Chapter 2

Humoral Anti-HLA Response
For Protection Against HIV-1

2.1 Review of literature

2.2 Objective

2.3 Study design

2.4 Materials and methods

2.5 Results

2.6 Discussion

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2.1: REVIEW OF LITERATURE

Immune system is a complex network of specialized cells and organs that has evolved to defend the host against "foreign" invaders such as bacteria, viruses, fungi, and parasites. Immune system does not normally act against self-tissue due to its remarkable capability of distinguishing between "self" and "nonself". Molecules that mark a cell as self are encoded by a group of genes known as Major Histocompatibility Complex (MHC). MHC genes are found in all mammalian species and are recognized by different names such as HLA (Human Leukocyte Antigen system) in human, H2 in mouse, DLA in canines, BoLA in cattles, FLA in felines, ELA in horses, SLA in swines, OLA in sheep and RTI in rats (MHC database; http://www.ebi.ac.uk/ipd/mhc). The molecules encoded by MHC genes show high degree of polymorphism as a result each member of the species carries near unique set of MHC genes. Therefore if a tissue is transplanted from one member to other member of same species (allograft), it is invariably recognized as "foreign".

Alloimmune response

Antigenic determinants that differ among members of same species such as MHC are called alloantigens. Exposure to alloantigens leads to induction of alloimmune response, which consists of cellular (CD4⁺ and CD8⁺ T cell) as well as humoral immune response. Humoral response further leads to antibody dependent cell-mediated cytotoxicity (ADCC) and complement activation.
Therefore before tissue/organ transplantation, careful matching of donor and recipient is vital. In absence of this knowledge, earlier efforts (before 20th century) of tissue transplantation were mostly unsuccessful.

Alloimmune response was first described in 1970 as a Mixed Lymphocyte Reaction (MLR). It was found that during co-culture of lymphocytes obtained from different donors, T cells undergo extensive proliferation and effector CTLs specific to alloantigens are generated. Rejection of transplant due to genetic difference between donor and recipient is example of alloimmune response in-vivo. In some cases, lymphocytes in the graft attack the recipient's tissue thereby causing Graft-Versus-Host disease (GVHD). GVHD is observed if the tissue from immunocompetent animal is transferred to immunosuppressed animal. The grafted lymphocytes proliferate, migrate to different organs and cause damage due to cytolytic mechanisms, which can result in life-threatening affliction.

**Human Leukocyte Antigens System (HLA)**

HLA proteins are classified as class I and II based on their structure and function. Class I protein comprises polymorphic peptide chain of 45 kDa and non-polymorphic peptide chain of 12 kDa (β2-microglobulin) whereas class II protein comprises two polymorphic peptide chains (α chain of 33 kDa & β chain of 28 kDa). HLA class I proteins are expressed on surface of all nucleated cells whereas expression of class II is usually restricted to antigen presenting cells. The genes that encode HLA proteins are arrayed within long continuous stretch of DNA on chromosome no. 6. There are three loci (referred as A, B & C) that encode HLA
class I proteins. Similarly another three loci (referred to as DP, DQ and DR) encode HLA class II proteins. Each individual codominantly expresses HLA A, B, C, DP, DQ and DR of maternal and paternal origin. Each of these genes is highly polymorphic and gives each individual a near unique haplotype. Every year several new alleles for these genes are being reported. There were 964 alleles known in year 1998 whereas their number has increased to more than 2500 in year 2007. The numbers of alleles known till year 2007 are presented in Table 2.1 (HLA database, version 2.17, released in April 2007; www.ebi.ac.uk/imgt/hla/stats.html).

Table 2.1: The number of alleles for each of the polymorphic loci of HLA known till year 2007.

<table>
<thead>
<tr>
<th>HLA</th>
<th>Loci</th>
<th>Chain</th>
<th>No of known alleles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Class I</td>
<td>A</td>
<td>α</td>
<td>545</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>α</td>
<td>894</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>α</td>
<td>307</td>
</tr>
<tr>
<td>Class II</td>
<td>DP</td>
<td>α</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td></td>
<td>β</td>
<td>126</td>
</tr>
<tr>
<td></td>
<td>DQ</td>
<td>α</td>
<td>34</td>
</tr>
<tr>
<td></td>
<td></td>
<td>β</td>
<td>83</td>
</tr>
<tr>
<td></td>
<td>DR</td>
<td>α</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>β</td>
<td>577</td>
</tr>
</tbody>
</table>
**Incorporation of HLA proteins by HIV-1**

HIV-1 buds out of the host cell using cellular ESCRT (Endosomal Sorting Complex required for Transport) that mediates outward vesiculation (Marsh and Thali, 2003). In this process, host cell plasma membrane embedded with viral proteins (gp160) forms HIV-1 envelope (outer lipid bi-layer) and encloses nucleocapsid. Therefore the envelope of HIV-1 contains large number of host cell proteins including HLA (Esser et al., 2001). The only host membrane protein that has not been reported in HIV-1 envelope is CD45 which may be because of its large cytoplasmic tail (Esser et al., 2001). Host cellular proteins found in HIV-1 envelope and their functions in HIV-1 pathogenesis are listed in Table 2.2. Cyclophilin A, actin and clathrin are the proteins that are found in HIV-1 envelope but their function in HIV pathogenesis is not known.

The number of HLA molecules incorporated by HIV-1 virions has been reported to exceed the viral Env (gp160) proteins (Arthur et al., 1992; Henderson et al., 1987; Trubey et al., 2003). Cantin et al showed that the amount of HLA DR proteins acquired by HIV-1 is virus strain and host cell type specific (Cantin et al., 1996). Cultivation of HIV-1 in PBMCs results in incorporation of substantially higher amounts of HLA class I and HLA DR proteins compared to monocyte-macrophages (Tsai et al., 1999). Presence of HLA-DR has been reported in HIV-1 derived from plasma of infected individuals indicating that HLA proteins are incorporated in vivo (Lawn and Butera, 2000). In a study by Arthur et al, the ratio of HLA DR to Gag protein was approximately 0.15 to 0.2 for HIV-1\textsubscript{MN} cultivated in H9 cells suggesting incorporation of large number of HLA proteins in HIV-1.
envelope (Arthur et al., 1992). In a recent study, HIV-1 was purified to remove microvesicles (which carry HLA proteins) before estimation of HLA proteins. In this study, the ratio of HLA DR to Gag was found to be 0.04 to 0.05. This finding suggests that there are about 50 to 63 HLA class II molecules per HIV-1 virion (Trubey et al., 2003).

Since HIV-1 carries HLA proteins of host cell and each individual may carry different set of HLA proteins, HIV-1 derived from different individuals may differ in their HLA proteins. Due to presence of HLA alloantigens on HIV-1, it has been suggested that alloimmune response may play role in protection against HIV-1 (Lehner et al., 2000; Shearer et al., 1993; Shearer et al., 1999).
Table 2.2: Cellular proteins reported in HIV-1 envelope

<table>
<thead>
<tr>
<th>Protein</th>
<th>Function</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD55, CD59</td>
<td>Protects from complement-mediated lysis</td>
<td>(Saifuddin et al., 1995)</td>
</tr>
<tr>
<td>ICAM-1, LFA-1</td>
<td>Increases viral infectivity</td>
<td>(Fortin et al., 1997; Liao et al., 2000)</td>
</tr>
<tr>
<td>HLA class I and class II</td>
<td>Increases viral infectivity</td>
<td>(Cantin et al., 1997a; Cantin et al., 1997b)</td>
</tr>
<tr>
<td>CD80, CD86</td>
<td>Facilitates viral attachment and entry process due to interaction with their two natural ligand CD28 and CTLA-4</td>
<td>(Giguere et al., 2004)</td>
</tr>
<tr>
<td>CD28</td>
<td>Increases infectivity of cells expressing CD80 and CD86</td>
<td>(Giguere et al., 2002)</td>
</tr>
<tr>
<td>Tsg-101</td>
<td>Required for HIV-1 budding</td>
<td>(Hammarstedt and Garoff, 2004)</td>
</tr>
<tr>
<td>Cyclophilin, actin, clathrin</td>
<td>Not reported</td>
<td>(Hammarstedt and Garoff, 2004)</td>
</tr>
</tbody>
</table>

**Alloimmune response and protection against HIV-1**

The possible association between alloimmune response and protection against HIV-1 was fortuitously discovered. Three groups of macaques were vaccinated with either inactivated SIV, SIV-infected C8166 cells (a human T-lymphoblastoid cell line which are used for cultivation of SIV) or uninfected C8166 cells. When macaques were challenged with SIV grown in C8166 cells, surprisingly animals from all groups were equally protected (Stott et al., 1990). However the studies in which macaques were challenged with SIV grown in monkey cells, protection was not observed (Cranage et al., 1992; Le Grand et al.,...
1992; Osterhaus et al., 1992). These findings suggested that the protection might be due to immune response against cellular proteins present on SIV cultured in human cells. Strong positive correlation was observed between protection of macaques and antibody response to HLA class I molecules (Chan et al., 1992). This observation was confirmed in subsequent studies in which macaques immunized with purified HLA class I (Chan et al., 1995) and class II (Arthur et al., 1995) proteins were protected against SIV cultured in human cells.

Arthur et al immunized groups of macaques with either purified HLA class I, class II (DR), β2M, HuT 78 cell lysate (cell line that was used for cultivation of SIV as well as for source of HLA proteins) and culture supernatant derived from uninfected HuT 78 cells (which contains microvesicles that carry HLA proteins). The animals immunized with HLA class I, β2M and HuT 78 cell lysate produced antibodies that immunoprecipitated ^125^I-β2M at >1:500 serum dilution. The serum of animals immunized with HLA-DR and culture supernatant of uninfected cells, immunoprecipitated ^125^I-HLA-DR at >1:500 dilution. When these macaques were challenged with SIV cultured in HuT 78 cells, all macaques except those that produced anti-HLA-DR antibodies got infected. However when these animals were challenged with SIV cultured in macaque PBMCs, protection was not observed (Arthur et al., 1995).

Chan et al vaccinated 4 macaques with purified HLA class I proteins. All four animals produced anti-HLA antibodies, which inhibited replication of SIVmac-32H in-vitro. When these animals were challenged with SIVmac-32H
grown in human cells, 2 of 4 macaques were protected whereas 4 of 4 control macaques got infected (Chan et al., 1995).

The HLA proteins incorporated in the membrane of various strains of SIV as well as HIV-1 were found to exceed viral envelope proteins (Arthur et al., 1992). Chan et al suggested that binding of antibodies to HLA molecules might block interaction of viral gp120 with CD4 receptor due to steric hindrance and inhibit viral entry into new host cell (Chan et al., 1992). This led to hypothesis that induction of alloimmune response may serve as protective mechanism against HIV/AIDS (Shearer et al., 1993).

**Reports of HIV-1 neutralization by human anti-HLA antibodies**

Some women show recurrent spontaneous abortions, which may be due to lack of tolerance to semiallogenic fetal cells. Leukocyte immunotherapy was given to those women to induce tolerance to paternal alloantigens that are expressed by fetal cells. The therapy consisted of multiple injections of husband’s leukocytes. Leith et al identified two women that produced antibodies against husband’s HLA proteins (Leith et al., 2003). One woman showed antibodies against HLA class I whereas other showed antibodies against both HLA classes. Serum IgG was purified from both women and was tested for neutralizing activity against HIV-1 grown in husband’s lymphocytes. IgG from woman that contained antibodies against both classes of HLA showed HIV-1 neutralizing activity.

Spruth et al and Wilflingseder et al, identified polytransfused patients producing antibodies against HLA proteins expressed by M8166 and H9 cell
lines. The anti-HLA antibody positive sera from these patients neutralized HIV-1 IIIB cultured in M8166 and H9 cells and activity was significantly enhanced in presence of complement (Spruth et al., 1999; Wilfingseder et al., 2003). Spear et al have reported similar finding using primary HIV-1 isolate (Spear et al., 2001). These authors showed that HIV-1 neutralization was due to viral lysis that occurred in presence of anti-HLA antibodies and complement.

Role of anti-HLA antibodies in protection against HIV-1 is still inconclusive

Polyanskaya et al immunized macaques with allogenic simian B cells and challenged with SIV cultured in macaque PBMCs. Alloimmunization induced anti-MHC antibody response but failed to protect animals against the challenge virus (Polyanskaya et al., 1997).

Lopalco et al, studied discordant couples (In which one partner is HIV seropositive but other remain seronegative despite repeated unprotected sexual intercourse for > 2 years) to identify mechanism of protection of uninfected partner. HIV-1 neutralizing activity was detected in few HIV-1 resistant individuals. However HIV-1 neutralizing activity did not correlate with presence of anti-HLA antibodies (Lopalco et al., 2000).

Transmission of HIV-1 from mother-to-child occurs in 15-30% cases in absence of any intervention (Newell, 2006). It was hypothesized that anti-HLA antibodies might be playing role in cases where HIV-1 is not transmitted. Luscher et al found that anti-HLA antibodies present in children were not associated with lack of HIV-1 acquisition (Luscher et al., 1998a).
Resistance to HIV-1 infection was observed among commercial sex workers (CSWs) from Nairobi. Therefore their immune responses were extensively studied. It was postulated that antibodies specific to HLA alleles of their clients might be the factor contributing to HIV-1 resistance. Luscher et al showed that anti-HLA antibodies were not associated with resistance to HIV-1 infection (Luscher et al., 1998b).

Although above studies do not provide evidence for protective role of anti-HLA antibodies in-vivo, studies carried out in-vitro suggest that anti-HLA antibodies may bind to HLA proteins incorporated by HIV-1 virions and reduce their infectivity (Leith et al., 2003; Spear et al., 2001; Spruth et al., 1999).

Other soluble factors that are elicited after exposure to alloantigens and can serve as potential mediators for protection against HIV-1

M-tropic strains of HIV-1 use CCR5 as co-receptor for entry into CD4\(^+\) T cell. Therefore $\beta$-Chemokines such as RANTES (regulated upon activation, normal T cell expressed and secreted), MIP1-\(\alpha\) (macrophage inflammatory protein) and MIP1-\(\beta\), which are ligands of CCR5 can block HIV-1 entry (Cocchi et al., 1995; Deng et al., 1996; Dragic et al., 1996). Induction of these soluble factors after exposure to alloantigens has been reported (Wang et al., 1998; Wang et al., 1999).

Wang et al, immunized macaques with inactivated SIV that was earlier cultured in human CD4\(^+\) T cells and subsequently challenged them with live SIV. These investigators reported that secretion of $\beta$-chemokines was significantly
higher in macaques that were protected against SIV compared to other macaques (p < 0.001) (Wang et al., 1998).

Wang et al obtained CD8⁺ T cells from 12 women before and after leukocyte immunotherapy (multiple injections of husband’s lymphocytes). The cells were stimulated with PHA and culture supernatant derived after cell stimulation was assessed for anti-HIV activity. Culture supernatant obtained after leukocyte immunotherapy contained elevated level of β-chemokines and suppressed HIV-1 replication in CD4⁺ T cells. The study also demonstrated that alloimmunization induced down-regulation of CCR5 and CXCR4 receptors on T-cells. Therefore rate of HIV-1 replication was significantly low in PBMCs obtained after leukocyte immunotherapy (Wang et al., 1999).

Anti-CCR5 antibodies have been reported in xenoimmunised macaques (Lehner et al., 1999) and alloimmunised individuals (Wang et al., 2002). These antibodies have been also detected in sera of multiparous women (Wang et al., 2002). It has been suggested that xeno or alloimmunization might break tolerance to CCR5 resulting in formation of anti-CCR5 antibodies (Wang et al., 2002). These antibodies may cause internalization of CCR5 and block entry of R5 strains of HIV-1.

Pinto et al reported that culture supernatant obtained after stimulation of PBMCs with allogenic cells inhibit HIV-1 replication. However the activity was found to be due to soluble factors other than β-chemokines (Pinto et al., 1998). Rugules et al analysed supernatants from mixed lymphocyte reaction for anti-HIV activity. These investigators found that inhibitory activity was heat resistant and
could be blocked by ribonuclease inhibitor. Therefore it has been proposed that induction of ribonuclease may be partly responsible for HIV-1 inhibition (Rugeles et al., 2003).

**Mucosal exposure to alloantigens and resistance to HIV-1 infection**

In majority of cases, HIV-1 is acquired due to exposure of mucosal tissues to cell free virions or virally infected cells. Therefore anti-HIV-1 immune response at mucosal site can play vital role in protection against HIV-1.

Peters et al studied whether unprotected sexual intercourse that induces alloimmune response at mucosal sites (due to presence of allogenic cells in semen and vaginal secretions), can mediate protection against HIV-1. Heterosexual couples with unprotected sexual activity were investigated. PBMCs from study participants were stimulated with irradiated lymphocytes derived from their sexual partner. Significantly higher stimulation was observed after stimulation with lymphocytes from their partner compared to unrelated lymphocytes. These investigators studied rate of HIV-1 replication in PBMCs of study participants. It was found that individuals practicing unprotected sex show significantly low level of HIV-1 replication compared to individuals practicing protected sex or no sex (Peters et al., 2004).

Bergmeier et al administered allogenic mononuclear cells ($1 \times 10^4$ to $10^7$) in rectal or vaginal mucosa of macaques. The macaques showed higher production of RANTES, MIP1-α, MIP1-β and anti-CCR5 antibodies compared to
controls. These responses were found to be associated with reduced SIV replication in their CD4$^+$ T cells (Bergmeier et al., 2005).

These observations suggest importance of mucosal alloimmune response in protection against HIV-1.

**Exploration of alloimmune response for protection against HIV-1**

Alloimmunization has been suggested as a potential strategy for protection against HIV/AIDS (Lehner et al., 2000; Shearer et al., 1993; Shearer et al., 1999). Anti-HLA antibodies may mediate protection against HIV-1 infection if they are present at the site of exposure and are able to neutralize the virus. Besides anti-HLA antibodies, secretion of β-chemokines and other soluble factors can also contribute to protection against HIV-1. The advantage of this approach is that the mechanisms of protection are not likely to be affected by genetic diversity of HIV-1. However for exploration of this strategy against HIV-1, further investigation has been suggested (McMichael and Hanke, 2003). To study potential of this strategy, a study was designed to investigate protective role of anti-HLA antibodies against HIV-1 in-vitro.
2.2: OBJECTIVE

To determine whether antibodies specific to HLA proteins incorporated in the HIV-1 envelope mediate virus neutralization.
2.3: STUDY DESIGN

Plasma from married, multiparous and HIV seronegative women were screened for anti-HLA antibodies by commercial ELISA kit. To confirm that anti-HLA antibodies are directed against HLA types of husband, plasma from each woman was incubated with husband’s PBMCs. After incubation, antibodies bound to PBMCs were detected by flow-cytometry. The levels of anti-HLA antibodies in plasma were measured by ELISA before and after incubation with husband’s PBMCs.

A primary HIV-1 isolate was cultured in PBMCs from husband of anti-HLA antibody positive woman so that the virus carried specific (husband’s) HLA types. To determine whether anti-HLA antibodies bind to HIV-1 that was cultured in husband’s PBMCs, the plasma and the virus were mixed and incubated. Binding of antibodies to the virus was detected by capturing the virus-antibody complex on a well coated with anti-human IgG. To determine neutralizing activity of anti-HLA antibodies, the plasma-virus mixture was used to infect GHOST cells (which produce fluorescence after HIV-1 infection) and infectivity of virus in presence and absence anti-HLA antibodies was determined (Figure 2.1). IgG was purified from two plasma samples and was tested against the virus at highest possible concentration. The role of complement in neutralization of virus carrying HLA proteins was also studied.

The prerequisite for anti-HLA antibody mediated HIV-1 neutralization is incorporation of HLA proteins by HIV-1. Therefore to determine the extent of
incorporation of HLA proteins by primary HIV-1 isolate; ratio between HLA proteins incorporated by HIV-1 and HIV-1 p24 protein was determined.

Figure 2.1: Assessment of anti-HLA antibody mediated HIV-1 neutralization.
2.4: MATERIAL AND METHODS

1. Study participants

Couples (HIV seronegative women with three or more full-term pregnancies, accompanied with their husbands) were identified at post-natal clinic of the Sassoon General Hospital, Pune. Hundred and twenty couples were counseled about the study objectives. Informed written consent was obtained from 65 women and their husbands that were willing to participate in the study. The study was approved by ethics committees of National AIDS Research Institute, Pune and B.J. Medical College-Sassoon General Hospital, Pune, India.

The women with history of organ transplantation or blood transfusion were excluded.

2. Blood sample collection

Ten ml blood was collected by venipuncture in sterile vacutainer (Beckton Dickinson, Franklin Lakes, NJ) containing K$_3$EDTA as an anticoagulant and was immediately transported to laboratory at room temperature.

3. Separation of PBMCs

Blood was processed within 4 hrs after collection. PBMCs were separated by Ficoll-hypaque density gradient centrifugation as described below.

1. Blood was layered on 4 ml of Histopaque-1077 (Mixture of polysucrose: 5.7 g/dl and sodium diatrizoate: 9.0g/dl with density of 1.077 ± 0.001 g/ml from Sigma) in a 15ml conical centrifuge tube.
2. Tube was centrifuged in swinging bucket rotor (Sorvall RT7) at 400g for 30 minutes at room temperature. During centrifugation, erythrocytes and granulocytes were sedimented at the bottom whereas mononuclear cells remained at plasma-histopaque interface.

3. After centrifugation, upper layer of plasma was aspirated and transferred to storage vials (0.5 ml/ vial). The PBMC layer was transferred to sterile 15 ml conical centrifuge tube.

4. 10 ml wash medium (RPMI-2; appendix A) was added and cell suspension was centrifuged at 250g for 10 minutes. After two more washes, the cell pellet was resuspended in 10 ml culture medium (RPMI-10; appendix A).

5. For counting of viable number of PBMCs, 20μl cell suspension was mixed with equal volume of trypan blue (Gibco, Grand island, USA; 0.4% prepared in PBS) and the mixture was filled in counting chamber of hemacytometer (Hausser Scientific, USA). The viable cells (that are not stained by trypan blue) in four WBC chambers (each chamber: 0.1 mm³) were counted under light microscope. PBMC count (cells / ml) was determined as:

   \[
   \text{Cells/ml} = \frac{(\text{Average number of cells in four WBC chambers}) \times \text{dilution factor}}{10^4}
   \]

4. Cryopreservation of PBMCs and plasma

   The cell pellet was suspended in chilled freezing medium (appendix A) at concentration of 5 x 10⁶ cells / ml and aliquots of 1ml were prepared in cryovials.
(Nalgene, NY, USA). The vials were immediately transferred to freezing container (Nalgene) and stored at -80°C. After 16 to 24 hrs, vials were transferred to liquid Nitrogen freezer (-186°C). The aliquots of plasma were stored at -80°C.

5. Heat inactivation of plasma

For heat inactivation, plasma vial was incubated at 56°C for 30 minutes in a waterbath. After inactivation, plasma was centrifuged at 1000g for 1 min. The supernatant plasma was transferred to another vial and used in all subsequent assays.

6. Detection of anti-HLA antibodies by ELISA

Plasma was tested for IgG antibodies against HLA class I & II proteins using commercially available ELISA kit (Quikscreen and B-screen respectively) from Genetic Testing Institute (GTI), Brookfield, WI, USA as per manufacturer’s instructions. Plate coated with purified diverse HLA class I & II proteins was provided in the kit. Plasma was added to the wells and antibodies bound to the well were detected using alkaline phosphatase labeled secondary antibody.

Procedure

1. Wash buffer (provided in the kit) was added to all wells (250 µl/ well) of ELISA plate and was kept at room temperature for 10 minutes. After soaking, the plate was decanted to remove the wash buffer.

2. Negative control serum, positive control serum (provided in the kit) and plasma were diluted 1:1 using the specimen diluent (provided in the kit).
50μl of diluted serum / plasma was added to the assigned wells in duplicate to the assigned well. One well was kept as blank. Plate was sealed and incubated at 37°C for 45 minutes.

3. After incubation, plate was washed four times using wash buffer (provided in the kit) to remove unbound antibodies and other proteins.

4. Anti-human IgG conjugated with alkaline phosphatase (provided in the kit) was diluted in specimen diluent (1:100) and 50μl was added to all wells except blank as per manufacturer’s recommendations. Plate was sealed and incubated at 37°C for 45 minutes.

5. After incubation, plate was washed four times using wash buffer to remove unbound secondary antibody.

6. PNPP (p-nitrophenyl phosphate) substrate was freshly prepared as per manufacturer’s instructions and 100μl substrate was added to all wells.

7. Plate was sealed and incubated in dark at room temperature for 30 minutes. After incubation, 100μl stop solution (3M NaOH) was added to each well.

8. Absorbance was measured at 450 nm and mean of absorbance from duplicate wells was determined. Signal to cut-off ratio was determined for each sample as:

   Mean absorbance of test / mean absorbance of negative control.
Validity criteria: Results were considered valid if following criteria were met as described by manufacturer.

1. Mean absorbance of negative control must be between 0.04 to 0.15 for Quickscreen and 0.1 to 0.25 for B-screen.

2. Mean absorbance of positive control must be >1.5.

Interpretation: Plasma samples showing absorbance equal or greater than twice of absorbance in negative control (signal to cut off ratio ≥ 2) were considered positive for anti-HLA antibodies.

7. Flow cytometric evidence of anti-HLA antibodies

Plasma from multiparous woman was incubated with husband’s PBMCs and autologous PBMCs. PBMCs were also stained with control (pool of plasma from 12 healthy, anti-HLA antibody negative individuals). Binding of antibodies to the lymphocytes was detected by flow-cytometry using FITC labeled anti-human IgG antibody.

Procedure

1. Heat inactivated plasma (100μl) was incubated with PBMCs (2 X 10^5 cells suspended in 100μl of culture medium) at 1:10 dilution for 45 minutes at room temperature.

2. PBMCs were washed 4 times using wash buffer-A (appendix A) and were resuspended in 100μl of same buffer.
3. FITC conjugated anti-human IgG (Bangalore Genei) was incubated with
the cells at 1:80 dilution (as per manufacturer’s recommendation) at room
temperature for 30 minutes in dark.

4. PBMCs were washed four times using wash buffer-A and were suspended
in PBS containing 3% formaldehyde. Cells were incubated at 4°C for at
least 30 minutes.

5. Cells were analyzed on flow-cytometer (FACsort, Beckton Dickinson)
using CellQuest software. During acquisition of cells on flow-cytometer,
electronic gate was formed for lymphocytes based on their forward and
side scatter (Figure 2.2). Ten thousand cells (lymphocytes) were acquired
within the gate.

6. Dotplots were obtained based on cell fluorescence intensity and quadrants
were set to separate cluster of stained and unstained lymphocytes as
shown in figure 2.2. The percent of stained lymphocytes (cells in lower,
right quadrant) was determined.

7. The histograms based on cell fluorescence intensity were drawn as shown
in figure 2.2. Median fluorescence intensity (MFI) of total lymphocytes
was determined. Specific Median Fluorescence Intensity (sMFI) was
calculated for each woman’s plasma as:

\[
\text{sMFI} = \frac{\text{MFI of lymphocytes stained with woman’s plasma}}{\text{MFI of lymphocytes stained with control}}
\]
Validity of the assay: Results were considered valid if number of PBMCs stained with plasma control were less than 10%.

Interpretation: sMFI greater than 2 was used as indicator for binding of antibodies to lymphocytes.
Figure 2.2: Acquisition and analysis of lymphocytes on flow-cytometer
8. Specificity of antibodies bound to husband's lymphocytes

In order to confirm that antibodies bound to husband's lymphocytes are HLA specific, level of anti-HLA antibodies in plasma of three women (40W, 43W and 65W) was measured before and after incubation with husband's PBMCs. Level of anti-CMV antibodies was also measured as control.

Procedure

1. Heat inactivated 0.3 ml plasma was mixed with husband's PBMCs (3X10^6 cells suspended in 0.3 ml PBS) or control (0.3ml PBS alone) and was incubated at 37°C for 1 hr.
2. The mixture was centrifuged at 200g for 10 minutes and supernatant was collected.
3. Level of anti-HLA antibodies in the plasma was determined by ELISA (as described earlier in section 2.4, page 2-59). The plasma were tested at 1:2 and 1:20 dilution.
4. For determination of level of anti-CMV antibodies, the plasma was tested at 1:100 dilution by commercially available ELISA kit (PLATEIA CMV IgG kit, BIO-RAD, France). The plasma samples were tested as per protocol provided by the manufacturer (appendix B).
9. HIV-1 culture

Primary HIV-1 isolate

Primary HIV-1 subtype C isolate (VB49) was obtained from virus repository of National AIDS Research Institute, Pune. The TCID50 of virus stock was $1 \times 10^3$/ml. The virus was cultured in the PBMCs as described below.

Procedure

1. Frozen PBMCs were thawed at $37^\circ$C and washed twice using wash medium (RPMI-2; appendix A) and suspended in culture medium (RPMI-10; appendix A). Viable cell count was determined as described earlier (section 2.4, page: 2-58). After revival of one vial of frozen PBMCs, about 3 to $3.5 \times 10^6$ viable cells were obtained.

2. The cell suspension was centrifuged at 250g for 10 minutes. The cell pellet was transferred to 5ml culture medium (RPMI-10; appendix A) containing $5 \mu$g/ml PHA-P (Sigma). Cells were incubated at $37^\circ$C in 5% CO2 atmosphere for 48 hrs.

3. The cell suspension was centrifuged. Supernatant was discarded and cell pellet was infected with 0.3ml virus (~200TCID50). The cells were incubated at $37^\circ$C in 5% CO2 atmosphere for 12 to 16 hrs.

4. After adsorption of virus, cells were washed thrice using 10 ml wash medium (RPMI-2; appendix A) to remove cell-free virus and were transferred to culture medium (RPMI-10) containing IL2 (20U/ml) and were incubated at $37^\circ$C with 5% CO2 and humid atmosphere for 7 days.
5. Culture supernatant (0.2ml) was collected immediately after resuspending the cells in IL2 medium (Zero day) and at 7 days post infection.

6. Culture supernatant collected on day zero and 7 were tested for HIV-1 p24 protein by commercial ELISA kit (Vironostica HIV-1 antigen microelisa system, Biomerieux, Netherlands) as described in appendix C.

7. Culture supernatant was harvested on day 7 when high concentration of p24 protein (absorbance > 2 at 1/200 dilution) was detected. The culture was centrifuged; the supernatant was aliquoted and stored at -70°C.

**Determination of TCID$_{50}$ of virus stocks**

TCID$_{50}$ for each virus stock was determined as described in NIAID ACTG laboratory manual http://aactg.com/LabManual.htm (appendix D).

10. **Binding of antibodies to HIV-1**

Anti-HLA antibody positive plasma was incubated with HIV-1 cultured in husband’s and autologous PBMCs. Binding of antibodies to virus was detected by capturing virus-antibody complex on a well coated with anti-human IgG. A pool of plasma from 12 healthy, HIV seronegative and anti-HLA antibody negative individuals was used as negative control whereas pool of plasma from HIV seropositive individuals was used as positive control.

**Procedure:**

1. Anti-human IgG antibody (Bangalore Genei) was diluted to 10 µg/ml in bicarbonate buffer (0.1M, pH: 9.2; appendix A) and added (100 µl / well)
to ELISA plate (96 well, flat-bottom, high protein affinity plates from Nalgene, USA). Plate was incubated overnight at 4°C.

2. After incubation, wells were washed 4 times with PBS (300μl / well).

3. To block free protein binding sites in the well, 200μl of PBS with 10% FBS was added to each well and plate was incubated at 37°C for 1 hr. After incubation, the plate was washed 4 times with PBS (300μl / well).

4. Plasma and virus were mixed in equal proportion (50 μl plasma + 50 μl virus). The mixture was incubated at 37°C for 1 hr.

5. The plasma-virus mixture was added to the well coated with anti-human IgG and incubated at 37°C for 1 hr.

6. After incubation, the wells were washed 6 times to remove free virus. After washing, 50 μl lysis buffer (appendix A) was added to each well. The wells were incubated at 37°C for 30 minutes.

7. The lysate was tested for HIV-1 p24 protein by commercial ELISA kit (Vironostika HIV-1 antigen microelisa system, Biomerieux, Netherlands) as described in appendix C.

11. HIV-1 neutralization assay

The neutralization assay was performed using GHOST cells as described by Trakola et al (Trkola et al., 1998). GHOST is genetically modified Human Osteosarcoma cell line that expresses HIV-1 receptor (CD4), one of the several HIV-1 co-receptor (e.g. CCR5, CXCR4) and Green Fluorescence Protein (GFP).
Expression of GFP is under control of HIV Tat protein. Hence, HIV-1 infected GHOST cells produce GFP and can be detected using flow-cytometer.

Procedure

1. GHOST cells were cultured as described in appendix E. The monolayer was detached (appendix E) and $6 \times 10^4$ cells were added to each well in a 24-well plate. Plates were incubated overnight at $37^\circ C$ in 5% CO$_2$ & humid atmosphere. After overnight incubation, plates were observed under inverted light microscope. The wells showing 60-70% confluent monolayer of GHOST cells were used for the assay.

2. The virus dilution required for the neutralization assay (that produced about 1000 fluorescent cells 4 days after infection) was determined as described in appendix F. During the assay, 50 $\mu$l of diluted virus (that corresponded to $150 \pm 100$ TCID$_{50}$) was incubated at $37^\circ C$ for 1hr with 50 $\mu$l of heat inactivated plasma (1:10 dilution), culture medium and controls (negative control: pool of plasma from 12 healthy, HIV seronegative individuals; positive control: pool of plasma from 4 HIV-1 seropositive individuals that showed neutralizing antibodies against VB49).

3. The mixture was added in duplicate to the wells carrying monolayer of GHOST cells. DEAE-dextran was added to all wells (8 $\mu$g/ml). Plate was incubated at $37^\circ C$ in 5% CO$_2$ & humid atmosphere.

4. On day 4, the plate was observed under inverted light microscope. The wells that were kept uninfected showed confluent monolayer whereas wells infected with virus alone showed cytopathic effect (Figure 2.3).
Figure 2.3: GHOST cell monolayer observed under inverted microscope

A: Uninfected cells

B: HIV-1 infected cells (4 days after infection)
5. After observing cytopathic effect, cell monolayer was washed once with DMEM supplemented with 2% FBS and 300μl of 2mM EDTA was added to all wells. After incubation for 30 minutes, single cell suspension of detached cells was prepared by repeat pipetting. The cell suspension was transferred to tubes containing 100μl of 6% formaldehyde (prepared in PBS). The tubes were incubated at 4°C for at least 30 minutes for fixing of the cells.

6. The cells were then analyzed on FACSort flow-cytometer (Becton Dickinson) using CellQuest software. During acquisition of cells on flow-cytometer, electronic gate was formed for GHOST cells based on forward and side scatter (Figure 2.4). Fifteen thousand GHOST cells were acquired within the gate.

7. The dotplots were drawn based of fluorescence intensity vs forward scatter and quadrants were set to separate uninfected cells from infected cells as shown in figure 2.4. The number of infected (fluorescent) cells (present in upper, right quadrant) was determined.

8. Percent neutralization was calculated using formula:

\[
(1 - \frac{\text{Number of infected cells in presence of plasma}}{\text{Number of infected cells in absence of plasma}}) \times 100.
\]

**Validity of the assay:** The assay was considered valid if negative control showed < 30% neutralization (cut-off value) and positive control showed >90% neutralization. The 30% neutralization (30% reduction in infectivity) was used as a cut-off value based on earlier preliminary work in which plasma from 20
normal, healthy individuals were tested against the HIV-1 isolate (VB49). Non-specific virus inhibition shown by these plasma samples was 5.2 ± 8.2 % (mean ± SD). Therefore 30% neutralization (mean + 3SD of background response) was considered as cut-off value.
Figure 2.4: Acquisition and analysis of GHOST cells on flow-cytometer
12. **HIV-1 neutralizing activity of IgG purified from anti-HLA antibody positive plasma**

**IgG purification**

IgG was purified by IgG purification kit (Bangalore Genei) as per manufacturer’s instructions as described below.

**Procedure:**

1. Plasma (700 μl) was mixed with equal volume of equilibration buffer (provided in the kit) and was loaded on the top of Protein A column. The mixture was passed through the column. Subsequently 25 ml equilibrium buffer was passed to wash unbound proteins.

2. 5 ml elution buffer (provided in the kit) was added on top of the column to elute IgG.

3. 0.5 ml fractions were collected in tubes containing 12.5 μl neutralizing buffer (provided in the kit).

4. Protein content of each fraction was determined by Lowry’s method using Protein estimation kit from Bangalore Genei as per manufacturer’s instructions.

5. The protein rich fractions were pooled together and dialyzed against distilled water using dialysis membrane bag with cut-off of 50,000. The dialysis bag was kept in distilled water on magnetic stirrer for 60 hrs. Water was replaced after every 12 hrs.

6. The dialyzed protein fraction was freeze dried. Lyophilized powder was suspended in 300 μl phosphate buffer (0.01M, pH: 7.2).
Testing of purified IgG for anti-HLA antibodies

Purified IgG (that was suspended in 300 µl phosphate buffer) and plasma samples (40W & 43W) were diluted (1:4, 1:8, 1:16) in phosphate buffer (0.01M, pH: 7.2). All specimens were tested for antibodies against both classes of HLA using Quickscreen and B-screen ELISA kits as described in section 2.4; page: 2-59.

Testing of purified IgG for HIV-1 neutralizing activity

Purified IgG (that was suspended in 300 µl phosphate buffer) from two plasma samples (40W and 43W) was tested against respective virus (40H and 43H) at minimal (1:2) dilution. IgG purified from negative and positive control plasmas (described above) were also tested. Neutralization assay was carried out as described above (section 2.4; page: 2-68).

13. HIV-1 neutralizing activity of anti-HLA antibody positive plasma in presence of complement

Two plasma samples (40W & 43W) were tested for neutralizing activity against viruses grown in husband’s PBMCs (40H & 43H) in presence of complement. Serum from 5 healthy individuals was pooled and used as source of complement. Equal volume (50µl) of inactivated plasma, virus and neat complement were mixed and incubated at 37°C for 1hr. Further assay was done as described above (section 2.4; page: 2-68).
14. Incorporation of HLA class I proteins by HIV-1

HIV-1 (VB49) was cultured in PBMCs of two healthy individuals. The virus (culture supernatant) was ultracentrifuged and virus pellet was lysed using lysis buffer (appendix A). HLA and HIV-1 p24 proteins present in the lysate were quantitated. The HIV-1 culture contains microvesicles that carry HLA proteins of host cell origin and are pelleted along with the virus during ultra centrifugation. To determine the proportion of HLA proteins present in microvesicles, culture supernatant from culture of control PBMCs (without HIV infection) was ultracentrifuged and amount of HLA proteins in the pellet was determined. For determination of extent of incorporation of HLA class I proteins by HIV-1, ratio of p24 and HLA class I proteins was determined after subtracting the amount of HLA proteins due to microvesicles.

HIV-1 culture and preparation of virus lysates

Procedure

1) PBMCs (30 x 10⁶) from whole blood of the two healthy individuals were obtained as described earlier on page 2-57. The cell suspension was centrifuged at 200g for 10 minutes. The cell pellet was transferred to 30ml culture medium (RPMI-10; appendix A) containing 5μg/ml PHA-P (Sigma). Cells were incubated at 37°C in 5% CO₂ atmosphere for 48 hrs.

2) The suspension of stimulated cells was equally distributed in two sterile 15ml tubes and centrifuged at 200g for 10 minutes. The cells in one tube were infected with primary HIV-1 isolate (VB49) and cultured as described before...
Cells in other tube were kept as uninfected cell control and were cultured similarly.

3) On day 7 post-infection, culture supernatant derived from infected as well as uninfected control cells was passed through 0.22 μm filter. The filtrate was ultracentrifuged at 40,000g for 4 hrs. The pellet in both tubes was suspended in 0.4 ml phosphate buffer (0.05M, pH 7.0). Equal volume of lysis buffer (appendix A) was added to lyse the virus.

**Quantitation of HIV-1 p24 protein present in virus lysate**

HIV-1 p24 protein in the lysate was quantitated by commercial ELISA kit (Vironostica HIV-1 antigen microelisa system, Biomerieux, Netherlands) as described in appendix C.

**Quantitation of HLA Class I proteins present in virus lysate**

HLA class I proteins were captured on a microwells coated with W6/32 antibody that recognizes non-polymorphic region of HLA class I (A, B & C) proteins. The captured proteins were detected using biotin-labeled anti-β2-microglobulin antibody; streptavidin-HRP (enzyme) conjugate and TMB/H2O2 (substrate). Serial dilutions of purified HLA protein (gift from GTI, Brookfield, WI, USA) were tested and absorbance was plotted against HLA protein concentration. Using straight-line equation of standard curve, concentration of HLA class I proteins in virus lysate was determined.

**Procedure:**

1. Rabbit anti-mouse IgG antibody (Bangalore Genei) was diluted to 5 μg/ml in the bicarbonate buffer (0.1M, pH: 9.2; appendix A). Diluted antibody
was applied (100µl/ well) to ELISA plate (96 well, flat-bottom, high protein affinity plates from Nalgene, USA). Plate was incubated overnight at 4°C.

2. The plate was washed 6 times using wash buffer (appendix A; 300 µl / well). W6/32 antibody (Biodesign International, USA) was diluted to 20 µg/ml in PBS. 100 µl of diluted antibody was added to each well (2 µg / well). Plate was incubated at 37°C for 2 hrs.

3. The plate was washed 6 times using wash buffer. 300 µl of 5% Bovine Serum Albumin solution (prepared in PBS) was added to each well for blocking of free protein binding sites. Plate was incubated at 37°C for 2 hrs.

4. After blocking, plate was washed 3 times using wash buffer.

5. Serial dilutions (200, 100, 50, 25 and 12.5 ng/ml) of pure HLA protein (gift from GTI, Brookfield, WI) were made in PBS. 100 µl of virus lysate and diluted standards were added in assigned wells. Plate was incubated at 37°C for 1 hr.

6. After incubation, plate was washed 6 times using wash buffer. 100 pg of biotin labeled, anti-β2-microglobulin antibody from Fitzgerald International, USA was added to all wells. Plate was incubated at 37°C for 1 hr.

7. Plate was washed 6 times using wash buffer and 100 µl of streptavidin-HRP (Bangalore Genei) was added to each well and plate was incubated at 37°C for 1 hr.
8. After incubation, plate was washed 6 times using wash buffer and 100μl of TMB/H₂O₂ (Bangalore Genei) was added in each well. Plate was incubated at room temperature for 20 minutes in dark.

9. Reaction was stopped by adding 50 μl of 2 N H₂SO₄ and optical density was measured at 450 nm. HLA protein concentration was plotted against absorbance (Figure 2.5).

10. The HLA protein concentration in virus lysate was determined by using straight line equation of the standard curve.

If y is absorbance shown by test specimen, then

\[ y = mX + C \]

in which m represents slope of the straight line, C represents Y-axis intercept and X is concentration of HLA proteins in the test specimen.
Figure 2.5: Standard curve for obtaining HLA concentration in test sample. Purified HLA proteins of known concentration (shown on X-axis) were tested in the ELISA. The absorbance obtained is shown on Y-axis.
2.5: RESULTS

1. Anti-HLA class I & class II IgG antibodies

Out of 65 multiparous women tested, 14 were found to be anti-HLA antibody positive by the ELISA. Out of these 14 women, 6 showed antibodies against HLA class I & II, 2 against class I alone and remaining against class II (Table 2.3). The absorbance for anti-HLA class I antibodies ranged from 0.72 to 1.89 whereas it was 0.48 to 1.94 for anti-HLA class II antibodies. The absorbance value and signal to cut off ratio is presented in Table 2.3.

Table 2.3: Detection of anti-HLA antibodies by ELISA

<table>
<thead>
<tr>
<th>Sr. No</th>
<th>Participant Sample No</th>
<th>Absorbance (Signal to cut off ratio)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>HLA class I</td>
</tr>
<tr>
<td>1</td>
<td>40W</td>
<td>1.89 (17.6)</td>
</tr>
<tr>
<td>2</td>
<td>41W</td>
<td>0.94 (8.7)</td>
</tr>
<tr>
<td>3</td>
<td>43W</td>
<td>1.46 (13.7)</td>
</tr>
<tr>
<td>4</td>
<td>49W</td>
<td>1.81 (12.7)</td>
</tr>
<tr>
<td>5</td>
<td>63W</td>
<td>0.92 (6.5)</td>
</tr>
<tr>
<td>6</td>
<td>65W</td>
<td>0.72 (5.1)</td>
</tr>
<tr>
<td>7</td>
<td>28W</td>
<td>1.84 (13.5)</td>
</tr>
<tr>
<td>8</td>
<td>45W</td>
<td>0.94 (6.6)</td>
</tr>
<tr>
<td>9</td>
<td>31W</td>
<td>0.07 (&lt;2.0)</td>
</tr>
<tr>
<td>10</td>
<td>33W</td>
<td>0.08 (&lt;2.0)</td>
</tr>
<tr>
<td>11</td>
<td>44W</td>
<td>0.09 (&lt;2.0)</td>
</tr>
<tr>
<td>12</td>
<td>47W</td>
<td>0.08 (&lt;2.0)</td>
</tr>
<tr>
<td>13</td>
<td>48W</td>
<td>0.10 (&lt;2.0)</td>
</tr>
<tr>
<td>14</td>
<td>56W</td>
<td>0.17 (&lt;2.0)</td>
</tr>
</tbody>
</table>

*Mean absorbance of test / Mean absorbance of negative control

A sample that showed a signal to cut-off ratio of ≥ 2 was considered positive (boldface type and in shaded box).
2. Plasma from multiparous women carried IgG antibodies that specifically bound to HLA expressed on lymphocytes from their husband

Plasma from 8 of 14 anti-HLA antibody-positive women (Table 2.3) carried antibodies that bound to lymphocytes from the husband. The sMFI values ranged between 2.6 to 147.6 (Figure 2.6). When plasma samples were incubated with autologous lymphocytes, sMFI was < 2 indicating that antibodies did not bind to autologous cells. Of these 8 samples, 6 were positive for antibodies against HLA class I & II and 2 were positive for antibodies against HLA class I in ELISA. The remaining (6 of 14) plasma samples were positive only for anti-HLA class II antibodies in ELISA (Table 2.3).

After incubation of wife’s plasma (40W, 43W & 65W) with her husband’s PBMCs, there was >75% reduction in level of anti-HLA antibodies. However no reduction was observed in the level of antibodies against unrelated Cytomegalovirus (CMV) antigen (Figure 2.7). This indicates that the antibodies that stained husband’s PBMCs are HLA specific.
Figure 2.6: Binding of antibodies from wife’s plasma to husband’s lymphocytes (For couple: 28, 40, 41, 43, 45, 49, 63 and 65). Wife’s plasma was incubated with husband’s PBMCs (Window H). PBMCs were also incubated with pool of anti-HLA antibody negative plasma (control). Husband’s lymphocytes (windows H) stained with wife’s plasma ( ■ ) showed higher fluorescence intensity (shown on X-axis) compared to control ( □ ). Wife’s plasma did not show binding to autologous lymphocytes (windows W).

*sMFI = Median Fluorescence Intensity (test)/ Median Fluorescence Intensity (control)
Figure 2.7: Confirmation of HLA specificity of antibodies that bound to husband's PBMCs. IgG antibodies against HLA class I, class II & Cytomegalovirus (CMV) were measured by ELISA in plasma from three women (40W, 43W & 65W) before and after incubation with husband’s PBMCs. The absorbance values are shown on Y-axis. All plasma showed >75% reduction in absorbance values for antibodies against HLA class I & II after incubation whereas no reduction was observed in the absorbance for antibodies against CMV.
3. HIV-1 culture in husband’s PBMCs

Primary HIV-1 subtype C isolate (VB49) was cultured in PBMCs obtained from husbands of women that showed anti-HLA antibodies. The virus cultured in PBMCs of husband of woman with sample No. 40W was designated as 40H. Similarly all other virus stocks were designated.

The TCID$_{50}$ of these virus stocks is shown in Table 2.4.

Table 2.4: TCID$_{50}$ of HIV-1 stocks cultured in husband’s PBMCs

<table>
<thead>
<tr>
<th>Sr. No</th>
<th>Virus Id*</th>
<th>TCID$_{50}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>40H</td>
<td>$47 \times 10^3$</td>
</tr>
<tr>
<td>2</td>
<td>41H</td>
<td>$16.6 \times 10^4$</td>
</tr>
<tr>
<td>3</td>
<td>43H</td>
<td>$6.6 \times 10^3$</td>
</tr>
<tr>
<td>4</td>
<td>49H</td>
<td>$257 \times 10^3$</td>
</tr>
<tr>
<td>5</td>
<td>63H</td>
<td>$66 \times 10^3$</td>
</tr>
<tr>
<td>6</td>
<td>65H</td>
<td>$3.6 \times 10^3$</td>
</tr>
<tr>
<td>7</td>
<td>28H</td>
<td>$41.6 \times 10^3$</td>
</tr>
<tr>
<td>8</td>
<td>45H</td>
<td>$2.6 \times 10^3$</td>
</tr>
<tr>
<td>9</td>
<td>31H</td>
<td>$166 \times 10^3$</td>
</tr>
<tr>
<td>10</td>
<td>33H</td>
<td>$10.7 \times 10^3$</td>
</tr>
<tr>
<td>11</td>
<td>44H</td>
<td>$104 \times 10^3$</td>
</tr>
<tr>
<td>12</td>
<td>47H</td>
<td>$66 \times 10^3$</td>
</tr>
<tr>
<td>13</td>
<td>48H</td>
<td>$2.6 \times 10^3$</td>
</tr>
<tr>
<td>14</td>
<td>56H</td>
<td>$66 \times 10^3$</td>
</tr>
</tbody>
</table>

*ID, identification number.

* HIV-1 stock that was grown in PBMCs of husband of respective woman.
4. Binding of antibodies to HLA proteins incorporated by HIV-1

The binding of anti-HLA antibodies in wife's plasma (28W & 49W) with HLA class I proteins on the HIV-1 cultured in husband's PBMCs was demonstrated by capturing the complex on a well coated with anti-human IgG antibody. The lysate of the captured virus showed absorbance 0.830 and 0.620 respectively when it was tested for HIV-1 p24 antigen by ELISA. However when these plasma were incubated with HIV-1 cultured in their autologous PBMCs, the virus was not captured and the lysate showed absorbance < 0.3 (Figure 2.8) in HIV-1 p24 ELISA. This indicates that the antibodies bound specifically to the virus that carried specific HLA types.

HIV-1 was also incubated with pooled plasma from anti-HLA antibody negative, HIV seronegative individuals as negative control and pooled plasma of HIV seropositive individuals as positive control. The HIV-1 was captured when it was incubated with HIV-1 seropositive plasma (positive control) but not with negative control (Figure 2.8).
Figure 2.8: Binding of antibodies from anti-HLA antibody positive wife’s plasma to HIV-1 cultured in husband’s PBMCs.

Wife’s plasma 28W (A) & 49W (B) was incubated with HIV-1 cultured in their husband’s (28H & 49H respectively) and autologous (28W & 49W respectively) PBMCs. The virus was also incubated with pooled plasma from healthy, anti-HLA antibody negative individuals (negative control) and pooled plasma from HIV seropositive individuals (positive control). The virus-plasma mixture was added and incubated in a well coated with rabbit anti-human IgG. After washing, virus bound to the well was lysed and HIV-1 p24 antigen was measured by ELISA. Wife’s plasma contained antibodies that specifically bound to HIV-1 cultured in husband’s PBMCs but did not show binding to HIV-1 cultured in autologous PBMCs.
5. Anti-HLA antibodies did not show HIV-1 neutralization

HIV-1 (VB49), cultured in lymphocytes from husband was incubated with anti-HLA antibody positive plasma from wife and reduction in virus infectivity (% neutralization) was determined. The reference positive control plasma showed > 90% neutralization in all experiments whereas negative control showed < 30% neutralization (cut-off value). The 30% reduction in infectivity was used as cut-off value based on earlier preliminary work in which plasma from 20 normal, healthy individuals was tested against the HIV-1 isolate (VB49). These plasma samples were tested at 1:10 dilution to determine background non-specific virus inhibition. Non-specific virus inhibition shown by these plasma samples was 5.2 ± 8.2 % (mean ± SD). Therefore 30% neutralization (mean ± 3SD of background response) was considered as cut-off value.

The mean % neutralization (± standard deviations) shown by plasma samples with antibodies against both HLA classes (classes I and II) ranged from -11.4 to 27.9% (Figure 2.9). The mean % neutralization shown by plasma samples with antibodies only against HLA class I ranged from 10.9 to 15.6%, whereas plasma samples with only anti-HLA class II antibodies showed 0 to 30% neutralization (Figure 2.9). Thus, none of the anti-HLA antibody positive plasma sample showed neutralization activity above the cutoff value (30% neutralization).

The purified and concentrated IgG from two plasma samples (40W and 43W) showed higher absorbances than undiluted plasma when tested for anti-HLA antibodies by ELISA (Figure 2.10). The purified IgG from the plasma
samples 40W and 43W showed 29.3 ± 13% and -15 ± 4.9% neutralizing activity, when tested at a minimal (1:2) dilution against the respective virus (Figure 2.11).

HIV-1 neutralization above the cutoff value was not observed, even after complement was added to the reaction mixture. Plasma samples 40W and 43W, when tested in the presence of complement, showed 27% and 29% neutralization, respectively (Figure 2.12). Thus, neither the purified and concentrated IgG nor the plasma tested in the presence of complement showed HIV-1 neutralization. Therefore, there was no evidence for HIV-1-neutralizing activity by anti-HLA antibodies.
Figure 2.9: Anti-HLA antibodies did not show HIV-1 neutralizing activity. Anti-HLA antibody positive plasma from multiparous women were tested for neutralizing activity (shown on Y-axis) against HIV-1 cultivated in husband’s PBMCs (shown on X-axis). Positive control showed >90% neutralization and negative control showed <30% neutralization in all assays. A: % neutralization shown by plasmas showing antibodies against HLA class I and II B: % neutralization shown by plasmas showing antibodies against HLA class I. C: % neutralization shown by plasmas showing antibodies against HLA class II.
Figure 2.10: Anti-HLA antibodies in IgG fraction and plasma obtained from two participants (40W & 43W). Purified IgG and plasma were tested for anti-HLA antibodies using commercially available ELISA kit. Absorbance shown by plasma and IgG is presented on Y-axis whereas X-axis shows reciprocal of dilution.
Figure 2.11: Purified anti-HLA antibodies did not show HIV-1 neutralization. IgG purified from plasma of two anti-HLA antibody positive women (40W & 43W) was tested for neutralizing activity against HIV-1 cultured in husband’s PBMCs. The Y-axis shows %neutralization whereas X-axis show virus against which antibodies were tested. Positive control showed >90% neutralization whereas negative control showed <30% neutralization. The plasma did not show neutralizing activity above cut-off value (30%).
Figure 2.12: Anti-HLA antibodies did not show HIV-1 neutralizing activity in presence of complement. Anti-HLA antibody positive plasma from two women (40W & 43W) was tested for neutralizing activity against HIV-1 cultured in husband’s PBMCs in presence of complement. The Y-axis shows %neutralization whereas X-axis show virus against which plasma was tested. Positive control showed >90% neutralization whereas negative control showed <30% neutralization. The plasma did not show neutralizing activity above cut-off value (30%).
6. Significant amount of HLA proteins were incorporated by HIV-1

After subtracting the amount of HLA proteins present in the microvesicles, lysate of HIV-1 cultured in PBMCs from donor 1 & 2 contained 69.8 & 118.2 ng/ml (i.e. 1.22 & 2.07 nmole/ml) of HLA class I proteins (Molecular weight of HLA class I protein: 57 kδ).

To estimate number of HLA proteins per virion we determined the ratio of HLA protein with HIV-1 p24 protein. Lysate of HIV-1 cultured in PBMCs from donor 1 and 2 contained 1637 & 906 ng/ml (i.e. 68.2 & 37.7 nmole /ml) p24 protein respectively (Molecular weight of HIV-1 p24 protein: 24 kδ). Therefore HLA class I: HIV-1 p24 molar ratio was 0.017 for donor 1 and 0.054 for donor 2 (Table 2.5). Since there are average of 2500 gag (p24) molecules per virion (Trubey et al., 2003), our estimate of HLA: p24 ratio suggests acquisition of 42 to 135 HLA class I molecules in each virion. Since there are ~14 envelope trimers per HIV-1 virion (Zhu et al., 2003; Zhu et al., 2006), our data suggest that the virions of primary HIV-1 isolate may carry more number of HLA molecules than envelope trimers.
Table 2.5. Incorporation of HLA class I proteins by primary HIV-1 isolate

| Donor | Protein in culture supernatant (ng/ml)* | | HLA class I protein/ Gag (p24) molar ratio*
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HLA class I</td>
<td>Gag (p24)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Infected cells</td>
<td>Control cells</td>
<td></td>
</tr>
<tr>
<td>Donor 1</td>
<td>150.2</td>
<td>80.4</td>
<td>1636.8</td>
</tr>
<tr>
<td>Donor 2</td>
<td>223.9</td>
<td>105.7</td>
<td>905.6</td>
</tr>
</tbody>
</table>

* The culture supernatant of HIV-1-infected cells and control cells was ultracentrifuged and the pellet was lysed. The amounts of HLA class I proteins were quantitated by ELISA in both pellets. For pellet of supernatant of infected cells, the amount of p24 protein was quantitated.

* After subtracting the amount of HLA class I proteins in the control
2.6: DISCUSSION

Although HIV infection leads to a strong humoral antibody response against HIV, not all antibodies are able to neutralize the virus in in-vitro neutralizations assays. Antibodies specific to Env (gp160) protein differ in their binding and neutralizing efficiency against different HFV-I strains. Broadly cross-reactive antibodies that are able to neutralize genetically diverse strains of HIV is the desired outcome of HIV vaccination. However, this has not been achieved so far. Therefore novel strategies for induction of broadly cross-reactive neutralizing antibodies are being investigated (described later in detail in chapter 3; Table 3.3). Anti-HLA antibodies have also been reported to mediate HIV-1 neutralization (Leith et al., 2003). Anti-HLA antibody mediated HIV-1 neutralization, if demonstrated conclusively, would offer anti-HIV strategy that would work independent of HIV-1 genetic variations. Considering this potential advantage, anti-HLA antibodies were assessed for their HIV-1 neutralizing activity.

HIV-1 acquires host HLA proteins while budding out of the infected cell. The number of HLA proteins incorporated by HIV-1 exceeds the number of viral Env (gp160) trimers (Arthur et al., 1992; Trubey et al., 2003). Due to the close proximity of HLA and Env trimers on virus surface, binding of antibodies to HLA proteins may sterically hinder the interaction of viral Env with its receptor (Wilfingseder et al., 2003). The hypothesis that steric hindrance for interaction between HIV-1 Env and CD4 receptor may render the virus non-infective has been supported by another experimental evidence (Ren et al., 2005). In this study, binding of an antibody to the region of HIV-1 envelope protein that does not play
role in virus entry into host cell was able to reduce infectivity of the virus. Similar mechanism of neutralization has been described for other viruses. For example, binding of monoclonal antibody 7-1-9 (which is not against receptor binding moiety) to rabies virus causes steric hindrance to virus-receptor interaction (Irie and Kawai, 2002). Binding of monoclonal antibody 4F2 to reovirus causes hindrance to interaction between virus and sialic acid on the host cell (Nason et al., 2001). Due to steric hindrance for virus-receptor interaction, these antibodies show virus-neutralizing activity. Similarly whether binding of antibodies to HLA proteins present on HIV-1 virions cause steric hindrance for interaction of its Env protein with the receptor and mediate virus neutralization was investigated.

Although the strategy of HIV-1 neutralization by anti-HLA antibodies can potentially overcome HIV-1 genetic diversity, there are two major concerns. It may not be possible to know which HLA types will be present on HIV-1 virions that are encountered by the persons at greater risk of HIV infection. In this context, it has been proposed that by appropriately selecting few HLA alleles, it may be feasible to induce an immune response that can overcome HLA diversity in a given population (Lehner et al., 2000). The other major concern would be safety of this strategy. It may be noted that induction of anti-HLA antibodies was found safe in more than 2,000 women undergoing leukocyte immunotherapy (TrialistsGroup, 1994). Recombinant vectors that encode different HLA proteins have been reported and can be used for induction of anti-HLA antibodies.

During pregnancy, women get exposed to semi-allogenic fetal cells that pass through maternal-fetal barrier and produce anti-HLA antibodies. Multiparous
women are repeatedly exposed to semi-allogenic fetal cells. Early characterization of HLA antigens was carried out using plasma from multiparous women. We screened plasma from women with multiple parity for anti-HLA antibodies. Plasma samples showing anti-HLA antibodies were used in the experiments conducted for examining anti-HIV activity of anti-HLA antibodies. Women with history of tissue transplantation were excluded to minimize possibility of anti-HLA antibodies of unrelated specificity.

Plasma from women was tested for anti-HLA antibodies by commercial ELISA kit and 14 women with anti-HLA antibodies were identified (Table 2.3). To confirm that the anti-HLA antibodies are specifically directed against HLA types of husband, wife’s plasma was incubated with their own (autologous) and husband’s PBMCs. The plasma contained antibodies that bound to husband’s lymphocytes but did not show binding to autologous lymphocytes in flow cytometric assays (Figure 2.6). After incubation of plasma with husband’s PBMCs, greater than 75% decrease was observed in the level of anti-HLA antibodies, indicating that antibodies detected in the plasma are specific for husband’s HLA proteins (Figure 2.7). The level of anti-CMV IgG antibodies measured as control did not show any decline confirming the specificity of binding.

A primary HIV-1 isolate was cultured in PBMCs from husbands so that the virus carried husband’s HLA proteins. HIV-1 carrying husband’s HLA proteins on the envelope was incubated with wife’s plasma to test whether anti-HLA antibodies in plasma neutralize virus-carrying HLA of matching
specificities. Pooled plasma from HIV seronegative, healthy individuals was used as negative control and pooled plasma of HIV seropositive individuals was used as positive control. The negative control showed less than 30% virus inhibition whereas positive control showed greater than 90% inhibition in all the assays. The 30% neutralization (30% reduction in infectivity) was used as a cut-off value based on earlier preliminary work in which plasma from 20 normal, healthy individuals were tested against the HIV-1 isolate (VB49). Non-specific virus inhibition shown by these plasma samples was 5.2 ± 8.2 % (mean ± SD). Therefore 30% neutralization (mean + 3SD of background response) was considered as cut-off value. None of the 14 plasma from multiparous women showed greater than baseline neutralization that was seen in HIV uninfected healthy control plasma. The results clearly indicate that in our experimental system anti-HLA antibodies did not show neutralization of HIV-1 carrying matching HLA proteins on the envelope.

Factors such as inadequate level of anti-HLA antibodies in the plasma or absence of optimal density of HLA molecules on the HIV-1 envelope may be responsible for lack of HIV-1 neutralizing activity. Leith et al have reported neutralization of HIV after incubation with anti-HLA antibodies (Leith et al., 2003). In this study, anti-HLA antibodies were obtained from women who had been immunized with multiple injections of $10^8$ allogenic lymphocytes (Leith et al., 2003). Anti-HLA antibodies were detectable in plasma from these women at 1:100 dilution. The study in which anti-HLA-DR antibody positive serum showed HIV-1 neutralization (Arthur et al., 1995), the titer of antibodies was high enough
to immunoprecipitate $^{125}\text{I}$-HLA-DR at $>1:500$ dilution. Although there may be relatively lower titer of anti-HLA antibodies in the plasma of multiparous women compared to those who have been deliberately alloimmunized, it was not possible to test them at dilution lower than 1/10 because at such low dilution, non-specific HIV-1 inhibition may be seen. IgG antibodies were purified from two plasma samples (40W & 43W). The purified IgG showed higher level of anti-HLA antibodies than the plasma (Figure 2.10). However, purified IgG did not show HIV-1-neutralizing activity (Figure 2.11) even when it was tested against respective virus at highest possible concentration. Although no evidence was obtained for HIV-1 neutralizing activity of anti-HLA antibodies from naturally alloimmunized multiparous women, one needs to consider possibility that high antibody titers that may arise out of deliberate alloimmunization may still afford some degree of protection.

The number of discordant HLA alleles between woman and her husband can influence on outcome of this study. If husband carries multiple discordant HLA alleles, then wife is likely to show polyspecific anti-HLA antibodies. More number of antibodies from plasma of such woman may bind to HIV-1 virions cultured in her husband’s cells than the plasma with mono-specific antibodies. The study in which plasma from deliberately alloimmunized woman showed neutralizing activity against HIV-1 cultured in husband’s PBMCs (Leith et al., 2003), the anti-HLA antibodies produced by woman were directed against three HLA alleles (A19, A24 and DR4) that were expressed by husband. Although in present study, HLA allele specificity of anti-HLA antibodies was not determined,
it is unlikely that all women carried monospecific antibodies. Out of 14 anti-HLA antibody positive samples, 6 showed antibodies against both classes of HLA (Class I & II) and remaining were positive for antibodies against either class of HLA alleles. Although some plasma samples showed antibodies against only one class of HLA alleles, they may be positive for multiple HLA alleles within that class.

Spruth et al and Wilfingseder et al reported neutralization of HIV-1 IIIB (laboratory adapted strain that was cultured in T cell lines) in presence of anti-HLA antibodies and complement (Spruth et al., 1999; Wilfingseder et al., 2003). The virus inhibition reported in those studies was partly due to complement-mediated virus lysis (Wilfingseder et al., 2003). It has been reported that HIV-1 derived from T-cell lines are highly susceptible to complement, due to low incorporation of complement-regulatory proteins (CD55 and CD59) (Stoiber et al., 1997). Another mechanism for enhanced neutralization in the presence of complement may be due to higher steric hindrance to virus entry by the complement-antibody complex than by the antibody alone as described for respiratory syncytial virus (Yoder et al., 2004). Therefore anti-HLA antibodies were tested against HIV-1 in presence of complement. HIV-1 neutralization by anti-HLA antibodies was not seen even in presence of complement (Figure 2.12). This might be due to resistance of primary HIV-1 isolate (which is cultured in PBMCs) to complement-mediated lysis. It has been shown that primary HIV-1 isolate, cultured in PBMCs incorporates complement-regulatory proteins
(Saifuddin et al., 1994; Saifuddin et al., 1995; Stoiber et al., 1997) that can confer resistance to complement-mediated viral lysis.

The prerequisite for anti-HLA antibody-mediated HIV-1 neutralization is acquisition of HLA molecules by HIV-1 in the envelope. It has been reported that the amount of HLA proteins acquired by HIV-1 may differ depending on viral strain and the type of host cell (Cantin et al., 1996). Therefore the extent of incorporation of HLA proteins by HIV-1 strain that was used in present study (VB49) was determined. The molar ratio of HLA class I protein to HIV-1 capsid (P24) protein was 0.017 & 0.054 for the virus (VB49) cultured in PBMCs of two healthy individuals (Table 2.5). Since there are average of 2500 capsid molecules per HIV-1 virion (Chertova et al., 2002; Trubey et al., 2003), our estimate of HLA: p24 ratio suggests acquisition of 42 to 135 HLA class I molecules by each virion. Since there are ~14 envelope trimers per HIV-1 virion (Chertova et al., 2002; Zhu et al., 2003; Zhu et al., 2006), our data suggest that the virions of primary HIV-1 isolate in our experimental system, carried more number of HLA molecules than envelope trimers. Similar findings have been reported earlier (Arthur et al., 1992; Trubey et al., 2003). Further it was determined whether anti-HLA antibodies present in plasma of multiparous women bind to HLA proteins incorporated in HIV-1 virions. When wife’s plasma was incubated with HIV-1 cultured in husband’s PBMCs, the virus could be captured on a well coated with anti-human IgG antibody. When these plasma samples were incubated with HIV-1 cultured in their autologous PBMCs, the virus was not captured (Figure 2.8).
This indicates that wife’s plasma contained antibodies that bound to the HIV-1 virions that carried husband’s HLA types.

Despite incorporation of significant number of HLA molecules by HIV-1 virions, anti-HLA antibodies were unable to reduce viral infectivity. The lack of HIV-1-neutralizing activity by anti-HLA antibodies may be attributed to the difference in size between HLA and HIV-1 envelope proteins. The HIV-1 gp160 trimer is 483 kDa, whereas HLA class I and II molecules are 57 and 61 kDa, respectively. Binding of IgG antibody (150 kDa) to HLA molecules can form a complex of ~210 kDa. Since the HIV-1 envelope molecule is relatively large and it forms a spike that bulges out of the membrane, binding of anti-HLA antibodies may not interfere with the interaction between viral gp160 and the CD4 receptor. Although this could be anticipated without experimental investigation, the complexity of the mechanism of viral entry into the target cell, experimental evidences of anti-HLA antibody mediated HIV-1 neutralization (Leith et al., 2003; Spear et al., 2001; Spruth et al., 1999; Wilfingeder et al., 2003) and the potential advantage of this approach prompted this investigation. The study shows that primary HIV-1 isolate cultured in PBMCs incorporates large number of HLA proteins. However binding of antibodies to HLA proteins does not mediate HIV-1 neutralization.

In all earlier studies where anti-HLA antibodies have been shown to mediate HIV-1 neutralization, the finding is based on not more than two plasma samples. Spruth et al (Spruth et al., 1999), Spear et al (Spear et al., 2001) each have reported two anti-HLA antibody positive plasma samples with HIV-1
neutralizing activity whereas Leith et al (Leith et al., 2003) could detect HIV-1 neutralizing activity only in one of two anti-HLA antibody positive plasma samples. In one of the study (Spruth et al., 1999) neutralizing activity was detected only against laboratory-adapted HIV-1 strain (HIV-1 IIIB). In the study presented here, 14 anti-HLA antibody positive plasma samples were tested for neutralizing activity against primary HIV-1 isolate. None of the plasma sample showed virus-neutralizing activity.

The finding reported here is in agreement with earlier report by Polyanskaya et al (Polyanskaya et al., 1997). In this study, macaques were vaccinated with simian allogenic B cells. The macaques showed antibody responses to MHC class I and II but they were not protected against SIV challenge. However in this study, the anti-HLA antibodies were not able to precipitate HLA molecules present in the challenge virus. Therefore there was no evidence whether anti-HLA antibodies bound to HIV-1 virions or not. In present study, it was found that although anti-HLA antibodies bind to HIV-1 virions (Figure 2.8), these antibodies did not mediate HIV-1 neutralization.

Although HIV-1 neutralization is reported by anti-HLA antibody positive plasma from deliberately alloimmunized women (Leith et al., 2003) and polytransfused patients (Spear et al., 2001; Spruth et al., 1999), those plasma samples were not tested for anti-CCR5 antibodies. Anti-CCR5 antibodies have been reported in xenoimmunised macaques (Lehner et al., 1999) and alloimmunised individuals (Wang et al., 1998). It has been suggested that xeno or alloimmunization might break tolerance to CCR5 resulting in formation of anti-
CCR5 antibodies (Wang et al., 2002). These antibodies may cause internalization of CCR5 and block entry of R5 strains of HIV-1. The studies where macaques were xenoimmunized and protected against SIV, factors such as secretion of β-chemokines, CD8 soluble factors and anti-CCR5 antibodies may have contributed in protection. Wang et al, immunized macaques with inactivated SIV that was earlier cultured in human CD4⁺ T cells and subsequently challenged them with live SIV. These investigators reported that secretion of β-chemokines was significantly higher in macaques that were protected against SIV (Wang et al., 1998).

Earlier studies of anti-HLA antibody mediated HIV-1 neutralization were inconclusive for various reasons. Therefore whether anti-HLA antibodies mediate HIV-1 neutralization was investigated. The study show that HIV-1 primary isolate incorporates significant amount of HLA proteins when cultured in PBMCs. However binding of antibodies to these proteins does not reduce virus infectivity.