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MATERNAL IMMUNE RESPONSES AND RISK OF INFANT INFECTION WITH HIV-1 AFTER A SHORT COURSE ZIDOVUDINE IN A COHORT OF HIV-1 INFECTED PREGNANT WOMEN IN RURAL KENYA
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ABSTRACT

Objective: **To investigate the effects of short-course nucleoside reverse transcriptase inhibitor (Zidovudine, ZDW/AZT) on maternal immune responses and risk of infant infection with HIV-1 among rural-based mothers in western Kenya.**

Design: **A prospective cohort study involving HIV-1 seropositive pregnant mothers and their infants.**

Subjects: **One hundred and seven HIV-1 seropositive asymptomatic pregnant women and their infants.**

Methods: **After informed consent, the women were enrolled at gestation age between 16-24 weeks. For cultural and economic reasons, all mothers were allowed to breast feed their infants. Short-course antepartum regime of AZT was administered to all mothers starting at 36 weeks gestation until start of labour. Maternal absolute CD4+ T cell subset assays were performed before 3rd trimester (about 36 weeks gestation) and after a 4-week therapy of AZT (at least one month post-nuptially). Infant HIV-1 status was determined by HIV-1 DNA polymerase chain reaction (PCR) on samples sequentially taken at 1, 2, 3, 4, 6 and 9 months and confirmed by serology at 18 months of age.**

Interventions: **Antepartum short-course orally administered AZT: 300mg twice-daily starting at 36 weeks gestation until start of labour, 300mg at labour onset and 300mg every three hours during labour until delivery.**

Main Outcome Measures: **Maternal CD4+ T cell counts before and after AZT treatment. Determination of infant HIV-1 infection status.**

Results: **Among 107 women sampled, only 59 received full dose of AZT and thus qualified for present analysis. Of these, 12 infected their children with HIV, while 47 did not. Comparison of CD4+ T cells before and after AZT treatment scored a significant rise in all mothers ($P = 0.01$). This increase in CD4+ T cells was not significant among mothers who infected their infants with HIV-1 ($P = 0.474$). However, a significant rise in CD4+ T cells following AZT therapy was observed only in mothers who did not transmit HIV-1 to their infants ($P=0.014$).**

Conclusion: **These data suggest that a rise in the CD4+ T cell counts following short AZT regimen, now widely in use in resource-weak countries, may be evidence of the active suppression of the replication of HIV. However, further studies to examine the multi-factorial effect of CD4+ lymphocytes and pregnancy on MTCT of HIV need to be carried out to help fully explain the effect of AZT on immune response and whether the CD4+T cell count can be used as a true test of immunological normalisation during antiretroviral therapy.**

INTRODUCTION

There is evidence that normal pregnancy and pregnancy complicated with HIV infection is associated with diminished immune responses(1-2). Reduced maternal immunity, especially low CD4+ T cell count has been associated with risk of having an infected child(3-5). The World health Organisation (WHO) estimates that of the 1.2 million children infected with HIV world-wide, most of them live in Africa and acquired their infection through mother-to-child

transmission (MTCT)(6). The estimated risk of MTCT in this region ranges from 25-30% (14-25% in the developed world), the near universal practice of breastfeeding explaining most of the difference(7-9).

Since 1994, when results of a large placebo-controlled trial of AZT use during second and third trimester in pregnant women and during the first weeks of life, in the absence of breastfeeding, demonstrated a significant reduction in MTCT (10-11). AZT has been the reference prophylaxis for mother to child HIV transmission(12-13). However, although AZT is said to increase circulating CD4+ T lymphocytes during HIV infection(14), its effects on immune reconstitution in pregnancy are still not very well understood(15-16). Any change in immune defences during pregnancy could be particularly important for HIV-1 seropositive women, in whom a significant degree of immunodeficiency may already exist. In the present analysis, we include CD4+ T cell counts before and after AZT administration and correlate this with risk of infant infection with HIV-1. This is done with the objective of delineating the reconstitutive effect of short-course AZT therapy on maternal immune response (as measured by CD4+ T cell count) during HIV infection in pregnancy and how this affects mother to child transmission of HIV-1.

MATERIALS AND METHODS

Study Participants: The sample population consisted of 59 pregnant HIV seropositive mothers participating in a MTCT study in four districts of western Kenya: Busia, Siaya, Bondo and Kisumu. This sample population was part of the 107 HIV-seropositive pregnant women who were participating in an MTCT study in the above districts. Seven centres in the four districts were used (Matayos, Khunyangu, Siaya, Usigu, Kombewa, Chulaimbo and Nyahera) as clinical evaluation centres. The sample population was aged 18 to 30 years, had a mean parity of three and a baseline CD4+ T cell count of 499 cells/microlitre of blood (cells/µl) and were drawn from the same socio-economic settings. After giving informed consent, the mothers were enrolled at gestation age between 16 and 24 weeks from June to November 1998. Although the mothers were counselled by trained counsellors on HIV and its attendant risks of mother to infant transfer through breastfeeding, a ban on the latter was not enforced. After enrolment, the mothers were followed and evaluated at six and eight months of pregnancy and at two and six months post partum. During each visit, the same group of project clinicians performed standardised physical examination for each mother and administered a confidential questionnaire.

The infants born to these mothers were seen by the project paediatrician after birth (at least within two calendar weeks) and thereafter at ages 1, 2, 3, and 4 months and subsequently every three months up to 18 months of age. At every visit blood specimen were obtained from the infants.

Laboratory methods: HIV antibody tests: The HIV-1 antibody status of the mother and infant was determined by testing serum/plasma with Particle Agglutination (PA Test, Fujirebio, Japan) and anti-HIV-1/2 antibody ELISA (Enzygnost^{R} anti-HIV 1/2 Plus ELISA, Behring-Marburg, Germany). Infant HIV-1 antibody status was confirmed using samples collected from 18 months of age. Reactive samples were confirmed by Western Blot (WB) assay (HIV Blot, Genelab, Diagnostics, USA).

Diagnostic PCR: For early diagnosis of HIV-1 in infants, we used nested PCR amplification of HIV-1 sequences on DNA extracted from three consecutive PBMCs obtained from children at age nine months. The quality of DNA was checked by β-globin gene amplification. Nested PCR employed the following primers: Envelope (ENV); ED3/14 and ED5/12, Polymerase (POL); HPOL 4320/4538, HPOL 4327/4481, Group Antigen (GAG); GAG-MZ13/14, MZ8/9. Based on our in-house experience (not published), the amplification of POL and ENV primers was used for early diagnosis of HIV-1. The amplified products were then run on a 2% Nusieve^{R} agarose gel stained with ethnidium bromide and visualised by an electronic gel illuminator (Toyobo-FASIII, Japan).

CD4+ T cell counts: Lymphocyte subset counts were quantitated by standard flow cytometry procedures(17) using monoclonal antibodies labelled with fluorochromes according to instructions provided by the kit manufacturers (Tritest^{TM} Becton-Dickinson, USA). Briefly, since CD4+ counts are dependent on total lymphocyte count, WBC and lymphocyte counts were performed on whole blood in EDTA using an electronic blood cell counter (Sysmex^{TM} Electronic cell counter, Japan). Aliquots of 50µl of whole blood in EDTA were incubated with 3-colour fluorochrome-labelled monoclonal antibodies (anti-CD3/45*PerP, anti-CD4*PE and anti-CD8*FITC) (Becton-Dickinson, USA). After incubation and lysis of red blood cells, flow cytometric analysis was performed on Facscalibur^{R} cytometer using an automatic acquisition and analysis program (Multiset, Becton-Dickinson, USA).

AZT administration: AZT was administered to HIV-1 confirmed mothers after informed consent and professional counselling. The regimen given was a modification of the short course ZDV recommended by the CDC for combination with formula feeding(18). All women chose to breastfeed their infants. The regimen involved the following:

(i). 1 AZT tablet (300mg) twice daily at 36 weeks of gestation until onset of labour.
(ii). 1 AZT tablet (300 mg) at onset of labour.
(iii). 1 AZT tablet (300 mg) every three hours during labour until delivery.

The same clinical officer/nurse team administered the drug every week. RBC cell counts were used as a monitor of side-effects, especially anaemia.

Ethical considerations: As part of our social contract with the study population, the project continued to follow-up all the children and especially HIV-infected children for medical treatments for their ailments. To enhance sustainability and avoid overdependence on the project for medical assistance, the project initiated partnerships with the local health delivery institutions where the study participants could join the on-going cost-sharing program for medical treatment.

Data collection: Data were collected on recruitment and subsequently at six and eight months of pregnancy and at two and six months post partum. Gestational age was determined by obstetric evaluation. Where this was not possible, antenatal ultrasonography by a portable system was used (Philips, Netherlands). Blood was drawn into EDTA-vacutainer tubes at every visit. Data collected included:

maternal age, complete blood count, gestational age, complete blood count, HIV diagnosis, 3-monthly CD4+ T cell counts, infant polymerase chain reaction (PCR) for HIV-DNA and HIV antibody test.

Data analysis: Data were managed and analysed by Statview Software (Abacus Concepts, Inc., Berkeley, CA, USA). Nominal/ discrete variables were analysed using Chi-square tests with Fisher's exact test to determine significance.

RESULTS

Sample size: Out of 107 mothers who tested HIV seropositive and were thus eligible for the present study, only 59 (55%) successfully completed the study. The rest were excluded from the present analysis for various reasons: eight (7.5%) died in the course of the study, 15 (14%) lost sick baby after illness and declined further participation in the study, 18 (17%) refused to have blood drawn and seven (6.5%) were lost to follow-up.

Characteristics of participating women: Table 1 shows characteristics of mothers studied stratified by the HIV status of their index child. There were no statistically significant differences between the transmitter and non-transmitter mothers with regard to; age, parity, age at birth and birth outcome and socio-economic status. The majority (51%) of mothers delivered between 38-40 weeks of gestation and at home. Mothers with no education were more likely to have their first-born deliveries at (p=0.018). Most of the mothers who delivered at home had educational level ranging from nil to upper primary.

Table 1

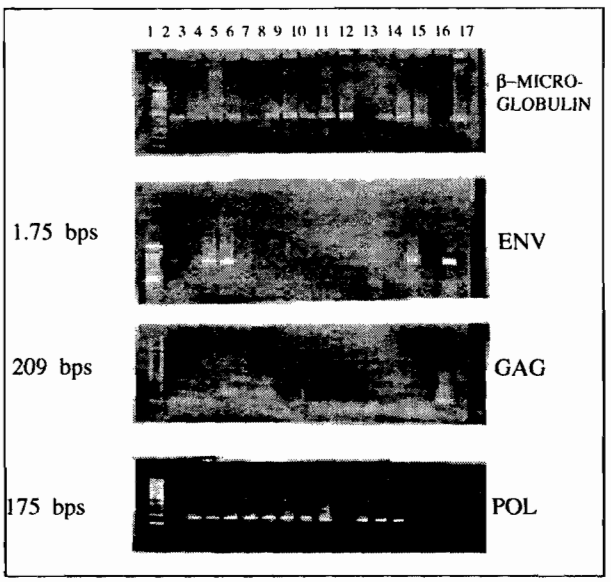
Characteristics of participant mothers by their child HIV-1 status

Variable	All (n=59)	Ψ Transmitters (n=12)	ϕ Non Transmitters (n=47)
Age Group (years)			
<19	6	0	6
19-24	21	4	17
25-29	10	2	8
30-34	5	1	4
35-39	1	0	1
>40	1	0	1
Missing	15	5	10
Mean Parity	2	2	3
Gestation age at birth (weeks)			
Full term (37-40)	30	3	27
Pre-term (<37)	7	2	5
Missing	12	12	8
Baby status			
Alive	38	2	36
Dead	15	6	9
Still Birth	1	1	0
Missing	5	3	2
Birth Outcome			
Boy	25	6	19
Girl	34	6	28
Boy/Boy	0	0	0
Girl/Girl	0	0	0
Boy/Girl	0	0	0
Baseline (CD4+μl)			
*Class 1 (>500)	21	4	17
*Class 2 (200- 499)	18	2	16
*Class 3 (<200)	4	1	3
Not Done	17	5	11
Place of Delivery			
Health Centre	15	2	13
Hospital	8	3	5
Home	30	6	24
Missing	6	1	5
Mode of Delivery			
Vaginal	54	12	42
Caesarean Section	0	0	0
Missing	5	0	5

Ψ Transmitters are mothers who infected their children with HIV-1. ϕ Non-transmitters are mothers who did not transfer HIV-1 infection to their children. * Classes 1-3 are CDC immunological categories based on CD4+ T cell counts (Centres for Diseases Control and Prevention (CDC). Revised Guidelines for Performing CD4+ T cell determinations in persons infected with human immunodeficiency virus, *MMWR*, 1997; **46 (RR-2):** 1-29).

Figure 1

Results of testing peripheral blood mononuclear cells (PBMC) DNA from children born to HIV infected mothers with four sets of nested primers. Track 17 is positive control sample from a known PCR positive mother amplifying all primers (note that the same sample is in track 14 on POL). Tracks 2,12, and 16 are consistently PCR negative samples from children born to HIV positive mothers. Most PCR positive samples amplified ENV and POL primers (tracks 5,6,13,15). Track 5 is one of the few samples amplifying all HIV primers in use. Track is molecular weight marker. Beta-microglobulin PCR was initially performed to test quantity of DNA extracts of each specimen



Mother-to child transmission of HIV-1 rate: The number of children with positive PCR (Figure 1) of consecutively processed samples under nine months, and later confirmed at age 18 months by the HIV

antibody particle agglutination (PA) test were 12 out of 59. This represented a vertical transmission rate of 20.3%.

Effect of AZT on CD4 T cell levels: The profiles of CD4+ T cell counts harvested at week 24 of gestation, through pregnancy to 10 weeks after delivery are illustrated in Figure 2. When the profiles were stratified by the HIV infection status of the child, it was clear that there was an increase of CD4+ T cells in all mothers following AZT therapy. Paired comparison of CD4+ T cell changes at different stages showed that the increase was more significant between week 34 of gestation and 10 weeks after delivery (p=0.01) for all mothers. The results also showed a more enhanced increase of CD4+ T cells in mothers who did not eventually transmit the HIV virus to their infants.

Figure 2

Absolute CD4+ cell counts (cells/microlitre) and 95% confidence intervals at specified times before and after AZT prophylaxis for HIV transmitter and non-transmitter mothers

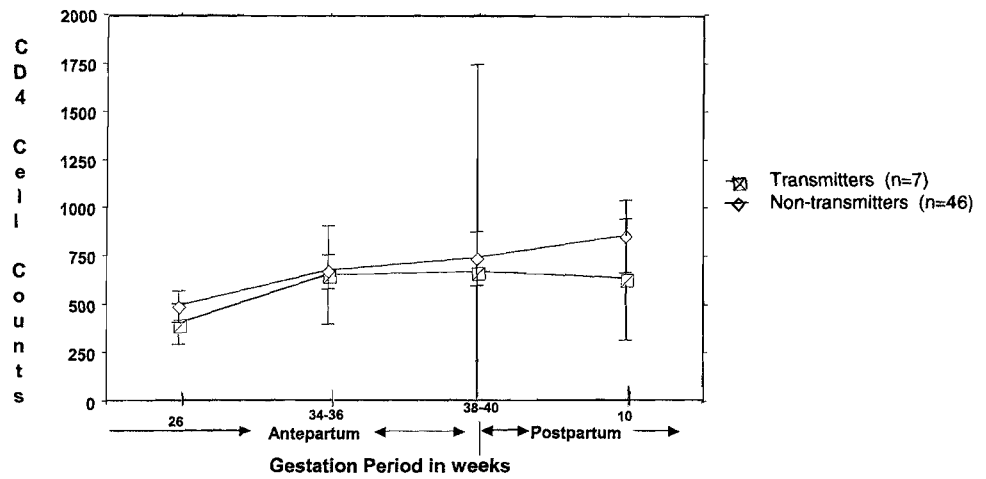
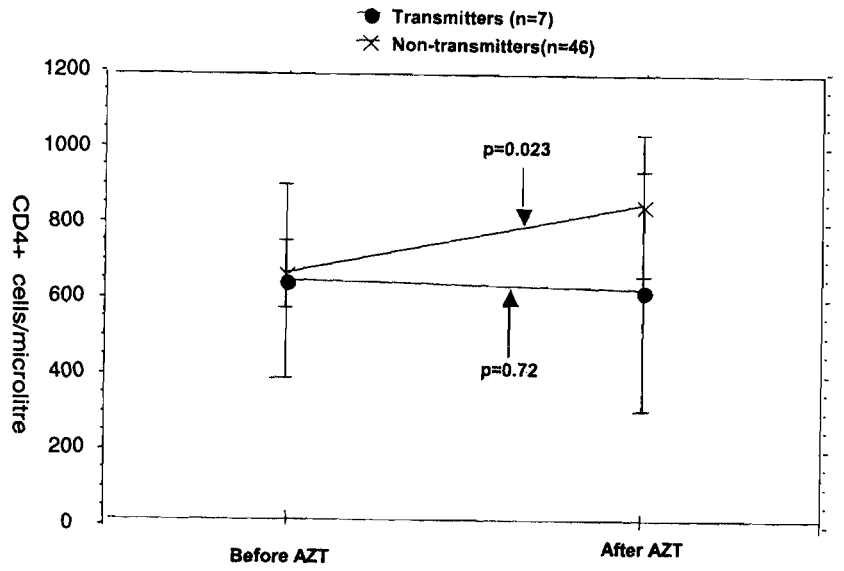


Figure 3

Absolute CD4+ cell changes and 95% confidence intervals at 2 points: between 34 weeks of gestation (just before AZT administration) and 10 weeks after delivery/AZT. The change is illustrated for the two categories of mothers



AZT-associated CD4+ changes: Paired t-test was used to compare the specific CD4+ T cell increases in the two categories of mothers (transmitters and non-transmitters of HIV) before and after AZT administration. The increase in CD4+ T cell counts was significantly associated with lack of transmission of HIV-1 (p=0. 0135) (Figure 3).

DISCUSSION

Out of 107 HIV-seropositive pregnant women enrolled in the study, only 59 were selected for the present analysis. These were mothers who had not only, completed the full dose of antenatal AZT, but also had consistent evaluations from recruitment through pregnancy and after delivery. Since repeated measurements were to be used in the analysis, the use of consistently collected data during the study period helped minimise potential bias. In addition, these mothers were selected because they consented to having their infants bled consecutively for virological assays.

We used absolute CD4+ T cell and not the percent CD4+ counts as a measure of immune status. This is because in our experience, alterations in maternal blood volume (haemodilution) in pregnancy and delivery did not significantly alter the absolute counts of this cohort (unpublished data). Also, given that CD4+ counts are known to be subject to significant regular individual variations, we considered overall trends in the CD4+ T cell counts which, in common experience of both clinicians and patients, may be more meaningful than percentage counts.

Of the 59 mothers given short course AZT prophylaxis to prevent mother to child HIV transmission, 12(20.3%) transmitted HIV-1 to their children while 47(79.7%) did not. Without interventions the risk of mother-to child of HIV-1 in sub-Saharan Africa is estimated to be between 25% and 45%(9). Breastfeeding has been reported to present an additional risk of mother to child transmission of HIV estimated at 5% to 12%(18). In this study, although both the infant formula supplements were recommended and counselling against breastfeeding done, all women chose to breastfeed their infants due to a combination of strong traditional attitudes and economic constraints favouring breastfeeding (unpublished data). Additionally, our study was based in a rural setting, where the absence of safe alternatives to breastfeeding makes the latter the most established and accepted method of infant feeding.

Considering that our cohort of women were derived from a small sample size and allowed to breastfeed their infants, the vertical infection rate of 20.3% compares with previously reported MTCT rates of 12.2% and 15.7% at ages three weeks and four months respectively in breastfeeding population(19). Although, the efficacy of AZT prophylaxis in a breastfeeding population in west Africa has been reported(15) one cannot be certain that infection could have been prevented in additional 15% of the children whose mothers had been discouraged from breastfeeding. This is because, the effects of different infant feeding options and their duration on the post-natal exposure to HIV-1 even during ARV is still not well understood(20). Other risk factors known to favour vertical transmission during AZT prophylaxis have been identified, including infant birth weight during study period, maternal HIV viral load, antepartum procedures and cervicovaginal secretions(21). In this study, these factors were not controlled for. This was because such factors were not within the study objectives and also because this study was situated in the rural area where such procedures could not be undertaken with ease.

Our results demonstrate a trend towards immune reconstitution following AZT prophylaxis (Figure 2). Considering that most of the participants in this study were asymptomatic, meaning early HIV disease, this numeric reconstitution of CD4+ T cells was expected(22). At this stage, the mechanisms of CD4+ T lymphocytes are still functional as a result of which antiretroviral intervention is expected to achieve maximum suppression of HIV replication. Thus, the immune system is expected to benefit from antiretroviral treatment more during the asymptomatic HIV infection than it does in late-stage disease, by which time significant immune damage has already occurred.

From these results, it can be inferred that the CD4+ T lymphocyte increase was in response to AZT prophylaxis. Additionally, the observed CD4+ T cell increase would be expected to provide "help" for the suppression of HIV replication thus leading to the observed reduced risk of HIV transmission from mother to child among the non-transmitters. However, our findings raise two significant issues which may limit the generalisability and interpretations of the results; was the reduced risk of transmission of HIV-1 among the non-transmitter mothers a function of a rise in CD4+ T cell only or was AZT administered at a gestation age where restoration of CD4+ T cell levels was more and hence risk of transmission lowest?

A multivariate analysis adjusting for maternal CD4+ T cell counts would probably have provided answers to the above questions. Our failure to control for the CD4+ T cell counts in the two groups of mothers is one of the limitations of this study. However, by the time of the study, strong and statistically significant protective effects of AZT against MTCT even after adjustment for maternal CD4+ T cell levels had already been well described(16). We also took cognisance of the earlier reports which had adjusted for CD4+ T cell count changes over the entire period of pregnancy. During such a long time of observation, the like-hood that the effects and causes of immunosuppression due to HIV-1 infection and pregnancy would confound CD4+ T cell changes due AZT is expected to be higher. In this study therefore, we decided to study changes in CD4+ T cell counts examined immediately before

and after AZT short-course therapy.

Although the numerical and phenotypic profiles of the CD4+ T cell are the most widely used marker of immune status during HIV infection, these profiles only reflect one aspect of immune system and T cell competence. The CD4+ T cells functions are very broad and can only be adequately assessed through their helper functions in terms of proliferation and alteration in the naive and memory subsets(23) and elaboration of cytokines that affect the entire immune system. Because of our study design, these functional assays of T cells were not performed. Instead we sought to evaluate the effect of short-term AZT on CD4+ T cells during late pregnancy in relation to transmission of HIV-1 to infant. We acknowledge the constrained interpretation of our findings given the small sample size. However, our observed significant association between prenatal use of AZT and increase in CD4+ T cell counts in non-transmitting mothers further corroborates the previous findings(13,24) and supports initiation of bigger cohort studies to identify the T cell subsets and their cytokines associated with ARV prophylaxis against MTCT. Already, a preferential loss of naive T cells (CD45 RA+) coupled with a relative increase in memory T cells (CD45RO+) during HIV disease progression has been reported(22). Clearly, any interventions that invoke the regeneration and activation of CD4+ T cell of naive phenotype should be a major goal of pMTCT of HIV-1 trials.

Decreased maternal CD4+ T cells have been correlated with risk of mother to child transmission of HIV-1(25). In this cohort the mothers did not differ significantly with regard to CD4+ T cell counts at baseline. Although the CD4+ T cell counts were low at baseline in both groups of mothers, they increased significantly more, between gestation week 36 and delivery among the HIV-non-transmitting than the transmitting mothers. This observation implies that further trials on the timing of short-course regimens of ARV against MTCT are still needed.

In conclusion these data suggest that a rise in the CD4+ T cell counts following short AZT regimen, now widely used in resource-weak countries, may be evidence of the active suppression of the replication of HIV. However, further studies to examine the multifactorial effect of CD4+ lymphocytes and pregnancy on MTCT of HIV need to be carried out to help fully explain the effect of AZT, and possibly other new ARVs, on immune response and whether the CD4+T cell count can be used as a true and more rapid test of immunological normalisation during intervention against MTCT.

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