CHAPTER 4: DISCUSSION

4.1 Importance of the quality of antigen preparations

Isolation of potentially useful mAbs is a lengthy selection process where the nature of the antigen used to screen is crucial. Preparation of antigens requires the production of large amounts of inactivated viruses. By using cell lysates derived from HIV-1 subtype C infected cell lines as the antigen source in our ELISA screening system, we aimed to capture viral particles in their near native state as far as possible. These viral particles may expose novel epitopes similar or identical to an *in vivo* situation in HIV-1 infection. We deliberately avoided purification of viruses as this process may result in significant losses of envelope proteins, e.g. trimeric gp120/41. So, using purified protein preparation may be inefficient in detecting antibodies to trimeric gp120/41 proteins and favour detection of other HIV antigens that may be present as a result of debris from shedding of trimeric structures. Since antigen preparation may contain heterogeneous population of fully, partially and non-denatured molecules, it was important to compare proteins prepared within the same ELISA screening system to establish identical standardized conditions for the antigen stocks. The choice of virus strain used as the source of envelope glycoprotein antigens for antibody screening is an important aspect of monoclonal antibody selection and production. Our approach for screening for new and novel antibodies from culture supernatant of EBV-transformed cells was to use antigen preparations derived from cell lysates.

We used the antigen preparations from various viruses in an ELISA screening, and the quality of these antigens was evaluated using A32, 1.7B, C11 and 7B2. The combination of

these mAbs were selected for use as tools for screening because they cover a wide range of epitopes and thus broaden our chance of capturing diverse mAbs. It was considered that further evaluation of the quality of the antigen preparation by other means (i.e. immunoprecipitation, quantitation etc) was unnecessary. Overall, these results suggested that the antigen stock raised from the viruses we used contained at least the C1-C4 and the CD4i epitopes as the viral preparations were able to bind to both A32 and 1.7B antibodies. In addition to binding to A32 and 1.7B, other antigen preparations used in our screening were found to bind well to both C11 and 7B2 (results not shown). Antigen preparations that bound poorly (OD_{450nm} <1.0) to these mAbs such as Du151 cell lysate were not used.

We tested the ability of isolated mAbs to bind to various antigen preparations in several experiments. Here we wanted to determine which antigen stock was suitable, in terms of the binding ability to be able to do further analysis and characterization of the mAbs. Our results indicated that all the Du23 mAbs were broadly cross-reative as they bound all antigen preparations.

4.2 Making human monoclonal antibodies

The identification of immunogenic sites within HIV-1 subtype C envelope glycoprotein and possibly define epitopes that are able to elicit potent neutralising antibodies against primary viral isolates was of special interest in this study. One such approach is by generation of monoclonal antibodies that are able to block HIV infection *in vitro.* These reagents are useful for the identification of the neutralization sensitive epitopes that would provide important information for designing HIV vaccines that aim to induce sterilizing immunity.

We proposed to generate monoclonal antibodies against HIV-1 subtype C epitopes by immortalisation of B cells from HIV-1 subtype C infected asymptomatic patients in the early stage of disease with relatively good neutralising antibody titres (Table 1) using EBV transformation, and then screen for anti-HIV-1 envelope antibodies. We used Triton solubilized viral lysate derived from T cell line infected cultures as antigens source. The rationale for using this type of antigen preparation was to try to keep the viral envelope intact, at its native state as far as possible. The antigen stock contained gp120 and gp41 in dissociated and solubilized states with disrupted trimers. The "native" state therefore would refer to antigen preparations that have not been denatured by reducing agents such as DTT or 2-mercaptoethanol. This design of our screening system limited us in terms of capturing mAbs that could bind to both monomeric and oligomeric forms of glycoproteins. The mAbs were characterized in terms of their binding specificity to the HIV-1 envelope glycoproteins as well as assessing them for their functional ability in blocking HIV-1 infection *in vitro*.

Several experiments were performed, and from one of them 5 cell lines 1.5D, 2.3C, 2.9D, 3.2C and 4.12E were established, and with the exception of 1.5D the other 4 clones were able to grow for months without loss of specificity. The reasons for loss or failure to obtain clones from other transformation experiments are discussed below.

4.3 Difficulties in making mAbs

Making monoclonal antibodies and selection of anti-HIV-1 clones presents many challenges. Before selection of a clone, the cultures are maintained over a long period of time and as such contamination could be one of the main problems. To mimimize this problem, we

maintained our cultures in a combination of antibiotics to suppress both bacteria and mycoplasma. This strategy seemed to have worked since we experienced few cases of contamination and four novel mAbs were successfully selected. Moreover, loss of potential clones could occur during the screening process. Non-producers could overgrow specific clones before anti-HIV-1 specific stable line are selected. Early and repeated subcloning steps, as was done in selecting our clones, is necessary for successful establishment of stable antibody producing cell lines. In our situation, the first subcloning steps were performed with cells in 96-wells at low cell density so that non-producers could not overgrow specific anti-HIV antibody-producing cell lines. In subsequent steps, anti-HIV positive wells were selected at high cell dilution and this sub-cloning step was repeated at least twice so that a cell line producing monoclonal antibodies could be established. Another problem is that cells could die at any stage of culturing.

The characteristic nature of the B-cells from HIV-1 infected subjects and the conditions used to immortalize them apparently affects the number and type of antibody-producing cell lines that grow out. Gorny and coworkers contend that the probability of generating stable cell lines from patients in early stages of disease is higher compared to using source material from those in the late stage of the disease (Gorny et al., 1989). The reactivity of antibodies produced by the transformed cells may also be affected, with cells derived from the sickest patients producing a higher frequency of antibodies that react nonspecifically or polyspecifically with HIV compared to the antibodies from cells derived from patients in whom the disease process is more limited. Moreover, few HIV-1 infected individuals make Nabs which make isolation of broadly neutralizing mAbs a difficult undertaking. Despite our

attempt to select suitable patients (asymptomatic subtype C infected individuals in early stage of disease), we observed considerable differences in transformation rates after EBV infection and they were not reproducible (Table 7).

No HIV-1-specific clones were isolated from the transformation experiments that showed poor transformation efficiencies ranging from 0-35%. About 5-15% of the circulating lymphoid pools are B-cells, defined by the presence of surface immunoglobulin (Roitt et al. 2001). In HIV-1 infected individuals those B-cells making anti-HIV-1-specific antibodies make up an average of 0.2% (range 0.03-0.46%) of these cells (Morris et al., 1998). So, it is conceivable that the pool of B-cells in these samples could have been poor or of low quality.

EBV-transformation is not a very stable system and this could affect efficient establishment and selection of stable anti-HIV-1 cell lines. The cell line may lose the antibody producing capability, either through loss of EBV transformation capability (genes being lost from integrated into the host target B-cell) or class switching. Another problem could be transient activation of lymphocytes by EBV without subsequent transformations. Several researchers indicate that B-cells from HIV-infected individuals are dysfunctional, polyclonally activated and display a low level of infectivity with EBV (Birx, Redfield, and Tosato, 1986; Gorny et al., 1989; Yarchoan, Redfield, and Broder, 1986). This could account, in part, for the results observed here.

In addition to the above, there are several other problems that could be encountered when generating monoclonal antibodies. Poor storage conditions of cryopreserved samples could

result in B cells dying further reducing a pool which is already low. It is reported that some antibody-producing clones could be lost if the quality of feeder cells is poor or if the feeders are not used during the transformation procedure (Birx, Redfield, and Tosato, 1986; Gorny et al., 1989; Yarchoan, Redfield, and Broder, 1986). In our situation, some transformation experiments failed possibly because of poor quality of macrophages. When all of these factors are taken into consideration it can be appreciated that producing stable anti-HIV-1 cell lines from a pool of PBMC derived from HIV-1 infected individuals is a difficult process.

Of the five clones that we generated, 1.5D failed to produce stable and sustained antibodies. A smear was observed for 1.5D sample on polyacrylamide gel. There are several varied reasons that could account for the loss of 1.5D clone. It may be possible that the cells stopped producing antibodies, or EBV-transformation capability was lost. The other factor could be that this cell line may have been overgrown by other cells not making anti-HIV-1 envelope glyocoprotein during the process of sub-culturing. One other way of preventing loss of a clone is to freeze a portion of cells early in the cloning process. However, this was not possible with 1.5D as it was a very slow grower. Where possible, clones could also be fused with suitable partner such as HMMA or CB-F7 to make a stable heteromyloma.

4.4 Characterization of Du23 mAbs

4.4.1 Subclass, purity and specificity

The purity of the Du23 mAbs was determined to be of good quality as there was no indication of contaminating proteins (Fig 16). Only a single antibody concentration of 10 μg/ml was used to determine the purity. Ideally, increasing concentrations gradient of

antibody would normally be used to determine the amount of impurities in the protein, if any, but this was considered unnecessary in the light of the quality of the results obtained. Pure mAbs are important for biotinylation and evaluation procedures. All four Du23 mAbs were determined to be of IgG_1 isotype and contain λ light chain, the predominat subtype in peripheral blood (Table 11) (Roitt et al. 2001).

Data presented here indicate that Du23 mAbs bind specifically to HIV-1 proteins. However, until these mAbs are tested against other reagents such as phospholipids, cardiolipids we cannot at this stage exclude the possibility that these mAbs could be polyreactive (Haynes et al., 2005). This is more a case for 2.9D than for other Du23 mAbs as the competition pattern for this mAb was puzzling. In many experiments 2.9D did not block itself, and yet competed with F91 and 1.5E. The reasons for this unusual phenomenon will be explored later.

4.4.2 Determining viral subtype restriction on binding of Du23 mAbs

We wanted to know if any of the Du23 mAbs could be strains specific in terms of their binding ability; are the epitopes for these mAbs conserved across viral strains and between subtypes B and C? The binding ability of the Du23 mAbs was evaluated against a range of antigen preparations derived from different viral strains and between subtypes B and C. We found that viral subtype did not appear to influence the binding ability of these mAbs. Although a lower OD_{450nm} signal was observed with Du179/STR5 compared to binding to Yu-2 gp120 and ADA, this might be a reflection of higher antigen concentration rather than the subtype of the virus. The protein concentration of the supernatant derived from virus infected cell lines was not determined. Yu-2 gp120 was used in purified form at a

concentration of 20 μg/ml while other viral preparations were used as culture supernatant. The ability of the Du23 mAbs to bind to subtype B SF162, ADA and 89.6 virus preparations and purified Yu-2 proteins was also evaluated. The binding ability of the mAbs to all of these antigen preparations was found to be relatively good, with both STR5/Du179 and the control v89.6 giving comparable results. Moreover, the Du23 mAbs were found to bind regardless of the viral subtype or strain from which the antigen preparation was derived. This showed that the epitope for all four Du23 mAbs are not restricted but are conserved across the subtypes B and C viral strains.

4.4.3 Reaction of clones to reduced and non-reduced gp120 protein

For the selected antigen preparations we observed that the Du23 mAbs required intact proteins for their binding. All four Du23 mAbs were able to bind well to conformationally intact protein preparations but failed to bind to proteins reduced by DTT. Thus conformational integrity of the protein is required for the Du23 mAbs to bind to their respective epitopes. Many gp120 mAbs that have been described in the literature are discontinuous and this presents challenges in identifying epitopes. Our observation with the Du23 mAbs may indirectly indicate that these mAbs bind to discontinuous epitopes on the intact protein.

4.4.4 Epitope mapping

4.4.4.1 Mutagenesis approach

One approach in elucidating the antibody binding site on gp120 is to examine smaller, biologically active fragments that may be more amenable to structural analysis, as has been

reported (Jeffs et al., 1996). However, in some instances deletion of part(s) of envelope protein may result in a series of incorrectly folded proteins. For instance, deletion of some NH_2-terminal amino acids results in a series of incorrectly folded proteins (Jeffs et al., 1996; Jeffs et al., 2002). Wyatt and coworkers reported that a V1/V2/V3 envelope deletion mutant of HXB2, expressed at the cell surface, bound CD4BS mAbs with up to 50-fold higher affinity than full-length protein (Wyatt et al., 1993). Hence, the variable loops mask some discontinuous conserved epitopes in both oligomeric and monomeric gp120. Most of these residues are unlikely to be directly involved in CD4 binding, but their presence may be required either to form supporting structures essential to maintain the conformation of the binding site or may be critical for the folding of the molecule into active conformation. The observation that most of the conserved sequences are required to maintain functional folding is consistent with the concept that many of these regions may be buried in the native structure. This further suggests that removal of variable regions may lead to enhanced exposure of conserved gp120 regions, which may normally be exposed only by conformational changes accompanying the fusion process. Thus, truncated forms of the gp120 molecule may be useful for vaccine development and may induce antibodies capable of recognizing epitopes conserved between divergent viral isolates.

We have used some of these approaches in order to map the binding site of the Du23 mAbs on gp120, particularly subtype C derived envelope. We found that most of the Du23 mAbs partially competed with A32 mAb and that C1 region is required for the binding of this mAb. We therefore sought to determine the extent of similarity or differences of the Du23 mAbs with A32 by deleting C1 and V1/V2 regions from Du151 gp150 protein to determine the

effect of such deletion on the binding ability of the mAbs. The binding of the mAbs 2.3C, 3.2C and 4.12E were shown to be affected by the deletion of C1 region whereas 2.9D was not. Since the epitope for A32 is C1-C4 as confirmed here, and based on our results, it it fair to conclude the epitope for all the Du23 mAbs is at least defined by C1 region.

The binding of all four Du23 mAbs was not abolished by the deletion of V1/V2 regions. Instead, there was 40-60% increase in binding ability of the Du23 mAbs when V1/V2 regions were deleted from Du151 gp150. This characteristics features have been described for A32 (Wyatt et al., 1995). In many respect the Du23 mAbs have shown features similar to A32, although they are different in some respect. For instance, the Du23 mAbs competed with F91 and 1.5E and yet A32 did not. The increased binding ability of the Du23 mAbs on Du151 gp150 with deleted V1/V2 suggested that the epitopes of these mAbs are better exposed with the deletion of the regions. The role of the variable loops in masking discontinuous conserved epitopes in both oligomeric and monomeric gp120 has been discussed. The binding pattern as observed for the Du23 mAbs suggests that removal of variable regions may lead to enhanced exposure of conserved gp120 regions, which may normally be exposed only by conformational changes accompanying the fusion process.

4.4.4.2 Competition analysis

Characterization of mAbs in a cross-competition pattern may indicate whether the test mAbs have shared epitope with others, or there is interference due to steric hindrance, of binding of one mAb to gp120 results in conformational changes that affect the binding of the other. It was not clear from our results which of these modes of competitions apply. Nonetheless, it

was interesting to note that A32 did not compete with F91 while Du23 mAbs showed reciprocal blocking with F91. This data indicated that the 2.3C, 2.9D and 4.12E mAbs while appearing similar to A32, they are in actual different mAbs with unique binding specificities. However, the Du23 mAbs did not compete with 1.7B and yet showed reciprocal competition with 1.5E. None of the Du23 mAbs were V3-specific antibodies as they failed to compete with any of the V3 mAbs (1.9B, 2.1E, 3.9F and KX16). All the Du23 mAbs showed reciprocal competition with a CD4BS mAb F91, which suggested that the binding sites may be proximal to each other. We have also found that the F91 mAb showed non-reciprocal competition with mAbs 1.7B (CD4i) and 1.5E (CD4BS). EH21 (N-terminus) or 2.3G (C-terminus) mAbs showed reciprocal competition with their respective counterparts, but did not compete with the Du23 mAbs or other subtype B-derived mAbs. These results are summarized in Table 18 below. It can be concluded that the binding sites for the Du23 mAbs require at least the presence of a C1 region. Furthermore, removal of V1/V2 regions results in better exposure of the binding sites for the Du23 mAbs.

2.9D mAb presents a different situation. Although this mAb showed reciprocal blocking with F91 and 15E, it could not block itself (non-reciprocal competition, beige coloured) in all cross competition we conducted. This lack of identical reactivity of unlabeled mAb with its biotinylated counterpart could suggest inherent problems with the mAb. Possibly, there was something wrong with the biotinylated batch we used at the time. However, this could not be confirmed as there was no other available sample. That repeated results consistently showed similar results with biotinylated 2.9D possibly suggested that this was a low affinity antibody. But then unlabeled F91 and 1.5E were able to block biotinylated 2.9D (Tables 12 and 14).

One of the indicators of clonality used in the screening system was the ability of the clone to show consistent results in terms of reactivity. To some extent this appears to have been demonstrated with 2.9D. An absolute assurance in this regard is actually re-cloning of 2.9D at 1 cell per well. This may be relatively easily achievable if a hybridoma is made by fusion of an appropriate partner. On the other hand, 2.9D may be a polyreactive antibody, binding to other proteins as well as to gp120. However, that it was able to block F91 and 1.5E suggested some specificity. It is also conceivable that the biotinylated form of 2.9D may have been contaminated with other mAb during the biotinylation process or the additives BSA may have affected the mAb in some other way. Until all other possibilities are explored, 2.9D remains a problematic clone to characterize.

Table 18. Summary of cross-competition analysis of the Du23 mAbs.

Biotin mAb		Relative reactivity with selected mAbs										
		2.3C	2.9D	3.2C	4.12E	A32	1.7B	1.5E	F91	EH21	2.3G	V3: 2.1E, KX16, 3.9F, 1.9B
B.2.3C		1	1	1	1	1	0	1	1	0	0	0
B.2.9D		2	2	2	2	1	0	1	1	0	0	0
B.4.12E		1	1	1	1	1	0	1	1	0	0	0
B.A32	C1-C4	2	2	2	2	1	2	0	0	0	0	0
B.1.7B	CD4i	3	3	3	3	3	1	2	2	0	0	0
B.1.5E	CD4BS	2	2	2	2	0	1	1	1	0	0	0
B.F91	CD4BS	1	1	1	1	0	2	1	1	0	0	0
B.EH21	N-terminus	0	0	0	0	0	0	0	0	1	0	0
B.2.3G	C-terminus	0	0	0	0	0	0	0	0	0	1	0
B.V3	2.1E, KX16, 3.9F, 1.9B	0	0	0	0	0	0	0	0	0	0	1

1. The biotinylated mAbs are listed vertically (y-axis) & unlabeled across (x-axis); V3 means all the V3 loop mAbs considered together.
2. The number 1 (yellow boxes) indicate that competition is complete and reciprocal (i.e. "A", on horizontal, blocks B, on vertical, and B blocks A); 2 (tan boxes) mean one-way non-reciprocal competition (whereby "A", blocks "B", but B does not block A); 3 (orange boxes) mean binding of unlabeled "A" enhanced binding of biotinylated "B"; 0 indicates no significant competition each way.

4.4.4.3 Effect of sCD4

Here we wanted to determine if any of the Du23 mAbs share sites with CD4BS mAbs. If not are the epitopes induced (CD4i) or better exposed upon sCD4 binding to gp120? The effect of sCD4 on the binding ability of the Du23 mAbs to gp120 was evaluated in several experiments. We observed that none of the Du23 mAbs was blocked by binding of sCD4 to gp120, and therefore they were found not to be CD4BS mAbs. None of the Du23 mAbs competeted with 1.7B, but the available data could not conclusively determined if any of the mAbs are CD4i. Soluble CD4 showed enhanced binding of the Du23 mAbs, suggesting that the binding of sCD4 to gp120 results in conformational changes that better exposes the epitopes for the mAbs.

4.4.5 Neutralization

Monoclonal antibodies capable of suppressing viral replication, that is Nab, are important because they can be used to guide vaccine development. In our study, it was envisaged that we will be able to generate such NAb. Our aim was that such antibodies could provide some light into defining epitopes in subtype C virus strains that could provide useful information in designing subtype C based vaccines. We used two different assays to evaluate neutralization activity of the isolated mAbs: R5 cell line grown viruses and pseudovirion assays. One could argue that the R5 (T-cell line grown) viruses are more comparable to primary viruses. There are differences in sensitivity to antibody-mediated neutralization in conventional assays between T-cell line strains and primary isolates of HV-1, but less is known about R5 grown viruses. The R5 strains are reported to be highly sensitive to neutralization and primary isolates are difficult to neutralize (Sawyer et al., 1994; Zolla-Pazner et al., 1995). It had been

a major challenge to HIV-1 vaccine development to explain why this is the case and what it means, and to determine whether it is real or merely a reflection of artificial design of *in vitro* assays.

There appears to be significant differences in the conformations of native oligomer between primary viruses and TCLA viruses. The accessibility of many epitopes on gp120 is said to be reduced on the native oligomer compared with monomeric gp120. The inaccessibility is shown to be more pronounced for primary viruses than for TCLA viruses. Antibodies to C1 and C5 regions have been found to react strongly with monomeric gp120 but not native oligomer, consistent with the idea that residues from these regions are involved in the interaction between gp120 and gp41 (Helseth et al., 1991). Why there is a strong human humoral response to these regions if they are sequestered on native virions? It is argued, as with gp41, that this reflects immunization with viral debris such as unprocessed gp160 where C1 and C5 regions are exposed, rather than native virus. The inaccessibility of many other gp120 epitopes in native oligomer has been shown not to be as great as that for the C1 and C5 regions, producing decreased affinity for the oligomer rather than abrogation of antibody binding. It is thought that his decreased affinity is likely to diminish neutralization potency. Antibodies elicited by gp120 in conformations other than the native envelope (unprocessed gp160 or shed gp120) may have high affinity for the eliciting antigen but lower affinity for native envelope and only poor neutralization ability.

It is therefore important to keep the above background information in interpreting neutralization data. Using the Du23 virus, all the Du23 mAbs including the control mAb

2.1E showed partial neutralization activity of 40-57%. This is rather low when compared to neutralization activity of >70% that is usually reported for 2G12, b12, 2F5 an 4E10 (Binley et al., 2004). Given the nature of the viruses used, it becomes problematic to interpret this data. Since the potency of antibodies is assessed by estimating the concentration required to reduce virus infection by 50%, 80% or 90% (Moore and Burton, 2004). These are taken as convenient concentrations to measure, and the biological value of achieving only 50% neutralization (inhibition) is said to be questionable. Against this background, it is safe to conclude that the Du23 mAbs reflected limited neutralization. For both Du151 and Du179 neutralization activity for the Du23 mAbs including the 2.1E mAb was between 42-69% with 3.2C mAbs showing neutralization of 29%. On the other hand, much lower neutralization activities of 2-16% were observed using SF162 virus for all the mAbs. It this stage, it is not clear why this discrepancy in the neutralization profile of the Du23 mAbs between subtype C versus subtype B viruses since the Du23 mAbs were shown not to be restricted in their binding abilities. But again there appear to be some differences in the range of neutralization profile within subtype C viruses. The control mAb 2.1E showed expected 98% neutralization activity with SF162 (subtype B) while in general this mAb gave a limited neutralization profile of around 40% with subtype C viruses. Neutralization results from two-fold viral dilutions and controls were consistent and reproducible as most of these experiments were repeated more than twice. Up to 30% neutralization was indicated with Du151.2 pseudovirions. This is low compared to the neutralization observed with the cell line grown viruses. It is possible that this inconsistency is related to the differences in the nature of the viruses used. Perhaps the pseudovirion assay in this particular case is not sensitive enough to detect Du23 mAbs neutralization or the differences in the Env sequence in the pseudovirion

and the cell line grown viruses account for discrepancies. The passage history of the viruses in which prolonged culturing could have rendered the viruses progressively more neutralization sensitive hence the apparent higher antibody inhibition as observed with Du23 mAbs in cell line grown viruses than in pseudovirion assay (Beaumont et al., 2004; Pugach et al., 2004). Such differences have also been reported in which there was neutralization in the pseudovirion assay but not in the PBMC assay (Binley et al., 2004). Neutralization work by binding of mAb(s) to the native, trimeric Env complex on the surface of infectious virions, thereby impairing the ability of viruses to infect target cells (Klasse and Sattentau, 2002; Parren and Burton, 2001). The Env on the surface of the pseudovirion may be derived from a molecular or biological clone different from that of highly passaged cell line grown viruses.

In summary, this data could indicate that neutralization sensitivity of the Du23 mAbs may be affected by the presence or absence of the relevant epitope(s) on the virion envelope, the exposure of the epitope on the intact virion, and the affinity of the antibody to the virus used. Additional factors that may influence neutralization sensitivity are density and or number of Env oligomers on the surface of a given virus. All of these factors contribute to the shape of the neutralization curve and the ultimate outcome of Ab-virus interactions (Parren et al., 1999). Given the available data, these Du23 mAbs could be considered non-neutralizing because they showed limited potency in both cell line grown viruses and pseudovirion assay.