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Altered heterochromatin organization after perinatal exposure to zidovudine

*Sho Senda[1,2], Stéphane Blanche[3,4], Dominique Costagliola[5,6], Christian Cibert[7], Fabienne Nigon[1,2], Ghilaine Firtion[8], Corinne Floch[9], Sophie Parat[10] and Evani Viegas-Péquignot[1,2]**

[1]U741, INSERM, Paris, France
[2]UMR-S741, Université Paris Diderot-Paris 7, Paris, France
[3]EA3620, Faculté René Descartes, Université Paris Descartes, Paris, France
[4]Unité d'Immunologie Hématologie Pédiatrique, Hôpital Necker-Enfants Malades, Assistance Publique-Hôpitaux de Paris, Paris, France
[5]U720, INSERM, Paris, France
[6]UMR-S720, Faculté de médecine, Université Pierre et Marie Curie-Paris6, Paris, France
[7]UMR CNRS 7592, Institut Jacques Monod, Université Paris Diderot-Paris7, Paris, France
[8]Maternité Hôpital Cochin-Port Royal, Assistance Publique-Hôpitaux de Paris, Paris, France
[9]Maternité, Hôpital Louis Mourrier, Assistance Publique-Hôpitaux de Paris, Colombes, France
[10]Maternité, Hôpital Necker-Enfants Malades, Assistance Publique-Hôpitaux de Paris, Paris, France

*Corresponding author: Tel: +33 1 44 27 28 15; Fax: +33 1 44 27 82 10; E-mail: viegas@ijm.jussieu.fr

Background: Zidovudine (3′-azido-3′-deoxythymidine, AZT), administered to pregnant women alone or in combination with other antiretroviral drugs, greatly reduces the mother-to-child transmission of HIV-1. The potential genotoxicity of these molecules is underestimated and wide-ranging evaluation of its biological and clinical consequences is required.
Methods: We investigated the nuclear organization of constitutive heterochromatin, a major domain participating in epigenetic regulation, in uninfected infants born to HIV-1-infected mothers treated with zidovudine and/or other nucleoside reverse transcriptase inhibitors (NRTIs) during pregnancy. We studied the organization of chromosome 1 heterochromatin (1q12) in peripheral leukocytes of 25 HIV-1-uninfected children (newborn to 9 years old): children born to HIV-1-infected mothers exposed to zidovudine and/or other NRTIs (*n*=15), children born to HIV-1-infected mothers not exposed to any NRTIs (*n*=6) and children born to HIV-1-uninfected mothers (*n*=4).
Results: Results differed significantly between NRTI-exposed and -unexposed children. By contrast, there was no difference between NRTI-unexposed children born to HIV-1-infected mothers and children born to HIV-uninfected mothers. The anomaly persisted in lymphocytes cultured for 48 h. There was no evidence of abnormal DNA methylation, a major feature of constitutive heterochromatin and associated with the loss of its structure. In a complementary sample of children, analysis of chromosome 11 and 16 heterochromatin suggests that the defect affects most of the other heterochromatic sites of the human genome. The heterochromatin defect persists long after the end of the exposure and appears in leukocytes of both myeloid and lymphoid lineages, suggesting that haematopoietic stem cells are affected.

Introduction

Antiretroviral therapy during pregnancy greatly reduces the risk of mother-to-child transmission of HIV-1 [1]. Zidovudine is the backbone of the prophylaxis administered to pregnant women, although it is frequently used in combination with other antiretroviral drugs. Zidovudine is also one of the most widely used drugs in prophylaxis programmes in low-income countries [2].

Zidovudine is a thymidine analogue that is incorporated into HIV proviral DNA and blocks the nucleoside-binding site of viral reverse transcriptase, thereby inhibiting viral genome replication by chain termination [3]. It is also incorporated into host cell DNA [4,5] and its genotoxicity was initially associated with genome instability and carcinogenic activity in mammalian cell lines and in animal model systems [6].

Animal models of *in utero* exposure to zidovudine, alone or in combination with other nucleoside reverse transcriptase inhibitors (NRTIs), show that these drugs can have adverse effects in fetuses and newborns.

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Zidovudine is incorporated into both nuclear and mitochondrial DNA (mtDNA) in several organs in the offspring of treated pregnant mice, monkeys and humans [7,8], indicating that the placental barrier is overcome. In monkeys and mice exposed *in utero* to zidovudine, alone or in combination with lamivudine at human-equivalent doses, mitochondrial dysfunction associated with mtDNA depletion has been observed in various fetal and newborn tissues [9,10]. In humans, clinically detectable acute or persistent mitochondrial dysfunction has been described, associated with frequent but transient asymptomatic hyperlactataemia and, rarely, a persistent severe neurological disease [11,12]. Telomere shortening was described in fetal tissues of mice after zidovudine exposure [7]. By contrast, in the primate *Erytrocebus patas* this type of effect appeared only after combined exposure to both zidovudine and lamivudine [13]. In humans, no significant telomere shortening was detected in the blood of zidovudine-exposed children [14]. In three independent transplacental carcinogenicity studies of zidovudine in CD-1 mice, one study determined a no-observed-effect level of zidovudine [15] but two studies identified treatment levels of zidovudine that caused dose-related increases in cancer of the liver, lung and reproductive tissues [7,16,17]. Despite investigations in two cohorts with a short follow up, there is currently no evidence in humans for any carcinogenic effects due to perinatal exposure to zidovudine alone [18,19]. These results suggest that the sensitivity might differ between mammalian species and, consequently, that studies performed in animal models cannot be directly used to evaluate human perinatal genotoxicity.

Early studies on hamster cell lines indicated that although both centromeric and telomeric regions incorporated substantial amounts of zidovudine, centromeric regions containing constitutive heterochromatin were particularly involved in chromosome rearrangements [20]. Given that telomeres of exposed children are not affected we assumed that constitutive fetal centromeric and juxtacentromeric heterochromatin, known to share some structural and functional properties with telomeres [21], might be sensitive to exposure to zidovudine and other NRTIs during pregnancy. Heterochromatin was initially defined as a portion of the eukaryotic chromatin that remains condensed throughout the cell cycle. By contrast, euchromatin is the fraction that remains less condensed at interphase and contains genes and low-repeat sequences. Constitutive heterochromatin is involved in chromosome stability and segregation, and in epigenetic gene silencing by *cis* or *trans* regulation [21]. Biochemical epigenetic changes, including DNA methylation, histone-tail modifications and the binding of specific non-histone proteins, reorganize the chromatin structure and consequently induce either transcription or silencing of individual genes, independent of mutations in the genomic sequence [22]. There is growing evidence that toxic agents can modify the patterns of gene expression by affecting chromatin epigenetics [23,24] and thus contribute to the establishment or the maintenance of new epigenetic states.

We therefore investigated the organization of constitutive heterochromatin in peripheral blood leukocytes from children perinatally exposed to zidovudine and/or other NRTIs. Here we report that the integrity of several heterochromatic sites of the human genome, identified by fluorescence *in situ* hybridization (FISH), is compromised by the *in utero* treatment.

Methods

Patients

The NRTI-treated cohort included children born to HIV-1-infected mothers and exposed during pregnancy to zidovudine and/or other NRTIs. The non-NRTI-treated control group was composed of children either born to HIV-1-uninfected mothers or to HIV-1-infected mothers never exposed to NRTIs. All the children enrolled were asymptomatic. PCR assays 6 weeks after birth were used to confirm the absence of HIV-1 infection.

The mothers and children received medical care at the Necker-Enfants-Malades Hospital (Paris), the Port Royal Maternity (Cochin Hospital, Paris) and the Louis Mourrier Hospital (Colombes). The study design received approval from the local ethics committee (Comité Consultatif de Protection des Personnes dans la Recherche Biomédicale, CCPPRB Paris Necker). Signed, informed consent was obtained from all parents.

Slide preparation for heterochromatin analysis

One blood sample of 5–10 ml was collected from each child in a sterile tube containing heparin. For direct fixation of leukocyte nuclei, 400 μl aliquots of whole blood were added to 10 ml of RPMI 1640 medium (Gibco, Invitrogen SARL, Cergy-Pontoise, France) and then fixed in 8 ml of fixative I (ethanol: chloroform:acetic acid [6:3:1]) and finally in 6 ml of fixative II (ethanol:acetic acid [3:1]). Aliquots of 400 μl of whole blood samples were also cultured: they were added to PB-MAX karyotyping medium (Gibco, as before, modified from the technique described by Moorhead *et al.* [25]) and cultured for 48 h at 37°C. To obtain metaphase chromosomes, cells were treated with colchicine (0.04 μg/ml) for the last 2 h of culture, then re-suspended in a hypotonic solution (human serum:water [1:6]) for 15 min at 37°C and fixed twice in fixative II. To obtain 48 h-cultured nuclei, samples were cultured similarly but without colchicine or hypotonic treatment, and then fixed twice in fixative II. One

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to three drops of the concentrated cell suspension were put on clean moist slides then dried for 24 h and kept at -20°C until use.

Fluorescence *in situ* hybridization (FISH)

A classical satellite 2 probe was used to detect juxtacentromeric heterochromatin of chromosome 1 (1q12; Q-BIOgene, Illkirch, France) and 16 (16q11; VYSIS, Rungis, France). A probe specific for classical satellite 3 was used to recognize chromosome 9 juxtacentromeric heterochromatin (9q12; CYTOCELL, Cambridge, UK). The centromeric heterochromatin of chromosome 11 was identified with a specific α-satellite probe (11p11-q11; Q-BIOgene, as before). Probes were labelled with fluorescein or rhodamine. The slides were immersed in 2×SSC (0.3M NaCl, 30mM sodium citrate, pH 7.0) at 37°C for 30 min, and the samples dehydrated with ethanol, denatured with 70% formamide/2×SSC (pH 7.0) at 70°C for 2 min and dehydrated again with cold ethanol. The probe (10 μl) was diluted 1:5 in the hybridization mixture and denatured at 96°C for 10 min, then added to each slide and incubated at 37°C for 16 h. The slides were then immersed in wash buffer (0.5×SSC, 0.1% SDS) at 37°C for 5 min and counterstained with DAPI (4′,6-diamidino-2-phenylindole dihydrochloride). The anti-fading solution, PPD (p-phenylenediamine), was added and the slides examined under a DMRA2 fluorescence microscope (Leica Microsystems, Wetzlar, Germany) using a high-resolution camera (Hamamatsu Photonics, Hamamatsu, Japan) and the Openlab imaging software (ImproVision, France).

Combined FISH and immunofluorescence with an anti-5-methylcytosine monoclonal antibody

After acquiring FISH images, the slides were immersed in wash buffer (0.5×SSC, 0.1% SDS) at 85°C for 5 min, rinsed twice for 5 min at room temperature in PBS, irradiated with UV light for 16 h and then immersed in cold PBT (PBS, 0.1% Tween 20, 0.4% bovine serum albumin). The slides were incubated for 45 min at RT (room temperature) with the anti-5-methylcytosine antibody (anti-5-MeC; Eurogentec, Seraing, Belgium; 1:1000 dilution in PBT), rinsed for 5 min in PBT, and then incubated with anti-mouse fluorescein-conjugated IgG antibodies (Sigma-Aldrich, Lyon, France; 1:40 dilution in PBT). After 45 min at RT, the slides were rinsed with PBS and examined as indicated above.

Southern blot analysis

Southern blot analysis was performed as described previously [26]. DNA from peripheral blood samples was digested with the methylation-sensitive restriction enzyme *Hpa*II and tested for hybridization using the Satellite 2 oligonucleotide-specific probe.

Statistical analysis of heterochromatin organization

At least 100 nuclei from each child were analysed. To assess heterochromatin anomalies, the number and the type of fluorescent signals (condensed spots [c] and dispersed spots [d]) corresponding to heterochromatin were recorded in each leukocyte nucleus. The percentages of nuclei with ≥1 dispersed spot were compared using non-parametric Mann–Whitney tests with SPSS 11 for Mac OS 10 (SPSS Inc., Chicago, IL, USA). *P*-values <0.05 were considered significant. For the juxtacentromeric region of chromosome 1, the results in the two types of controls (unexposed children born to HIV-1-infected and HIV-1-uninfected mothers) were compared prior to comparison between exposed and unexposed children. Comparisons were also performed separately for round and polymorphic nuclei to assess the impact of exposure to NRTIs in both types of cells.

Results

We first analysed the organization of the juxtacentromeric heterochromatin of chromosome 1 in sample sets of 15 NRTI-exposed children and ten unexposed control children (Table 1, patients P1–P15 and controls C1–C4 and C7–C12). Then, using a smaller sample set, the analysis of heterochromatin organization was extended to other heterochromatic sites located in chromosome 9, 11 and 16 (Table 1, patients P1, P7 and P16–P19 and controls C2–C6).

Characteristics of the sample sets

Nineteen NRTI-exposed children and 12 unexposed control children were studied (Table 1). Six of the children (P1–P5 and P19) were exposed to zidovudine alone, ten (P6–P12 and P16–P18) to zidovudine in combination with various NRTIs, protease inhibitors (PIs) or non-nucleoside reverse transcriptase inhibitors (NNRTIs) and three (P13–P15) to a combined NRTI treatment without zidovudine. Six of the 19 NRTI-exposed children were treated throughout the pregnancy (P7, P8, P10, P11, P13 and P15); for nine (P1–P6, P9, P12 and P14) treatment started between 8 and 33 weeks, and for four children (P16–P19) no information was available. All women received an intravenous infusion of zidovudine during labour. After birth, all the children received a 4- or 6-week course of zidovudine, except for P14 and P15. P14 received didanosine and a single dose of nevirapine, and P15 a combination of stavudine and lamivudine.

The control sample was composed of twelve children. Six (C1–C6) were born to HIV-1-uninfected mothers. The other six (C7–C12) were born to HIV-1-infected mothers; most of them received no prophylaxis at all (C7, C8, C10 and C11) and two (C9 and C12)

were exposed to a nucleoside analogue-free prophylaxis (PI plus NNRTI).

Analysis of heterochromatin in peripheral leukocytes of perinatally exposed children

Two types of nuclei were observed in fixed leukocytes from peripheral blood: round nuclei, mainly from lymphocytes, and polymorphic nuclei, mostly from neutrophils, but also from eosinophils and basophils. In all these nuclei, two types of hybridization signal were observed: condensed spots and a dispersed set of small spots (Figure 1). Constitutive heterochromatin in normal diploid somatic nuclei is generally believed to be condensed, but no quantitative information is available. Here, we quantified the distribution of the hybridized fluorescent signal in the nuclei of each patient and each control (about 100 nuclei for each individual). Nuclei were classified as follows: (i) those harbouring two spots, whether condensed or dispersed, each corresponding to one homologous chromosome

Table 1. Children's characteristics and antiretroviral exposure

	Age	Mother's treatment	Start of the mother's treatment, weeks of pregnancy	IV AZT treatment at delivery	Child's treatment (duration)	Heterochromatic regions analysed
NRTI-exposed children						
P1	3 months	AZT	8	+	AZT (4 weeks)	1, 9, 11, 16 and 1c
P2	2 years	AZT	33	+	AZT (4 weeks)	1
P3	7 years	AZT	24	+	AZT (6 weeks)	1 and 1c
P4	9 years	AZT	28	+	AZT (6 weeks)	1
P5	8 years	AZT	28	+	AZT (6 weeks)	1
P6	6 months	AZT+3TC	20	+	AZT (4 weeks)	1
P7	3 years	AZT+3TC+ABC	a	+	AZT (6 weeks)	1, 16 and 1c
P8	6 months	AZT+3TC+NFV	a	+	AZT (6 weeks)	1 and 1c
		AZT+3TC+RTV+indinavir	24*			
P9	12 months	AZT+3TC+NVP	24	+	AZT (4 weeks)	1
P10	18 months	AZT+3TC+NVP	a	+	AZT (6 weeks)	1 and 1c
P11	4 years	AZT+3TC+indinavir	a	+	AZT (4 weks)	1 and 1c
P12	4 years	AZT+3TC+NFV	19	+	AZT (6 weeks)	1
P13	3 months	3TC+d4T+RTV+SQV	a	+	AZT (6 weeks)	1
P14	2 years	ddI+APV+RTV+NVP	12	+	ddI (4 weeks)+NVP sd	1
P15	5 years	d4T+3TC+NFV	a	+	3TC (6 weeks)+d4T (24 h)	1
P16	2 years	AZT+3TC+NVP	NA	+	AZT (6 weeks)	9, 11 and 16
P17	2 years	AZT+3TC+NVP	NA	+	AZT (6 weeks)	9, 11 and 16
P18	3 years	AZT+3TC+RTV+SQV	NA	+	AZT (6 weeks)	9 and 11
P19	1 year	AZT	NA	+	AZT (6 weeks)	9
Controls (non-NRTI-exposed children)						
Children from HIV-negative mothers						
C1	22 months	–	–	–	–	1 and 1c
C2	10 months	–	–	–	–	1, 9, 11, 16 and 1c
C3	New born	–	–	–	–	1, 9, 11, 16 and 1c
C4	New born	–	–	–	–	1, 9, 11, 16 and 1c
C5	New born	–	–	–	–	9, 11 and 16
C6	14 years	–	–	–	–	9
Children from HIV-positive mothers						
C7	10 years	–	–	–	–	1 and 1c
C8	1 year	–	–	–	–	1 and 1c
C9	22 months	RTV+SQV	20	–	NVP sd	1
C10	6 years	–	–	–	–	1
C11	6 months	–	–	–	–	1
C12	6 months	SQV+NFV	26	–	NVP sd	1

The nucleoside reverse transcriptase inhibitors are zidovudine (AZT), lamivudine (3TC), abacavir (ABC), stavudine (d4T) and didanosine (ddI). Nevirapine (NVP) is a non-nucleoside inhibitor of the viral reverse transcriptase. Nelfinavir (NFV), ritonavir (RTV), saquinavir (SQV), amprenavir (APV) and indinavir are inhibitors of the viral protease. 'a' denotes that the mother had started the therapy before pregnancy. *The mother of P8 had started therapy with AZT+3TC+ nelfinavir before pregnancy and then changed to AZT+3TC+ ritonavir+ indinavir at 24 weeks of pregnancy. 1, 1q12; 9, 9q12; 11, 11p11–q11; 16, 16q11; 1c, 1q12 (after 48 h culture); C, control; IV, intravenous; NA, not available; P, patient; sd, single dose.

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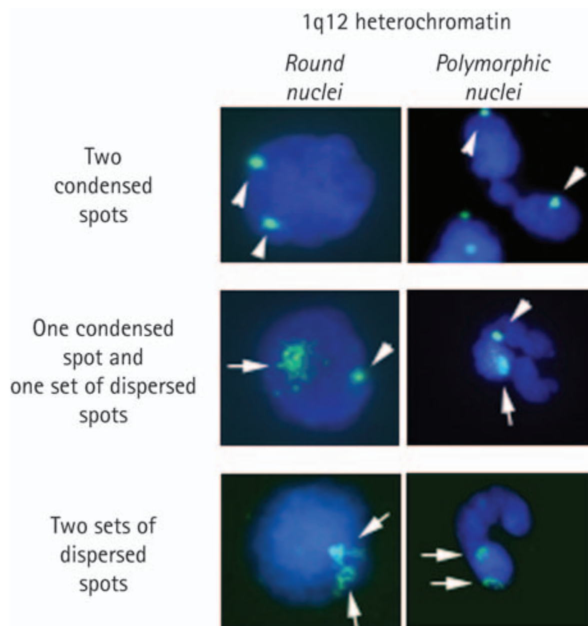
(c:c, c:d and d:d); and (ii) those harbouring only one homologous chromosome condensed or dispersed spot (c:– and d:–).

Analysis of chromosome 1 heterochromatin. Fifteen NRTI-exposed children and ten control children (six born to non-exposed HIV-1-infected mothers and four to HIV-1-uninfected mothers) were analysed with the chromosome 1 satellite 2 probe detecting the 1q12 region (Table 1). Proportions of total nuclei (round and polymorphic) with at least one dispersed spot (c:d+d:d+d:–) differed significantly ($P<0.001$) between the exposed and control children (Figure 2; Table 2). Thus, perinatal exposure to NRTIs (mainly zidovudine) was associated with an increase in the frequency of nuclei with dispersed 1q12 heterochromatin in peripheral blood leukocytes. No difference was observed between the two control groups: the group of children born to HIV-1-infected mothers and the group of children born to uninfected mothers ($P=0.476$; Figure 2), indicating that the viral infection in mothers does not appear to affect the heterochromatin organization in their children. Comparisons performed separately in round and polymorphic nuclei showed that, in both types of nuclei, there was a significantly higher proportion of nuclei with at least one dispersed heterochromatin in the NRTI-exposed children than in the control children (Table 2). The difference between NRTI-exposed and -unexposed children was larger for polymorphic nuclei (63% vs 46%; $P<0.001$) than for round nuclei (70% vs 66%; $P=0.031$).

Analysis of chromosome 1 heterochromatin in PHA-stimulated T-lymphocytes. Heterochromatin integrity in nuclei of phytohaemagglutinin (PHA)-stimulated T-lymphocytes cultured for 48 h in NRTI-free medium was examined. Samples from six exposed children and six controls were analysed by FISH using the 1q12 probe (Table 1). The proportion of nuclei with at least one dispersed spot was higher in the exposed than control children ($P<0.05$) (Table 2). Thus, heterochromatin modifications persisted in T-lymphocytes cultured *in vitro*.

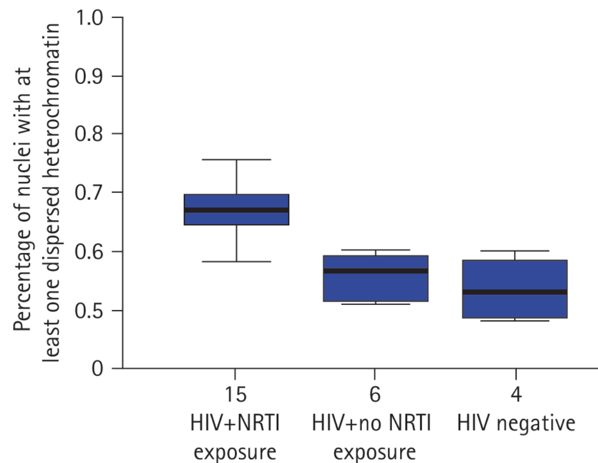
Analysis of other heterochromatic regions. To evaluate the extent of the heterochromatin defect, we used peripheral blood leukocytes from a smaller number of children to study the organization of other heterochromatic domains in the juxtacentromeric regions of chromosomes 9 and 16 and the centromeric region of chromosome 11. The juxtacentromeric heterochromatin

Figure 1. *In situ* hybridization of a heterochromatin 1q12-specific probe to leukocyte nuclei from peripheral blood



Green fluorescent spots reveal hydridized sites. Nuclei were counterstained with DAPI (blue fluorescence). Round (mainly lymphocytes) and polymorphic nuclei show condensed spots (arrowheads) and dispersed sets of spots (thin arrows). The pattern on constitutive heterochromatin in normal somatic cells is predominantly condensed spots.

Figure 2. Box plots of the percentage of nuclei with at least one dispersed 1q12 spot in the three groups of children



Results for children born to NRTI-exposed mothers (HIV+NRTI exposure), to HIV-1-infected unexposed mothers (HIV+no NRTI exposure) and HIV-1-uninfected mothers (HIV negative) are shown. The median value is the line in bold, and the box limits correspond to the interquartile range.

Table 2. Percentage of nuclei with at least one dispersed signal in NRTI-exposed and -unexposed control children

Heterochromatic region analysed	Nucleus population	Exposed*	Control*	*P*-value (Mann–Whitney test)
Chromosome 1 satellite 2 (1q12)	Total	67 (65–70), *n*=15	56 (51–60), *n*=10	<0.001
	Round	70 (66–81), *n*=15	66 (58–70), *n*=10	0.031
	Polymorphic	63 (58–69), *n*=15	46 (44–49), *n*=10	<0.001
Chromosome 16 satellite 2 (16q11)	Total	65 (59–71), *n*=4	56 (54–58), *n*=4	0.029
Chromosome 11 α-satellite (11p11-q11)	Total	71 (61–74), *n*=4	53 (50–58), *n*=4	0.043
Chromosome 9 satellite 3 (9q12)	Total	67 (57–73), *n*=5	53 (51–59), *n*=5	0.095
Chromosome 1 satellite 2 (1q12) after culture	Total[†]	63 (54–71), *n*=6	41 (37–49), *n*=6	0.002

*Values given as median percentage (interquartile range). [†]Phytohaemagglutinin-stimulated T-lymphocyte.

of chromosomes 9 and 16 are composed of repeats of satellites 3 and 2, respectively. The centromeric heterochromatin of chromosome 11, as well as centromeric regions of all the other chromosomes, is composed of α-satellite repeats. We analysed four exposed and four control children for chromosome 16 and chromosome 11 and five exposed and five control children for chromosome 9 (Table 1). The proportion of dispersed heterochromatin in all the nuclear regions examined was higher in NRTI-exposed children than in controls (Table 2). The difference between exposed and unexposed control children was significant ($P<0.05$) for chromosome 16 and chromosome 11, but was not significant for chromosome 9 ($P=0.095$). These results indicate that the effect of perinatal exposure to NRTIs on heterochromatin organization is not limited to the juxtacentromeric region of chromosome 1.

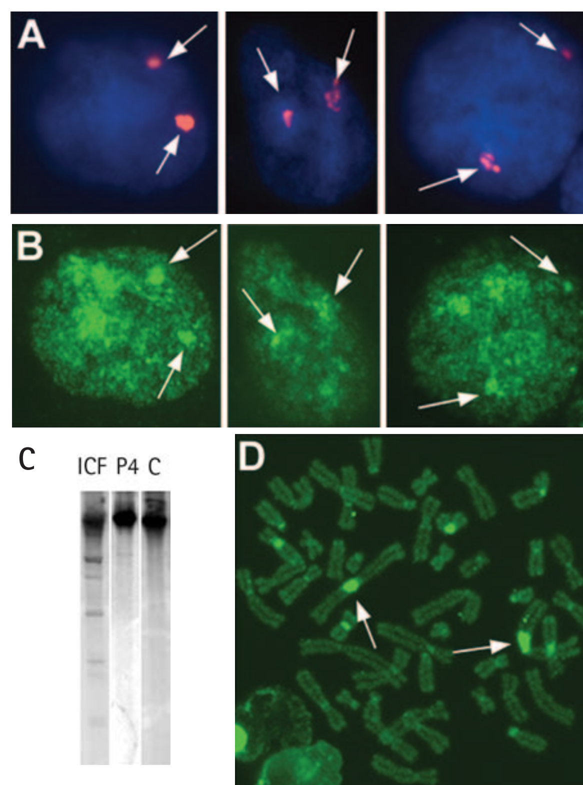
Analysis of the DNA methylation profile of the 1q12 heterochromatic domain in children exposed to NRTIs

The methylation profile in dispersed heterochromatin of leukocyte nuclei from peripheral blood of two exposed children and of PHA-stimulated T-lymphocyte nuclei of two exposed children was assessed by *in situ* hybridization and anti-5-MeC immunofluorescence (Figures 3A, 3B). No abnormality was detected. Southern blot analysis confirmed the normal methylation pattern of the 1q12 heterochromatin in peripheral leukocyte DNA of exposed children (Figure 3C). Anti-5-MeC binding to chromosomes in PHA-stimulated T-lymphocytes of exposed children indicated that the heterochromatin was methylated and condensed normally (Figure 3D).

Discussion

We show here that perinatal exposure to zidovudine alone or in combination with other NRTIs can affect the organization of the constitutive heterochromatin domains of various committed peripheral blood leukocytes in HIV-1-uninfected children. Dispersed organization of the heterochromatin domain of chromosome 1

Figure 3. DNA methylation patterns in AZT-exposed children



Combined *in situ* hybridization with the 1q12-specific probe **(A)** and immunofluorescence with a 5-methylcytosine (MeC) antibody **(B)** in leukocytes of zidovudine (AZT)-exposed children. The condensed and dispersed spots (red fluorescence, arrows) co-localize with the methylcytosine-stained spots (green fluorescence, arrows). Southern blot analysis using the methyl-sensitive enzyme *Hpa*II of chromosome 1 heterochromatin **(C)** of patient 4 (P4, middle lane), an ICF patient (demethylated control, left lane) and normally methylated control (right lane). P4 DNA is as methylated as the normally methylated control DNA. 5-MeC immunofluorescence in metaphase chromosomes of exposed children obtained after 48 h of culture **(D)**. Arrows indicate the normally condensed and methylated 1q12 heterochromatin of both chromosomes 1. ICF, immunodeficiency, centromeric region instability, facial anomalies syndrome.

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(1q12), the largest heterochromatin site of the human genome, occurred more frequently in NRTI-treated children than in non-treated controls. This defect persists long after treatment has been terminated (up to 9 years of age) and persists in cultured T-lymphocytes. By contrast, there was no difference between the two control groups: children born to HIV-1-uninfected mothers and those born to non-treated HIV-1-infected mothers. Chromosome 1 heterochromatin is not the only heterochromatic domain affected by the NRTI treatment: other heterochromatin sites, in the juxtacentromeric region of chromosome 16 (16q11) and in the centromeric region of chromosome 11 (11p11-q11), also presented the same type of anomaly. Chromosomes 1 and 16 heterochromatin are composed of satellite 2 and chromosome 11 of α-satellite, so several major types of satellite DNAs that compose human heterochromatin are susceptible to this defect. For chromosome 9, composed of satellite 3, the frequency of the anomaly was higher in NRTI-treated children than in untreated children, but not significantly so. A more exhaustive analysis should be performed for chromosome 9.

The dispersed heterochromatin was not restricted to a specific type of leukocytes, suggesting that haematopoietic stem cells (HSCs) are affected by the treatment. Indeed, all mature blood cells originate from a small population of self-renewing pluripotent HSCs stored in the fetal liver, and, after birth, in bone marrow. Presumably, our patients acquired HSC abnormalities during fetal exposure, and consequently, the heterochromatin in circulating cells derived from lymphoid and myeloid lineages was modified. Indeed, damage to HSCs during fetal development would explain the persistence of abnormal heterochromatin long after the end of the treatment. Were this the case, both lymphoid and myeloid stem cells and/or committed progenitors and precursors of haematopoietic cells would be damaged. The important point is that the anomaly was memorized at some stage during early haematopoiesis and subsequently perpetuated through differentiation and proliferation of the cells.

The frequency of heterochromatin anomaly observed after 48 h of culture of T-lymphocytes from exposed children was significantly higher than in those from the controls, indicating that the nuclear anomaly is perpetuated *in vitro* through cell divisions. However, metaphase chromosomes obtained after PHA-stimulation showed no evidence of abnormal heterochromatin organization. Thus zidovudine and other NRTIs appear to target cellular factors involved in the interphase organization of constitutive heterochromatin, but not those involved in its metaphase organization. The protein complexes and epigenetic markers associated with heterochromatin differ depending on the phase of the cell cycle [27,28]. For instance, some proteins are present only during mitosis and are concentrated in centromeric heterochromatin [29], whereas others move from euchromatin to heterochromatin at the onset of mitosis, possibly in order to favour equal distribution to the daughter cells [30].

Other than the binding of specific non-histone proteins, the establishment and maintenance of silent heterochromatin involves a series of biochemical modifications to DNA and histone proteins [22]. Any of the epigenetic markers of heterochromatin might be involved in the defective organization we describe herein. However, we excluded the involvement of DNA methylation, a major marker of heterochromatin: satellite 2, located in the heterochromatin of chromosomes 1 and 16, was normally methylated in the DNA from the exposed children. Therefore, the mechanism involved in the abnormal configuration of heterochromatin is different from those previously described in patients with immunodeficiency, centromeric region instability, facial anomalies (ICF) syndrome [31], in tumours [32] and in cells treated with the demethylating agents 5-azacytidine or 5-aza-2′-deoxycytidine [33,34].

The clinical consequences of this persistent anomaly are difficult to evaluate despite the functional role of heterochromatin in genome regulation. Several severe clinical symptoms associated with constitutional epigenetic defects have been described, including increased risk of cancer, severe neurological disease and haematopoiesis defects [35–37]. The potential link between treatment and a long-lasting haematopoiesis defect observed in independent cohorts of zidovudine-exposed children might be relevant [38,39]. This mild but intriguing persistent inhibition affecting three different blood cell lineages (polynuclear neutrophils, lymphocytes and platelets) is consistent with the hypothesis that perinatal administration of zidovudine and NRTIs affects haematopoietic stem cells. Note that the nuclear organization of heterochromatin is important for gene silencing and determining cell fate [40]. The three-dimensional organization of the nuclear centromeric compartment changes during haematopoietic differentiation and distinct myeloid and lymphoid configurations have been described [41,42]. In addition, there is evidence from activated B-lymphocytes [43] and differentiating helper T-cells [44] that heterochromatin regulates gene silencing during mammalian haematopoiesis. Induction of silencing is associated both with the repositioning of the target genes to nuclear heterochromatin and with chromatin modifications usually associated with a repressive state. Indeed, Su *et al.* [45] observed changes and two-directional spreading of histone 3 lysine 9 (H3K9) methylation and histone 4 deacetylation in the

promoter region of the mouse terminal transferase gene (*Dntt*), which is silenced and repositioned to pericentromeric heterochromatin during thymocyte maturation. The acquisition of epigenetic markers associated with an inactive chromatin state reinforces and heritably maintains the gene expression pattern characteristic of each differentiated cell type.

In conclusion, we report the description of a new genotoxic effect of perinatal exposure to zidovudine and other NRTIs. The molecular factor involved in this abnormal organization of heterochromatin is not yet known. However, the well documented involvement of heterochromatin in genome regulation justifies further studies to evaluate the long-term consequences of the persistent heterochromatin disturbance.

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