains (Fig. 2). The highest reactivity of HuMAb MN215 was found with the HIV-1 Universal consensus and the HIV-1 MN sequences. It also reacted with V3 domain peptides derived from the HIV-1 SC and SF2 strains. Since solid phase peptide ELISA values may not be considered to represent an adequate measurement of the relative affinity of an antibody for a given peptide, we also performed a peptide inhibition ELISA with the peptides showing reactivity with HuMAb MN215 (Fig. 3). HuMAb MN215 showed a relatively high affinity for the HIV-1 MN peptide and for the HIV-1 Universal V3 and HIV-1 SC peptides, but it bound with a much lower affinity to the HIV-1 SF2 peptide.

Minimum epitope recognized by HuMAb MN215

The minimum epitope recognized by HuMAb MN215 was determined by Pepscan analysis using 8 to 16 amino acid peptides, overlapping by all but one amino acid, spanning the HIV-1 MN V3 loop. The minimum epitope was defined as the shortest common sequence found in peptides reactive with HuMAb MN215. No reactivity could be found in this Pepscan analysis with overlapping peptides of 8 to 14 amino acids. The shortest peptide showing reactivity with HuMAb MN215 was a 15 amino acid peptide spanning the tip of the loop (GPGR) and amino acids at the C- and N-terminal side of this structure (Fig. 4*a*). A similar analysis was carried out with 7 to 13 amino acid peptides with the Dutch consensus sequence. The minimum epitope recognized by HuMAb MN215 with this V3 loop sequence proved to be 9 amino acids long and consisted of amino acids at the N-terminal side of the tip of the V3 loop only (Fig. 4*b*).

Individual amino acids involved in HuMAb MN215 binding

Differences between donor #658 and the Dutch consensus V3 domain sequences were introduced in a set of peptides based on the Dutch consensus sequence for Pepscan analysis. Substitutions S to G at position 13, T to A at position 28 and E to D at position 30, which were observed in donor #658 as compared to the Dutch consensus, did not influence the binding capacity of HuMAb MN215 (data not shown). The role of individual amino acids at both sides of the tip of the loop was determined by using peptide analogues which differed in only 1 amino acid from the peptide with the Dutch consensus sequence peptides in a separate Pepscan analysis (KSIHIGPGRAFYT) (Fig. 5).

Five single amino acid substitutions caused improved binding capacity of HuMAb MN215:

1) Substitution G to N at position 19 and R to K at position 20, which are both well tolerated at that position in a type II β turn (Wilmot & Thornton, 1988; Ghiara *et al.*, 1994).

2) F to L at position 23, which results in a smaller surface area and more rotational freedom.

3) Y to E and T to E at position 24, both resulting in a more negative charge on the C-terminal side of the tip of the loop.

Substitution of amino acids at the tip of the loop for amino acids with low frequency in known type II β turns were not well tolerated in the Pepscan with MN215 (20 P → L, 21 G → P, 21 G → A and 22 R → P; Wilmot & Thornton, 1988; Langedijk *et al.*, 1991). Reactivity of HuMAb MN215 with the V3 loop therefore seems to be dependent on the type II β turn at the tip of the loop (LaRosa *et al.*, 1990; Ghiara *et al.*, 1994).

A peptide was subsequently constructed in which all these mutations from the Dutch consensus sequence (KSIHIGPNKALEE) had been applied. HuMAb

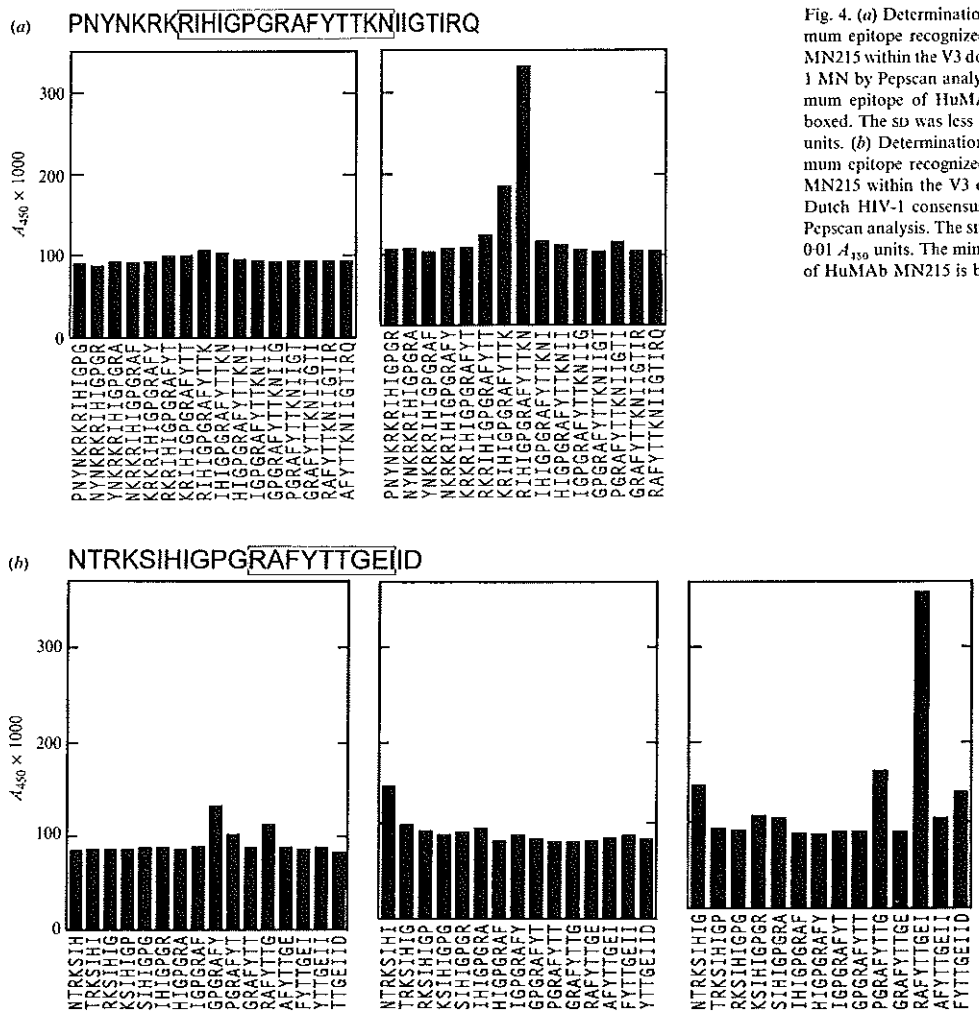


Fig. 4. (*a*) Determination of the minimum epitope recognized by HuMAb MN215 within the V3 domain of HIV-1 MN by Pepscan analysis. The minimum epitope of HuMAb MN215 is boxed. The SD was less than 0·01 A_{450} units. (*b*) Determination of the minimum epitope recognized by HuMAb MN215 within the V3 domain of the Dutch HIV-1 consensus sequence by Pepscan analysis. The SD was less than 0·01 A_{450} units. The minimum epitope of HuMAb MN215 is boxed.

MN215 failed to bind to this peptide in ELISA (data not shown).

Preferential reactivity of HuMAb MN215 with glycoprotein of NSI origin

The data obtained with the Pepscan analysis suggested a preferential reactivity of HuMAb MN215 for HIV-1 strains of the NSI phenotype. We therefore tested HuMAb MN215 in the peptide inhibition ELISA with V3 domain sequences derived from recombinant envelop proteins of cloned NSI and SI viruses from two donors, #16 and #320, of the Amsterdam cohort (Fig. 6). HuMAb MN215 showed a significantly higher relative affinity for the NSI- than for the SI-derived V3 peptides of both donors. We subsequently tested HuMAb MN215 together with three other HuMAbs directed against conserved epitopes on HIV-1 glycoproteins with the recombinant glycoproteins derived from the SI and NSI variants of donors #16 and #320. We also included glycoproteins from the HIV-1 MN isolate in this analysis. A significantly higher reactivity of HuMAb MN215 was found with the NSI glycoproteins as compared to the SI glycoproteins from both the donors (Table 1). HuMAb MN215 only recognized the HIV-1 MN (SI) glycoprotein

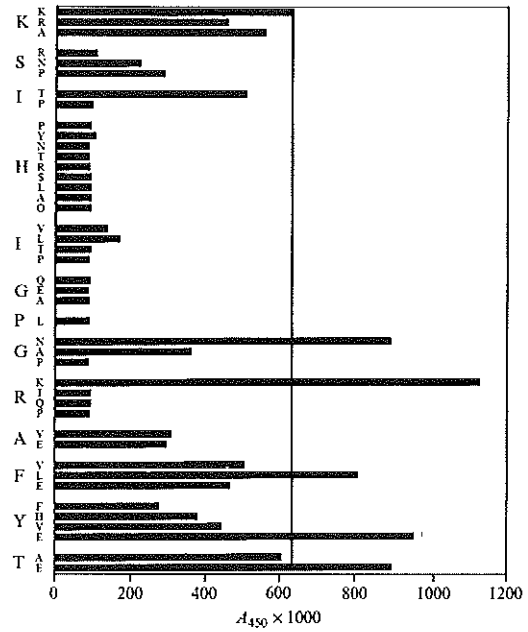


Fig. 5. Reactivities of HuMAb MN215 in a Pepscan analysis with peptides modified from the Dutch consensus sequence, by replacing one amino acid compared to the native sequence (KSIHIGPGRAFYT). The SD was less than 0.01 A_{450} units.

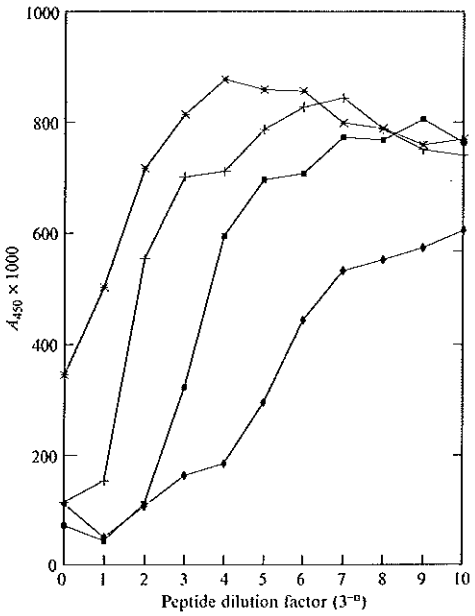


Fig. 6. Peptide inhibition ELISA of HuMAb MN215 V3 loop peptides derived from NSI and SI viruses from two individuals (donors 16 and 320). A_{450} values were obtained in a solid phase HIV-1 MN V3 peptide ELISA with a standard concentration of HuMAb MN215 incubated with serial threefold dilutions of the respective peptides. These were derived from sequences of cloned SI and NSI viruses from donors 16 and 320 from the Amsterdam cohort [#16 SI (+), #16 NSI (◆), #320 SI (*), #320 NSI (■); see Fig. 1]. A starting peptide concentration of 3×10^{-11} mol was used.

poorly in this assay. The other HuMAbs directed against more conserved epitopes on gp160 did not clearly distinguish between the glycoproteins derived from viruses with the two phenotypes.

VN activity of HuMAb MN215 towards SI strains

It has been shown by others that HIV-1 NSI strains are highly resistant to VN activity by human antibodies (Bou-Habib *et al.*, 1994). In accordance with these observations we failed to demonstrate inhibition of entry by chimeric HIV-1 carrying the recombinant 16.4 NSI glycoprotein in the virus entry inhibition assay (> 90% of c.p.m. without HuMAb at 200 µg/ml of HuMAb MN215). However, despite the low relative affinity of HuMAb MN215 for the SI HIV-1 glycoprotein from donor #16, it did inhibit entry of chimeric virus carrying the 16.2 SI glycoprotein, albeit at high concentrations (about 60 µg/ml for 50% inhibition and < 200 µg/ml needed for 90% inhibition). As expected HuMAb K14 failed to inhibit entry of these chimeric viruses (Table 2).

Table 1. *Relative affinity of HuMAbs for glycoproteins of SI and NSI HIV-1 strains*

HuMAb	Epitope	#320 SI	#320 NSI	#16 SI	#16 NSI	MN
MN215	V3 domain	< 4*†	48	< 4*	84	< 4*
257-D	V3 domain	250	250	125	125	128
GP13	CD4 bd	84	100	84	33	84
K14	gp41	42	10	84	55	42

* Dilution could not be determined because reactivity did not reach plateau value at the concentrations tested.

† Dilutions of the respective HuMAbs, starting at 1 µg/ml, at which 50% of the maximum binding was reached in the antibody capture ELISA with the cell lysates of rVV-infected HELA cells.

Discussion

We have described an HIV-1-neutralizing HuMAb (HuMAb MN215; IgG1) that proved to react with the V3 domain of a selection of subgroup B virus strains. This HuMAb displayed a higher affinity for NSI than for SI glycoproteins within the same host.

Variation in the V3 domain within the host-delimited

Table 2. *Inhibition of entry of chimeric HIV-1, carrying the #16.2 SI glycoprotein, by HuMAb MN215 as measured in the virus entry inhibition assay*

HuMAb concentration (μg/ml)	Percentage of maximum c.p.m. using HuMAb K14	Percentage of maximum c.p.m. using HuMAb MN215
0	100*	100
25	105	82·9
50	98	55·7
100	98	39·2
200	91	21·9

* Percentage from c.p.m. using 16.2 SI *env* gene without addition of HuMAb [(c.p.m. with HuMAb/c.p.m. without antibody) × 100].

quasispecies distribution has been shown to be concentrated at the N- and C-terminal sides of the tip of the loop (Epstein *et al.*, 1991; Kuiken *et al.*, 1992). Both the tip and the base of the loop are relatively conserved (LaRosa *et al.*, 1990). Variation within the V3 domain is believed to be largely driven by the VN antibody response. Consequently, an important part of this response seems to be directed against both sides of the loop. The relatively long minimum epitope determined by Pepscan analysis with the HIV-1 MN sequence (15 amino acids) and the mutational analysis of the MN215 epitope indicated that amino acids involved in binding of HuMAb MN215 are also primarily concentrated in these areas with high variation. HuMAb MN215 is therefore directed against those parts of the V3 domain against which most of the VN antibody response in seropositive individuals is directed.

Recently, two other HuMAbs have been described with a minimum epitope on the HIV-1 MN V3 loop sequence of 15 amino acids in length (Gorny *et al.*, 1993). We were able to narrow the core epitope down to 9 amino acids at the C-terminal side of the type II β turn by using a sequence more related to the V3 sequence derived from the virus of donor #658. It should, however, be stressed that the type II β turn and the N-terminal parts of the V3 loop also contribute to the optimum binding of the HuMAb.

Combining all amino acids giving improved binding of HuMAb MN215 in one peptide did not result in a peptide recognized by HuMAb MN215, indicating the importance of intramolecular interactions for optimum epitope presentation. Combining all the criteria for the 'best fitting' MN215-binding peptides with actually described V3 sequences of HIV-1 (LaRosa *et al.*, 1990) resulted in the following V3 sequence: KS/GIHIGPGKAFYTTGE/DI. This sequence exhibits a striking similarity with the conserved consensus V3 loop sequence of macrophage-tropic HIV-1 strains (Chesebro *et al.*, 1992). Previous reports have shown that all NSI strains isolated so far have the combination of no charge at position 13 and negative or no charge at position 30 of the V3 loop, whereas SI HIV-1 strains have a positively charged residue at either one of these positions (Fouchier *et al.*, 1992). Others have shown that negative charge at the C-terminal side of the V3 loop is associated with less SI capacity (de Jong *et al.*, 1992). Negative or uncharged residues at these positions are also important for binding of HuMAb MN215 to V3 peptides. On the basis of the Pepscan data it can be concluded that changes within the V3 loop resulting in more SI capacity for the virus will decrease the affinity of HuMAb MN215. Indeed, when tested in a peptide inhibition ELISA and in a catching antibody ELISA, with whole solid phase gp160, HuMAb MN215 showed a significantly higher affinity for the V3 domains of NSI than for the V3 domains of SI viruses within the two donors tested. The other HuMAbs tested, directed against more conserved epitopes of gp160, did not discriminate between the peptides and glycoproteins representing different phenotypes from the two donors. The V3 loop of the SI recombinant envelop proteins from donor #16 have been shown to transfer the phenotype when exchanged with the V3 loop of an NSI recombinant envelop protein from donor #16 (Andeweg *et al.*, 1993). The amino acid differences between the SI and NSI V3 domains are therefore believed to be important for the SI capacity of HIV-1 strains. On the basis of the data presented it is very likely that HuMAb MN215 has a higher affinity for the NSI and macrophage-tropic HIV-1 strain within a host-delimited quasispecies distribution than for the SI and T cell line-tropic strains. Despite the fact that HuMAb MN215 was selected for reactivity with a HIV-1 MN V3 loop peptide and despite its high affinity for the HIV-1 MN V3 loop peptide in both solid phase peptide ELISA and peptide inhibition ELISA, HuMAb MN215 only bound with low affinity to the HIV-1 MN V3 loop when it was presented in the context of the whole HIV-1 MN glycoprotein. These findings indicate that antigenicity data generated with isolated peptides in solid phase assays (Moore, 1993) and also with isolated peptides in solution may not be representative of the antigenicity of these structures in their natural configuration. It has been shown that macrophage-tropic HIV-1 strains need a certain conformation of their V3 loop (Innocenti-Francillard *et al.*, 1994), which is apparently recognized by HuMAb MN215. This may explain why the 'suboptimum' V3 sequence of donor #320 NSI, which is not recognized by HuMAb MN215 in Pepscan analysis (data not shown), still results in a glycoprotein that is more efficiently recognized than the #16 SI or the #320 SI glycoproteins. The low relative affinity of HuMAb MN215 for the HIV-1 MN glycoprotein is in accordance with the low reactivity in FACScan analysis of this antibody observed

with HIV-1 MN-infected SupT1 cells (unpublished results) and the relatively low VN capacity of this antibody towards HIV-1 MN and SF2 (A. McKnight, personal communication). We also showed that HuMAb MN215 inhibited entry of a chimeric HIV-1 strain carrying the recombinant 16.2 SI HIV-1 glycoprotein, albeit very inefficiently. The CD4-binding domain (CD4 bd)-specific HuMAb GP13, which neutralizes T cell line-adapted HIV-1 strains (Schutten *et al.*, 1993), did not neutralize the 16.2 SI chimeric virus at the concentrations tested (data not shown) although this antibody has a significantly higher relative affinity for the 16.2 SI glycoprotein than HuMAb MN215. This stresses the relatively high VN capacity of antibodies directed against the V3 domain. It was unexpected that HuMAb MN215 did not inhibit entry of a chimeric virus carrying the 16.4 NSI glycoprotein, which it preferentially recognizes. However, an affinity-purified immunoglobulin preparation from seropositive individuals (HIVIG; Prince *et al.*, 1991) also failed to do so (data not shown). The inability of HuMAb MN215 to neutralize an NSI virus under conditions at which it does neutralize an SI virus, which it recognizes with lower affinity, should therefore be considered in the light of general difficulties encountered with the neutralization of non-T cell line-adapted NSI HIV-1 strains (Bou-Habib *et al.*, 1994).

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CHAPTER 4

Molecular characterization of variable heavy and light chain regions of five HIV type 1-specific human monoclonal antibodies.

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Molecular Characterization of Variable Heavy and Light Chain Regions of Five HIV Type 1-Specific Human Monoclonal Antibodies*

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ABSTRACT

We have reported the generation and characterization of four HIV-1 neutralizing human monoclonal antibodies. Three antibodies recognize a conformational epitope within the CD4-binding site of HIV-1 gp120 and one recognizes a linear epitope located within the hypervariable V3 domain of gp120. In the present study we report the nucleotide sequences of the cDNAs encoding the variable regions of the heavy and light chains of these antibodies. Molecular characteristics, closest germline genes, and the putative extent of somatic mutation are presented. Two of the four heavy chain variable (V_H) regions are derived from the V_H1 gene family, one from the V_H3 gene family, and one from the V_H5 gene family. In addition, the V_H chain of a previously described human monoclonal antibody, directed against HIV-1 gp41, is derived from the V_H3 gene family. The degree of nucleotide variation between these five antibodies and their closest germline counterparts ranges from 4 to 12%, mainly located in the complementarity-determining regions. Significant nucleotide sequence homology with previously described germline diversity (D) genes could be found for only two of five antibody D segments. Joining (J_H) gene segments utilized are J_H4 or J_H6. Two light chain variable (V_L) regions are derived from a $V_\kappa1$ gene segment, one from a $V_\kappa4$, one from a $V_\lambda2$, and one from a $V_\lambda6$ gene segment.

INTRODUCTION

ACQUIRED IMMUNODEFICIENCY SYNDROME (AIDS) is the late-stage disease of infection with human immunodeficiency virus (HIV). Counter-AIDS strategies include the development of active immunization protocols to prevent HIV infection, and passive immunization protocols for postexposure therapy. Passive immunization in particular may be important in preventing transmission of HIV from infected mothers to their offspring[1] and in preventing infection after accidental exposure. These goals may be achieved by the administration of a cocktail of human monoclonal antibodies (MAbs), capable of neutralizing a variety of HIV strains.

HIV-1 infection elicits at least two major types of neutralizing antibodies directed against gp120. One is directed against the hypervariable V3 domain, the other against the conserved CD4-binding site (b.s.). Antibodies directed against the V3 domain are found already in the early phase of infection. Initially it was proposed that V3 domain-specific antibodies would predominantly neutralize the eliciting HIV-1 strain.[2-5] However, it has been demonstrated that several V3 domain-specific antibodies have much broader reactivities than previously suggested.[6-9] Emini *et al.* showed that chimpanzees, passively immunized with an HIV-1 IIIB neutralizing V3 domain-specific antibody, were protected against infection with the homologous HIV strain.[10,11] Antibodies directed against this side are therefore likely candidates for passive immunization. Antibodies directed against the CD4 b.s. are detected later in infection and have a wide range of neutralizing activity against HIV-1 strains, owing to the conserved nature of the CD4 b.s.[12] The neutralizing capacity of these antibodies seems to be generally lower, as compared to the V3 domain-specific antibodies.[13] Equimolar mixtures of human MAbs, directed against either of these two sites, may have a synergistic HIV-neutralizing effect.[14-16] We described the generation and characterization of four HIV-1-neutralizing human MAbs: one V3 domain-specific antibody (MN215) and three antibodies directed against the CD4 b.s. (GP13, GP44, and GP68).[9,17] GP13, GP44, and GP68 display

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*The sequences reported in this article have been submitted to the GenBank database.

broadly HIV-1-neutralizing activity, whereas MN215 reacts predominantly with macrophage-tropic and non-syncytium-inducing (NSI) HIV-1 strains.[9] During the asymptomatic stage of the HIV-1 infection predominantly HIV-1 strains of the NSI phenotype are found.[18] A low efficiency of the primary immune response in eliminating NSI/macrophage-tropic HIV-1 strains or a preferential transmission of these HIV-1 strains has been suggested.[19] This makes MN215 a likely candidate to be used in preparations for early passive immunization therapies. Here we report the molecular characterization of the variable heavy and light chain regions of these four human MAbs, and of a previously described broadly reactive human MAb (K14) directed against an epitope on HIV-1 gp41.[20] Although this is a nonneutralizing MAb, it was included in these studies because of a possible synergistic therapeutic effect in a cocktail of human MAbs.

Molecular characterization of the V genes used by HIV-neutralizing, -nonneutralizing, and -enhancing human MAbs will lead to a better understanding of the interaction between HIV and the antibody repertoire. Furthermore, molecular data are necessary for the *in vitro* construction of broadly reactive, high-affinity HIV-neutralizing human antibodies and in discriminating between neutralizing and enhancing antibodies. These data will provide a valuable contribution to the development of an efficacious anti-HIV vaccine to be used in passive immunization protocols. Therefore, in the present study we give a detailed molecular characterization of the V regions of the five antibodies mentioned above. Remarkably, two of five HIV-specific human MAbs presented here express a V_H3 gene segment, whereas data suggest a superantigen-like binding of gp120 to (membrane) immunoglobulin V_H3 gene products and the subsequent deletion of V_H3-expressing B cell clones in AIDS patients.[21,22]

MATERIALS AND METHODS

HIV-1-specific human monoclonal antibodies

Human MAbs to HIV-1 were isolated from Epstein-Barr virus (EBV)-transformed B cell lines, derived from peripheral blood mononuclear cells from asymptomatic HIV-1-seropositive donors, as described previously.[17,20] Briefly, EBV-transformed B cells producing IgG antibodies specific for HIV-1 were selected by screening for reactivity in enzyme-linked immunosorbent assay (ELISA) with either gp120, gp160, or V3 loop peptides.[9,17] Five IgG_1 MAbs from three donors were studied: GP13, GP44, and GP68, recognizing a conformational epitope partly overlapping with the CD4 b.s. of gp120[17]; MN215, recognizing the principal neutralizing domain (V3 domain) of gp120 of the MN isolate[9]; and K14, recognizing an epitope on gp41.[20] Monoclonal antibodies GP13 and GP68 have been demonstrated to neutralize various HIV-1 laboratory isolates *in vitro*, GP44 neutralizes the SF2 isolate, and MN215 neutralizes the MN and SF2 isolates.[9,17]

Oligonucleotides

The oligonucleotides used in the PCR amplifications were synthesized on an Applied Biosystems (Foster City, CA) DNA synthesizer. Sequences of the oligonucleotides are shown in Table 1.

Single-stranded cDNA synthesis and polymerase chain reaction

Total RNA was extracted from 10^7 EBV-transformed B cells by the RNAzol method (CINNA/Biotex Laboratories, Inc., Houston, TX). Single-stranded cDNA (ss-cDNA) was synthesized by using Moloney leukaemia virus (M-MLV) H^- reverse transcriptase superscript (GIBCO-BRL/Life Technologies, Gaithersburg, MD) and an oligo(dT) primer. Polymerase chain reactions (PCRs)[23] were done essentially via the method recommended by the manufacturer (Perkin-Elmer Cetus, Norwalk, CT). The PCR cycles were as follows: denaturation at 96°C for 1 min, annealing at 60°C for 2 min, and extension at 72°C for 1 min 30 sec, controlled in a DNA thermal cycler (Perkin-Elmer Cetus).

Isolation, cloning, and sequencing of amplified products

Amplified DNA was digested with *Sst*I and *Hind*III and size selected on a 1% ethidium bromide agarose gel. The purified

TABLE 1. SEQUENCES OF OLIGONUCLEOTIDES USED IN POLYMERASE CHAIN REACTION AMPLIFICATIONS

Oligonucleotide	*Sequence*
V_H1 leader	5′ ATAGAGCTCATGGACTGGACCTGGAGG 3′[a]
V_H3 leader	5′ ATAGAGCTCTGGAGTTTGGGCTGAGCTGG 3′[a]
V_H3 BACK	5′ ATAGAGCTCGAGGTGCAGCTGGTGGAGTCT 3′[a]
V_H5 leader	5′ ATAGAGCTCTCGCCCTCCTCCTG 3′[a]
$V_κ1$ leader	5′ ATAGAGCTCCTGCTGCTGCTGTGGCTGCCC 3′[a]
$V_κ1$ leader	5′ ATAGAGCTCATGGACATGAGGGTCCCC 3′[a]
$V_κ4$ leader	5′ ATAGAGCTCATGGTGTTGCAGACCCAG 3′[a]
$V_λ2$ leader	5′ ATAGAGCTCTGGACTCCCCTCCTCCTCACT 3′[a]
$V_λ6$ leader	5′ ATAGAGCTCCTCACTCACTGTACTGGTTCT 3′[a]
$C_γ$	5′ CTCAAGCTTCAGGGGAAGACCGATGG 3′[b]
$C_κ$	5′ CTCAAGCTTAACAGAGGCAGTTCCAGACTT 3′[b]
$C_λ$	5′ CTCAAGCTTTGTGGCCTTGTTGGCTTG 3′[b]

[a]The *Sst*I restriction site is underlined.
[b]The *Hind*III restriction site is underlined.

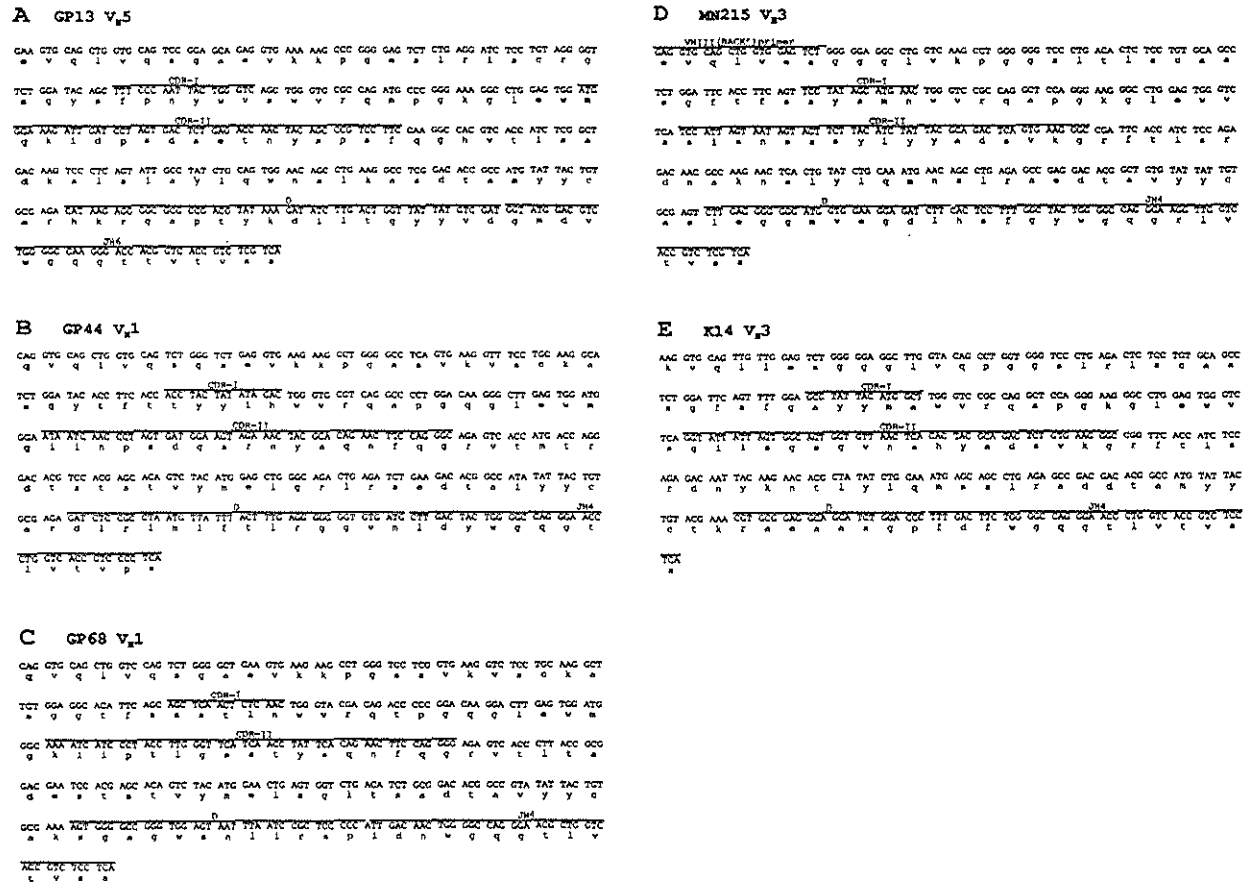

FIG. 1. cDNA sequences of the heavy chain variable regions of HIV-1-specific human monoclonal antibodies. The CDR-I, CDR-II, D, and J_H segments are denoted. cDNA sequences of the heavy chain of monoclonal antibodies GP13 (A), GP44 (B), GP68 (C), MN215 (D), and K14 (E) are shown.

product was ligated into the *SstI/HindIII* restriction site of a Bluescript phagemid vector and transformed into $CaCl_2$-competent XL1-blue bacteria. Several recombinant clones were selected and sequenced in both orientations using nonradioactive dye-labeled T3 and T7 oligonucleotide primers (Applied Biosystems) on a 370 A automated sequencer (Applied Biosystems).

Owing to limited availability of patient materials we were not able to obtain genomic DNA for the isolation of the respective germline gene counterparts. Therefore the latest update of the total EMBL/GenBank database was searched to identify expressed as well as germline genes displaying the highest nucleotide sequence similarities with the V genes presented in these article. Primary amino acid sequences were deduced and alignments were carried out using the DNAstar program (DNAstar, Inc., Madison, WI).

RESULTS

Analysis of the expressed V_H genes encoding HIV-1-specific human monoclonal antibodies

The complete nucleotide sequences determined for the V_H region of each antibody are shown in Fig. 1. As a result of the databank searches each of the expressed V_H regions could be compared with its closest germline counterpart (Fig. 2). An overview of the five HIV-1-specific human MAbs and their characterization is given in Table 2.

The GP13 V_H gene contains an open reading frame, has all the features characteristic of a functional V_H gene, and is most homologous to the previously described V_H5 germline gene V_H32 (94.2%).[24] There are two nucleotide differences in framework (FR) I, one being silent and one causing an amino acid substitution, and there is one silent mutation in FR II. The most extensive variation is found in the two CDRs (8–22%), which is indicative for an antigen-driven immune response. More than 60% of the total mutations in the complementarity-determining regions (CDRs) result in amino acid substitutions. An unusual number of nucleotide differences ($n = 6$) was observed within framework III of GP13 V_H as compared to the analogous framework of V_H32. Three of the six nucleotide differences in framework III resulted in amino acid substitutions (Fig. 3A). Furthermore, the degree of variation (6%) is higher than the usual mutation rate described for frameworks (i.e., 2%), indicating a possible role for the framework residues in HIV-1 binding.[25]

The GP44 V_H gene segment is a member of the V_H1 gene family and is most homologous to the HV1f10 germline gene (94.9%).[26] Furthermore, there is 88.7% nucleotide sequence

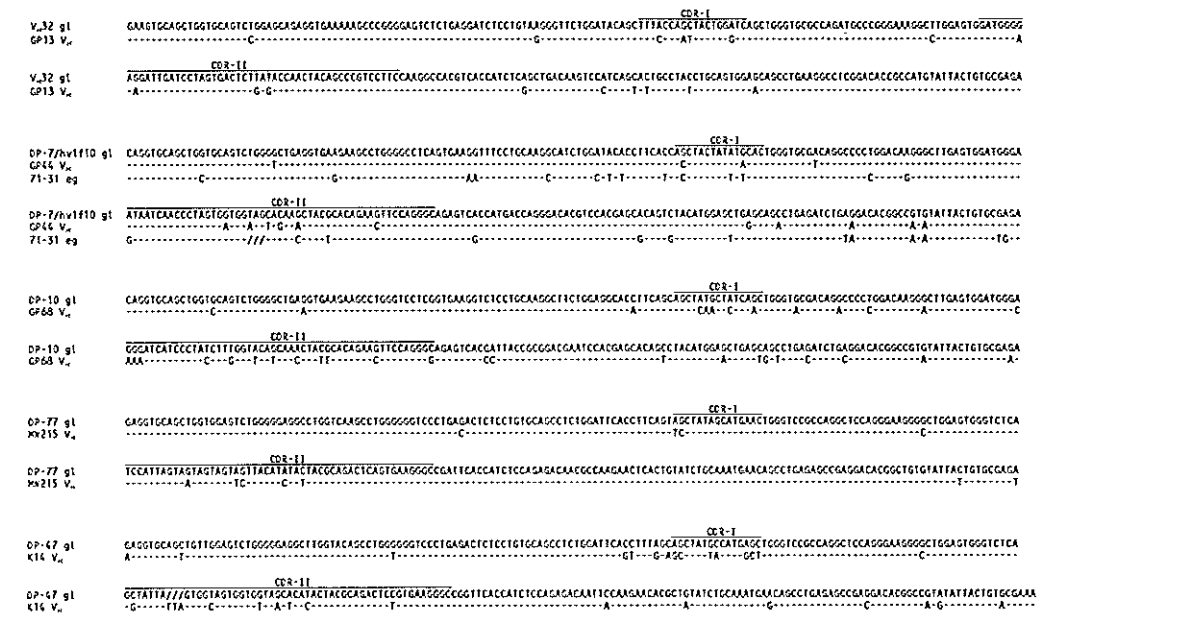

FIG. 2. Nucleotide sequence of each HIV-1-specific heavy chain variable region compared to the nucleotide sequence of the closest germline genes. Identities between sequences are indicated by dashes. (A) Nucleotide sequence of GP13 compared to the V_H32 germline sequence. (B) Nucleotide sequence of GP44 compared to the identical DP-7 germline and hv1f10 germline sequences and to the expressed gene 71-31. (C) Nucleotide sequence of GP68 compared to the DP-10 germline sequence. (D) Nucleotide sequence of MN215 compared to the DP-77 germline seqence. (E) Nucleotide sequence of K14 compared to the DP-47 germline sequence. gl, Germline; eg, expressed gene.

TABLE 2. CHARACTERISTICS OF FIVE HIV-1-SPECIFIC HUMAN MONOCLONAL ANTIBODIES

Antibody	Specificity	Virus neutralizing	V_H	V_L	% homology with closest germline gene: V_H	% homology with closest germline gene: V_L
GP13 (IgG_1,κ)	CD4 b.s.	Yes	V_H5	$V_\kappa4$	94.2% V_H32	94.1% $V_\kappa4$
GP44 (IgG_1,λ)	CD4 b.s.	Yes	V_H1	$V_\lambda2$	94.9% HV1f10	88.1% IGLV21
GP68 (IgG_1,κ)	CD4 b.s.	Yes	V_H1	$V_\kappa1$	87.8% DP-10	93.6% HK102
MN215 (IgG_1,λ)	V3 domain	Yes	V_H3	$V_\lambda6$	96.3% DP-77	—[a]
K14 (IgG_1,κ)	gp41	No	V_H3	$V_\kappa1$	89.8% DP-47	89.0% HK137

[a]—, No significant homology.

similarity with a previously described V_H1 gene, 71-31, expressed by an HIV-1-specific human MAb.[27] However, this MAb is directed against an epitope on HIV-1 p24. The GP68 V_H gene is also derived from the V_H1 gene family and is most homologous to the DP-10 V_H1 germline gene (87.8%).[28]

The MN215 V_H gene is derived from the largest gene family (V_H3) and is most homologous to the DP-77 germline gene (96.3%).[28] The K14 V_H gene is also derived from the V_H3 gene family and is 89.8% identical to the DP-47 germline gene.[28] A remarkable difference between K14 V_H and DP-47 is three additional nucleotides encoding an isoleucine residue in CDR-II of K14. Therefore it is unlikely that DP-47 would be the germline counterpart of K14 V_H.

Owing to the relatively high number of still unidentified members of the V_H1 and V_H3 gene families it remains difficult to determine whether our expressed GP44, GP68, MN215, and K14 V_H genes represent somatically mutated or as yet unidentified germline genes.

Analysis of expressed V_L genes

The complete nucleotide sequences determined for the V_L region of GP13, GP44, GP68, MN215, and K14 are shown in Fig. 4. Each of the expressed V_L regions was compared with the respective, closest germline sequence obtained from the EMBL/GenBank database (Fig. 5).

The GP13 V_L gene contains an open reading frame that has all the features characteristic of a functional $V_\kappa4$ gene segment. There is 94.1% homology with the previously described $V_\kappa4$ germline gene,[29] which is the only $V_\kappa4$ gene found on the human Ig κ locus. There are six nucleotide differences within the frameworks, all causing an amino acid substitution, except for the one in FR III (Fig. 3B). The variation in the three CDRs ranges from 5 to 16%, with most of the nucleotide differences accumulated within CDR-I. Furthermore, 75% of the nucleotide mutations in the CDRs result in amino acid substitutions.

The GP44 V_L gene segment is a member of the $V_\lambda2$ gene family and is 88.1% identical to the IGLV21 germline gene.[30] However, it is unlikely that the IGLV21 germline gene would be the germline counterpart of the GP44 V_L gene, because there is 94.6 and 94.2% homology with two other expressed V_L genes, WLR[31] and PV6,[32] respectively. Furthermore, there is 92.5% homology with the expressed gene HBW4-1,[32a] also encoding an HIV-1 gp120-specific human MAb. The GP68 V_L gene is derived from the $V_\kappa1$ gene family and is 93.6% identical to the HK102 germline gene[33] and 94.7 and 95.4% identical to the expressed genes 3D6[34] and kalc6,[35] respectively. Gene 3D6 also encodes an HIV-1-specific human MAb, but this MAb is directed against gp41, a transmembrane glycoprotein of HIV-1. The MN215 V_L gene segment is a member of the $V_\lambda6$ gene family. However, significant homology could be found only with two expressed genes, H95.EBV (93.9%)[36] and EB4V_λVI (94.5%).[37] The K14 V_L gene is also derived from the $V_\kappa1$ gene family and is 89.0% homologous to the HK137 germline gene.[38] Again, better homology was found with two expressed genes, HGQ (95.1%)[39] and A20 (95.2%),[40] suggesting another germline gene for K14 V_L. There is only 87.6% homology with the expressed gene No. 86,[32a] also encoding an HIV-1 gp41-specific human MAb.

Analysis of D, J_H, and J_L gene segments

Figure 6 shows the sequences of the expressed D segments, compared to their closest germline counterpart[41] or to other previously described expressed D segments.[42–46] Part of the GP13 D segment may be derived from the D_{XP1} germline. This part could be preceded by another unknown D segment, causing a

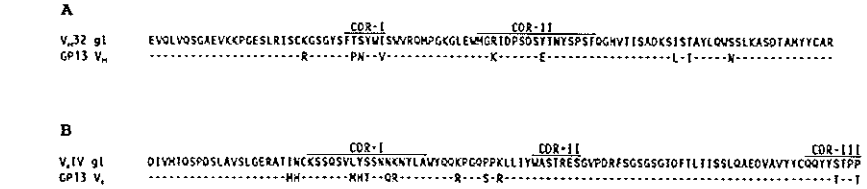

FIG. 3. Deduced amino acid sequences of the heavy (A) and light (B) chain variable regions of GP13 compared to the amino acid sequences of the closest germline genes. Identities between sequences are indicated by dashes.

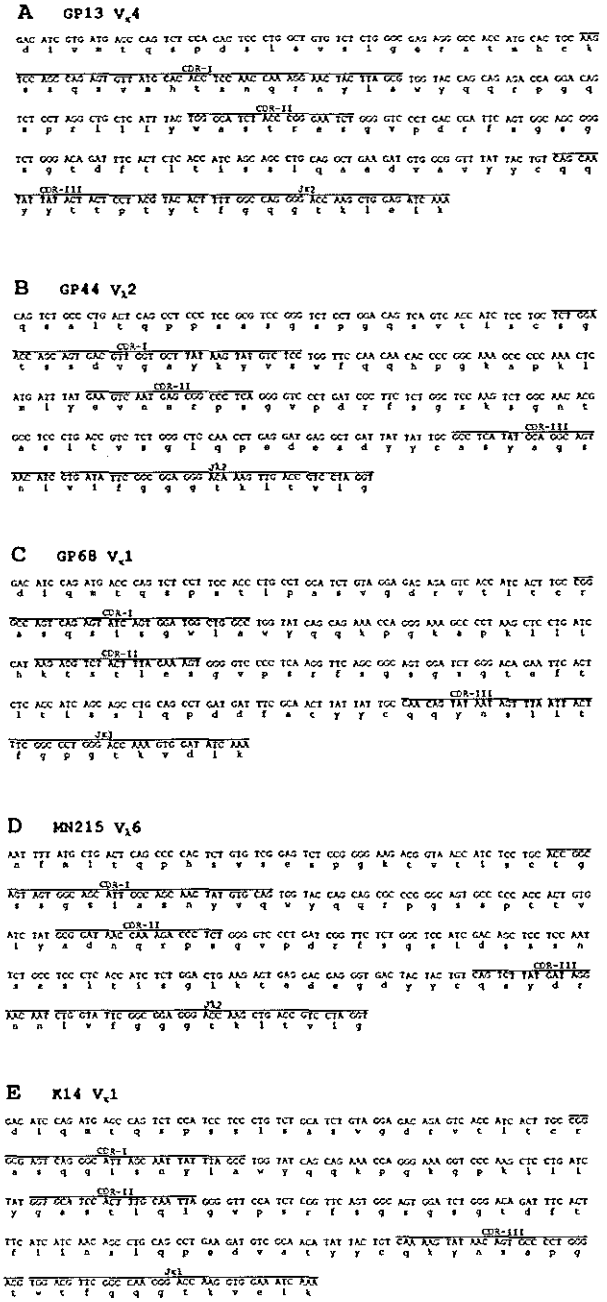

FIG. 4. cDNA sequences of the light chain variable regions of HIV-1-specific human monoclonal antibodies. The CDR-I, CDR-II, CDR-III, and J_L segments are denoted. cDNA sequences of the light chain of monoclonal antibodies GP13 (A), GP44 (B), GP68 (C), MN215 (D), and K14 (E) are shown.

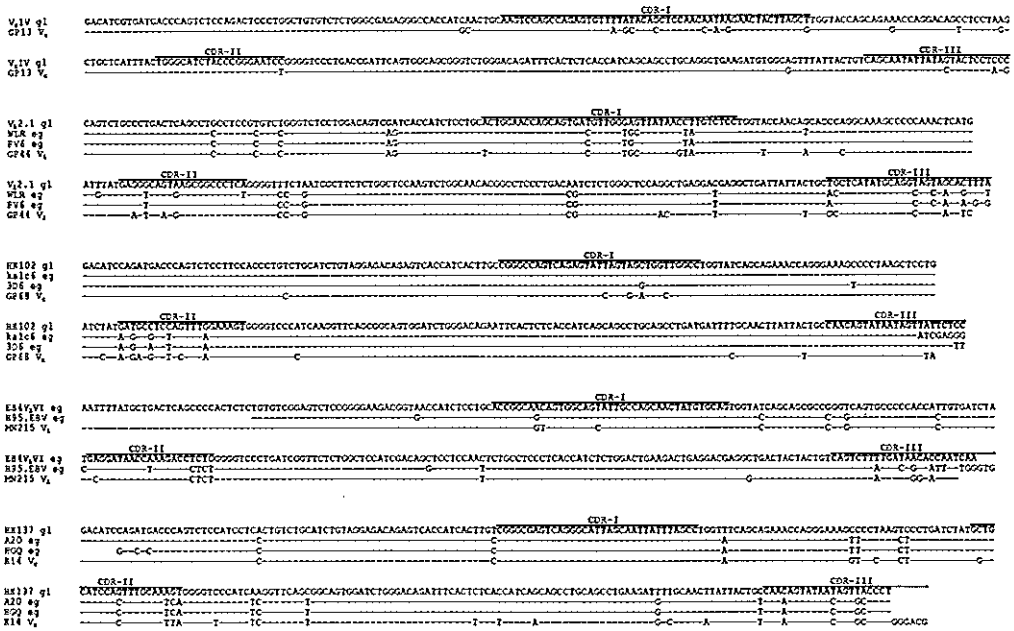

FIG. 5. Nucleotide sequence of each HIV-1-specific light chain variable region compared to the nucleotide sequence of the closest germline genes or expressed genes. Identities between sequences are indicated by dashes. (A) Nucleotide sequence of GP13 compared to the V_K4 germline sequence. (B) Nucleotide sequence of GP44 compared to the $V_\lambda2.1$ germline sequence and to the expressed genes WLR and PV6. (C) Nucleotide sequence of GP68 compared to the H102 germline sequence and to the expressed genes kalc6 and 3D6. (D) Nucleotide sequence of MN215 compared to the expressed genes EB4Vλ6 and H95.EBV. (E) Nucleotide sequence of K14 compared to the HK137 germline sequence and to the expressed genes A20 and HGQ. gl, Germline; eg, expressed gene.

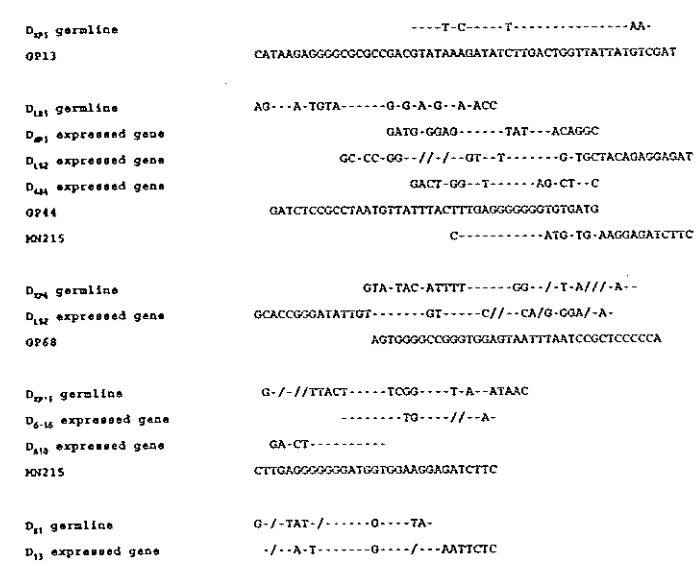

FIG. 6. Possible origins of the D segments of each HIV-1-specific human monoclonal antibody. Identities between sequences are indicated by dashes.

D–D fusion,[47] or by an N segment addition. The last three nucleotides (GAT) could not be derived from a J_H segment and are likely N segment additions. The D segment of K14 may be derived from the D_{N1} germline. For GP44, GP68, and MN215 we found little homology between the expressed D segments and the known germline D segments. Instead, higher homologies with other expressed D segments were observed (Fig. 6).

In Fig. 7 the expressed J_H gene segments are compared with their germline counterparts.[48] Four of the five antibodies express the J_H4 gene segment and one expresses J_H6. Nucleotide differences were observed within all four expressed J_H4 segments, in some cases resulting in amino acid substitutions. Except for MN215, they all miss the first five nucleotides. All four expressed J_H4 segments displayed the same allelic polymorphism, A to G, as described before; only the GP68 J_H4 and MN215 J_H4 segments may display another polymorphism, C to G.[49] Remarkable is the absence of the nucleotide stretch at the 5′ end of GP13 J_H6, normally encoding the five tyrosine residues, characteristic of a J_H6 segment. Furthermore, two nucleotide differences are observed, neither one causing an amino acid substitution.

K14 expresses the $J_\kappa1$ gene segment and GP13 expresses $J_\kappa2$ (Fig. 8). Both J_κ segments are unremarkable except for the last three nucleotides (CGT), which are absent. GP68 expresses the $J_\kappa3$ gene segment, which also misses these last three nucleotides. Besides, two nucleotide differences at the 5′ end cause an amino acid substitution. GP44 and MN215 both express the $J_\lambda2$ gene segment. For GP44 three nucleotide differences are observed, of which the first one causes an amino acid substitution. For MN215 only the first nucleotide is changed, causing an amino acid substitution.

DISCUSSION

In the present article we have reported the complete nucleotide sequences of the heavy and light chain variable regions of five human MAbs, directed against the envelope glycoproteins of HIV-1. Two heavy chains of these antibodies are derived from the V_H1, two from the V_H3, and one from the V_H5 gene family. For the light chains, two are derived from the $V_\kappa1$, one from the $V_\kappa4$, one from the $V_\lambda2$, and one from the $V_\lambda6$ gene family. Only for GP13 were we able to identify the germline counterparts of the V genes. The GP13 V_H gene is derived from the V_H5 gene family, which consists of three members, one of which is a pseudogene.[24] It is most likely that V_H32 is the V_H5 germline counterpart of GP13 V_H. Because it has been shown that the smaller human V_H gene families (V_H4, V_H5, and V_H6) display remarkably little polymorphism,[50] the observed nucleotide differences are most likely caused by somatic mutations. The relatively high number of mutations in FR III may indicate a possible role for some of the FR III amino acids in antigen binding, as has been suggested before.[15] The GP13 V_L gene is derived from the $V_\kappa4$ gene family, which consists of only one germline gene. Most of the somatic mutations are concentrated in the CDRs. Therefore, the extensive somatic variation in the V regions of GP13 indicates an antigen-driven (i.e., HIV-1) immune response.

The expressed V_H and V_L genes of the other four human MAbs are derived from V gene families containing an unknown number of still unidentified germline genes. For example, the expressed GP44 $V_\lambda2$ gene segment shows more homology with two expressed $V_\lambda2$ genes (WLR and PV6) than with any $V_\lambda2$ germline gene known at present (Fig. 5). The pattern of nucleotide differences in all three sequences is similar in the CDRs, and even more in the frameworks, suggesting that these three $V_\lambda2$ genes may originate from another, as yet unidentified $V_\lambda2$ germline gene. A similar observation has been made for the expressed K14 $V_\kappa1$ gene and two other expressed $V_\kappa1$ genes (A20 and HGQ) (Fig. 5).

Extensive computer analysis was performed on the V region nucleotides as well as the deduced primary amino acid sequences of the human MAbs presented in this article. Comparisons were also made with the sequences of all HIV-1-specific human antibody V regions known to data[27,32a,34,52] and

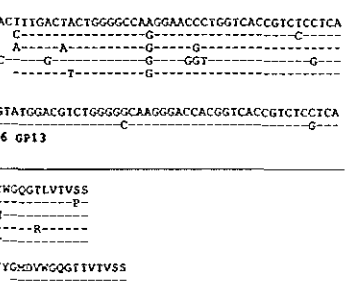

FIG. 7. (A) Nucleotide sequences of the J_H segment of each HIV-1-specific human monoclonal antibody compared to the nucleotide sequence of the closest J_H germline gene.[48] (B) Comparison of the deduced amino acid sequences of the J_H segments as described in (A). Identities between sequences are indicated by dashes.

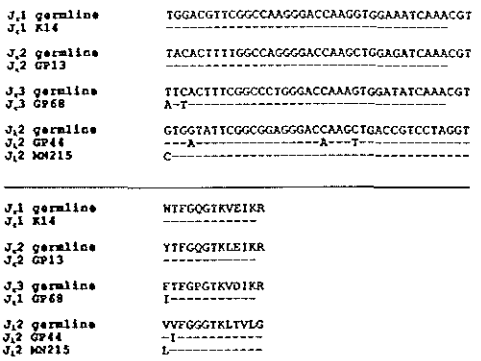

FIG. 8. (A) Nucleotide sequences of the J_L segment of each HIV-1-specific human monoclonal antibody compared to the nucleotide sequence of the closest J_L germline gene.[48] (B) Comparison of the deduced amino acid sequences of the J_L segments as described in (A). Identities between sequences are indicated by dashes.

with the sequences of the V regions of human Fab fragments from combinatorial libraries, recognizing either the CD4 b.s. or the V3 domain of HIV-1 gp120.[53] Also, the corresponding D segments were compared. However, information on antigen–antibody binding could not be obtained by these analyses. Instead of using linear amino acid sequences, three-dimensional modeling of antibody V regions may provide useful structural information.

To date only little information is available on the immunoglobulin variable region gene repertoire used by HIV-1-neutralizing human MAbs (see Refs. 27, 32a, 52, and this article). It was reported that in AIDS patients there is a clonal deficit of V_H3-expressing B cells.[21,22] Surprisingly, we found two V_H3-expressing HIV-1-specific human MAbs, MN215 and K14, obtained from two different HIV-1-seropositive donors. It is unlikely, that this could however, be due to the reported expansion of the V_H3 B cell pool in early clinical stages of HIV infection,[22] because MN215 and K14 were obtained from the donors 3–4 years after seroconversion. So far only one other V_H3-expressing HIV-1 gp41-specific human MAb has been reported.[34] A superantigen-like binding of gp120 to membrane immunoglobulin V_H3 gene products has been suggested.[22] Also serum V_H3 IgM from uninfected individuals was shown to bind to gp120. However, binding of gp120 to V_H3 IgG from uninfected individuals was significantly lower.[22] MN215 and K14 are both V_H3 IgG antibodies, and therefore we suggest a high affinity in binding of MN215 and K14 to their respective specific antigenic sites on HIV-1. This in contrast with a lower binding affinity of gp120 as a superantigen for the immunoglobulin V_H3 gene products MN215 and K14. This may provide an explanation for the presence of V_H3-expressing B cells in later stages of HIV infection. Additional V region nucleotide sequences of HIV-neutralizing human MAbs are necessary to obtain significant structural information which will eventually lead to a better understanding of HIV neutralization by antibody–antigen interactions. Furthermore, potential clinical benefits may be associated with understanding the extent of the V gene repertoire directed against specific HIV antigens presenton infection.[32a] In the near future broadly HIV-neutralizing engineered antibody preparations may play an important role in passive immunization or as therapeutic agents.

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CHAPTER 5

Enhancement of infectivity of a non-syncytium inducing HIV-1 by sCD4 and by human antibodies that neutralize syncytium inducing HIV-1.

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Enhancement of Infectivity of a Non-Syncytium Inducing HIV-1 by sCD4 and by Human Antibodies that Neutralize Syncytium Inducing HIV-1

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Schutten M, Andeweg AC, Bosch ML, Osterhaus ADME. Enhancement of Infectivity of a Non-Synctium-Inducing HIV-1 by SCD4 and by Human Antibodies that Neutralize Syncytium-Inducing HIV-1. Scand J Immunol 1995;41:18–22

Enhancement of virus infectivity after sCD4 treatment has been documented for SIVagm and HIV-2. It has been suggested that a similar phenomenon may play a role in HIV-1 infection. In the present study we have analysed biological activities of virus neutralizing polyclonal and monoclonal human antibodies and of sCD4, towards HIV-1 chimeras with envelope proteins derived from one donor, which display different biological phenotypes. The antibodies, which recognize the V3 and/or the CD4 binding domains of the glycoproteins of these viruses and also sCD4 showed different levels of virus neutralizing activity toward the syncytium inducing HIV-1 strains. In contrast, they all dramatically enhanced the infectivity of an HIV-1 chimera with an envelope glycoprotein displaying the non-syncytium-inducing phenotype. Given the relatively conserved nature of non-syncytium-inducing HIV-1 surface glycoproteins early after infection, these data suggest a major role for antibody mediated enhancement of virus infectivity in the early pathogenesis of HIV-1 infection.

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INTRODUCTION

The identification and characterization of virus neutralization inducing (VNI) antigenic sites on the glycoproteins of HIV-1 have been the focus of many studies (for review see [1]). For practical reasons, HIV-1 strains and primary HIV-1 isolates which efficiently replicate in T cells have predominantly been used to study virus neutralization [1]. In this context it is important to note that macrophage-tropic non-syncytium-inducing (NSI) variants with low efficiency of replication in T cells predominate in the asymptomatic stage and T cell line-tropic syncytium inducing (SI) variants are almost exclusively found in late stages of the infection [2, 3]. The macrophage-tropic NSI strains have been shown to exhibit a relatively high level of conservation in their VNI V3 loop [4]. It has been suggested that escape from virus neutralizing (VN) antibodies is based on the relatively high mutation rate generally observed in the virus envelope glycoprotein. Given the fact that also the CD4 binding domain (bd) is relatively conserved, another mechanism of escape from VN antibodies may be postulated for the relative success of NSI/macrophage-tropic viruses to persist in the host. To study the basis of this apparent paradox, we have used an *env* complementation assay in which the biological activities could be compared of human HIV-1-specific monoclonal and polyclonal antibody preparations towards identical HIV-1 strains only differing in their glycoproteins which displayed an SI or an NSI phenotype.

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MATERIALS AND METHODS

Human antibodies, sCD4 and HIV-1 envelope glycoproteins. The human monoclonal antibodies (HuMoAbs) used are directed against the CD4 binding domain (GP13 and GP68 [5]), a highly conserved conformational epitope on gp41 (K14 [6]) and the HIV-1 gp120 V3 loop (257-D [7]). Furthermore sCD4 (kindly provided by Dr I. Jones through the MRC AIDS directed programme) and HIVIG [8]) (kindly provided by Dr H. Schuitemaker) were used as VN agents. The *env* genes used [16.1 (SI), 16.2 (SI), 16.4 (NSI), MN and IIIB] for

the production of envelope glycoproteins in the capture antibody ELISA and for the production of chimeric viruses have previously been characterized and described [9, 10–12].

Capture antibody ELISA for measurement of relative affinity. The relative affinity of the HuMoAbs used in the present study was determined in a previously described ELISA. In short, ELISA plates (Costar, Cambridge, MA 02140, USA, cat. no. 3590), coated with affinity purified sheep immunoglobulins directed against the carboxy terminus of gp120 (Aalto BioReagents, Dublin, Ireland, cat. no. D7324), were incubated for 2 h with cell lysate of recombinant vaccinia virus (rVV) infected HELA [13] cells (16 (NSI) and 16 (SI)) or HIV-1 infected SupT1 [14] cells (MN [11] and IIIB [12]). Plates were subsequently washed and incubated with serial dilutions of previously described HuMoAbs (GP13, GP68 [5] and 257-D [7]). The ELISA was completed and read as previously described [10]. The relative amount of antigen bound to the ELISA plates was calibrated by using the same ELISA protocol with serial dilutions of the antigen and a standard excess concentration of polyclonal serum from seropositive individuals. The data presented are the mean values of duplicates in one assay which was performed at least twice with similar results.

Envelope trans-complementation assay. This assay was based on a previously described *trans*-complementation assay in which an *env*-defective HIV-1 provirus, encoding the bacterial CAT reporter gene [15], was complemented for a single round of replication by a set of recently described HIV-1 envelope glycoproteins [9]. The *env* genes encoding these glycoproteins were derived from biologically cloned SI or NSI viruses, isolated from one single donor (number 16) at a single point in time in natural target cells (peripheral blood mononuclear cells [PBMC]). In the complementation assay the genes encoding these envelope glycoproteins were co-transfected into a $CD4^-$ cell line (COS [16]) together with the *env*-defective molecular clone of the HIV-1 IIIB strain. This resulted in the production of virions carrying heterologous envelope glycoproteins which could infect $CD4^+$ cells, like the SupT1 cells or PHA stimulated PBMC used in this study. After 3 days cell free supernatants were collected by centrifugation and frozen at −135°C. For infection the amount of p24 antigen, as determined with a p24 ELISA kit (V5, Organon Teknika, Boxtel, The Netherlands) was calibrated and the level of envelope expression was checked with the capture antibody ELISA (see above). Infection was performed overnight at 37°C in 1 ml medium. After 24 h the cells were washed and cultured for 72 h. CAT activity in these cells was measured in a previously described CAT assay using a two-phase extraction system [17]. CAT activities expressed in the cells were considered a direct measure of virus infectivity in these assays.

Table 1. Relative affinities of HuMoAbs for different HIV-1 envelope glycoproteins

		HIV-1 strain (phenotype)				
HuMoAb	Specificity	16.1 (SI)	16.2 (SI)	16.4 (NSI)	MN (SI)	IIIB (SI)
K14	gp41	83*	67	67	33	67
GP13	CD4 bd	83	83	33	250	250
GP68	CD4 bd	67	67	67	125	250
257-D	V3 domain	125	125	125	125	–†

* Reciprocal of dilutions of MoAbs, starting at 1 μg/ml, still giving 50% of maximal binding with the respective envelope glycoproteins in a capture antibody ELISA as a measure of relative affinity. The data presented are the mean values of duplicates in one assay which was performed at least twice with similar results.

† No binding: OD450 nm values remained at background levels at the concentrations tested.

RESULTS

Relative affinity of HuMoAbs for HIV-1 envelope glycoproteins

The HuMoAbs used in these experiments were selected on basis of their epitope specificities and relative affinities in the capture antibody ELISA for different HIV-1 envelope glycoproteins (Table 1). The same recombinant envelope glycoproteins were used in the *env* complementation assay (Table 2). Both CD4 bd specific HuMoAbs GP13 and GP68 showed an overall higher relative affinity for the glycoproteins of the T cell line adapted HIV-1 strains IIIB and MN than for the glycoproteins derived from the donor number 16 HIV-1 strains. The V3 loop specific HuMoAb 257-D showed a high relative affinity for the glycoproteins of the MN-like HIV-1 strains and no binding was observed in this ELISA with the glycoproteins of HIV-1 IIIB (Table 1).

Virus neutralization and enhancement of viral infectivity by sCD4 and human antibodies

The VN activities of human antibodies and sCD4 toward T cell line adapted strains HIV-1 MN and HIV-1 IIIB (Table 2) have been documented previously [10–13]. The CD4 bd specific HuMoAbs GP13 and GP68 as well as HIVIG and sCD4, inhibited infectivity of HIV-1 16.2 (SI) only to a limited extent (< 50%) when tested at a concentration (15 μg/ml) that significantly inhibited infectivity of HIV-1 IIIB in the *env* complementation assay (reduction to 15%, 12%, 9% and 8% respectively) (Table 2). The V3 domain specific HuMoAb tested at the same concentration, neutralized HIV-1 16.2 (SI), as was shown by a reduction of CAT activities to 0.7% (Table 2). No major biological activity of any of the antibodies tested or sCD4, was observed against HIV-1 16.1 (SI) as was shown by a reduction of CAT activity with 2–9% only, using 15 μg/ml antibody (Table 2).

In contrast to the VN activities observed with the SI viruses, the same concentration of all these VN antibodies and sCD4 caused a dramatic enhancement of infectivity of HIV-1 16.4 which has a NSI envelope (> six-fold increase in CAT activity) (Table 2). In order to demonstrate the dose dependency of these phenomena, dose response curves were made with the V3 domain specific HuMoAb 257-D, which exhibited the highest affinity for all the envelop proteins

Table 2. Virus neutralizing and enhancement activities of human antibody preparations and sCD4

Antibody preparation	Epitope	Virus strain (biological phenotype) MN* (SI)	IIIB (SI)	IIIB (SI)	16.1 (SI)	16.2 (SI)	16.4 (NSI)
none		...‡	—	100§	100	100	100
				72.461*	55.065	54.428	8.239
K14	gp41	—	—	103	98	106	96
GP13	CD4 bd	+	+	15	97	65	732
GP68	CD4 bd	+	+	12	93	65	741
sCD4	CD4 bd	+	+	9	92	84	620
257-D	V3	+	—	97	91	0.7	655
HIVIG	poly	+	+	8	95	95	744
		VN assay		*env* complementation assay			

‡ Due to differences in the VN assays used, biological activities are indicated as high (+) or undetectable (−).

§ Percentage of CAT activity relative to the CAT activity observed in the absence of antibody. The mean value within one experiment obtained with SupT1 cells is given. The antibody preparations were tested at least twice with the same chimeric virus yielding similar results.

* CAT activity in counts per minute (cpm), background values range from 300 to 800 cpm.

tested (Table 1). Clear dose related VN and enhancement activities were found with this antibody in the *env* complementation assay with HIV-1 16.2 (SI) and HIV-1 16,4 (NSI) respectively (Fig. 1). When these experiments were repeated in PHA stimulated PBMC essentially the same phenomena were observed. The overall differences were however less pronounced in this system (average enhancement about 50%) probably due to a limited cellular host range of this virus (A. C. Andeweg *et al*, unpublished observations).

DISCUSSION

Using an assay that is highly sensitive for measuring viral entry we have shown in the present study that both sCD4 and human antibodies which neutralize SI HIV-1 strains may enhance infectivity of NSI HIV-1 strains.

HIVIG, sCD4 and the CD4 bd specific HuMoAbs GP13 and GP68 readily neutralized the HIV-1 IIIB strain whereas limited or no VN activity was observed against the primary HIV-1 strains from donor number 16. This supports previous observations that sCD4, CD4 bd specific HuMoAbs and HIVIG have little or no neutralizing activity against primary virus strains (M. Schutten, personal observation and [18]). It has been shown by several groups that VN capacity of V3 loop specific MoAbs toward T cell line adapted HIV-1 strains is dependent on the affinity of the antibody for the V3 loop of the HIV-1 strain used [7, 19]. However, in the present study we showed that VN activity of V3 loop specific HuMoAb 257-D toward the SI HIV-1 strains from donor number 16 is not directly related to its relative affinity for the glycoproteins involved: identical relative affinities for the 16.1 (SI) and 16.2 (SI) envelope glycoproteins were observed, but reductions in the *env* complementation assay proved to be 9 and 99.3% respectively. This was unexpected since both envelopes exhibit SI capacity and a 95% overall predicted amino acid sequence homology exists between these envelopes [9]. Since we have previously shown that other domains than the V3 domain are involved in membrane fusion [10], it may be speculated that HIV-1 16.1 is less dependent on the V3 domain to establish membrane fusion and infection.

The efficiency of entry into T cell lines of the NSI HIV-1 strain 16.4 was significantly lower than that of the SI HIV-1 strains 16.1 and 16.2 as shown by a > six-fold lower CAT activity. The infectivity of the NSI HIV-1 strain was however enhanced to levels comparable to those of the SI HIV-1 strains by the administration of sCD4 or antibodies that neutralized these SI HIV-1 strains. Different mechanisms for HIV-1 specific antibody mediated enhancement have been described, including Fc receptor- and complement-mediated enhancement [20, 21]. Two observations argue against the same mechanism in our experiments. Firstly, SupT1 cells do not express Fc receptors [14] and complement was not present in the assays. Secondly, enhancement of HIV-1 16.4 (NSI) was also observed with sCD4 (Table 2). An explanation for the observed enhancement can be found in a mechanism previously described for the enhancement of non-cytopathic SIV agm infectivity and induction of HIV-2 mediated cell fusion of $CD4^-$ cells, by sCD4. In these systems sCD4 does not induce dissociation of surface glycoprotein (SU) from infected cells [22, 23]. In contrast, sCD4 does

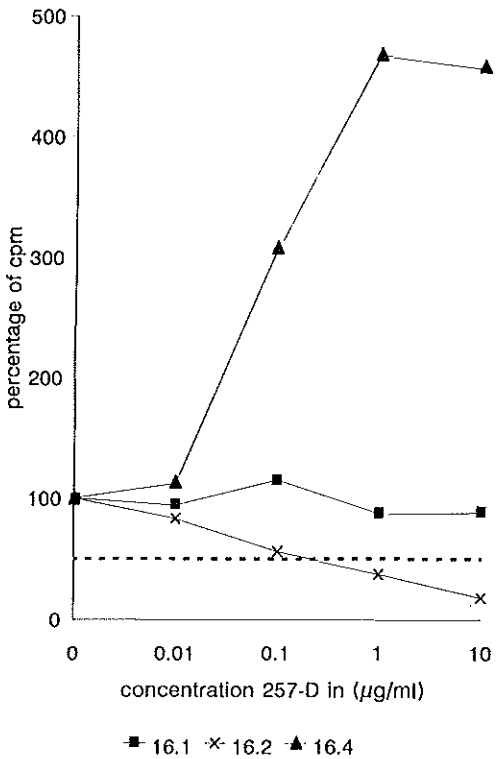

Fig. 1. Virus neutralizing and infectivity enhancing activities of serial dilutions of HuMoAb 257-D using SupT1 cells in the *env* complementation assay with HIV-1 16.1 (SI), HIV-1 16.2 (SI) and HIV-1 16.4 (NSI) chimeric viruses. Counts per min (cpm) obtained in the assays with antibodies, are expressed as percentages of cpm obtained in the assay carried out in the absence of antibodies. Dotted line represents 50% inhibition of virus infectivity.

induce dissociation of SU from cells infected with T cell line adapted HIV-1 strains which are readily neutralized by sCD4 [24]. We hypothesize that both sCD4 and the antibodies we studied, cause conformational changes in the viral envelope complex of certain NSI/macrophage-tropic HIV-1 strains exposing cryptic fusion sites as was also shown in the SIVagm and HIV-2 systems [22, 23]. These changes would allow a more efficient virus-cell fusion that is less dependent on the presence of CD4 on the target cell membrane.

Taken together our data suggest that naturally occurring antibodies to the so-called HIV-1 VNI antigenic sites, may cause either neutralization or enhancement dependent on the biological phenotype of the virus recognized. If indeed our findings are a reflection of a more general phenomenon of enhancement also found in infections with other lentiviruses like HIV-2 [23] and SIVagm [22], they may explain why early after HIV-1 infection predominantly viruses with an NSI/macrophage-tropic phenotype are found and why these viruses are able to persist in the infected host despite the conserved nature of their 'VNI' antigenic sites. The observed enhancement would actually favour replication of viruses displaying the NSI/macrophage-tropic phenotype, whereas viruses displaying the SI/T cell line-tropic phenotype are neutralized.

These observations may not only have implications for our understanding of the pathogenesis of HIV-1 infection but also for the development of immunization strategies. If this observation is the reflection of a more general phenomenon, the induction or administration of antibodies that neutralize viruses of the SI/T cell-tropic phenotype, which is a commonly followed strategy at present, may actually be counter productive.

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CHAPTER 6

Modulation of primary HIV-1 envelope glycoprotein mediated entry by human antibodies.

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Modulation of primary HIV-1 envelope glycoprotein mediated entry by human antibodies

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Recently we and others have shown that the interaction between envelope specific antibodies and primary HIV-1 isolates may result in either inhibition or enhancement of viral entry. The outcome proved to be determined by the virus isolate rather than by the specificity of the antiserum used. To study the mechanism underlying this phenomenon, a series of HIV-1 envelope glycoproteins from closely related primary virus isolates of different syncytium inducing phenotypes and chimeras of these proteins, were tested in an envelope trans-complementation assay for their sensitivity to either antibody mediated inhibition or enhancement of HIV-1 entry.

Based on the observation that, in contrast to the inhibition of HIV-1 entry, antibody mediated enhancement was not temperature dependent and could not be mediated by F(ab) fragments, we concluded that the mechanisms underlying both phenomena are essentially different and that antibody mediated enhancement of HIV-1 entry is largely if not exclusively mediated by HIV-1 glycoprotein cross-linking. The susceptibility of the envelope glycoprotein chimeric viruses to neutralization or enhancement of infectivity proved to be primarily determined by the configuration of the V3 loop and the affinity of the antibodies to monomeric HIV-1 gp160 molecules, proved to be of quantitative importance only.

One human monoclonal antibody directed against gp41 (IAM 2F5), inhibited entry of all the viruses studied, irrespective of their phenotype, and directly proportional to its affinity to monomeric HIV-1 gp160.

INTRODUCTION

The HIV-1 envelope glycoprotein complex of T cell line adapted (TCLA) HIV-1 strains has been shown to mediate virus entry by a pH-independent mechanism, which is initiated by a high affinity interaction between the surface glycoprotein (SU, gp120) and the cellular receptor CD4 (Stein *et al.*, 1987). Apart from this high affinity interaction, a lower affinity interaction with a second receptor is required for HIV-1 entry (Maddon *et al.*, 1986). These interactions of the HIV-1 glycoproteins with cell surface molecules lead to conformational changes within the HIV-1

glycoprotein complex and shedding of gp120, which eventually results in the fusion of viral and cellular membranes (Allan, 1991; Moore *et al.*, 1991). Both soluble CD4 (sCD4) and antibodies directed against the envelope glycoproteins may interfere with this process of virus entry in several ways. They may block the interaction between gp120 and the cell surface molecules, induce conformational changes which disable the envelope glycoprotein complex to exert further functions, or induce premature shedding of gp120 (Clapham *et al.*, 1989; Moore *et al.*, 1990; Orloff *et al.*, 1993; Poignard *et al.*, 1996).

It has become clear that the glycoprotein complex of HIV-1 isolates that have only been propagated in peripheral blood mononuclear cell (PBMC) cultures differs from that of TCLA HIV-1 strains in several respects. The so-called primary HIV-1 isolates are less susceptible to virus neutralization by sCD4 and antibodies directed to the V3 loop or the CD4 binding domain (CD4 bd) (Moore *et al.*, 1995; Schutten *et al.*, 1996). Furthermore, as was also shown for HIV-2 and SIVagm, primary HIV-1 isolates do not readily shed gp120 upon binding to sCD4 (Allan, 1991; Moore *et al.*, 1992). We and others have recently shown that sCD4 may enhance primary HIV-1 envelope glycoprotein mediated entry (Schutten *et al.*, 1995a; Sullivan *et al.*, 1995), a phenomenon that has also been described for HIV-2 and SIVagm strains (Clapham *et al.*, 1992; Werner *et al.*, 1990). This enhancement of HIV-1 envelope glycoprotein mediated entry is also mediated by polyclonal HIV-1 specific antibody (HIVIG) and by HIV-1 gp120 specific human monoclonal antibodies (HuMAb's) directed against either the V3 loop or the CD4 bd. It may be speculated, that this antibody mediated enhancement (AME), is a more common strategy of these closely related lentiviruses, to escape from - or even take advantage of - virus neutralizing antibodies (Moore *et al.*, 1991, Kostrikis *et al.*, 1996). Why such a common strategy has not been identified previously, may be due to a selection against AME sensitive viruses, in HIV-1 isolation procedures. The use of mitogen stimulated PBMC in the absence of virus specific antibodies, may be expected to favour the isolation of T cell tropic viruses (Innocenti-Francillard *et al.*, 1994).

In the present paper we present results of studies into the mechanism underlying AME, which phenomenon is essentially different from previously described Fc receptor and complement receptor mediated antibody dependent enhancement mechanisms of HIV-1 infectivity (Eaton *et al.*, 1994; Robinson, Jr. *et al.*, 1990; Schutten *et al.*, 1995a). To this end a series of HIV-1 envelope glycoproteins from closely related primary HIV-1 biological clones of different *in vitro* biological phenotypes and chimeras of these proteins, were tested in an envelope trans-complementation assay (ETCA) for their sensitivity to either antibody mediated inhibition of HIV-1 entry (IE) or AME.

METHODS

Human antibodies and HIV-1 envelope glycoproteins. The antibodies used in this study are the CD4 bd specific HuMAb's GP13 (Schutten *et al.*, 1993) and IgG1-b12 (Burton *et al.*, 1994), the combinatorial phage display library F(ab) fragment FAB12 (Roben *et al.*, 1994) which was used to generate IgG1-b12, V3 loop specific HuMAb's 257-D (Gorny *et al.*, 1993) and MN215 (Schutten *et al.*, 1995b), HuMAb K14 directed against a highly conserved conformational epitope on gp41 (Teeuwsen *et al.*, 1990) and IAM 2F5 which is specific for a relatively conserved epitope on gp41 (ELDKWAS) (Muster *et al.*, 1993). The use of the TCLA HIV-1 envelope gene derived from the HIV-1 IIIB isolate (HXB2-D) in the envelope trans-complementation assay has previously been described (Helseth *et al.*, 1990). The previously described (Andeweg *et al.*, 1992; Groenink *et al.*, 1992) HIV-1 *env* genes derived from primary biological HIV-1 clones from the donors #16 and #320 from the Amsterdam cohort of seropositive individuals designated 16.1 (SI), 16.2 (SI), 16.4 (NSI), 320.2A.6 (NSI) and 320.2A.7 (SI) were used for the production of viruses expressing the respective envelope glycoproteins. The *env* gene from the HIV-1 biological clone 168.10 (SI) had been

generated using a similar protocol (de Jong *et al.*, 1992; Schuitemaker *et al.*, 1993). The generation and characterization of the SI/NSI envelope chimeras have been described in more detail by Andeweg *et al.* (Andeweg *et al.*, 1993).

Affinity of the HuMAb's used for the monomeric recombinant glycoproteins. The affinity of the HuMAb's for the monomeric form of the envelope glycoproteins expressed by the chimeric HIV-1 virions used in the env-complementation assay, was determined in a previously described capture antibody ELISA (D7324, Aalto Bioreagents Ltd., Dublin, Ireland) (Andeweg *et al.*, 1993; Moore, 1990). In short, the primary envelope glycoproteins were expressed in COS cells (Gluzman, 1981) using a vaccinia expression system. Vaccinia infected cells were lysed with 1% Empigen and the amount of HIV-1 glycoprotein in the cell lysate was estimated using the capture antibody ELISA. Subsequently equal amounts of HIV-1 glycoprotein were coated and the concentration of HuMAb was determined where half maximum binding was reached in the capture antibody ELISA. This concentration was taken as relative affinity of the HuMAb for the HIV-1 envelope glycoprotein tested.
Despite the pronounced amino acid variation in the hyper variable regions of the envelope glycoproteins, all HuMAb's showed a similar relatively high affinity for the envelope glycoproteins ((Schutten *et al.*, 1995b) + unpublished results). HuMAb IAM 2F5 (gp41) however, showed low affinity for the 16.4 (NSI) envelope glycoprotein and HuMAb MN215 (V3 loop) showed low affinity for all SI envelope glycoproteins (Schutten *et al.*, 1995b). The low affinity of HuMAb IAM-2F5 for the #16.4 (NSI) envelope glycoprotein may be explained by the lysine to threonine change in the 16.4 (NSI) amino acid sequence (ELDTWAS) (Andeweg *et al.*, 1992) relative to the optimal binding sequence for IAM 2F5 (ELDKWAS) (Muster *et al.*, 1993).

Envelope trans-complementation assay (ETCA). The ETCA was based on a previously described assay in which an envelope gene defective HIV-1 provirus, encoding the bacterial CAT reporter gene was complemented for a single round of replication by the set of HIV-1 envelope genes (Helseth *et al.*, 1990). In the ETCA the plasmids encoding the envelope glycoproteins were co-transfected by electroporation into a CD4$^-$cell line (COS) (Gluzman, 1981) together with the envelope gene defective molecular clone of the HIV-1 IIIB strain. This resulted in the production of virions carrying heterologous envelope glycoproteins which may infect CD4$^+$ cells. Cell free supernatants were collected after three days of culture by centrifugation and frozen at -135°C. The amount of virus particles used for infection was adjusted by calibrating the amount of p24 antigen, as determined with a p24 ELISA kit (V5, Organon Teknika, Boxtel, The Netherlands) to a standard amount of p24. The level of envelope expression was checked with the capture antibody ELISA described above. For infection the Sup T1 T cell line (Smith *et al.*, 1984) and three days PHA (PHA-M, Boerhinger Mannheim, Mannheim, Germany; 15 µg/ml) prestimulated PBMC were used. Infection of SupT1 cells (2x10^6/ml) and PHA prestimulated PBMC (5x10^6/ml) was routinely performed overnight at 37°C in 1 ml RPMI 1640 (GIBCO BRL, Life Technologies BV, Breda, The Netherlands) with 10% fetal calf serum, penicillin and streptomycin in the absence or presence of 15 µg HuMAb or 30 µg FAB12. To determine the temperature sensitivity of IE and AME, infection was performed at 4°C, 20°C and 37°C for five hours. After infection the cells were washed and cultured for another 72 hours. CAT activity, measured in cpm, was determined in a previously described CAT assay using a two phase extraction system (Seed & Sheen, 1988). CAT activities expressed in the cells were considered a direct measure of the capacity of the envelope gene product to mediate virus entry. The mean value of duplicates within one experiment is given. Each antibody virus combination was tested at least in two separate experiments yielding similar results.

RESULTS

Antibody mediated inhibition and enhancement of entry using a set of chimeric viruses carrying primary HIV-1 glycoproteins.

The efficiency of HIV-1 envelope glycoprotein mediated entry into the SupT1 T cell line and the capacity of the respective HuMAb's at a given concentration (15 µg/ml) to inhibit or enhance the process of entry mediated by these envelope glycoproteins, was determined in the ETCA (Figure 1). The capacity of the HuMAb's to inhibit entry of HXB2-D envelope complemented virus was proportional to their previously reported capacity to neutralize the HIV-1 strain IIIB (Burton *et al.*,

1994; Gorny *et al.*, 1993; Muster *et al.*, 1993; Schutten *et al.*, 1993; Teeuwsen *et al.*, 1990).

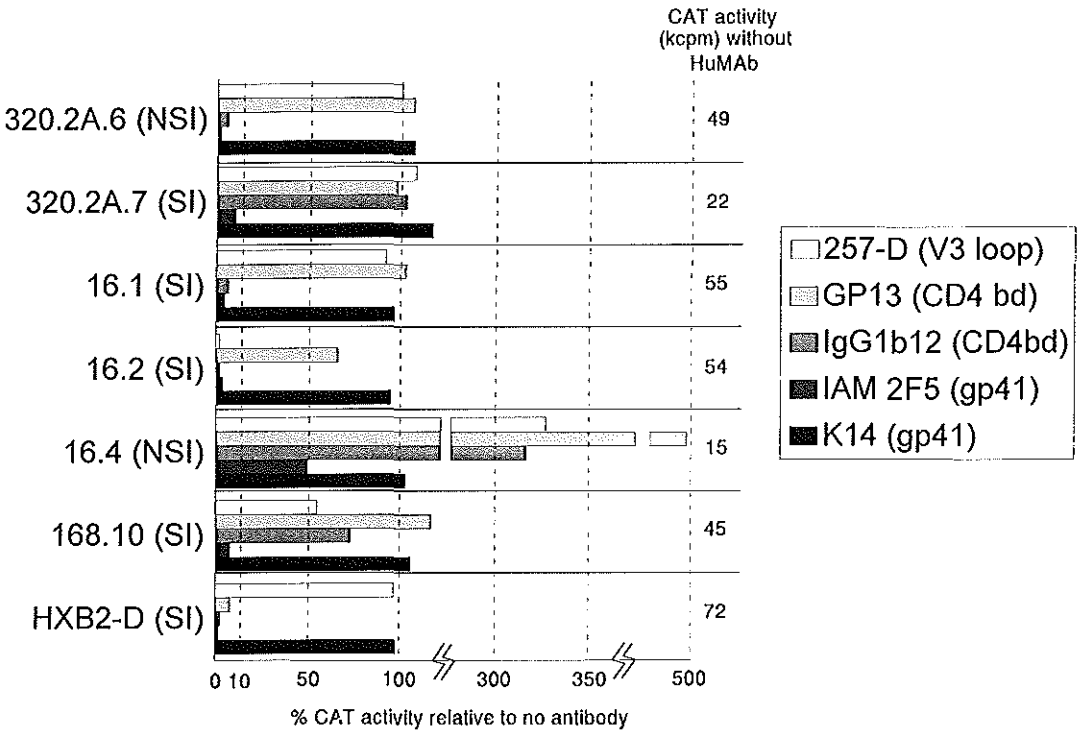

Fig. 1. Efficiency of entry into Sup T1 cells of primary and TCLA HIV-1 glycoprotein complemented chimeric virus in the absence and presence of HIV-1 envelope glycoprotein specific HuMAb's determined in the ETCA. On the X-axis IE or AME mediated by HIV-1 envelope glycoprotein specific HuMAb's are given as the percentages of CAT activity obtained with 15 µg/ml of HuMAb relative to CAT activity obtained with the same HIV-1 envelope glycoprotein complemented virus without HuMAb. On the right CAT activities obtained with the chimeric viruses without using HuMAb are presented.

HuMAb K14, which does not neutralize TCLA viruses in *in vitro* assays, did not influence entry mediated by any of the primary envelope glycoprotein complemented viruses. The V3 loop specific HuMAb 257-D efficiently inhibited entry (inhibition of entry >90%) of one of the six chimeric viruses, which expressed the envelope glycoprotein derived from primary HIV-1 strain 16.2 (SI), and enhanced envelope 16.4 (NSI) mediated entry (enhancement of entry >200%). The CD4 bd specific HuMAb GP13 inhibited envelope 16.2 (SI) mediated entry about 50% and enhanced envelope 16.4 (NSI) mediated entry about fivefold. IgG1-b12, which is also directed against the CD4 bd, inhibited entry of the envelope 320.2A.6 (NSI), 16.1 (SI) and 16.2 (SI) complemented viruses and enhanced envelope 16.4 (NSI) mediated entry about threefold. HuMAb IAM 2F5 directed against the proximal putative alpha helix of gp41, inhibited entry into Sup T1 cells of all the chimeric viruses more than 90% with the exception of envelope 16.4 (NSI) chimeric virus to which it displayed a much lower affinity. Entry of this chimeric virus was reduced about 50% by HuMAb IAM 2F5.

Inhibition and enhancement of viral entry in relation to HIV-1 envelope glycoprotein cross-linking by antibodies.

IgG1-b12 and the F(ab) fragments (FAB12), which had been used to generate IgG1-b12, were used to evaluate whether IE and AME of chimeric 16.2 (SI) and 16.4 (NSI) viruses respectively, required cross-linking. IgG1-b12 and its F(ab) fragments were selected since HuMAb IgG1-b12 showed broad IE activity against the primary HIV-1 envelope glycoprotein complemented viruses (Figure 2). The level of IE of the envelope 16.2 (SI) chimeric virus mediated by FAB12 and IgG1-b12 were in the same order of magnitude. However, enhancement of envelope 16.4 (NSI) mediated entry was only observed with IgG1-b12, whereas the F(ab) fragments of IgG1-b12 failed to enhance entry mediated by this envelope glycoprotein. Since IgG1-b12 does not bind to Sup T1 cells in FACS analysis, which is consistent with the lack of Fc receptors on SupT1, the lack of AME by FAB12 could not be attributed to the mere absence of the Fc portion of the antibody.

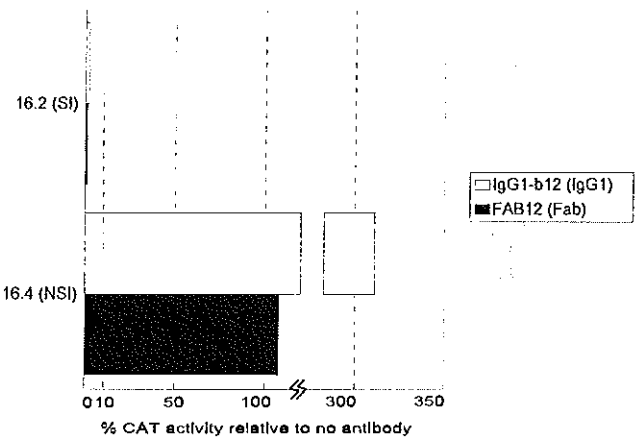

Fig. 2. Efficiency of entry into Sup T1 cells of chimeric viruses 16.2 (SI) and 16.4 (NSI) in the presence or absence of HuMAb IgG1-b12 and it's F(ab) fragment FAB12. On the X-axis IE or AME are given as the percentages of CAT activity obtained with 15 µg/ml HuMAb and 30 µg/ml of F(ab) relative to CAT activity with the same chimeric virus obtained without using HuMAb or F(ab).

Antibody mediated inhibition and enhancement of entry at different temperatures.

HuMAb 257-D was selected to study the effects of different temperatures on IE and AME, since this antibody recognizes a linear epitope (IHIGPG) that is most probably less dependent on the conformation of the envelope glycoprotein (Gorny *et al.*, 1993). Furthermore, probably due to the high affinity of HuMAb 257-D for the recombinant glycoproteins derived from donor #16, HuMAb 257-D completely blocked entry of envelope 16.2 (SI) chimeric virus. AME of envelope 16.4 (NSI) chimeric virus mediated by HuMAb 257-D typically resulted in levels of CAT activity which were similar to those found for envelope 16.2 (SI) chimeric virus when tested in the absence of HuMAB (Figure 1). At decreasing temperatures CAT activity levels declined for envelope 16.2 (SI) and envelope 16.4 (NSI) complemented viruses (Figure 3). This was probably due to the lower efficiency of entry at lower temperatures although limited cell death upon five hours incubation at lower temperature was also observed. The decrease in maximum CAT activities at

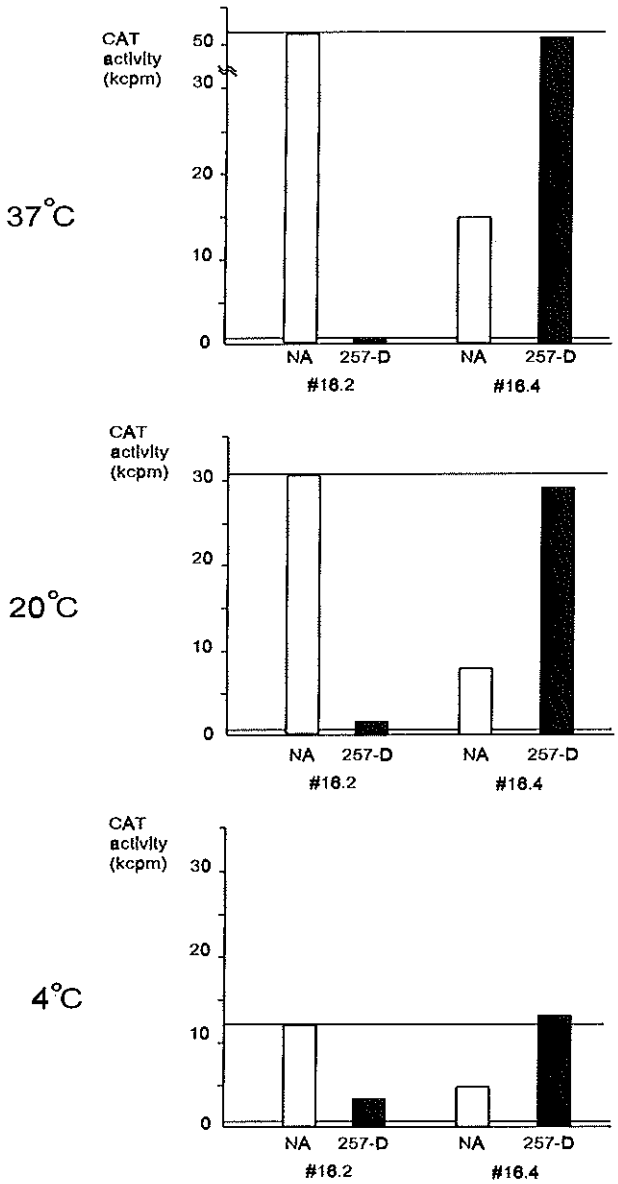

Fig. 3. Efficiency of entry into Sup T1 cells of chimeric viruses 16.2 (SI) and 16.4 (NSI) at different temperatures in the presence or absence of HuMAb 257-D. On the X-axis IE or AME are given as the CAT activities obtained with 15 µg/ml HuMAb 257-D. Infection was carried out at 37°C, 20°C and 4°C, respectively. CAT activity values in kcpm are presented on the X-axis. NA: no antibody.

lower temperatures obscures the efficiency of inhibition and enhancement of entry if these are given as ratio's. In figure 3, in contrast to figures 1,2 and 4, actual CAT activity values are therefore given. To determine the efficiency of inhibition and enhancement of entry by HuMAb 257-D, CAT activity values obtained in the presence of HuMAb 257-D at a given temperature are compared with the minimum and maximum CAT activity values respectively at that given

temperature. IE and AME mediated by HuMAb 257-D with envelope 16.2 (SI) and envelope 16.4 (NSI) chimeric viruses, at decreasing temperatures were tested in parallel (Figure 3). The level of IE of envelope 16.2 (SI) chimeric virus proved to have decreased significantly at 20°C (CAT activity about two times background) and even more at 4°C (CAT activity about five times background) when compared to the complete block of entry at 37°C (background CAT activity). In contrast, the level of AME of envelope 16.4 (NSI) chimeric virus proved not to be affected by the lower temperatures: The CAT activity values obtained with envelope 16.4 (NSI) chimeric virus at different temperatures in the presence of HuMAb 257-D were virtually identical to the values obtained with envelope 16.2 (SI) chimeric virus at the same temperature in the absence of antibody. Collectively these results show that in contrast to the level of IE, the level of AME is not significantly influenced by decreasing temperatures.

Antibody mediated inhibition and enhancement of entry in a set viruses carrying hybrid SI/NSI constructs.

Using a series of HIV-1 envelope glycoprotein with different biological phenotypes, originating from one patient at one time point, and hybrids generated from these proteins, an ETCA was carried out with a V3 loop specific (MN215) and a CD4 bd specific (GP13) HuMAb in Sup T1 cells (Figure 4). The ratio of the CAT activity measured in Sup T1 cells versus the CAT activity measured in PHA stimulated PBMC was taken as a measure of the relative efficiency of entry of the respective chimeric viruses into Sup T1 cells.

The sensitivity of envelope 16.2 (SI) to IE was transferred to envelope 16.1 (SI) and envelope 16.4 (NSI) proteins by the transfer of the envelope 16.2 (SI) aa 1-610 fragment (12AX and 42AX). These two constructs mediated efficient entry into Sup T1 cells, suggesting that both the efficiency of entry into Sup T1 cells and the sensitivity of the chimeric viruses to either IE or AME mediated by both HuMAb's is a trait of gp120. The transfer of the V3-V5 region of envelope 16.2 (SI) (StuI-AvrII, aa 211-610) to envelope 16.4 (NSI) (24SA), resulted in a hybrid envelope mediating relatively high efficiency of entry into SupT1. This exchange also changed the sensitivity of the envelope 16.4 (NSI) from AME sensitive to IE sensitive. We have previously shown that within the StuI-AvrII fragment of envelope 16.2 (SI), the V3 domain (aa 272-378, HindII-CvnI) alone proved to be sufficient to transfer the SI capacity of env 16.2 (SI) to a NSI background (Andeweg *et al.*, 1993). In line with this finding the env 16.2 (SI) based construct containing the 16.4 (NSI) V3 loop (42HC) showed a relatively low efficiency of entry into SupT1 and entry mediated by this construct envelope was still significantly enhanced by both HuMAb's. These data suggest that within the 16.2 (SI) and 16.4 (NSI) HIV-1 envelope glycoproteins the V3 loop may not only determine the efficiency of Sup T1 entry but also the sensitivity to IE or AME.

However, in contrast with this observation, exchange of the 16.4 (NSI) V3 domain for the 16.1 V3 domain (14HC) failed to significantly increase the efficiency of entry into SupT1, and also failed to change the AME phenotype of the envelope 16.4 (NSI) chimeric virus (Figure 4).

DISCUSSION

Naturally occurring changes within the HIV-1 *env* gene contribute to the *in vitro* biological characteristics used to describe the phenotype of HIV-1 strains. These biological characteristics include the syncytium inducing capacity, cellular host range, replicative capacity and the ability to induce single cell lysis. In the present paper we have shown that also the sensitivity of HIV-1

strains to IE or AME is determined by naturally occurring changes in envelope regions, which directly influence the SI capacity and efficiency of viral entry.

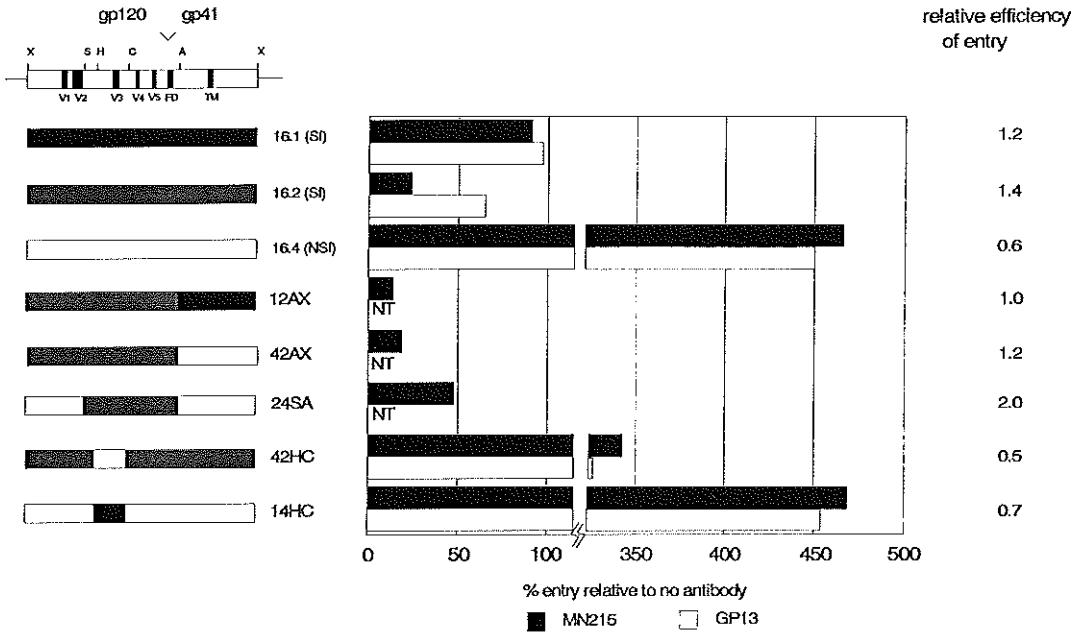

Fig. 4. Efficiency of entry into Sup T1 cells of a set of parental HIV-1 envelope glycoproteins (16.1, 16.2 and 16.4) and chimeric HIV-1 envelope glycoproteins derived from these SI and NSI HIV-1 envelope glycoproteins (12AX, 42AX, 24SA, 42HC and 14HC) complemented chimeric viruses in the absence or presence of HuMAb MN215 (V3 specific) and HuMAb GP13 (CD4 bd specific). The complete envelope gene is represented by a bar in which the variable domains (V1 to V5) and the fusion domain (FD) are indicated. The restriction enzymes used for the construction of the chimeric envelope are indicated (A=*AvrII*, C=*CvnI*, H=*HindII*, S=*StuI*, X=*XhoI*). On the X-axis IE or AME mediated by HIV-1 glycoprotein specific HuMAb's are given as the percentages of CAT activity obtained with 15 µg/ml of HuMAb relative to CAT activity obtained with the same HIV-1 envelope glycoprotein complemented virus without HuMAb. The relative efficiency of entry into Sup T1 cells is given on the right hand side of the plot as the ratio CAT activity found for the respective chimeric viruses with Sup T1 cells and PHA stimulated PBMC. N.T. not tested

HuMAb 257-D and HuMAb IgG1-b12 only inhibited entry mediated by a limited number of the HIV-1 envelope glycoproteins tested (one and three out of six, respectively) (Figure 1), although their affinities for the monomeric HIV-1 envelope glycoproteins used proved to be within the same range. The lack of biological activity of these HuMAb's towards the other viruses suggests that they either do not bind to the oligomeric HIV-1 glycoprotein complex of these viruses or do not exert biological activity after binding. The former explanation seems consistent with our previous FACS studies using vaccinia expressed HIV-1 envelope glycoproteins, which showed that VN inducing sites are less well presented by oligomeric primary HIV-1 glycoprotein complexes used in the present study than by their monomeric counterparts (Sattentau *et al.*, 1994). However, HuMAb's directed against these sites were shown by others to bind oligomeric HIV-1 glycoprotein complexes present on primary HIV-1 infected cells (Zolla-Pazner *et al.*, 1995). Indicating that the conformation of the HIV-1 glycoprotein complex present on HIV-1 virions may be essentially different from vaccinia expressed cell membrane associated envelope glycoproteins

(Q. Sattentau, personal communications). Therefore, lack of biological activity after binding may still be a more likely explanation. This may either be due to the absence of involvement of the antibody binding site of these viruses in the process of entry, or alternatively, binding of these antibodies does not induce the changes needed to inhibit entry of these viruses. The results of the ETCA experiments with the SI/NSI hybrid constructs, presented in this study, seem to favour the explanation that the binding sites are not involved in the process of entry of these viruses. It has recently been shown that NSI and SI HIV-1 strains use different second receptor molecules during envelope mediated entry (Deng *et al.*, 1996; Feng *et al.*, 1996). The CXCR-4 αchemokine receptor proved to be the second receptor for SI or T cell line adapted HIV-1 strains, whereas NSI/macrophage-tropic HIV-1 strains required expression of the β chemokine receptors CCR-2a, CCR-3 or CCR-5 for entry (Doranz *et al.*, 1996; Choe *et al.*, 1996). Despite the fact that T cells express different α and β chemokine receptors including CCR-5, NSI HIV-1 strains replicate usually less efficient in T cells as compared to SI HIV-1 strains (Schutten *et al.*, 1995a; Sullivan *et al.*, 1995). The differential requirements of these HIV-1 strains for second receptor molecules proved to be determined at least in part by the configuration of the V3 loop (Choe *et al.*, 1996). It may therefore be speculated that the inability of the HIV-1 envelope glycoproteins expressing the 16.4 (NSI) V3 loop to mediate entry via the CXCR-4, may be overcome by adding HIV-1 gp120 specific antibodies. It should be realized that the number of amino acid differences between these closely related proteins is limited (Andeweg *et al.*, 1992). Still despite the high degree of homology, in particular in those regions that apparently govern syncytium induction and entry, the HIV-1 chimeric viruses differ significantly in their sensitivities to IE or AME.

The temperature dependent nature of IE (Figure 3) suggests that the induction of conformational changes in the HIV-1 envelope glycoprotein complex after binding of the HuMAb's are essential for IE. This has also been suggested for the neutralization of TCLA HIV-1 strains by gp160 specific antibodies (Poignard *et al.*, 1996; Sattentau & Moore, 1991). The observation that HuMAb's with relatively low affinity to 16.4 (NSI) envelope glycoprotein induce levels of AME similar to those with high affinity (M. Schutten, unpublished observation), together with the apparent temperature independent nature of AME (Figure 3), led us to conclude that the changes induced by antibodies and sCD4, leading to AME, have a minimal energy requirement. Furthermore, IE and AME both proved to be dependent on the concentration of antibody or sCD4 (Schutten *et al.*, 1995a; Sullivan *et al.*, 1995): IE reached a plateau value at increasing concentrations whereas AME increased with concentration to an optimum level. Collectively these data indicate that the mechanisms which underly IE and AME are fundamentally different and that antibody mediated cross-linking is essential for AME. This was confirmed by the observation that FAB12 did not induce AME although its IE inducing capacity toward IE sensitive viruses was equal to that of IgG1-b12 (Figure 2). In this light it is interesting to note that sCD4 exhibits the same pattern of IE and AME with the respective chimeric viruses as the V3 domain specific antibodies and sCD4 induces conformational changes within the HIV-1 glycoprotein complex similar to V3 loop specific antibodies (Poignard *et al.*, 1996). Moreover, the concentration profiles of sCD4 and antibody induced AME were essentially the same, suggesting that cross-linking is required for sCD4 induced AME (Sullivan *et al.*, 1995). It is not clear however how sCD4, which is a monomer (Malvoisin & Wild, 1994), may cause cross-linking. We postulate that either sCD4 dimerizes with membrane associated proteins that also bind to HIV-1 gp120, or that more than one recognition site exist between gp120 and sCD4. The former explanation seems most likely since only recently it was shown that a D1-D2 fragment of CD4 bound to CCR-5 which also binds HIV-1 gp120 (Wu *et al.*, 1996). Since sCD4 activated HIV-1 and HIV-2 glycoproteins mediated membrane fusion has

been suggested to parallel gp120-CD4 activated membrane fusion (Allan, 1991), the high affinity interaction of HIV-1 gp120 with CD4 is probably required to establish a stable complex between the oligomeric HIV-1 glycoprotein complex and cellular CD4. This interaction then induces conformational changes allowing the gp120/CD4 complex to bind to members of the α and β chemokine receptor family (Choe *et al.*, 1996; Wu *et al.*, 1996). This complex interaction may subsequently induce conformational changes within the gp120-CD4 complex, resulting in a fusogenic state of the whole envelope-CD4 complex.

The capacity of HuMAb IAM 2F5 to inhibit entry of all the chimeric viruses tested (Figure 1), indicates that this antibody, in contrast to the gp120 specific HuMAb's used in these studies, blocks an essential step in the process of entry of both TCLA and primary viruses. This antibody binds to the relatively conserved proximal part of a putative alpha helix, which is essential for HIV-1 IIIB envelope glycoprotein mediated entry. Therefore we conclude that the site recognized by HuMAb IAM 2F5, rather than those involved in AME, may be the most suitable target for antibody mediate immune intervention.

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CHAPTER 7

Human antibodies that neutralize primary HIV-1 in vitro do not provide protection in an in vivo model.

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Human antibodies that neutralize primary human immunodeficiency virus type 1 *in vitro* do not provide protection in an *in vivo* model

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Recently, conflicting data have been published about the ability of antibodies which efficiently neutralize T cell-adapted human immunodeficiency virus type 1 (HIV-1) strains to neutralize primary HIV-1 strains *in vitro* and *in vivo*. Here we present data indicating that such antibodies fail to neutralize primary HIV-1 strains *in vivo*. To this end, a newly developed chimeric human-to-mouse model was used, in which several aspects of primary HIV-1 infection are mimicked. Poly- and monoclonal antibodies protected the grafted human cells, in a dose-dependent way, from infection with T cell-adapted HIV-1 in this system. A human monoclonal antibody specific for the CD4 binding domain that efficiently neutralizes HIV-1 IIIB *in vitro* did not protect the human graft from HIV-1 IIIB infection. None of the antibodies provided protection in the *in vivo* model against infection with primary HIV-1 strains, although they were able to neutralize these same strains *in vitro*.

Introduction

Studies aimed at the development of a vaccine against human immunodeficiency virus type 1 (HIV-1) have largely focused on the induction of antibodies which neutralize HIV-1 strains adapted to replication in T cell lines. It was shown that the neutralizing capacity of such antibodies was significantly lower or absent against primary HIV-1 strains, directly isolated in peripheral blood mononuclear cells (PBMC) from HIV-1-seropositive individuals (Moore *et al.*, 1995). The significance of these antibodies for *in vivo* protection and consequently the need to induce such antibodies by vaccination has been subject of fundamental debate (Schutten *et al.*, 1995; Moore, 1995). Evaluation of the potential of antibodies and antiviral compounds to interfere with the replication of primary HIV-1 strains *in vivo* has largely been hampered by the limited availability of suitable animal models. Besides the chimpanzee (*Pan troglodytes*) and the pigtail macaque (*Macaca nemestrina*), human–mouse chimeric models for HIV-1 infection have been described. These are based on the creation of a human type haematological environment by grafting human PBMC or haematopoietic tissue into SCID mice (Mosier *et al.*, 1991; Namikawa *et al.*, 1988); subsequently, the activated human graft is infected with HIV-1. In these models it was demonstrated that polyclonal hyperimmune globulins from seropositive donors (HIVIG), a monoclonal antibody (MAb) directed against the V3 loop on HIV-1 gp120 and 3′-azido-2′,3′-dideoxythymidine (AZT) inhibited the replication of T cell line-adapted (TCLA) HIV-1 strains (Safrit *et al.*, 1993; Shih *et al.*, 1991; McCune *et al.*, 1990). In infected individuals HIV-1 has been shown to replicate predominantly in activated macrophages and T lymphocytes resulting in high virus loads in lymphoid organs (Embretson *et al.*, 1993; Pantaleo *et al.*, 1993). With this in mind an alternative chimeric human-to-mouse model was developed in which human PBMC were grafted intraperitoneally (i.p.) into gamma-irradiated CBA/N mice. This results in an acute xenogenic-graft versus host disease (xeno-GvHD) providing a system in which human cells of both the monocytic and lymphocytic cell lineages become highly activated (Huppes *et al.*, 1992, 1993). In order to infect the grafted cells in a quiescent state, infection with HIV-1 was carried out within 1 h of grafting. Here we describe the replication kinetics of primary HIV-1 strains in this model. Furthermore, we assess the potential of different human HIV-1 neutralizing antibodies to interfere with the replication of TCLA and primary HIV-1 strains in grafted human cells.

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Methods

Viruses and antibodies. The antibody preparations used for passive immunization studies with the TCLA and primary HIV-1 strains were selected for a broad and high affinity for the HIV-1 strains used. They included: CD4 binding domain (bd)-specific human monoclonal antibody (HuMAb) GP13 (Schutten *et al.*, 1993); the HIV-1 IIIB V3 loop-specific mouse MAb F58H3 (Broliden *et al.*, 1992; Hinkula *et al.*, 1994); the HIV-1 V3 loop-specific HuMab 257-D (Gorny *et al.*, 1993); the gp41-specific HuMAb K14 (Teeuwsen *et al.*, 1990); and HIVIG (Prince *et al.*, 1991).

HIV-1 IIIB was kindly provided by the MRC AIDS Directed Programme and the virus stock was expanded using the CB15 $CD4^+$ T cell line (Gallo *et al.*, 1983; Nick *et al.*, 1993). The primary HIV-1 molecular clones 320.2A.1.2 [SI (syncytium inducing/non-macrophage tropic)] and 320.2A.2.1 [NSI (non-syncytium inducing/macrophage tropic)], and the primary HIV-1 strains ACH 172.BA-L (NSI) and ACH 168.10 (SI), were provided by H. Schuitemaker from the Central Laboratory for the Blood Transfusion Service in Amsterdam. Their *in vitro* passage history has been described previously (Schuitemaker *et al.*, 1992*a*, *b*, 1993). The full-length infectious molecular clones HIV-1 320.2A.1.2 (SI) and HIV-1 320.2A.2.1 were transfected into the $CD4^-$ cell line COS and cell-free supernatants were obtained after 3 days. A high titred virus stock from HIV-1 320.2A.1.2 (SI) was made within 2 weeks of primary infection using the CB15 cell line. During this short single passage it is unlikely that the genotype and phenotype of the HIV-1 molecular clone changed significantly (Back *et al.*, 1993; Gartner & Popovic, 1990). Since HIV-1 320.2A.2.1 (NSI) does not replicate to high titres in phytohaemagglutinin (PHA)-stimulated PBMC and immortalized T cell lines, xeno-GvHD mice were infected with HIV-1 320.2A.2.1 (NSI) and after 2 weeks peritoneal tissues from these mice were cultured for another 6 days to produce high titre virus stocks. Virus stocks from ACH 168.10 (SI) and ACH 172.BA-L were produced using PHA-stimulated human PBMC according to established procedures (Schuitemaker *et al.*, 1992*a*).

Xeno-GvHD mice. Xeno-GvHD mice were generated as previously described by Huppes *et al.* (1992). In brief, 4 to 5-week-old CBA/N/Rij mice (Harlan CPB, Zeist, The Netherlands), bred and kept under specific pathogen-free conditions, were conditioned by total body irradiation (9 Gy) with haematological support of 5×10^5 syngeneic bone marrow cells intravenously. For grafting 2×10^7/g bodyweight human PBMC isolated on a Ficoll gradient were used; this has been shown to induce an acute GvHD in 100% of cases (Huppes *et al.*, 1992, 1993). Mice within an experiment received PBMC from one individual only. The data plotted in Figs 2 and 3 represent the results of one experiment with data measurements at a single time-point. All experiments were performed in duplicate with similar results. Each virus–antibody combination was therefore tested with PBMC from at least six different donors.

Virus load. The virus load of the human lymphocytes isolated from the xeno-GvHD mice infected with the different HIV-1 strains was determined with an infectious centre test (ICT). Human lymphocytes from peritoneal lavages of each mouse were counted and titrated individually in duplicate starting at $2{\cdot}5 \times 10^6$ per well in 96-well round bottom plates. Human PBMC that had been prestimulated with PHA for 3 days were also added at a concentration of 5×10^4 per well. Cells were cultured for 1 week in RPMI 1640 (GIBCO BRL), 10% heat inactivated fetal bovine serum (Hyclone Laboratories Inc.), penicillin (100 U/ml), streptomycin (100 μg/ml) and 50 units of recombinant human IL-2/ml. (Proleukin, EuroCetus). After 7 days, 50 μl of supernatant per well was tested in a p24 antigen ELISA (V5 p24 antigen ELISA kit; a kind gift of Organon Teknika, Boxtel, The Netherlands) according to the manufacturer's instructions. The minimum number of cells from the peritoneal lavage from each individual mouse that was required to detect viral antigen in more than 50% of the wells was taken as a measure of viral load. Both peritoneal tissue and spleen were also cultured using the same culture medium for detection of productively infected cells.

Immunohistochemistry and *in situ* hybridization. CD45 (human leukocyte marker) and CD68 (human macrophage marker) immunohistochemistry was performed as described previously (Tenner-Racz *et al.*, 1994). Sequential series of 4 μm sections were stained for HIV-1 in combination with CD45 and CD68. For detection of productively infected cells by *in situ* hybridization, a ^{35}S-labelled antisense RNA probe of HIV-1 and a ^{35}S-labelled sense RNA probe as negative control (Lofstrand Labs) were used. Sections (4 μm) of formalin-fixed paraffin-embedded tissues were digested with Proteinase K (Sigma) at 37 °C for 15 min. The tissue sections were subsequently acetylated in 0·25% acetic anhydride–0·1% trietholamine pH 8·0 at room temperature for 5 min and subsequently prehybridized with 50% formamide, 0·5 M-NaCl, 10 mM-Tris–HCl pH 7·4, 1 mM-EDTA, 0·02% Ficoll–polyvinyl pyrrolidone–BSA and 2 mg/ml tRNA for 2 h at 45 °C. Radiolabelled probe (2×10^6 d.p.m./μl) in prehybridization mixture + 10% dextran sulphate was added to the slides and incubated overnight at 45 °C. After extensive washing and RNAse digestion (Boehringer Mannheim) tissue sections were dehydrated and incubated with Kodak NTB-2 emulsion and developed in Kodak D-19 developer; the sections were then counter-stained with haemalum and mounted. Also, photographs were taken of the *in situ* hybridization slides using excitation at one specific wavelength to identify the silver grains showing HIV-1-specific *in situ* hybridization.

Results

HIV-1 infection in xeno-GvHD mice

Xeno-GvHD mice were infected i.p. with 30 xeno-GvHD mice infectious doses 50% ($XeID_{50}$) of primary and TCLA HIV-1 strains with SI and NSI phenotypes. These included the TCLA HIV-1 strain IIIB and the primary HIV-1 strains 320.2A.1.2 (SI), 320.2A.2.1 (NSI), ACH.172.BA-L (NSI) and ACH 168.10 (SI). Directly after development of acute xeno-GvHD, combined *in situ* hybridization and immunohistochemistry studies were carried out, using an HIV-1 probe and CD45 and CD68 specific conjugates, on peritoneal tissues and lymphoid organs of mice infected with the primary HIV-1 strains. Acute xeno-GvHD consistently started between 6 to 14 days after grafting, depending on the numbers of human PBMC grafted. The HIV-1-specific staining showed a significant increase between days 6 and 12 after grafting (not shown). Fig. 1(*a*–*c*) shows a sequential series of sections from part of the diaphragm stained for the macrophage marker CD68 and HIV-1. Large numbers of human macrophages were present in the peritoneal tissues of these mice. In mice infected with the NSI, HIV-1 strains, like HIV-1 320.2A.2.1 (Schuitemaker *et al.*, 1992*a*), HIV-1-specific staining was found mainly in these areas. HIV-1-specific staining of T cell-rich areas was also observed, although to a lesser extent since HIV-1 320.2A.2.1 also infects $CD4^+$ T cells. In mice infected with SI HIV-1 strains like 320.2A.1.2 and 168.10, which do not infect monocyte-derived macrophages *in vitro* (Schuitemaker *et al.*, 1992*a*), HIV-1-specific staining was significantly lower in the

macrophage-rich areas (Fig. 1*d–f*). However, in the lymphoid organs of these mice, as in the periarteriolar lymphoid sheath shown in Fig. 1(*g–i*) where predominantly human T cells and hardly any human macrophages were present, abundant HIV-1 staining could also be demonstrated. The number of productively infected cells per field at the same magnification was about ten times higher in these T cell-rich areas compared to tissues in the peritoneal cavity. The numbers of HIV-1-infected human cells in peritoneal lavages of those mice whose tissues were used for immunohistochemistry and *in situ* hybridization were determined in the ICT. For the primary HIV-1 strains, ratios between infected and non-infected cells increased from about 10^{-4} at day 6 to more than $10^{-1.5}$ at day 12 after grafting. This proved not to be related to the capacity of the virus to induce syncytia *in vitro*. HIV-1 IIIB infection in the xeno-GvHD model proved to be self-limiting since the ratio of infected and non-infected cells in the peritoneal lavage cells was 10^{-4} at day 6 and diminished to an undetectable level at day 11 (Fig. 2*a, b*). Infectious HIV-1 could not be detected at day 11 in spleen or peritoneal tissues of mice infected with HIV-1 IIIB.

Passive immunization studies with HIV-1 IIIB

Intraperitoneal administration of 20 mg/kg poly- or monoclonal antibody preparations, 1 h prior to grafting of the PBMC, followed by infection with 30 $XeID_{50}$ HIV-1 IIIB, resulted in different patterns of interference with virus replication (Fig. 2*a*). Administration of the CD4 bd-specific HuMAb GP13, which neutralizes HIV-1 IIIB *in vitro* [inhibitory concentration 90% (IC_{90}) = 2 μg/ml] (Back *et al.*, 1994)] had no effect on the virus load in human cells at day 6 in the peritoneal lavages. Administration of the V3 loop-specific mouse MAb F58H3 either alone or in the presence of HuMAb GP13 (both administered at 20 mg/kg), and administration of HIVIG, resulted in the absence of detectable HIV-1 IIIB infected cells in peritoneal lavages, peritoneal tissues and spleen. The minimum concentration of mouse MAb F58H3 that interfered with the numbers of detectable HIV-1 IIIB infected cells was determined by titrating the antibody *in vivo*. Between 10 and 20 mg/kg was needed to abolish the presence of HIV-1 IIIB-infected cells (Fig. 2*c*). At day 11, HIV-1 IIIB was detected by ICT in the peritoneal lavage cells from xeno-GvHD which had received the CD4 bd-specific HuMAb GP13. This is surprising since peritoneal lavage cells, spleen and peritoneal tissue collected at day 11 from xeno-GvHD mice that had received identical numbers of PBMC from the same blood donor and the same dose of HIV-1 IIIB were negative in the ICT (Fig. 2*b*).

Passive immunization studies with primary HIV-1

In contrast, i.p. administration of 20 mg/kg of the HuMAbs or HIVIG, prior to infection of the human PBMC graft with 30 $XeID_{50}$ of the primary HIV-1 strains, did not result in a significant reduction in the virus load in any of the antibody–primary HIV-1 strain combinations tested (Fig. 3*a–d*). The antibody preparations used in these assays included the following.

CD4 bd-specific HuMAb GP13, which neutralizes TCLA HIV-1 strains *in vitro* (Back *et al.*, 1994).

V3 loop-specific HuMAb 257-D, which neutralizes the primary HIV-1 strain 320.2A.1.2 *in vitro* with an IC_{50} of 10 μg/ml and an IC_{90} of 35 μg/ml.

A combination of equal amounts of these two antibodies, which neutralized HIV-1 320.2A.1.2 (SI) *in vitro* better than HuMAb 257-D alone, with an IC_{50} of 6 μg/ml and an IC_{90} of 20 μg/ml.

Polyclonal HIVIG preparation, which had previously been shown to neutralize HIV-1 IIIB *in vitro* and *in vivo* (Prince *et al.*, 1991) (Fig. 1) and primary HIV-1 strains *in vitro* (H. Schuitemaker, personal communication).

Discussion

Recently, conflicting data have been published about the capacity of antibodies which efficiently neutralize T cell-adapted HIV-1 strains to neutralize primary HIV-1 strains *in vitro* and *in vivo*. Here, we present data indicating that such antibodies fail to neutralize primary HIV-1 strains *in vivo*.

In the HuPBL-SCID and HuCBL-SCID models it has been shown that in more than 50% of the grafts HIV-1 IIIB fails to establish a persistent infection (Reinhardt *et al.*, 1994; Mosier *et al.*, 1991). It has been speculated that the self-limiting nature of this infection is due to the relatively high cytopathogenicity of HIV-1 IIIB resulting in the elimination of all permissive cells in infected foci (Mosier & Sieburg, 1995). In our model we also observed clearance of HIV-1 IIIB infection as early as 11 days post-infection. However, a persistent infection was observed in mice which also received the CD4 bd-specific HuMAb GP13. We speculate that this is due, on the one hand, to the ability of the antibody to inhibit syncytium induction of HIV-1 IIIB (Schutten *et al.*, 1993), and, on the other hand, to the inability to inhibit virus entry (Fig. 1*a*), collectively resulting in diminished *in vivo* cytopathogenicity. All other antibody preparations tested efficiently neutalized HIV-1 IIIB infection in this *in vivo* model.

The concentrations of antibodies used in the *in vivo* neutralization assays with the primary HIV-1 strains were higher than the concentrations of mouse MAbs F58H3 and HIVIG needed to neutralize HIV-1 IIIB in the same system (Fig. 1). They were in the same range as the concentrations used to neutralize HIV-1 IIIB in chimpanzees with other poly- and monoclonal antibodies (Emini *et al.*, 1992; Prince *et al.*, 1991). In the xeno-GvHD mouse model for HIV-1 infection we have recently also tested negatively charged succinylated human serum albumins (Suc-HSA), which neutralize TCLA and

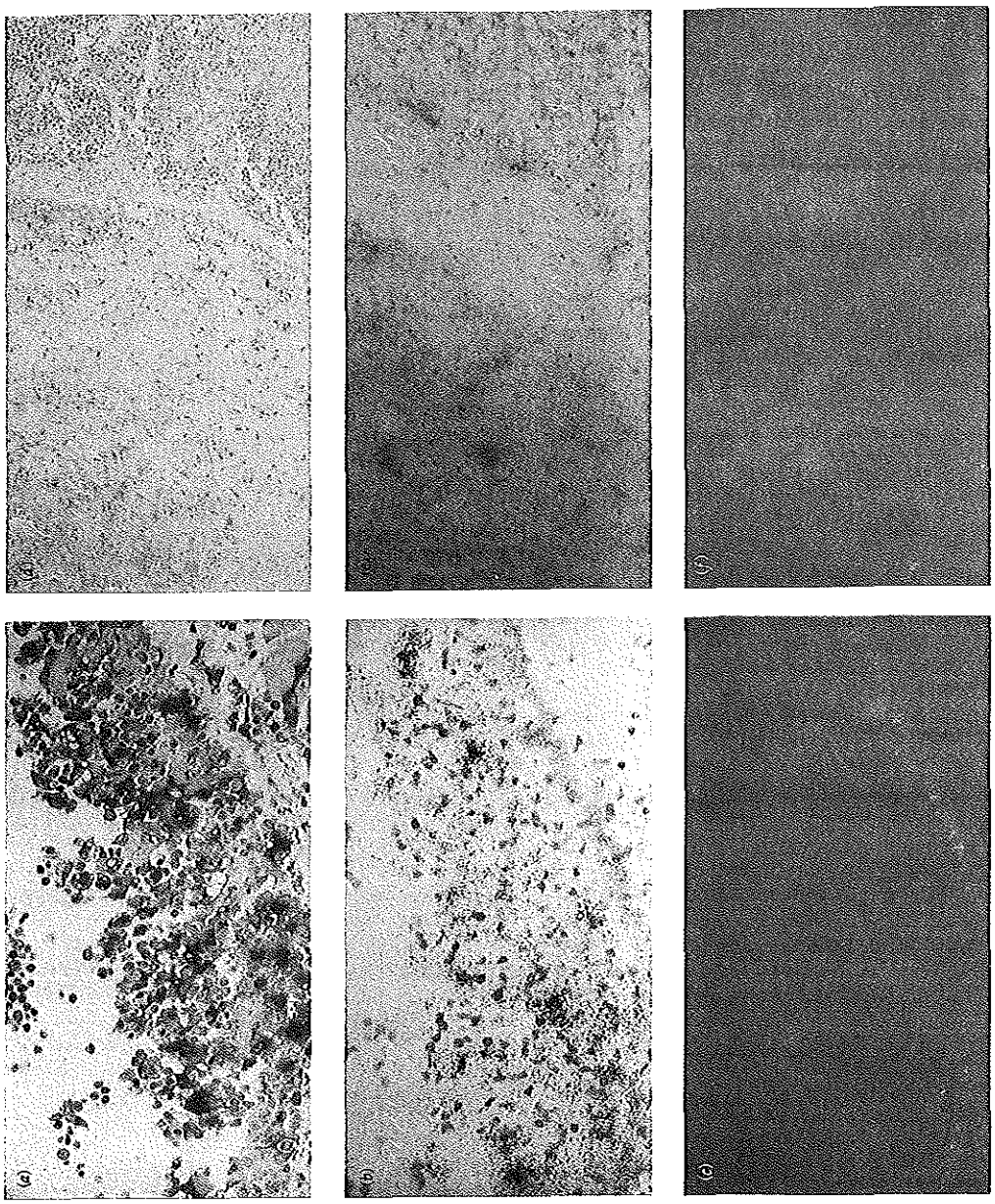
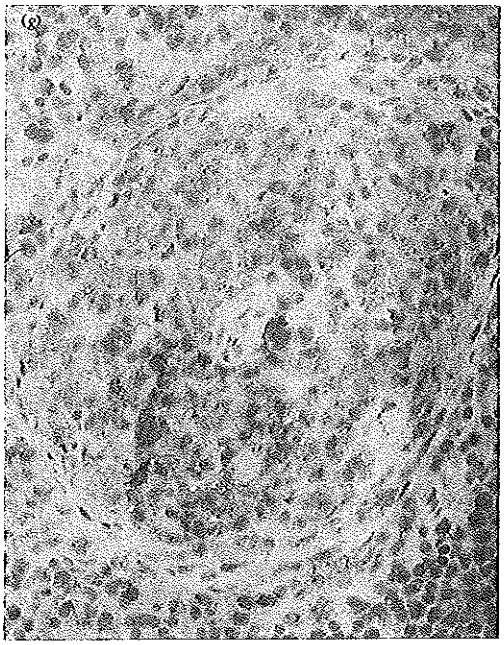
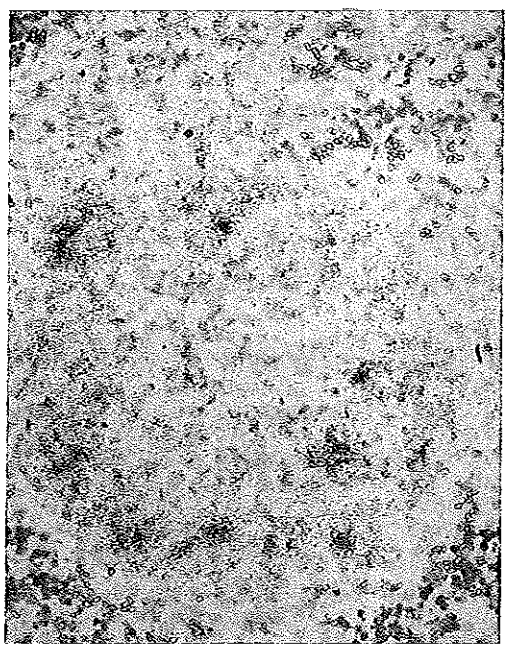


Fig. 1 (a–f). For legend see opposite.





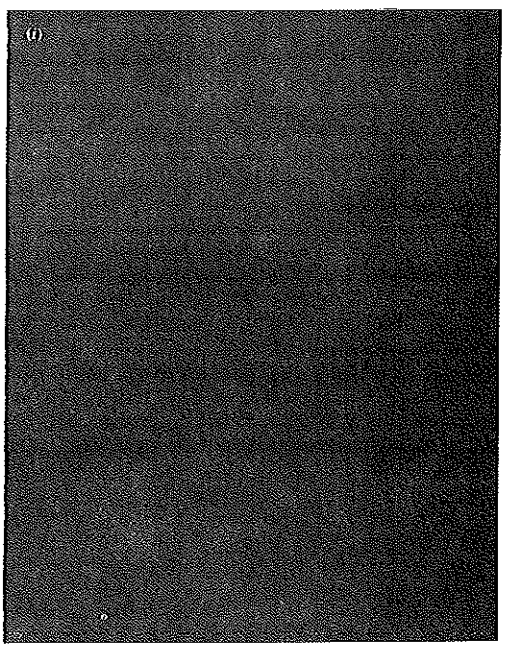


Fig. 1. HIV-1 *in situ* hybridization, CD45 and CD68 immunohistochemistry of spleen and peritoneal tissue from xeno-GvHD mice infected with either 320.2A.1.2 (SI, non-macrophage tropic) or 320.2A.2.1 (NSI, macrophage tropic). (*a–c*) Sections from the diaphragm of xeno-GvHD mice infected with HIV-1 320.2A.2.1 and stained for CD68 (*a*) or HIV-1 RNA (*b*) and epifluorescence of the same HIV-1 RNA *in situ* hybridized section (*c*) (magnification 220 ×). (*d–f*) Sections from the spleen (at the right-hand side of the figure) and pancreas (at the left-hand side of the figure) infected with HIV-1 ACH 168.10 and stained for CD68 (*d*) or HIV-1 RNA (*e*), and epifluorescence of the same HIV-1 RNA *in situ* hybridized section (*f*) (magnification 44 ×). (*g–i*) Sections from the spleen of HIV-1 320.2A.1.2-infected xeno-GvHD mice, stained for CD45 (*g*) or HIV-1 RNA (*h*), and epifluorescence of the same HIV-1 RNA *in situ* hybridized section (*i*) (magnification 430 ×).

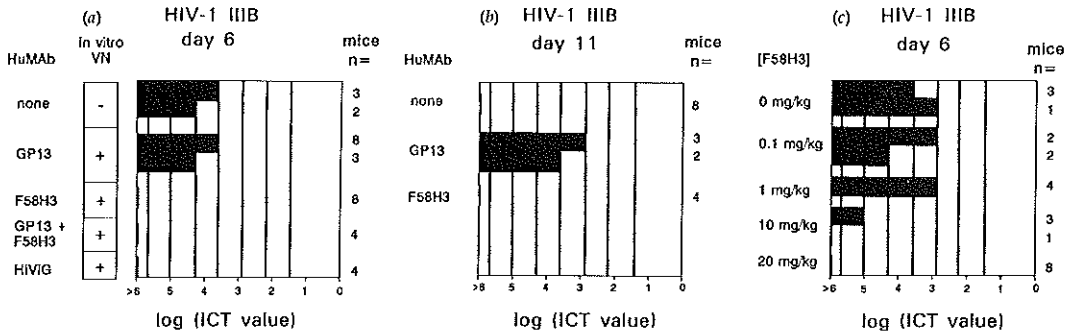


Fig. 2. Cell-associated virus load determined by ICT in human cells from peritoneal lavages of xeno-GvHD mice at the day xeno-GvHD was observed in mice infected with 30 $XeID_{50}$ HIV-1 IIIB. Prior to infection, MAbs or HIVIG were administered i.p. at 20 mg/kg. In the antibody mixtures (V3 specific MAb + CD4 bd specific HuMAb) 20 mg/kg of each antibody was given. In (c) different concentrations of the V3 specific MAb F58H3 were tested as indicated on the left-hand side of the plot. The number of mice used per group is indicated on the right-hand side of each plot (n). On the horizontal axis the logarithm of the numbers of cells needed to yield positive cultures in the ICT are given. Solid grid lines indicate the actual dilutions made (5-fold dilution steps starting at 5×10^5 cells). Where $> 10^6$ cells is indicated, none of the mouse tissues tested in culture (peritoneal tissue, spleen, ascitic fluid cells) proved to be positive for HIV-1 p24 after 7 days of culture.

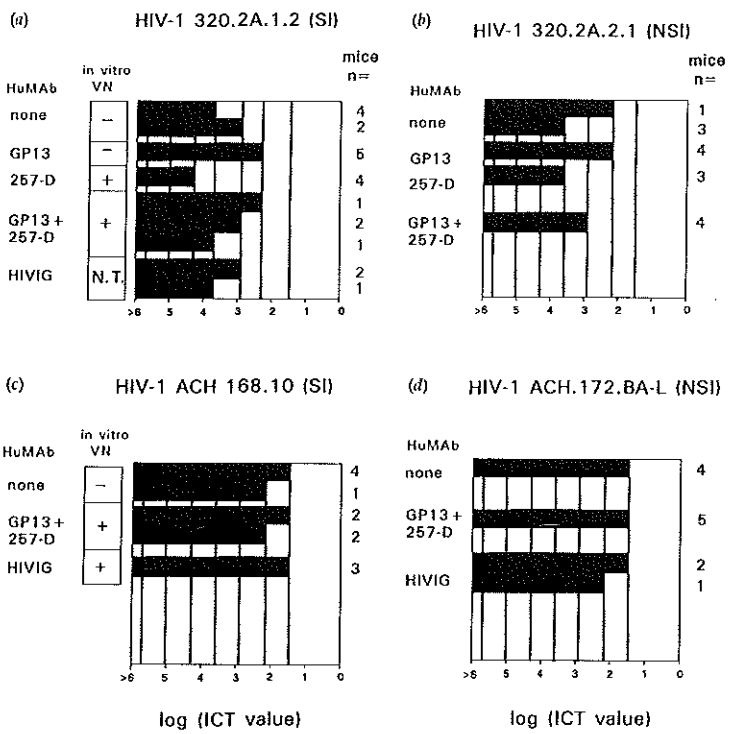


Fig. 3. Cell-associated virus load determined by ICT in human cells from peritoneal lavages of xeno-GvHD mice at the day xeno-GvHD was observed in mice infected with HIV-1 320.2A.1.2 (SI), HIV-1 320.2A.2.1 (NSI), HIV-1 ACH 168.10 (SI) and HIV-1 ACH 172.BA-L (NSI). Prior to infection, MAbs or HIVIG were administered i.p. at 20 mg/kg. In the antibody mixtures (V3 specific MAb + CD4 bd specific HuMAb) 20 mg/kg of each antibody was given. Data are presented as indicated in Fig. 2.

primary HIV-1 strains by binding to the V3 loop of gp120 (Jansen *et al.*, 1993). The concentration of Suc-HSA needed to neutralize HIV-1 IIIB in the xeno-GvHD mouse system was approximately the same on a molar basis as that of the V3 loop-specific mouse MAb F58H3 (Fig. 1) (Kuipers *et al.*, 1996). Preliminary data, however, showed that a 100-fold higher concentration failed to provide neutralizing activity towards primary HIV-1 strains. This concentration is more than 50 times the *in vitro* IC_{50} against HIV-1 320.2A.1.2 used in this study (unpublished results). Therefore, we conclude that the inability of the V3 loop-specific antibodies to neutralize primary HIV-1 strains *in vivo* cannot be attributed to the use of insufficiently high concentrations of these compounds in our *in vivo* model.

These data extend *in vitro* observations showing that primary HIV-1 strains are more resistant to virus neutralization in human PBMC than TCLA strains (Moore *et al.*, 1995; Bou-Habib *et al.*, 1994; Schutten *et al.*, 1995). The relevance of the *in vivo* data generated with primary HIV-1 strains in the xeno-GvHD model in which no protection was found, as compared to the *in vitro* data showing limited neutralization of these strains, is determined by the similarities between HIV-1 infection in humans and HIV-1 infection in this model. Like HIV-1-infected human lymphoid tissues, the model provides a substrate of highly activated cells of the monocytic and T lymphocytic lineages, of which the latter are skewed toward a $CD4^+$ subpopulation (Huppes *et al.*, 1992, 1994). This allows high levels of HIV-1 replication, a feature that is thought to be essential for persistence of HIV-1 infection in humans (Wei *et al.*, 1995; Ho *et al.*, 1995). Furthermore, the close interaction between lymphoid cells and antigen-presenting cells, which is a hallmark of the development of acute xeno-GvHD, also seems to be essential in the pathogenesis of HIV-1 infection (Gartner *et al.*, 1986; Huppes *et al.*, 1992). Therefore, the xeno-GvHD HIV-1 model provides a human lymphoid environment which clearly exhibits more similarities with HIV-1-infected human lymphoid tissues (Fig. 2) (Embretson *et al.*, 1993; Pantaleo *et al.*, 1993) than *in vitro* HIV-1-infected PBMC cultures. Furthermore, macrophages are thought to play an important role during primary HIV-1 infection in humans. The *in situ* hybridization experiments with HIV-1 320.2A.2.1 (NSI) showed that at the site of primary infection, macrophages were productively infected. In order to mimic primary HIV-1 infection in humans more closely and in contrast to the other chimeric models (Namikawa *et al.*, 1988; Mosier *et al.*, 1991), human cells were challenged soon after grafting, long before the characteristic activation of the grafted cells could be demonstrated.

The reason why primary HIV-1 strains appear to be more resistant to the neutralizing activity of antibodies and Suc-HSA *in vivo* than their TCLA HIV-1 counterparts is not clear. A major difference between both virus types is that the former do not shed gp120 as readily (Moore *et al.*, 1992; Groenink *et al.*, 1995). It may be speculated that after binding of the virus to the receptor, V3-specific antibodies may at least temporarily prevent the process of fusion between the virus- and cellular-membranes. This leaves the TCLA viruses in particular vulnerable to gp120 shedding without being able to penetrate the cell membrane, which eventually results in more effective neutralization of TCLA HIV-1 strains. Finally, it should be realized that other mechanisms of antibody-mediated enhancement of infectivity have been described. At least one of these may also be dependent on the phenotype of the virus and the cell type involved (Schutten *et al.*, 1995). The lack of neutralizing activity found in the xeno-GvHD mouse model with the antibody preparations tested may therefore also partly be due to the interference of enhancing antibody activity.

Taken together, our data show that primary HIV-1 strains cannot easily be neutralized in an *in vivo* system that closely mimics HIV-1 infection in humans. Since induction of virus-neutralizing antibodies is still one of the major targets in the development of candidate HIV-1 vaccines, we propose to use for their evaluation assay systems like the xeno-GvHD mouse model for HIV-1 infection.

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CHAPTER 8

General Discussion
in part taken from:
Antibody mediated enhancement of lentivirus infection: Stumbling block for vaccine development or *in vitro* artefact?

submitted.

General discussion

The induction of HIV-1 specific antibodies that prevent HIV-1 infection or limit the spread of the virus during the initial stages of the infection, is thought to be an essential feature of future HIV-1 vaccines. *In vitro* and *in vivo* studies on the virus neutralizing capacity of HIV-1 envelope specific monoclonal antibodies and sera obtained from seropositive individuals have shown that factors determined by the virus strain and the assay system used, may have a pronounced influence on the outcome of the antibody-virus interaction[1-4]. The primary goal of the studies presented in this thesis was the evaluation of factors that influence the virus neutralizing capacity of HIV-1 specific antibodies.

In chapter 2, 3 and 4 the generation and characterization of human monoclonal antibodies (HuMAB's) directed against the V3 loop and the CD4 binding domain (CD4bd) are described. By pepscan analysis of the linear epitope of the V3 loop specific HuMAb MN215 it was shown that the "best fitting" V3 loop derived sequence for HuMAb MN215 exhibited striking similarities with the relatively conserved V3 loop consensus sequence of macrophage-tropic HIV-1 strains[5]. Furthermore, uncharged residues at position 13, uncharged or negatively charged residues at position 30 and negatively charged residues at the C-terminal side of the V3 loop proved to be important for a high affinity interaction with HuMAb MN215. Non syncytium inducing (NSI) HIV-1 strains have also been shown to exhibit such charge characteristics at these positions, whereas syncytium inducing (SI) strains tend to have more positively charged residues at these positions within the V3 loop[6-8]. It is therefore conceivable that HIV-1 strains within a host delimited quasispecies distribution, that have acquired mutations within the V3 loop, improving the fusogenic capacity of the envelope glycoprotein complex, will be recognized with lower affinity. Only recently HuMAb MN215 was tested in a peptide inhibition ELISA with peptide derivates of the subtype B consensus sequence with an early NSI, late NSI and SI character. HuMAb MN215 proved to have the highest affinity for late NSI derived peptides, a somewhat lower affinity for early NSI derived peptides and low affinity for SI derived peptides (Dr. F. Barin personal communication). Indeed when tested in a peptide inhibition ELISA and catching antibody ELISA with solid phase whole gp160, HuMAB MN215 showed significantly higher affinities for the NSI as compared to the SI derived V3 domains from donors #16 and #320 from the Amsterdam cohort.The NSI and SI HIV-1 derived peptides and envelope glycoproteins were obtained from biologically cloned primary NSI and SI strains obtained at the time point where the NSI to SI phenotype switch was observed in the donor. We have recently tested the affinity of HuMAb MN215 with one early NSI and a SI derived envelope glycoprotein from donor #168 from the Amsterdam cohort (ACH 168.1 and ACH 168.10 respectively[7,9]). The 168.1 and 168.10 envelope glycoproteins were recognized with similar affinities by HuMAb MN215, despite the fact that it was predicted, on basis of the pepscan criteria for the "best fitting" V3 loop sequence for HuMAb MN215, that HuMAb MN215 would have it's highest affinity for the early NSI derived glycoprotein (M. Schutten *et al.*, unpublished data). It may therefore be postulated that within early NSI HIV-1 glycoproteins, the V3 loop is less accessible for HuMAb MN215 binding as compared to late NSI and SI derived glycoproteins. This suggests that during evolution of the virus within the infected host from slowly replicating stable NSI strains towards fast replicating SI variants[10], exposure of the V3 loop within the envelope glycoprotein complex precedes the acquisition of amino acid changes required for the SI phenotype.

The V3 loop of HIV-1 has been shown to play a role in determining the SI capacity of both TCLA and primary HIV-1 strains[6-8,11]. Naturally occurring amino acid changes within the V3 loop

have been shown to influence the SI capacity of the envelope complex, HIV-1 cell tropism and the capacity of V3 loop specific antibodies and polyclonal HIV-1 specific sera to neutralize the virus[6,7,12-14]. Mutations within several other regions of HIV-1 gp120 (V1, V2, C4) also exhibit the capacity to change these characteristics of the virus[15-19]. Furthermore specific insertion of potential glycosylation sites by AAT triplet reiteration within the V4 and V5 domains suggests that these domains are also under selective antibody pressure, which may point towards a role of these domains in viral entry[20]. The envelope trans-complementation assay and the envelope genes derived of biological HIV-1 clones from donors #16, #168 and #320 were used to study the mechanism of primary HIV-1 entry, and the way in which envelope specific antibodies modulate this entry. In chapter 5 it was shown that the soluble form of the primary receptor of HIV-1 (sCD4) significantly enhanced entry mediated by the 16.4 (NSI) derived envelope glycoprotein. Similar observations had been described for SIVagm infection of the MOLT4 clone 8 $CD4^+$ T cell line and HIV-2 infection of $CD4^-$ cell lines. This suggests that enhancement of entry by the soluble form of the primary receptor is a more common phenomenon in lentivirus infections[21,22]. It is therefore conceivable that more HIV-1 strains will be found that are susceptible to sCD4 mediated enhancement[23]. Indeed recently our observations were confirmed by Sullivan *et al.* using the envelope genes derived from the HIV-1 YU2 (NSI) and ADA (NSI) strains[24]. Moreover entry of 16.4 (NSI) and YU2 (NSI) mediated entry in SupT1/PHA-PBMC and MOLT4 clone 8 cells respectively was significantly enhanced by gp120 specific monoclonal antibodies and purified hyperimmune immunoglobulines of HIV-1 seropositive individuals (HIVIG). Only recently in an extensive study on the biological activity of polyclonal sera of HIV-1 seropositive individuals against HIV-1 strains isolated from these individuals, it was shown that 41% of the HIV-1 strains were susceptible to enhancement of infection rather than to neutralization[4]. It was suggested that the enhancement observed is similar to that described in our studies and in the study of Sullivan *et al.*. This indicates that indeed enhancement of HIV-1 entry by a mechanism that parallels sCD4 mediated enhancement, is a common mechanism in HIV-1 infection.

In vitro studies on the biological activity of HIV-1 glycoprotein specific antibodies may provide valuable information on the molecular mechanisms underlying viral entry which may allow the identification of steps within the process of HIV-1 entry that are essential for HIV-1 strains with different biological phenotypes. Further studies conducted to gain insight into the molecular mechanisms of entry in the absence and presence of envelope specific antibodies are described in chapter 6. The susceptibility of the glycoprotein complex to neutralization versus enhancement of entry by gp120 specific antibodies proved to be determined by properties of gp120. By exchanging parts of the genes encoding the glycoproteins susceptible to either neutralization or enhancement of entry, it was shown that the V3 loop and an as yet undetermined part of gp120, determined the level of entry measured in this assay, and the susceptibility to neutralization versus enhancement. Alternatively an interaction between V3 and another region located within the V3 to V5 region might have been at the basis of the observed differences in entry and susceptibility to neutralization versus enhancement. Preliminary data confirm that this is indeed the case. Furthermore, the induction of conformational changes required for enhancement proved to have a minimal energy requirement, and cross-linking was shown to be a prerequisite for the enhancement observed with the HuMAb's. Based on the observations described in chapter 6, the following working hypothesis may be postulated for primary HIV-1 entry:
Conformational changes in gp120 after CD4 binding, result in the exposure of positively charged loops like the V3 loop and possibly other regions like the V1/V2 region[25-27]. Depending on the flexibility and the conformation of these variable regions, which seems to be determined at least

in part by inter- or intramolecular interactions[18], these domains may interact with CD4, like the previously reported V3/CDR3 interaction[28], or with other cellular proteins. This secondary interaction induces additional conformational changes by intermolecular cross-linking, required for the induction of the fusion attack complex. Within the fusion attack complex, the ability of the glycoprotein complex to bind to members of the C-C and C-X-C chemokine receptor subfamilies of the G-protein binding receptor superfamily determines the efficiency with which the HIV-1 particle infects different target cells[29-31].

The role of HIV-1 specific antibodies in protection against HIV-1 challenge in passive and active immunization strategies and protection against disease in HIV-1 induced pathogenesis, has been difficult to asses due to limitations of *in vitro* and *in vivo* models for HIV-1 infection. HIV-1 infection of cells of the monocytic cell lineage and infection of compact lymphoid tissues are thought to be essential factors for a reliable model for HIV-1 infection. Furthermore infection of quiescent lymphoid cells and subsequent virus replication, specifically activates the human immune system, which then in turn allows efficient HIV-1 replication[32,33]. With this in mind, a human to mouse model was developed in which human PBMC were grafted intraperitoneally into immuno-suppressed CBA/N mice: the "Xeno-GvHD model". This results in an acute xeno-graft versus host reaction providing a system, in which human cells of the monocytic and lymphocytic cell lineages become highly activated[34,35]. To infect the grafted human cells in a quiescent state, HIV-1 infection was carried out within one hour after grafting. In chapter 7 it was shown that a relatively high percentage of the human macrophages and T lymphocytes in compact tissues of these chimeric mice then become productively infected. Furthermore, protection against HIV-1 IIIB challenge could be achieved by administering HIVIG and a HIV-1 V3 loop specific monoclonal antibody, which is in line with previous studies in chimpanzees and HuPBL-SCID mice[3,36,37]. In our xeno-GvHD mouse model, none of the antibody preparations tested were able to protect against a challenge with primary HIV-1 strains of a different biological phenotype. This despite the fact that antibody concentrations were well above the *in vitro* IC_{50} and in some cases (HIV-1 320.2A.1.2 with HuMAb 257-D in combination with GP13) most probably reached the *in vitro* IC_{90}. However, the antibody concentrations used may still have been too low to achieve protection since it has been shown in the HuPBL-SCID model that at least the IC_{90} should be reached to achieve protection against TCLA HIV-1 strains[3]. The relevance of this observation seems questionable however, since *in vitro* determined inhibitory doses may vary up to 100 fold between laboratories using the same virus strain in the same VN assay[38,39]. To further address the difference in susceptibility to VN of TCLA and primary HIV-1 strains in this model, we also tested negatively charged human serum albumins. We were able to show that despite the rapid clearance of these compounds in the xeno-GvHD mice, a single dosage of succinilated human serum albumins protected the human graft from a challenge with the TCLA strain HIV-1 IIIB[40,41]. The efficiency with which this compound protected the human graft against a HIV-1 IIIB challenge on a molar basis proved to be similar to the capacity of a monoclonal antibody directed against the V3 loop. A 100-fold higher concentration as compared to the minimally required dose that neutralized HIV-1 IIIB still proved to be ineffective against a challenge with primary HIV-1. This despite the fact that more than 50 times the *in vitro* IC_{50} against HIV-1 320.2A.1.2 used in this study, was reached during the primary infection (Chapter 7, M. Schutten *et al.*, unpublished data). On basis of these experiments we speculate that the susceptibility to shedding of gp120 after CD4 binding is at the basis of the difference in susceptibility to VN of TCLA and primary HIV-1 strains in this model. Binding of TCLA HIV-1 strains to membrane associated CD4 in the presence of fusion inhibiting compounds, may result in the irreversible inactivation of the virus due to shedding

of gp120 from the glycoprotein complex[42]. Primary HIV-1 strains on the other hand may remain associated with the cell membrane since they do not shed gp120[43,44]. Decrease of the concentration of the inhibitory compound below the minimally required concentration at which neutralization is achieved, may in turn result in infection and overt replication of these HIV-1 strains. This phenomenon may have been at the basis of recent failure of passively administered HuMAb IAM-2F5 to protect against a challenge with a primary HIV-1 strain in the HIV-1/Chimpanzee model (H. Katinger personal communication). In this study temporary protection was observed with HuMAb IAM-2F5 against a challenge with primary HIV-1. HIV-1 infection could be demonstrated, approximately 80 days later as compared to the negative controls, when levels of HuMAb IAM-2F5 started to decline. From that point onward the kinetics of primary infection proved to be similar to that observed in the included control animals. The observations in the xeno-GvHD model and in the HIV-1/chimpanzee model have major implications for vaccine development strategies and indicate that much may still be learned about mechanisms of HIV-1 entry in the absence and presence of HIV-1 specific antibodies. Further insight in this subject may facilitate the development of a vaccine against AIDS.

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ANTIBODY MEDIATED ENHANCEMENT OF LENTIVIRUS INFECTION: STUMBLING BLOCK FOR VACCINE DEVELOPMENT OR *IN VITRO* ARTEFACT?

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Antibody dependent enhancement (ADE) of infection with enveloped viruses via Fc or complement (C') receptors is a feature commonly observed in *in vitro* systems (for review see Morens[1]). However, in only few virus infections, ADE has been shown to play a role *in vivo*. Active and passive immunization strategies which induce or transfer antibodies exhibiting Fc- or C' mediated ADE *in vitro*, still proved to be effective in providing protection in several *in vivo* viral systems, casting doubt on the relevance of ADE measured *in vitro*[2]. Recently we and others have shown that antibodies against the primate lentiviruses HIV-1, HIV-2 and SIVagm, may mediate enhancement of viral infectivity via another mechanism, that closely resembles normal activation of the virus-cell fusion (receptor activated membrane fusion (RAMF))[3-5]. Although data on the *in vivo* relevance of this type of antibody mediated enhancement are still lacking, there are indications that enhancement of infection by envelope glycoprotein specific antibodies may indeed play a role in the pathogenesis of lentivirus infections which may thus frustrate vaccine development efforts[6,7]. Accelerated virus replication and exacerbation of disease progression has been observed upon vaccination with experimental vaccines against the lentivirus equine infectious anaemia virus (EIAV)[8] and recently we observed a similar phenomenon upon experimental vaccination against feline immunodeficiency virus (FIV)[9]. This paper reviews the presently known mechanisms of antibody mediated enhancement of lentivirus infectivity and their possible relevance for the pathogenesis of lentivirus infections and vaccine development.

ANTIBODY MEDIATED ENHANCEMENT OF VIRUS-CELL ADHESION: Fc-ADE AND C'-ADE

Until recently two mechanisms of ADE have been described *in vitro* which are mediated by antibodies that recognize the envelope glycoprotein complex of lentiviruses: Fc receptor and C' receptor mediated antibody dependent enhancement (Fc-ADE and C'-ADE). In lentivirus systems these mechanisms have been studied most extensively for HIV-1 infection. It has been shown that HIV-1 infection enhanced by antibodies and C' may be either dependent or independent on the interaction of the glycoprotein complex and CD4, the primary receptor for primate lentiviruses[10-13]. This suggests that, two distinct mechanisms of this type of ADE exist. In CD4-dependent Fc-ADE and C'-ADE the physical interaction between the virus and the cell may be facilitated by the binding of the cellular Fc- and C' receptor to the opsonized virus particle. This in turn results in a more efficient receptor mediated membrane fusion[14]. These findings have been confirmed in binding studies using HIV-1 and $CD4^+$ cells in the absence and presence of antibody and C'[15,16]. Antibody and C' have also been reported to enhance infectivity of HIV-1 in a CD4-independent

manner. Fc- and C' receptors may therefore act as "pseudo" primary receptors[13]. The actual mechanism involved in the subsequent fusion of the virus with the cellular membrane is not known at present.

ANTIBODY MEDIATED MODULATION OF RECEPTOR ACTIVATED VIRUS-CELL FUSION: VN VERSUS AAMF

The soluble form of the primary receptor of HIV-1 (sCD4) and a large number of HIV-1 envelope glycoprotein specific antibodies inhibit entry of T cell line adapted (TCLA) HIV-1 strains[17,18]. It has been shown that the VN capacity of V3 loop specific antibodies positively correlated with the affinity of the antibody for the V3 loop[19]. VN mediated by sCD4 and these antibodies involves dissociation of the non-covalently linked surface glycoprotein from the transmembrane glycoprotein part of the complex ("shedding")[20]. Antibodies directed against other VN inducing sites on gp120, the surface part of the envelope glycoprotein (SU) do not induce shedding in most cases. The induction of conformational changes and/or interference with binding of the glycoprotein complex to a cellular counterpart also contributes to the neutralizing potential of these antibodies and V3 loop specific antibodies[21]. The interaction between sCD4 or antibodies with monomeric and multimeric SU glycoprotein, induces a variety of conformational changes, including changes that influence the exposure of gp120 and gp41 regions and the dissociation of gp120[22,23]. To what extent and how these conformational changes contribute to sCD4- and antibody-mediated neutralization is not known at present. Primary HIV-1 strains do not readily shed their SU after sCD4 and V3 loop specific antibody binding[24-27]. The (premature) induction of conformational changes alone appears to be sufficient to neutralize these viruses.

In contrast, entry of certain strains of HIV-2 and SIVagm, which also utilize the CD4 molecule as their primary receptor, is enhanced by sCD4[3,4]. We and others have recently shown that entry of certain primary HIV-1 strains of the non syncytium inducing (NSI) phenotype, is also enhanced by sCD4[5,28]. Furthermore, HIV-1 SU specific antibodies that neutralize TCLA HIV-1 strains proved to induce enhanced infectivity of HIV-1 strains susceptible to enhancement by sCD4, by a mechanism different from Fc-ADE and C'-ADE. We propose to call this mechanism that closely resembles sCD4 induced enhancement, antibody activated membrane fusion (AAMF). Enhancement through AAMF may be a commonly observed phenomenon for primary HIV-1 strains[29]. It was furthermore shown that the outcome (VN versus AAMF) in a neutralization assay with polyclonal sera from HIV-1 seropositive individuals was determined by the susceptibility of the virus to either mechanism. The relatively strong association of SU and the transmembrane part of the envelope glycoprotein (TM) of primary HIV-1 strains and HIV-2 and SIVagm strains susceptible to sCD4 and SU specific antibody induced enhancement of infectivity, has been postulated to be at the basis of this phenomenon[30-32]. This would allow the induction of a stable sCD4-glycoprotein complex or in the case of AAMF a stable antibody-glycoprotein complex, with increased capacities to fuse with the cell membrane. It seems unlikely however, that this is the only factor responsible for the observed enhancement, since the association between SU and TM is also high for primary HIV-1 isolates which are neutralized by sCD4 and glycoprotein specific antibodies, albeit to a lesser extent than TCLA HIV-1 strains[25]. Besides the strong association between SU and TM, the exact nature of the conformational changes induced by sCD4- and antibody binding, are likely to determine the final outcome of the interaction: VN versus AAMF. This hypothesis is supported by the observation that conformational changes after sCD4 binding, like the exposure of the V3 loop, are significantly different between HIV-1 strains susceptible to

neutralization and enhancement[27]. Unlike sCD4 mediated enhancement of HIV-2 entry, recent data on AAMF of primary HIV-1 strains indicate that the interaction of the envelope glycoprotein complex with membrane associated CD4 is still required for entry, since CD4 specific antibodies directed against the SU binding site, blocked the entry that was enhanced by SU specific antibodies (M. Schutten *et al.*, unpublished data). Studies on the conformational changes within the glycoprotein complex induced by CD4 binding, are seriously hampered by the fact that crystal structures of the envelope glycoprotein in it's native conformation and in the fusion attack complex, have sofar proved elusive. The present knowledge about the conformation of the glycoprotein complex is largely limited to the TM part of the glycoprotein complex. Based on peptide inhibition experiments, and in line with the model proposed for the HA2 part of the influenza virus heamagglutinin in it's native form and as a fusion attack complex, Matthews *et al.* proposed a model for the formation of a HIV-1 TM fusion attack complex[33-37]. In this model the formation of a central coiled coil core structure by the leucine zipper regions of the TM glycoprotein, is an essential step in the fusion process. Formation of this central core in the native state is inhibited by the putative distal alpha helix[38]. In parallel with the HA2 model, this alpha helix may be part of the central core in the fusion attack complex by placing itself on top of the central core, thereby bridging the space between viral and cellular membranes[36]. Insertion of the fusion related sequence into the adjacent membrane is thought to destabilize the cell membrane, which eventually allows fusion of both membranes to take place. It may be anticipated that the conformational changes induced by the binding of sCD4, in the glycoprotein complex of HIV-1, HIV-2 and SIVagm strains susceptible to sCD mediated enhancement, parallel those induced by membrane associated CD4 which lead to the induction of the fusion attack complex[31,32]. Further studies the conformational changes and aspects that determine the difference in susceptibility to either VN or AAMF, may provide valuable information to resolve the molecular mechanism of entry.

MOLECULAR MECHANISMS UNDERLYING LENTIVIRUS ENTRY AND SUSCEPTIBILITY TO VN OR AAMF

The molecular mechanism of envelope glycoprotein mediated entry has been studied most extensively for the human lentiviruses HIV-1 and HIV-2. which both use the CD4 molecule as primary receptor. Primary NSI HIV-1 and HIV-2 strains require expression of one of the β chemokine receptors CCR-2A, CCR-3 or CCR-5 to infect $CD4^+$ cells[39-41]. TCLA and primary SI strains may also infect $CD4^+$ cells that express the α chemokine receptor CXCR-4[42]. HIV-1 and HIV-2 strains may utilize more than one of these molecules as second receptors. Binding of the envelope glycoprotein complex to CD4 induces conformational changes within the SU and the CD4 molecules allowing regions of both molecules to bind to one of these second receptor molecules[43,44]. Several HIV-2 strains may also infect CD4 -cell lines by a direct interaction with CXCR-4, whereas other HIV-2 strains may infect these cell lines after preincubation with sCD4[45]. We have recently shown that CD4 independent infection via CXCR-4 of a HIV-2 isolate was dependent on one amino acid change, A526T, close to the predicted coiled coil domain of TM[46]. This amino acid change was essential but not sufficient for CD4 independent entry. Changes flanking the V4 domain of this HIV-2 SU glycoprotein, in combination with the A526T TM change, on the other hand, also increase the susceptibility to sCD4-mediated enhancement of infectivity, presumably by lowering the "activation threshold" for conformational changes leading to the fusion process[46]. A fourth mutation in V3, when combined with the TM and V4 changes, further increases

the susceptibility to sCD4 mediated enhancement and allows efficient infection of some CD4 negative cells[46]. These four amino acid changes therefore produce an envelope protein complex which is easily triggered into the "fusogenic" confirmation, perhaps akin to those influenza virus mutants which are capable of fusing at a higher than normal pH[47]. The CD4-independent tropism of some HIV-2 strains may therefore be linked to the increased propensity of their envelope glycoproteins to "flip" into a conformation, which allows them to bind to CXCR-4 independent of the conformational changes induced by CD4. In HIV-2, the same mutations which increase RAMF also allow CD4-independent entry, and the two phenomena therefore seem to be related, or in fact, identical.

Several differences have been observed between sCD4 mediated enhancement of HIV-1 and of HIV-2. First sCD4 enhanced entry of HIV-1 infection still requires the interaction of the envelope glycoprotein complex with membrane associated CD4[44] (see above). Second, sCD4 and antibody mediated enhancement of HIV-1 does not allow these strains to bind to CXCR-4 (C. Guillon, manuscript in preparation). sCD4 and antibody mediated enhancement however seems to increase the affinity of the envelope glycoprotein complex for different members (CCR-2 and CCR-5) of the β chemokine receptor family[43]. For primary HIV-1, domains of the glycoprotein complex determining the susceptibility to VN or AAMF, have only been defined to a limited extent. However, we have recently shown that analogous to HIV-2, one or more domains of SU are involved in determining this susceptibility[48]. An interaction of the V3 domain with an as yet unidentified domain within the C3-V5 region were shown to be involved in determining the susceptibility to VN or AAMF. Mutations in the V3 loop of HIV-1, as well as a region encompassing V4-V5 have been shown to contribute to the CD4-independent tropism for some neural cell lines of certain HIV-1 strains[49]. It is not known whether, as for HIV-2, the same mutations are responsible for AAMF/RAMF and CD4-independent tropism.

Although in other than primate lentiviruses, little is known about the mechanism governing viral entry, VN and AAMF, and the regions involved in these phenomena, some interesting similarities between the FIV and the primate lentiviruses have recently been identified. Similar to HIV-1 and HIV-2 also FIV utilizes members of the β chemokine receptor family[50]. We and others have shown, that like in HIV-1, the ability of FIV to replicate in established cell lines, is determined by the V3 region of SU[51,52]. Furthermore, preliminary *in vitro* data show the existence of a mechanism of enhancement different from Fc-ADE and C'-ADE in the FIV system. Using a series of two highly homologous molecular FIV clones and chimeras constructed on the basis of these clones, we demonstrated that the V4 and V5 regions, are also involved in determining the susceptibility to VN[52,53] versus antibody mediated enhancement of infectivity (Siebelink *et al.*, unpublished data).

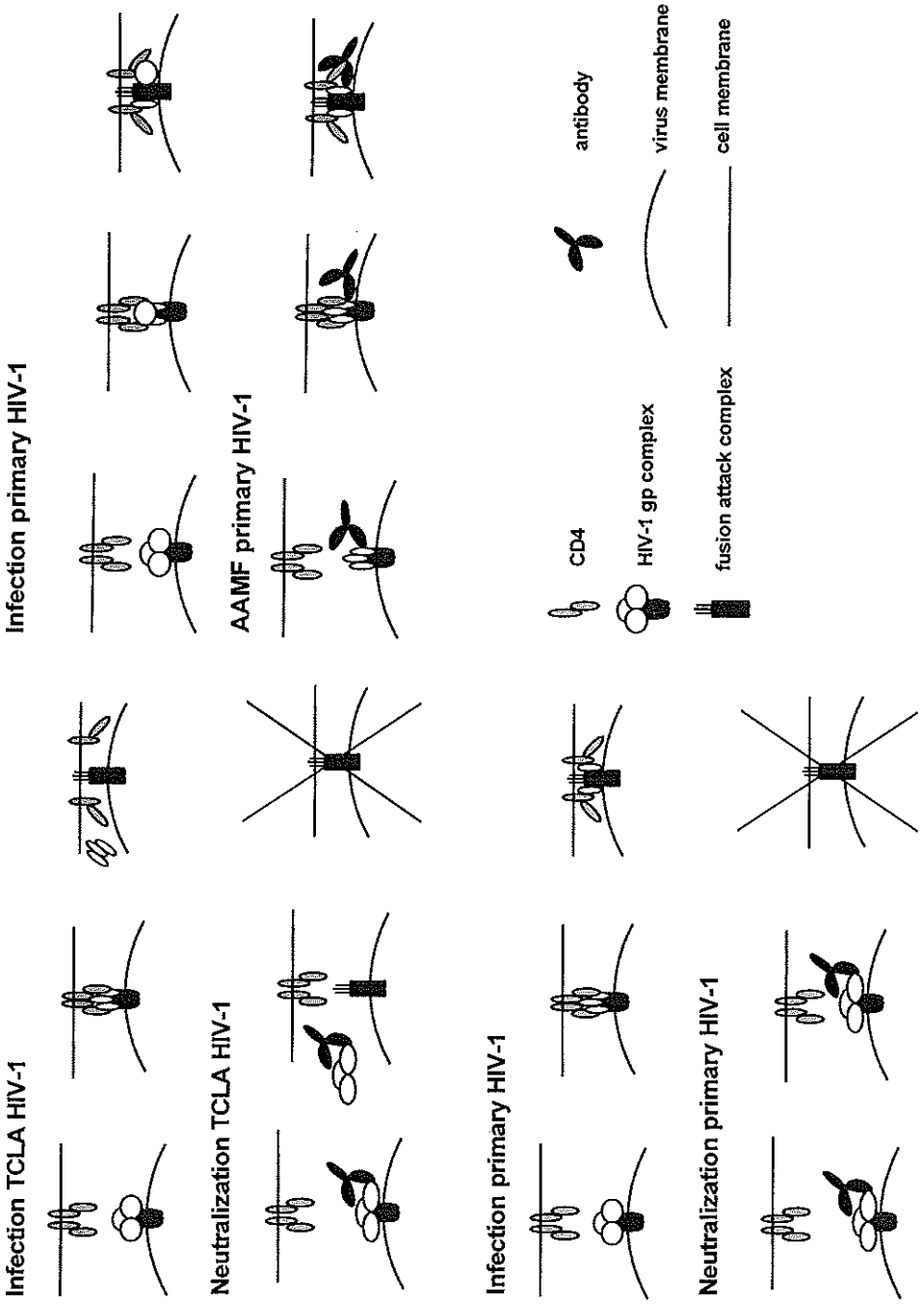
Collectively the *in vitro* data concerning the mechanism underlying VN and AAMF, indicate major similarities between the respective lentivirus systems. The molecular mechanisms underlying CD4 activated virus-cell fusion seem to govern the apparently closely related phenomena of cell tropism, syncytium induction and susceptibility to antibody mediated neutralization and enhancement of virus infectivity in these systems (Figure 1). The conformation of the lentivirus glycoprotein complex determines the ability to infect different cell types, most probably by allowing the glycoprotein complex to bind to different members of the recently identified C-C and α and β chemokine receptor families. Furthermore, changes in the SU glycoprotein seem to determine the nature of the conformational changes induced by sCD4 and antibodies and thereby the susceptibility of these lentiviruses to either VN or AAMF.

ANTIBODY MEDIATED ENHANCEMENT OF LENTIVIRUS INFECTIONS *IN VIVO*

Although studies in *in vitro* systems may provide valuable information about the interaction between antibodies and replicating viruses, it should be realized that the *in vivo* situation is generally more complicated. For example, we and others have shown that the use of established T cell lines or mitogen stimulated peripheral blood mononuclear cells, to isolate and propagate HIV-1 and FIV, may lead to the selection of viruses that are genotypically different from cell free virus found *in vivo*[54,55]. Our current knowledge on the role of antibody mediated VN and enhancement of lentivirus infectivity in the pathogenesis of lentivirus infections and in vaccination studies is limited. This is largely due to the lack of relevant experimental *in vivo* models. Vaccine studies in the EIAV and FIV animal models, have shown that the induction of enhancement of lentivirus infectivity by experimental vaccination may be a problem. The first study, clearly showing antibody mediated enhancement of lentivirus infectivity *in vivo*, used monomeric EIAV SU based vaccine preparations to vaccinate ponies[8]. After EIAV challenge the ponies which had developed SU specific antibodies, developed viraemia and disease most rapidly. Similarly we have shown that domestic cats vaccinated with certain candidate FIV vaccines developed an accelerated viraemia after FIV challenge as compared to the controls vaccinated with non-FIV related SIV glycoproteins[9]. This enhancement effect could be transferred to naive kittens with plasma collected at the day of challenge, suggesting that it is indeed antibody mediated. Although the mechanisms of enhancement in both systems has not been conclusively identified yet, it is interesting to note that with infectious molecular clones of FIV, we identified a mechanism of enhancement that closely resembles AAMF (see above). Therefore we speculate that this mechanism of enhancement has played a role in our FIV vaccination experiments. However, we cannot fully rule out that also Fc-ADE or C'-ADE have played a role in the observed enhancement, since intramuscular FIV challenge infection was used in these experiments. For HIV-1 it has been shown that in contrast to a intravenous infection, this route favours the replication of macrophage-tropic NSI viruses[56]. If indeed this would also be true for FIV infection, the replication of FIV strains susceptible to FC'-ADE or C'-ADE would increase in the presence of FIV specific Fc-ADE or C'-ADE mediating antibodies induced by vaccination.

It is likely that enhancement of virus infectivity by envelope glycoprotein specific antibodies, plays a role in the natural pathogenesis of lentivirus infections. There are indications that the currently used HIV-1 isolation procedures favour replication of viruses susceptible to VN rather than those susceptible to enhancement *in vivo*[55,57]. Although only few studies have analysed amino acid sequence differences of *in vivo* cell free viruses with viruses that can be isolated *in vitro* from the same individual, it has been shown that SI HIV-1 strains are only rarely found *in vivo* as cell free viruses, even in the later stages of the disease when the immune system is seriously compromised[55,57]. Viruses isolated from these individuals however consist of only SI strains in approximately 50% of the cases. It was furthermore shown that virus strains isolated *in vitro* from serum or plasma were recognized with low affinity by antibodies present in the body at the same time, whereas the *in vivo* cell-free viruses were recognized with high affinity[55]. This makes it unlikely that the circulating viruses are neutralized by those antibodies *in vivo*. Moreover the HIV-1 strains isolated *in vitro* seem highly susceptible to VN *in vivo*. The observation that in a HIV-1 infected individual, SI strains constitute approximatelly 50% of the strains found in monocytes and only 0.5% of the strains found in T lymphocytes, indicates that the NSI strains that are isolated less frequently *in vitro* in the absence of antibodies, require HIV-1 specific antibodies for optimal

Figure 1


Infection TCLA HIV-1
Neutralization TCLA HIV-1
Infection primary HIV-1
Neutralization primary HIV-1
Infection primary HIV-1
AAMF primary HIV-1
CD4
antibody
HIV-1 gp complex
virus membrane
fusion attack complex
cell membrane

Legend to Figure 1

A simplified model of TCLA and primary HIV-1 entry, VN and AAMF. For simplicity reasons the requirement of multiple envelope glycoprotein/CD4 interactions and the involvement of the second receptor are not depicted in this model.
TCLA HIV-1: HIV-1 envelope glycoprotein-membrane associated CD4 interactions have been shown to result in the induction of conformational changes in the envelope glycoprotein complex eventually leading to the formation of the fusion attack complex and shedding of gp120 from the complex. Insertion of the fusion related sequence of HIV-1 gp41 into the cell membrane is thought to destabilize the cell membrane allowing fusion to take place. HIV-1 gp120 V3 loop specific antibodies induce conformational changes within the envelope glycoprotein complex leading to shedding of gp120. This in turn leads to the irreversible inactivation of the virus particle.
Primary HIV-1 susceptible to VN: The conformational changes of primary HIV-1 strains susceptible to VN largely seem to parallel those of TCLA HIV-1 strains although some differences may be observed depending on the configuration of the different domains involved in the fusion process (V1/V2, V3 and amino acids 610-701). Primary HIV-1 strains do not readily shed gp120 after sCD4 and antibody binding. Neutralization of primary HIV-1 is therefore thought to be the result of the premature induction of conformational changes leaving the envelope glycoprotein complex unable to exert further functions.
Primary HIV-1 strains susceptible to AAMF: A relatively low efficiency of entry into the target cell of these HIV-1 strains as compared to TCLA and primary HIV-1 strains susceptible to VN is thought to result from the inefficient induction of conformational changes within the envelope glycoprotein complex by membrane associated CD4. Binding of gp120 specific antibodies and subsequent cross-linking (not represented in the figure) leads to the induction of a stable complex with increased capacity to mediate fusion after binding to membrane associated CD4.

infection of T lymphocytes[57]. Collectively, these findings suggest that antibody mediated enhancement, indeed favours the selection *in vivo* of a population of viruses susceptible to enhancement, with phenotypic characteristics different from those isolated *in vitro*. Therefore it is reasonable to postulate that their *in vivo* role has been underestimated sofar.

Most antigen preparations currently evaluated in vaccination strategies against HIV-1, are based on the complete gp120/gp160 proteins or alternatively on fragments containing antigenic sites, which elicit antibodies that neutralize certain HIV-1 strains. However, as discussed above, these antibodies may also be involved in the enhancement of the infectivity of certain virus strains. Therefore it seems more appropriate, in the framework of vaccine development activities, to pay more attention to the elucidation of the molecular mechanism which determine whether entry is blocked or enhanced by glycoprotein complex specific antibodies. Especially understanding the changes within the glycoprotein complex and CD4, which allow the process of virus-cell fusion to take place, may lead to the identification of antibodies which interfere with crucial events in this process. This would probably lead to a more rational approach for the induction of truly VN antibodies in the framework of lentivirus vaccine development.

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Samenvatting

De inductie van HIV-1-specifieke antilichamen die infectie met HIV-1 kunnen voorkomen of verspreiding van het virus gedurende de primaire fase van de infectie limiteren, is waarschijnlijk een belangrijke eigenschap van een te ontwikkelen succesvol vaccin tegen HIV-1. De virusneutraliserende activiteit van HIV-1-specifieke antilichamen wordt onder andere bepaald door aspecten van het virus. Daarnaast is deze activiteit afhankelijk van de celcultuur waarmee de virusneutralisatietest uitgevoerd word. Het primaire doel van het in dit proefschrift beschreven onderzoek is om meer inzicht te verkrijgen in de factoren die de uitkomst van de interactie tussen antilichaam en virus bepalen. Hiertoe werd onderzoek uitgevoerd naar de moleculaire mechanismen waarmee HIV-1 zowel in de af- als aanwezigheid van HIV-1-specifieke antilichamen $CD4^+$ cellen infecteert. Daarnaast werd een model voor *in vivo* HIV-1-infectie opgezet dat diverse aspecten gemeen heeft met primaire HIV-1-infectie in de mens. In dit model werd de antivirale activiteit van HIV-1-specifieke antilichamen bestudeerd.

In de introductie wordt aan de hand van een overzicht van de internationale literatuur over HIV-1 achtergrondinformatie gegeven over aspecten van het virus die betrekking hebben op de in dit proefschrift beschreven onderzoeken.

In hoofdstuk 2 worden humane monoclonale-antilichamen (HuMAb's) beschreven die een antigeen gebied op HIV-1 gp120 herkennen, dat overlapt met de CD4-bindingsplaats. Deze HuMAb's herkennen HIV-1-stammen die tot verschillende subtypen behoren. Tevens wordt aangetoond dat aminozuren, gelokaliseerd in verschillende constante regio's van gp120, een bijdrage leveren aan het tot stand komen van de epitopen die herkend worden door deze HuMAb's. De HuMAb's bleken tevens in staat om HIV-1-stammen die meerdere malen *in vitro* gepasseerd zijn in geïmmortaliseerde T-cellijnen, te neutraliseren.

In hoofdstuk 3 wordt de karakterisering van een HuMAb beschreven dat is gericht tegen het derde hypervariabele (V3)-domein van HIV-1 gp120 (HuMAb MN215). Er is uitvoerig onderzocht welke aminozuren in het V3-domein van HIV-1 bijdragen aan de binding van HuMAb MN215. Tevens is de bindingssterkte bepaald van HuMAb MN215 met monomeer glycoproteinen die afkomstig zijn van HIV-1-stammen met verschillende biologische fenotypen. Tenslotte is de virusneutraliserende activiteit tegen primaire HIV-1-stammen vastgesteld. HuMAb MN215 bleek in staat te zijn om te discrimineren tussen niet-syncytiuminducerende (NSI) en syncytium-inducerende (SI) HIV-1-stammen.

De DNA-sequentieanalyse van de variabele gedeeltes van de zware en lichte ketens van de HuMAb's worden beschreven in hoofdstuk 4. Ook worden de mogelijke consequenties hiervan besproken voor enerzijds de inductie van een immuunrespons gericht tegen HIV-1 in geïnfecteerde individuen en anderzijds de pathogenese geïnduceerd door HIV-1-infectie.

In de hoofdstukken 5 en 6 worden experimenten beschreven die tot doel hebben meer inzicht te verkrijgen in de manier waarop HIV-1 envelop-specifieke antilichamen het proces van envelop gemedieerde *entry* moduleren. Zowel de oplosbare vorm van de primaire receptor voor HIV-1 (sCD4) als HIV-1 envelopspecieke antilichamen die in meer of mindere mate in staat bleken SI-virusstammen te neutraliseren, bleken een verhoogde capaciteit van NSI-envelop gemedieerde *entry* teweeg te brengen. De gevolgen van antilichaam/antigeen-interactie in biologische zin (neutralisatie versus verhoging van infectiviteit) bleek bepaald te worden door de configuratie van specifieke regio's op gp120 enerzijds en inter/intramoleculaire-interacties anderzijds. De door *cross-linking* geïnduceerde veranderingen in het envelopeiwit, die leidden tot verhoging van infectiviteit, bleken een minimale energiebehoefte te hebben.

Om meer inzicht te verkrijgen in de virusneutraliserende capaciteit van HIV-1-specifieke antilichamen in de mens, werd een chimeer mens/muis-model (xeno-GvHD muismodel) voor *in vivo* HIV-1 infectie opgezet (Hoofdstuk 7). Bij het ontwikkelen van dit model werd nadrukkelijk rekening gehouden met aspecten die van belang worden geacht voor HIV-1-infectie in de mens. Antilichaampreparaten die *in vitro* zowel T-cellijn-geadapteerde, als primaire HIV-1-stammen neutraliseerden, bleken in het xeno-GvHD muismodel alleen in staat de T-cellijn-geadapteerd HIV-1-stammen te neutraliseren. De primaire HIV-1-stammen bleken in hoge mate resistent te zijn tegen neutralisatie.

In de algemene discussie in hoofdstuk 8 wordt verder ingegaan op de implicaties van de studies die zijn beschreven in dit proefschrift, voor *in vivo* HIV-1-infectie in de mens en voor HIV-1 vaccinontwikkeling. De belangrijkste conclusie die getrokken kan worden, is dat verhoging van infectie *in vivo*, geïnduceerd door HIV-1-specifieke antilichamen opgewekt met kandidaatvaccins, als een reëel probleem gezien dient te worden. Het is daarom belangrijk om in het kader van onderzoek aan HIV-1 vaccinontwikkeling speciale aandacht te besteden aan de moleculaire mechanismen van *entry* van primaire HIV-1-stammen. Een beter inzicht in deze moleculaire mechanismen kan leiden tot een rationelere ontwikkeling van antigeenpreparaten die in staat zijn om antilichamen te induceren die HIV-1-stammen met verschillende fenotypen zowel *in vitro* als *in vivo* kunnen neutraliseren.

Curriculum Vitae

Martin Schutten werd geboren op 20 januari 1965 te Purmerend. In 1984 werd het OVWO diploma gehaald aan de Rijks Scholen Gemeenschap Noord Kennemerland in Alkmaar. Datzelfde jaar werd aangevangen met de propaedeuse Biologie aan de Universiteit van Amsterdam, welke in 1985 afgerond werd. Na deze propaedeuse werd begonnen aan de vrij doctoraal studie Medische Biologie aan de Universiteit van Amsterdam. Tijdens deze studie werd een stage van 11 maanden gelopen bij het Laboratorium voor Klinische (Viro) Immunologie van het Centraal Laboratorium voor de Bloedtransfusie Dienst te Amsterdam (stagebegeleiders Prof. Dr F. Miedema en Dr R. Gruters). Februari 1990 werd de vrij doctoraal studie afgerond waarna aansluitend gestart werd als onderzoeker in opleiding aan het Laboratorium voor Immunobiologie van het Rijks Instituut voor de Volksgezondheid en Milieuhygiëne in Bilthoven (hoofd Prof. Dr A.D.M.E. Osterhaus). Vanaf april 1994 is hij werkzaam als wetenschappelijk onderzoeker aan de Afdeling Virologie van de Erasmus Universiteit te Rotterdam (hoofd Prof. Dr A.D.M.E. Osterhaus).

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