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MOTHER-TO-CHILD TRANSMISSION OF HIV-1: THE ROLE OF HIV-1 VARIABILITY AND THE PLACENTAL BARRIER*

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The acquired immunodeficiency syndrome (AIDS), which is caused by the human immunodeficiency virus (HIV), was first described in the United States of America in 1981 [1]. The worldwide spread of HIV has soon been recognized and AIDS has become one of the most alarming infectious diseases of our days. Its impact has been tremendous, high morbidity and mortality has caused a reversal of socioeconomic gains previously recorded in several developing countries, especially those in Sub-Saharan Africa [2].

Epidemiological data about the HIV and AIDS pandemic are updated by the Joint United Nation Programme on HIV/AIDS, UNAIDS (http://www.unaids.org). Their latest report from December 2000 states that in year 2000 approximately 5.3 million people have become newly infected with HIV, of which 2.2 were women and 600 000 children younger than 15 years of age. The estimated number of people living with HIV/AIDS globally is 36.1 million, of which 16.4 million are women and 1.4 million are children younger than 15 years of age. Approximately 25.3 million (70%) of these HIV infected people live in Sub-Saharan Africa, 5.8 million in South- and South-East Asia (15%), and 1.4 million in Latin-America (5%). During year 2000, 3 million people died of AIDS (1.3 million women and 500 000 children younger than 15 years of age). This means that an estimated total of 21.8 million persons have died of AIDS since the beginning of the epidemic, including 4.3 million children younger than 15 years of age.

Keywords: HIV-1, HIV-1 variability, vertical transmission, placental barrier

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The course of HIV-1 infection

HIV-1 infection in adults can be divided into three clinical stages: 1) an acute phase with pronounced viremia and with or without clinical symptoms of primary HIV-1 infection, 2) a chronic phase with minimal, but detectable, clinical and immunological abnormalities in the presence of less viremia and 3) a late stage with profound immunodeficiency which is often accompanied by opportunistic infections and/or neoplasias [3].

Disease progression is mainly monitored by registration of clinical symptoms and measurements of plasma HIV-1 RNA level and CD4+ and CD8+ T-lymphocyte counts. A decrease in CD4+ lymphocyte counts and CD4+/CD8+ lymphocyte ratios indicates progressive disease. The immunological deterioration is an important marker for disease progression, but all available data indicate that the plasma HIV-1 RNA level is the best prognostic marker [4] and the best instrument for monitoring antiretroviral therapy [5, 6].

The rate of CD4 cell decline is closely linked to the plasma HIV-1 levels, but the underlying reasons for CD4 cell destructions are incompletely understood (for review see [7]). However, it is clear that interdependent host, viral and environmental factors are of importance. Here we will focus on HIV-1 infection in the pregnant woman and in children.

HIV-1 infection during pregnancy

In normal pregnancy, the humoral response is normal but the cellular immunity has been shown to be impaired [8–10]. Thus, the number of CD4 cells and CD4+/CD8+ ratios are decreased, especially in early pregnancy [11–13]. This is thought to be favourable for accepting the fetus, which carries foreign paternal antigens.

In women with intercurrent disease such as diabetes, glomerulonephritis and cardiopathy, pregnancy can accelerate the course of the disease. Likewise, some infectious diseases such as tuberculosis, malaria and polio have been reported to carry increased morbidity and mortality in pregnant women in comparison to non-pregnant women [9]. This question has been carefully considered also in HIV-1 infected pregnant women.

Early case reports of HIV infected women with opportunistic infections during pregnancy suggested an acceleration of disease progression [14, 15]. However, these women had an advanced HIV disease. These findings have not been confirmed in later studies on mainly asymptomatic women [16–19]. Nevertheless, immunological markers are subject to large variability during pregnancy in both uninfected and HIV-1

infected women. It should also be mentioned that development of AIDS is more problematic in a pregnant HIV-1 infected woman, because the clinical complications may be more difficult to manage during pregnancy.

HIV-1 infection in children

Pediatric HIV-1 infection is primarily due to mother-to-child transmission of the virus. The first child with HIV-1 infection was described in 1982 [20]. It soon became evident that diagnosing HIV infection in children was more complicated than in adults. The course of infection and the clinical characteristics of AIDS in children appeared also different from those in adults [21–28].

Mother-to-child transmission of HIV-1

Rate of transmission

In the absence of any intervention, the reported rates of mother to child transmission of HIV-1 ranges from 15–25% in Europe and the U.S. [29] to 25–42% in Africa [30–32]. It has been discussed if these differences, at least in part, may be due to methodological differences and proposed that standardized methods should be used to estimate rates of mother-to-child transmission of HIV-1 [33]. Nevertheless, transmission rates in Africa still appear to be twice as high as in Europe. These geographic differences are not fully understood, but may be influenced by differences in frequency of breastfeeding, other concomitant infections in the mother, as well as other differences between host and viral factors.

Timing of transmission

Transmission of HIV-1 from mother to child can occur in utero, at the time of delivery (intrapartum) and postnatally through breastfeeding.

1. In utero *transmission*

In utero transmission is supported by studies based on PCR and *in situ* hybridisation. Thus, HIV-1 has been detected in fetal tissues obtained during the first and second trimester [34–36]. Lewis et al. showed that embryonic blood cell precursors were infected *in vitro* by HIV-1 in 8-week fetuses [37]. An important study showed that 2 of 100 fetuses were HIV-1 infected at half pregnancy [38]. This means that

approximately 10% of all perinatally infected children become infected in early pregnancy.

2. Transmission at time of delivery

Suggestive evidence of late or intrapartum transmission came first from observations from a study on HIV-1 infected pregnant women and their offsprings. HIV-1 was easily detected in the plasma of the mothers, but not in their newborn children or in the fetuses, but easily in the child at 6 months of age [39]. Shortly after, observations from a register of twins was published, which found that the first-born twin had a twofold higher risk of becoming HIV-1-infected than the second-born twin [40]. Exposure of the fetus to virus in cervico-vaginal secretions or maternal blood is thought to play a role, although the same phenomenon was observed for twins delivered by Cesarean section. There are more recently reports indicating that the mode of delivery affects the transmission rate. Thus, delivery by elective Cesarean section prior to labour and rupture of membranes decreased [41, 42], whereas prolonged rupture of membranes (>4 hours) increased the risk of transmission [43]. Thus available data indicates that a large proportion of infections occurs at time of delivery or in late pregnancy [44], for review see [45, 46].

3. Transmission through breastfeeding

A meta-analysis of studies of transmission through breastfeeding showed the additional risk of transmission to be between 7 and 22% [47]. A recent study conducted in Durban, South Africa showed that the risk of vertical transmission was considerably lower during exclusive breastfeeding than during mixed feeding [48]. It is believed that the lower transmission rate in exclusively breastfed infants is explained by the fact that these babies develop a healthier gut epithelium, which acts as a viral barrier [49–51]. This is an important finding since it is the first time that the risk associated with exclusive and mixed breastfeeding has been distinguished. Infant feeding practice needs further investigation since this study was not randomized. It has also been suggested that late weaning increases the risk of postnatal transmission [52, 53].

4. Definition of in utero *vs. intrapartum infection*

A working definition for the classification of the timing of transmission has been proposed, which is based on the time of HIV-1 detection in the infant. If virus is detected within 48 hours of birth, an infant is considered to have been infected *in utero*,

while intrapartum infection is assumed if viral studies are negative during the first week of life, but become positive between 7 and 90 days of life [54]. If infected around the time of delivery, the incubation time from infection to becoming PCR positive appears to be around one week [44].

Table I

Factors affecting mother-to-child transmission of HIV-1

	Factors	References
Viral	•RNA level in plasma	[55–57]
	•Viral genotype: minor or major viral population from the mother can be found in the infant	[58, 59]
	•Viral phenotype: NSI viruses transmitted to the infant SI viruses associated with transmitting mother	[60]
	•Viral resistance	[61]
Maternal	•CD4 cell count	[62]
	•Neutralizing antibodies	[63, 64]
	•Nutritional status	[65]
	•Clinical status	[24]
	•Behavioural factors	[66, 67]
	•Antiretroviral treatment	[68, 69]
Obstetrical	•Prolonged rupture of membranes more than 4 hours	[70, 71]
	•Mode of delivery	[42, 72]
	•Intrapartum haemorrhages	[66]
	•Obstetrical procedures	[43, 62]
	•Invasive fetal monitoring	[73]
Fetal	•Prematurity	[74]
	•Genetic factors	[75–77]
Infant	•Breastfeeding	[47, 78]
	•Immature immune system	[79]

Factors affecting mother-to-child transmission

Transmission of HIV-1 from mother to child is affected by a number of factors, of which many have not been fully elucidated. These can be divided into viral, maternal, obstetrical, fetal and infant factors (Table I). Here we are going to consider the influence of the viral genotype and phenotype on transmission of HIV-1 from mother to child.

Co-receptor usage and genetic subtype of HIV-1

Genetic variability is one of the major characteristics of HIV-1. In fact, HIV-1 has been described as a quasi-species, which consists of a cloud of closely related but distinct genetic variants around a central point ("the master-variant") [80]. HIV-1 can be regarded as a pool of genetic variants, from which pre-existing variants with favourable characteristics can be selected providing a potential for rapid adaptation.

At present, three HIV-1 groups have been identified: M (main), O (outlier) [81], and N (non-M-non-O) [82]. Group M viruses cause the global HIV-1 epidemic, whereas group O and N viruses occur at low frequency mainly in certain regions of Central Africa. Group M is divided into nine subtypes denoted A, B, C, D, F, G, H, J and K [83–85]. Subtypes E and I have been dropped because they turned out to be recombinant viruses [83]. The genetic distances within and between subtypes depend both on the region of the genome studied and the method used to measure the genetic distances. Generally members of the same subtype differ by less than 10% and those of different subtypes by more than 10% in the gag gene. Considering variable region 3 (V3) of the viral envelope, variation within an infected individual may be as high as 10–15%, between individuals carrying virus of the same genetic subtype about 30%, and different subtypes may differ more than 50% in nucleotide sequence [86].

Biological variability is another major characteristic of HIV-1. Primary HIV-1 isolates can be divided into different biological phenotypes according to specific *in vitro* properties, such as replicative and syncytium inducing capacities and cellular tropism [87–89]. The discovery that chemokine receptors may function as co-receptors when HIV-1 enters cells [90–93] was the missing link to our understanding of the molecular background of HIV-1 biological variation. Thus, the chemokine receptor CXCR4 serves as the major co-receptor for viruses earlier called rapid/high or syncytium inducing (SI), while CCR5 serves as the major co-receptor for slow/low or non-syncytium inducing (NSI) viruses. This allowed the introduction of a unifying nomenclature that classify HIV-1 isolates on the basis of their co-receptor usage [94]. The issue of biological variation and co-receptor usage has been dealt with in an earlier article [95].

Fig. 1. Co-receptor usage of 322 virus isolates from plasma or PBMC representing nine different genetic subtypes. Several isolates were obtained from the same patients. Co-receptor usage was determined by scoring for syncytium formation, expression of green fluorescent protein, GFP (in the GHOST assay) and HIV-1 p24 antigen production. Both panels of indicator cells were kindly provided by Dr. Dan L. Littman at the Skirball Institute for Biomolecular Medicine, New York University, but are now available from the NIBSC, AIDS Reagent Programme, MRC, UK and the NIH AIDS Research and Reference Reagent Programme, USA

Several studies have demonstrated that the severity of immunodeficiency *in vivo* correlates with the biological phenotype of virus isolates in vitro [87, 96–98]. Following the discovery of co-receptors, it could be pinpointed that, in analogy with the previously described phenotypic patterns for HIV-1 of subtype B, viruses using CCR5 (R5 viruses) predominate in early stages of HIV-1 infection, while X4 and dual-tropic R5X4 variants appear at late stages [99]. Also, among subtype B infected mothers those with CXCR4-using virus were at higher risk of transmission of HIV-1 to their children than mothers harbouring R5 virus [60] and unpublished. The importance of the co-receptors *in vivo* was further reinforced by studies on polymorphisms in genes coding for the co-receptors and their ligands. The most important polymorphism occurs in the CCR5 gene, CCR5Δ32 [100–103], a deletion that causes a defect membrane expression of CCR5. Consequently, homozygosity for CCR5Δ32 protects against infection with HIV-1 using CCR5 for cell entry. Heterozygotes are not protected against infection but show a slower rate of HIV-1 disease progression [104].

We were interested in the question whether different genetic subtypes differ in co-receptor usage and thereby may differ in important biological properties, such as virulence and transmissibility. Overall we have tested 322 primary HIV-1 isolates

representing eight different subtypes (A, B, C, D, F, G, H and J) and one recombinant type (CRF01-AE) on U87.CD4 and/or GHOST(3) indicator cells engineered to express CD4 and either of the chemokine receptors CCR1, CCR2b, CCR3, CCR5 or CXCR4 or the orphane receptors Bonzo or BOB [105-107]. We found that CXCR4 co-receptor usage correlated with the rapid/high, SI phenotype of the HIV-1 isolates and was independent of genetic subtype (Fig. 1).

In course of these studies we and others observed that the CXCR4-positive phenotype was underrepresented among subtype C isolates, even if the viruses were obtained from AIDS patients [105, 108–110]. The finding opened up the possibility that subtypes, in this case subtype C, may differ in virulence. Nevertheless, according to clinical studies, no difference was seen in disease progression for HIV-1 subtype C infected individuals [110–112]. Conceivably, other characteristics of subtype C viruses, such as extra NFκB sites in LTR, lack of a potential glycosylation site at the base of the V3 loop or an extra cysteine residue in Nef that is expected to alter the folding of the protein [113–118] may compensate for the R5 predominance, which alone would reduce virulence.

Subtype C had a low prevalence in the early phase of the HIV pandemic but is now spreading rapidly worldwide. At present, HIV-1 subtype C has been estimated to account for more than 50% of HIV-1 infections worldwide (htpp://www.unaids.org). In the context of mother-to-child transmission it is important to remember that in the Swedish material among 11 mothers with infected children, HIV-1 of subtype C was not overrepresented. Six different subtypes were present in this group and two of these were subtype C [119]. In another study examining the placental barrier [107], among nine HIV-1 infected mothers with uninfected children, four mothers carried subtype C virus. From two of the mothers virus with R5 and R5X4 phenotype could be isolated at delivery. This material is small, nevertheless no difference to other subtypes could be detected.

Virus isolates were also tested for usage of the orphan receptors Bonzo/STRL33/TYMSTR and BOB/GPR15 on GHOST(3) cells [106]. Among the Cameroonian women [106], we found that subtype A isolates (by envelope sequence) from five pregnant women and one child could use Bonzo in addition to CCR5. Bonzo has been shown to support efficient cell-cell fusion by a wide variety of SIV env proteins [120], but was inefficient at mediating infection by HIV-1 isolates [120, 121]. In addition to our findings, only isolates from one mother-infant pair infected with subtype B could use Bonzo as co-receptor in the GHOST cell system [122, 123]. BOB could also be used as co-receptor by some isolates in a panel representing five different genetic subtypes (A, B, C, D and F) [124]. Our data suggest that some HIV-1 isolates

may use Bonzo or BOB for productive infection in vitro, but the *in vivo* significance of this finding remains to be established.

Evolution of co-receptor usage during pregnancy

Evolution in co-receptor usage, i.e. a broadening in the capacity to use several co-receptors, including CXCR4, during clinical progression is a well-known phenomenon [99, 125]. Whether evolution of co-receptor use may be favored by the particular hormonal and immunological changes during pregnancy has since long been a matter of discussion. The Cameroonian cohort consisting of 28 women followed during pregnancy [106], gave us the possibility to address this question. Four mothers showed changes in co-receptor usage over time. Virus from two of these mothers acquired the capacity to replicate in Bonzo expressing cells. Both of these mothers transmitted the infection to their children. In one non-transmitting mother the virus lost the capacity to replicate in Bonzo expressing cells, while in another mother the virus showed fluctuations in its capacity to use CCR3. Comparison of the Cameroonian subtype A viruses with subtype B viruses (from Italy) showed that, in both cases, among transmitting mothers dual- or multitropic viruses were more frequent than in the group of non-transmitting mothers (Table II). While multitropism with subtype B involves in all cases CXCR4 use, subtype A viruses use Bonzo (in combination with CCR3 and/or CCR1). Taken together, 36.8% of transmitting mothers and 16.2% of non-transmitting mothers harbour multitropic viruses (Fischer exact test, p=0.007). The data suggets that presence of multitropic virus in the mother may increase the risk of HIV-1 transmission to the child.

In what way could the described changes in co-receptor use of HIV-1 be related to pregnancy? Alterations in certain cytokines and chemokines have been observed during pregnancy, which conceivably could modify HIV-1 replication [126]. In particular, IL-10 production, which has been reported to down-regulate HIV-1 replication [127], increases during pregnancy. Consequently, IL-10 and other cytokines may influence the milieu of the virus and thus favour growth of certain virus variants. Furthermore, all published Bonzo-using HIV-1 isolates that we are aware of, have been isolated from pregnant women and their infants. In this context, it is interesting to note that, expression of Bonzo mRNA is restricted to lymphoid tissue, PBMC and placenta [128–130]. Thus, trophoblasts from early placentae have been documented to express CCR5, CXCR4, Bonzo and GPR1 [131], whereas term trophoblasts do not express CXCR4, CCR5, CCR3, CCR2b or Bonzo [132]. Furthermore, there is evidence of bidirectional traffic of leukocytes across the placenta during pregnancy [133]. Naive CD45RA+/CD62L+ cells that express Bonzo are much more abundant in cord blood than in adult peripheral blood [134] and between these naïve cells and maternal blood

may be a route of infection. Clearly, much more work is needed to clarify if pregnancy favours the outgrowth of virus with certain biological properties.

Table II

Comparison of co-receptor use of HIV-1 env *subtypes A and B*

Genetic subtype	Mother	No. of mothers	Biological phenotype close to delivery	
			R5 only	Dual/multitropic
A	Transmitting	4	2	2 R5 Bonzo
	Non-transmitting	18	14	2 R5Bonzo 2 R5R1
B	Transmitting	15	10	3 R5X4 2 R5R3X4
	Non-transmitting	19	17	1 R5X4 1 R5R3X4

Interventions reducing mother-to-child transmission/ethical aspects

In industrialized countries

The ACTG076 study, which was published in 1994 [68], showed that zidovudine prophylaxis significantly reduces mother-to-child transmission of HIV-1 by approximately 2/3, from 26 to 8%. Consequently, zidovudine treatment has been recommended from gestational week 26. At delivery, intravenous zidovudine is given. The child is also treated from birth and during 6 to 8 weeks. Blanche et al. reported a possible adverse effect, i.e. mitochondrial dysfunction, in zidovudine-exposed children [135]. However, this has not been confirmed in the trials from the United States [136]. Nevertheless, this underlines the potential risk of giving a toxic drug to children, who would remain uninfected in the majority of cases also without treatment. When zidovudine prophylaxis is given together with elective Cesarean section the transmission risk is reduced to less than 2% [42].

In resource poor settings

UNAIDS has recognised that pregnant women have a right to free HIV testing and, if they are found to be positive, to proper counselling (VCT) [137]. Numerous clinical trials using short-term treatments have shown very promising results, especially HIVNET012 a randomised trial of the antiretroviral drug, nevirapine given as a single dose [69]. Nevirapine lowered the risk of HIV-1 transmission during the first 14–16

weeks of life by nearly 50% in a breastfeeding population. This simple and inexpensive regimen could decrease mother-to-child transmission of HIV-1 in less-developed countries.

Ethical aspects

The Declaration of Helsinki is a statement on research ethics, which was written on the initiative of the World Medical Association in 1964. The goal of the declaration is to protect the subjects taking part in biomedical research from abuse and exploitation [138]. Some clinical trials on mother-to-child transmission of HIV-1 have been argued to be in conflict with the Helsinki declaration [139]. After the publication of ACTG076 trial in 1994, all placebo-controlled clinical trials can be considered to be unethical, but nevertheless such studies have been continued because they give rapid and valid assessment of an alternative drug regimen to prevent vertical transmission [139, 140]. In order to avoid this type of problem, journal editors should routinely require that every paper reporting results of medical research contain a section on ethical considerations and clearance.

The placental barrier

Structure and function of the placenta

The placenta is a fetal organ of exchange, interposed between the fetal and maternal circulations [141]. The basic functional unit of the placenta is the chorionic villus. Chorionic villi are simple structures that have a core of loosely arranged mesenchyme, which is covered by a double layer of trophoblastic cells (Fig. 2). Hofbauer cells, fibroblasts, lymphocytes, macrophages and fetal capillaries are scattered within the mesenchyme. The inner layer of the trophoblast is made up of cuboidal cells called cytotrophoblasts, which generate and maintain the outer layer. The outer layer of trophoblastic cells is termed the syncytiotrophoblasts and is a continuous multinucleated epithelium in direct contact with maternal blood. The placental barrier which separates the maternal and fetal blood systems is composed of the two cell layers of trophoblasts, the connective tissue of the chorionic villi and the endothelium of the fetal villous vessel. The structure of the placenta is established in the first trimester and has acquired a definite form by the end of the fourth month. As the placenta grows and ages, certain histological changes are suggestive of an increase in the efficiency of transport to meet the growing fetal metabolic requirements. Such changes involve a decrease in thickness of the syncytiotrophoblastic layer, a partial disappearance of the

cytotrophoblasts, a decrease in stromal volume, and an increase in the number of capillaries and their approximation to the syncytial surface.

Fig. 2. Schematic view of a chorionic villus

Trophoblasts are the fetal tissue directly in contact with maternal blood. Class II MHC antigens are absent from trophoblasts at all stages of gestation, which is important for maternal acceptance of the fetus. The only Class I antigens expressed in human cytotrophoblasts is the nonpolymorphic HLA-G [142]. HLA-G expression may be crucial for invasion of the trophoblasts in the endometrium-decidua by helping to avoid killing by maternal NK-like cells (large granular lymphocyte). The syncytiotrophoblast layer expresses neither HLA class I nor HLA class II antigens [143].

The placenta in HIV-1 infected women

Upon macroscopic examination, the placenta in HIV-1 infected pregnant women often appears normal. Increased placental weight has been observed, but may not be directly related to HIV-1 infection [144]. Chorioamnionitis is the only histopathologic abnormality found with increased frequency, 43% in HIV-1-infected mothers vs. 20% in an uninfected control group [144, 145]. No abnormalities have been reported in the villous architecture of the placentae from HIV-1-infected mothers.

Immunohistochemical techniques and *in situ* hybridisation have been used in the evaluation of HIV-1-infected placentae from mothers without antiretroviral therapy. The results are contradictory. Expression of HIV-1 p24 antigen and presence of HIV-1 nucleic acid has been seen in trophoblasts from early and term placentae [37, 146, 147]. Hofbauer cells have also been found infected [37, 146, 147]. However, in these studies, the cell types were distinguished by morphology only, which makes identification uncertain. Other studies did not detect viral proteins in early and term placentae [148, 149]. Electron microscopy has detected retroviral particles, but not budding viruses [144]. These findings could be related to endogenous retroviruses [150] and need further investigation. In all cases, HIV-1 expression in the placenta did not correlate with HIV-1 infection of the fetus.

Possible mechanisms of *in utero* transmission of HIV-1 from mother to infant

Several different possible scenarios have been proposed and intensely discussed (see Fig. 3 and below). An important basis for these discussions is the fact that the virus population is genetically quite homogeneous early after transmission and that virus with R5 phenotype is much more common than virus with X4 or R5X4 phenotype [151, 152]. Many researchers have interpreted these findings as a sign of selection for virus with certain biological properties (i.e. R5 phenotype) during or immediately after transmission. The findings and discussion concerning sexual transmission of HIV-1 are very similar, but it is unclear if there are differences in mechanisms of perinatal and sexual transmission.

HIV-1 infection of the placenta

The trophoblast and syncytiotrophoblast layer, which is the outer layer of the placenta, is directly exposed to maternal blood. It has been extensively discussed whether this direct exposure of cells to HIV-1 infected blood could infect these cells. We addressed this question by using highly purified placental trophoblasts obtained from HIV seropositive mothers undergoing antiviral therapy and tested these by PCR amplification of HIV DNA and RNA [127]. We found the immunomagnetically purified trophoblast preparations HIV negative.

The cell fractionation procedures whereby trophoblasts are obtained seem to be crucial [153] and the level of contaminating nontrophoblastic cells will strongly influence the outcome of HIV detection. Other studies have detected HIV sequences in placental cell preparations [154, 155]. These cell preparations were obtained after only one round of immunomagnetic cell depletion using a single anti-CD45 monoclonal

antibody. In contrast, we carried out immunomagnetic cell separations in three sequential steps and used specific monoclonal antibodies for each cell type [107].

Fig. 3. Possible mechanisms for *in utero* transmission of HIV-1

HIV DNA was not detected in our immunomagnetically purified trophoblast preparations. This conclusion is supported by the finding of Kilani et al. [156] that pure placental trophoblasts resist infection by several different cell-free primary HIV-1 isolates. In fact, we were able to identify the HIV-1 DNA positive cells as CD3+ or CD14+, i.e. contaminating T-lymphocytes or macrophages, respectively. This is in line with previous studies demonstrating that non-trophoblastic placental cells carry HIV infection *in vivo* and are susceptible to infection *in vitro* [34, 149, 157, 158].

Another problem with inadequate separation techniques in these types of experiments is that the non-trophoblastic cell fraction of the placenta will consist of a mixture of maternal and fetal cells. Consequently, the demonstration of HIV infection in non-trophoblastic cells may simply be a reflection of the fact that the maternal blood in the placenta contains HIV infected CD4+ lymphocytes and monocytes. De Andreis and colleagues detected HIV provirus in more than 70% of placentae from HIV-1 infected mothers, who did not receive antiretroviral therapy [159]. They used DNA polymorphism analysis, based on identification of hypervariable regions in the human genome to rule out maternal contamination. However, this method can confirm, but not exclude, maternal contamination due to limitations in the sensitivity of detection of minor sequence variants. This means that significant contamination by HIV infected maternal blood cells may have gone unnoticed [Chamberlain, 1980 #1045]. We used two approaches to test the origin of HIV infected cells. First, the amount of HIV DNA

in each cell fraction was determined by a semi-quantitative, limiting dilution assay. For all mothers, PBMC and placental CD3+ T-lymphocytes carried similar amounts of HIV-DNA suggesting that the viral DNA was of maternal origin. Second, we determined the origin of cells in the different cell preparations by microsatellite analysis. Indeed, in our experiments we could confirm that the different non-trophoblastic placental fractions consisted of mixtures of maternal and fetal cells.

While CD3+ T-lymphocytes were regularly HIV positive, DNA in CD14+ cells of the placenta could be detected in three cases only. These findings are in line with a report of relative refractoriness of the placental macrophages to *in vitro* infection with primary isolates [160]. Taken together, our results and those of others strongly indicate that the trophoblastic barrier remains uninfected in a majority of HIV-1 infected pregnant women [34, 156].

All mothers in our study were receiving zidovudine therapy according to the ACTG076 protocol and some mothers in addition received other reverse transcriptase inhibitors. We cannot exclude that the absence of HIV-1 infection in the trophoblasts in our study may have been influenced by the antiretroviral therapy. However, the antiviral therapy was clearly sub-optimal in three mothers who had relatively high plasma HIV RNA levels. This suggests that the antiretroviral therapy is not the sole explanation for the absence of HIV DNA in the trophoblasts. It is also important to mention that all children born by mothers in our study appear to be uninfected. Thus, we cannot formally exclude the possibility that trophoblasts may be infected in pregnancies which result in HIV transmission to the fetus.

Our results indicate that the trophoblastic barrier remains uninfected in full-term placentae of HIV-seropositive mothers undergoing antiviral therapy. Possible other mechanisms of *in utero* transmission will be discussed in the followings. A schematic illustration of possible mechanisms involved in *in utero* transmission of HIV-1 is presented in Figure 3.

Selective infection or transport through the placenta?

The placenta is an obvious barrier between the mother and the child that could potentially select for virus with certain biological properties. Virus could pass the intact placenta either by some type of transport or by direct infection of the cells in the placenta. Our results clearly argue against infection of trophoblasts [107]. This is in line with the receptor expression pattern on trophoblastic cells. Trophoblasts do not express CD4 (mRNA or cell membrane antigen) [161–163]. Trophoblasts from early placentae have been shown to express CCR5, CXCR4, Bonzo and GPR1 [131], whereas no expression of CXCR4, CCR5, CCR3, CCR2b, Bonzo has been detected on term trophoblasts [132]. Thus, if trophoblasts are infected they would have to be

infected through a CD4-independent pathway, such as via Fc-receptors. The presence of Fc-receptors on the trophoblast is well documented [164].

Trancytosis of cell-bound, but not cell-free, HIV-1 through an artificial trophoblastic barrier has been described *in vitro* [165]. Once transported across the trophoblastic layer, virus could potentially spread to stromal cells, such as Hofbauer cells, which have been shown to sometimes be infected in placentae of HIV-1-infected mothers. The Hofbauer cells are placental macrophages of probable fetal origin that express CD14 and CD4, but not CCR5 or CXCR4 [160, 166, 167]. Placental macrophages have been shown to be permissive for infection with laboratory strains [158, 168, 169], but refractory to infection with various primary isolates [160].

Non-selective transmission through the placenta: mechanical breaks in the placental barrier or chorioamnionitis

A second hypothesis, which we favour states that transmission through the placenta is non-selective, but that there may be selective outgrowth of R5 virus in the infant after transmission. We have been studying co-receptor usage of isolates from 11 Swedish HIV-1 infected mother-child pairs [119]. The results showed that there was a predominance of R5 virus early after perinatal infection. A CXCR4-switch was later documented in three of the children. Importantly, the mothers of two of the children that displayed a CXCR4-switch also carried CXCR4 using virus, while all other mothers carried R5 virus. The fact that two of three children with late CXCR4-switches had mothers with documented CXCR4 using virus suggests that these variants were in fact transmitted, but that they were suppressed in the children during the first years of life [119].

The failure of the placenta to maintain absolute integrity of the fetal and maternal circulations is documented by numerous reports on the passage of cells between mother and fetus in both directions [141]. It has been reported that leukocytes from the fetus can survive maybe also divide in the mother; thus leukocytes with Y-chromosomes have been identified in blood of women up to five years after they have given birth to a son [170]. It has also been shown that maternal leukocytes can pass into the fetus [171]. The mechanism of passage of the cells is poorly understood. Breaks in the continuity of the syncytiotrophoblastic surface can occur from early pregnancy to delivery, and these defects are repaired by fibrin. Thus, it is very possible that HIV-1-infected cells may also cross from mother to child through physical breaks in the placenta [172].

As mentioned, chorioamionitis is the only histopathologic abnormality, which is regularly found in placentae of HIV-1-infected mothers. It is not clear whether these findings are HIV-1 specific or manifestations of other infections [145]. If

chorioamionitis is HIV-1 specific, it is possible that the fetus could become infected through an ascending route or by swallowing and aspiration of infected amniotic fluid [173].

According to the second hypothesis there is no selection of viral variants at the level of the placenta. Selection, if it occurs, would be more likely to occur in the host. We have indications that X4 and R5 virus were both transmitted from two mothers, but that R5 virus replicated in the blood compartment of the infant during the first months of life [119]. We will use molecular techniques to try to clarify if the CXCR4 variants in the infants were transmitted from the mothers or arose *de novo* in the infant by evolution from CCR5 variants.

Conclusions

With the global increase in human immunodeficiency virus 1 (HIV-1) infection in women of childbearing age, there has also been an alarming increase in the number of mother-to-child transmissions of HIV-1. Although antiretroviral therapy and Elective Cesarian section have been demonstrated to significantly decrease the vertical transmission rate of HIV-1, these interventions are not widely available in the developing world. Therefore, studies of the mechanisms of vertical transmission are important.

We have been studying genetic and biological variability of HIV-1 in prospectively followed HIV-1-infected patients, especially pregnant women, in Sweden and in Cameroon. The general rule emerging from these studies was that CXCR4 usage determines the biological phenotype (slow/low, NSI or rapid/high, SI) for all subtypes, but the frequency of CXCR4-usage may vary between HIV-1 subtypes. Notably, CXCR4 use was rare among subtype C isolates. This finding was not due to differences in clinical status, CD4 count or treatment. Likewise, subtype C infection was not overrepresented among mothers with infected children.

Earlier work with subtype B suggested that presence of multitropic virus (R5X4 or R5R3X4) in the mother may increase the risk of HIV-1 transmission to the child. We now found R5Bonzo-using virus in some pregnant HIV-1 envelope subtype A infected women from Cameroon [106]. Comparison of the Cameroonian subtype A viruses with subtype B viruses showed that, in both cases, multitropic viruses are more frequent among transmitting mothers than in the group of non-transmitting mothers (37% and 16%, respectively).

The placental barrier function was tested in nine term placentae from a cohort of pregnant women in Sweden, identified as infected by HIV-1 subtype A, B or C. All

mothers underwent antiretroviral therapy and all children remained uninfected. HIV-1 sequences were detected by PCR in the mother's blood and in enriched placental trophoblastic cell preparations. After several rounds of immunomagnetic cell separation of these mixed populations, the purified trophoblasts were overall negative. Proviral DNA was found in the non-trophoblastic placental cells, mainly T-lymphocytes, which were shown to be a mixture of maternal and fetal cells. This study indicates that the placental barrier, i.e., the trophoblastic layer, does not become HIV infected. If HIV-1 infection of the fetus occurs, it is likely to be through other routes, such as breaks in the placental barrier.

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