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T-cell re-population in HIV-infected children on highly active anti-retroviral therapy (HAART)

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SUMMARY

In this pilot study, we address the nature of the re-population of the T-cell compartment in HIV-1^+ (Human Immunodeficiency Virus 1), vertically infected children placed on successful regimens of HAART (highly active anti-retroviral therapy) incorporating 2 NRTI and a protease inhibitor. The clonality of the T-cell compartment and the abundance of RTEs (Recent Thymic Emigrants) were determined 2 weeks before and 20 weeks after initiation of HAART in a subgroup of children taking part in the PENTA (Paediatric European Network for the Treatment of AIDS) 5 trial. Analysis of the clonality of the circulating T-cell compartment was assessed using CDR3 spectratyping and analysed using the Kolmogorov–Smirnov two sample test. This revealed that a high degree of T-cell clonal restriction still exists 5 months into therapy, despite the appearance of previously undetectable T-cell clones within the periphery. We detected no increase in RTE abundance in this 5 month period, as determined by PCR detection of TRECs (T-Cell Receptor Excision Circles). We conclude that the observed re-population of T cells within the periphery of treated children is heavily reliant upon the maintenance/expansion of pre-existing cells during the 5 month period immediately following the initiation of therapy.

Keywords Paediatric HAART HIV TCR TREC

INTRODUCTION

The immune system of children is less well understood than that of adults [1,2], and it is not clear how they respond to HIV infection and what therapeutic strategies should be used. It is important to evaluate how the paediatric T-lymphocyte compartment is impaired by HIV infection and how this responds to HAART.

Prior to the initiation of therapy, HIV-infected children show a substantial up-regulation of the activation induced apoptotic marker CD95 (APO-1) [3,4] on their T cells compared with uninfected children. It is conceivable that this contributes significantly to the pathology of the infection [5], decreasing the average life span of the child's T cells and de-populating the peripheral pool [6,7]. Such a de-population has been shown to result in a clonally-restricted and dysfunctional T-cell pool in adults, which is not immediately repairable through the application of anti-retroviral therapy [8].

It has already been shown that following the introduction of HAART (Highly Active Anti-Retroviral Therapy) in adults, the initial re-population of the peripheral T-cell compartment is highly dependant upon a composite of T-cell re-distribution from other sites and proliferation of pre-existing cells [9–12]. In children, the thymus has a far higher output of T cells than in the adult, with a decrease only setting in dramatically after puberty [13]. It is therefore feasible that the high level of thymic output evident in children could rapidly 're-establish' a diverse T-cell compartment in such patients responding successfully to HAART. To determine whether this was the case, the clonality of the T-cell compartment before and after therapy was assessed in four children, using CDR3 spectratyping [14–17]. This technique is a PCR-based method for visualizing the composition of the T-cell receptor (TCR) repertoire. Primers specific for elements within the T-cell receptor produce measurable products, corresponding to the length of the Vβ CDR3 (Complementarity Determining Region 3) chain. By analysing the amount of product generated for each possible CDR3 chain within each TCRVβ family, the entire T-cell pool can be finely examined.

In this study, we analyse the apparent reconstitution of the T-lymphocyte population in children taking part in the PENTA (Paediatric European Network Treatment AIDS) 5 trial [18], placed on successful regimens of HAART, incorporating 2 NRTI and a protease inhibitor.

MATERIALS AND METHODS

Subjects

Four previously untreated, vertically HIV-infected children

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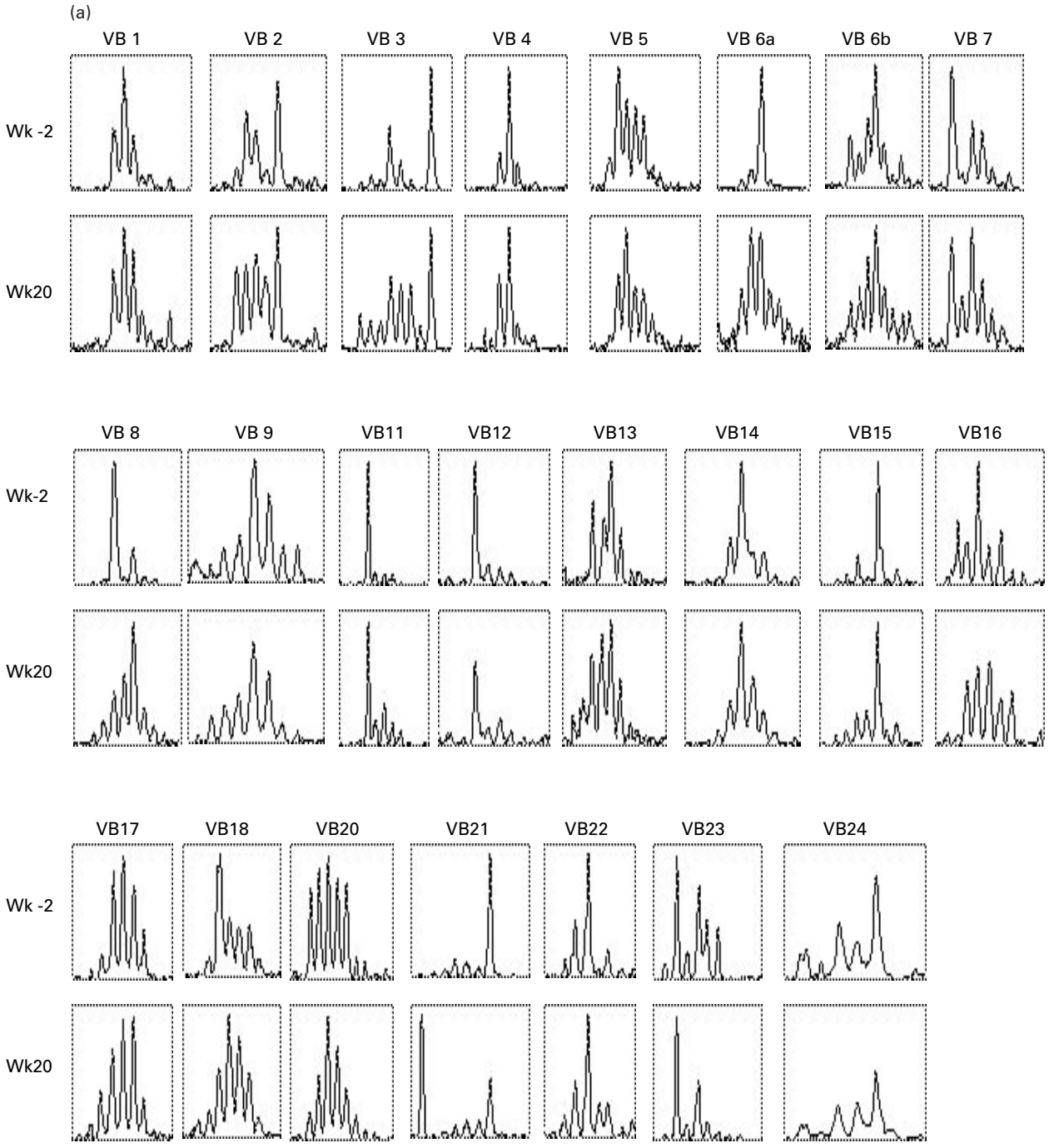
Table 1 Patient profiles before, and 20 weeks into, HAART. Clinical data for the four children studied, 2 weeks before initiation and 20 weeks after initiation of HAART. CD4 numbers are expressed as cells /mm^3 and VL is expressed in HIV RNA copies/ml.

Patient	Age (years)	CD4		CD8		VL		Change in TREC post-therapy (arbitrary units)
		Pre-HAART	HAART	pre-HAART	HAART	pre-HAART	HAART	
P5/1	3	796	1280	1990	1520	1900 000	104	− 0·139
P5/2	5	183	382	1029	819	314 000	203	− 0·248
P5/3	9	158	287	998	1370	25 000	< 50	− 0·290
P5/4	13	44	199	493	678	128 000	151	− 0·038



participating in the PENTA 5 trial were included. Treatment consisted of 2 NRTI (2 of Abacavir, ZDV or 3TC) plus Nelfinavir. All responded well to therapy, having a significant reduction in viral load (VL) (Roche Amplicor/Roche Ultrasensitive) and an increase in the $CD4^+$ T-lymphocyte number (Table 1).

Samples

Frozen samples of PBMCs (Peripheral Blood Mononuclear Cells) were obtained. The samples from each of the children consisted of paired samples taken 2 weeks before and 20 weeks after the initiation of HAART.

Sample preparation

PBMCs were thawed and lysed by resuspension in Tri-Reagent (Sigma). DNA and RNA were extracted as per the manufacturer's instructions. The RNA was used to create cDNA, primed with 16-mer oligo dT, as per the manufacturer's recommendations (all reagents Perkin-Elmer Applied Biosystems, Warrington, UK).

CDR3 spectratyping

The PCR to amplify each of the functional Vβ CDR3 regions was set up as follows. Reactions (25 μl) containing 2 μl 25 mM magnesium chloride, 2·5 μl ×10 PCR buffer, 0·5 μl mixed 10 mM dNTP, 0·2 μl cDNA, 0·6 U Taq polymerase, and previously published primers specific for the constant region (Cβ) and the variable region (Vβ) of interest [14–17], were run under the conditions of 95°C for 30 s, then 50 cycles of 95°C for 25 s, 60°C for 45 s and 72°C for 45 s, before a final elongation of 72°C for 5 min. The PCR products generated from the above reaction were subjected to flurophore-labelled, single primer elongations. The primers were specific for the constant region (Cβ) of the TCR β chain or to individual Joining region (Jβ) segments. These were performed under identical cycling conditions to the amplification PCR for between five and 10 cycles. The labelled products were loaded onto an ABI 310 Automate (Perkin-Elmer Applied Biosystems, UK), which measures the intensity of fluorescence (the amount of PCR product), and, by reference to the detection time of the internal size standards, the length of the PCR products was determined. The profiles or 'CDR3 spectratypes' generated from this may then be compared visually with one another to detect expansions, or assessed statistically to determine alterations in the composition or levels of perturbation, as described below.

Statistical analysis

Statistical analysis of the CDR3 spectratype data was conducted using a two sample Kolmogorov–Smirnov test. This test determines whether two samples of data may reasonably be assumed to possess the same distribution. Being non-parametric, the Kolmogorov–Smirnov test does not require the assumption that the population is normally distributed. Gorochov *et al*. [19] used this equation to estimate CDR3 spectratype deviation of clinical samples from a theoretical 'normal', thereby gauging the level of perturbation. Here, we used this test to determine directly the difference between samples taken from the children at

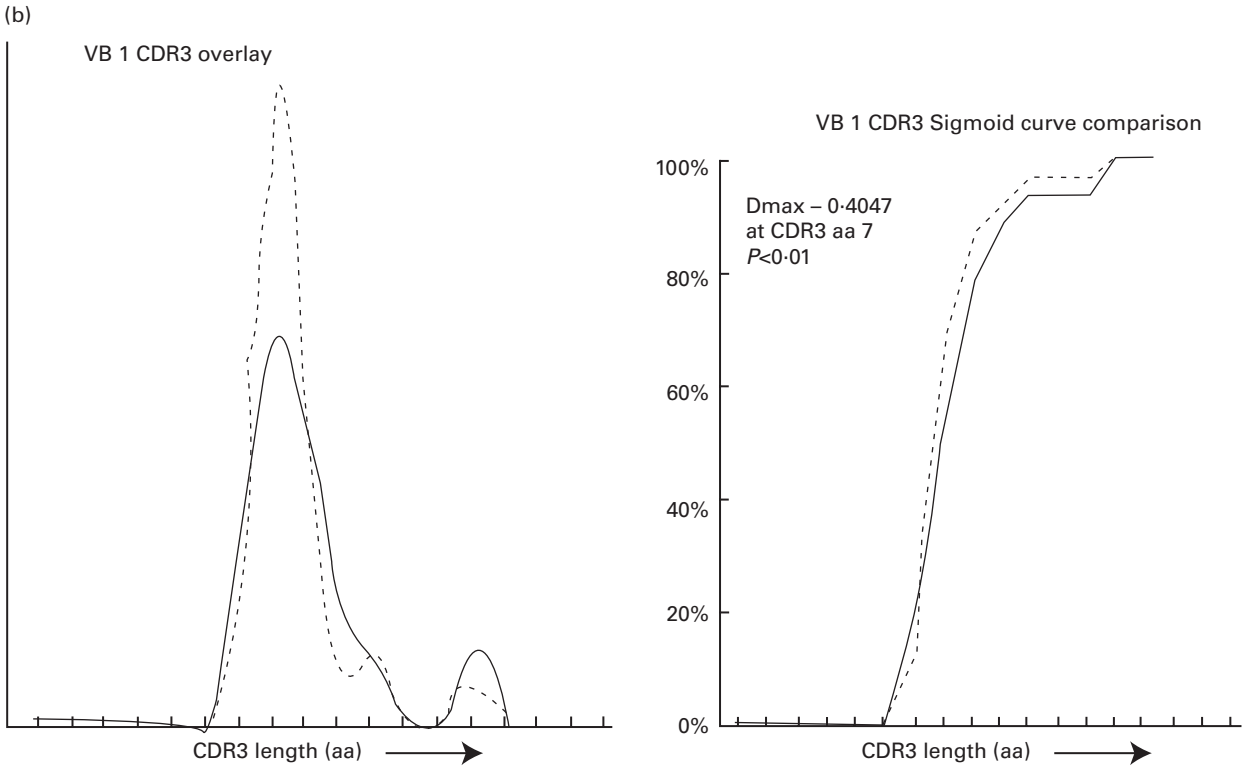


Fig. 1. CDR3 spectratypes. (a) A representative complete set of CDR3 spectratypes generated from the PENTA 5 children (subject P5/1 displayed) before and after therapy are shown. The differences in clonal distributions (Vb1) are assessed using the non-parametric Kolmogorov–Smirnov test, the sigmoid curve of the population distributions being compared with each other. (b) An example of the VB1 family of subject P5/1 is shown. The maximum difference (Dmax, ranging between 0 [no difference] to 1 [absolutely different]) between the two spectratypes is shown, along with the length of the CDR3 chain (aa) at which this occurs and the degree of significance.

Table 2 Kolmogorov - Smirnov 2 sample test results for PENTA 5 Subjects. Compiled Dmax values along with *P*-values for each of the children's Vβ families 20 weeks after initiation of HAART compared with 2 weeks prior to HAART.

	P5/1		P5/2		P5/3		P5/4	
VB	*P*-value	Dmax	*P*-value	Dmax	*P*-value	Dmax	*P*-value	Dmax
1	<0·01	0·405	ns	0·185	ns	0·190	<0·01	0·791
2	<0·01	0·627	ns	0·246	ns	0·143	<0·01	0·724
3	<0·01	0·663	<0·01	0·433	<0·01	0·807	<0·01	0·467
4	<0·05	0·270	<0·05	0·366	ns	0·088	<0·01	0·657
5	<0·05	0·377	ns	0·185	ns	0·209	<0·01	1
6a	<0·01	0·827	<0·01	1	<0·05	0·397	ns	0·062
6b	ns	0·187	ns	0·309	<0·05	0·342	ns	0·194
7	<0·01	0·455	ns	0·146	ns	0·151	<0·01	0·332
8	<0·01	0·762	<0·01	0·630	ns	0·314	<0·01	0·602
9	ns	0·197	<0·05	0·309	ns	0·161	<0·01	0·348
11	<0·01	0·341	<0·05	0·442	ns	0·129	<0·01	0·708
12	ns	0·244	<0·01	0·386	ns	0·149	ns	0·248
13	<0·01	0·523	<0·01	0·608	ns	0·204	<0·01	1
14	<0·01	0·372	ns	0·225	<0·01	0·480	<0·01	0·463
15	ns	0·266	<0·01	0·457	<0·01	0·469	<0·01	0·984
16	<0·01	0·652	<0·01	0·820	<0·01	0·403	<0·01	0·969
17	ns	0·202	ns	0·277	ns	0·029	<0·01	0·650
18	<0·01	0·578	<0·01	0·646	ns	0·157	<0·01	1
20	<0·01	0·510	<0·01	0·493	ns	0·113	<0·01	0·798
21	<0·01	1	<0·01	0·604	<0·01	0·758	<0·01	1
22	<0·05	0·300	<0·05	0·333	ns	0·260	<0·01	0·801
23	<0·05	0·346	<0·01	0·793	<0·05	0·379	<0·01	0·750
24	ns	0·234	<0·01	0·478	ns	0·314	<0·01	1
Mean		0·449		0·451		0·289		0·676
s.d.		0·223		0·224		0·200		0·292

different time points, and attempted to gauge the magnitude of any change by reference to the Dmax (maximum difference) in each VB family. Dmax tends to be small (tending towards 0) in healthy controls (Data not shown).

TREC analysis

Using the previously published primer sequences and PCR conditions for the detection of Signal Joint TRECs [20], excision circle abundance within the T-cell compartment was assessed. P^{32}-labelled dATP was incorporated into the PCR products by inclusion of Redivue αP^{32}-dATP (Pharmacia, Little Chalfont, UK) as 5% of the total nucleotides. Following agarose gel electrophoresis and ethidium bromide staining, the bands were excised from the gel and incorporation of P^{32} was measured using a Wallac 1205 betaplate-counter (Perkin-Elmer Life Sciences, Warrington, UK). The P^{32} TREC signal was standardized against an internal β-actin P^{32} PCR signal. Triplicates of positive control samples, incorporating DNA extracted from purified $CD4^+$ cells, were run simultaneously at 1:1, 1:2 and 1:10 dilutions to assure assay performance and sensitivity (data not shown).

RESULTS

T-cell clonality

To assess the clonality of the T-cell compartment, the CDR3-specific PCR described above was employed, generating CDR3 spectratypes for the functional Vβ families (Fig. 1a). Kolmogorov–Smirnov statistics were then used to determine the difference (Dmax, ranging between 0 and 1) between the individual Vβ families in each child, before and after therapy (Table 2), to reveal any alterations in the composition of the circulating T-cell compartment. This revealed a noticeable difference in the clonal composition of the T-cell compartment before and after treatment, with the average Dmax for P5/1 being 0·449, P5/2 0·451, P5/3 0·289 and P5/4 0·678. The high Dmax values indicate that a large difference exists between the 2 weeks prior to HAART and 20 weeks post HAART initiation time points analysed.

Conserved clonal T-cell expansions

Despite the prompt and sustained reduction in viremia and the rise in the number of peripheral $CD4^+$ T cells during 5 months of HAART (Table 1), over-represented CDR3 PCR products of identical length were found before, and 5 months into, therapy in a number of TCRVβ CDR3 clusters in all four children (Fig. 2).

By probing the products of the pre-labelled PCR reactions with flurophore-bound primers specific for each of the Jβ segments, we were able to dissect further the TCRVβ families into their constituent 13 Jβ elements, so that their clonality could be more closely evaluated. This technique was used to examine the TCRVβ clusters where an expansion of the same dominant CDR3 lengths was present in both the 2 weeks prior to HAART and 20 week post initiation of HAART time points. This revealed that these conserved TCRVβ expansions are maintained within the same Jβ families between the two time points (22 weeks time span), providing strong evidence for their clonal nature (Fig. 3).

TREC level in paediatric group

During the rearrangement of TCR genes to create the TCR, excisional, stable circles of DNA are generated. These fail to duplicate during mitosis and are subsequently diluted out in cellular populations during proliferation [20]. As such, PCRs targeting regions of these circles may be used to assess the replicative history of a cellular population, or rather, the contribution of recent thymic emigrants to the T-cell compartment. DNA extracted from the same cells as the RNA was used in PCR reactions to assess relative TREC levels in the paediatric samples.

By standardizing the sjTREC products against an internal marker, no increase in the relative abundance post therapy (Table 1.) in any of the children was observed. Therefore, no substantial increase in the number of recent thymic emigrants in the peripheral circulation due to HAART was recorded.

DISCUSSION

The TCRVβ repertoire studies undertaken here display alterations in the clonal composition of the repertoire in four children after 5 months of successful HAART (Fig. 1, Table 2). The fact that initially undetectable CDR3 spectratype products of certain TCRVβ families are found following therapy is encouraging (Figs 1 and 2), suggesting the re-establishment of truly naive cells within the periphery [13,20], a decrease in the size of the expansions within the $CD8^+$ compartment [21] allowing the detection of less frequent CDR3 lengths, or the redistribution of sequestered cells from other sites following the alleviation of viral burden [9]. Should redistribution of sequestered cells be a major cause of this change in peripheral T-cell pool composition, it

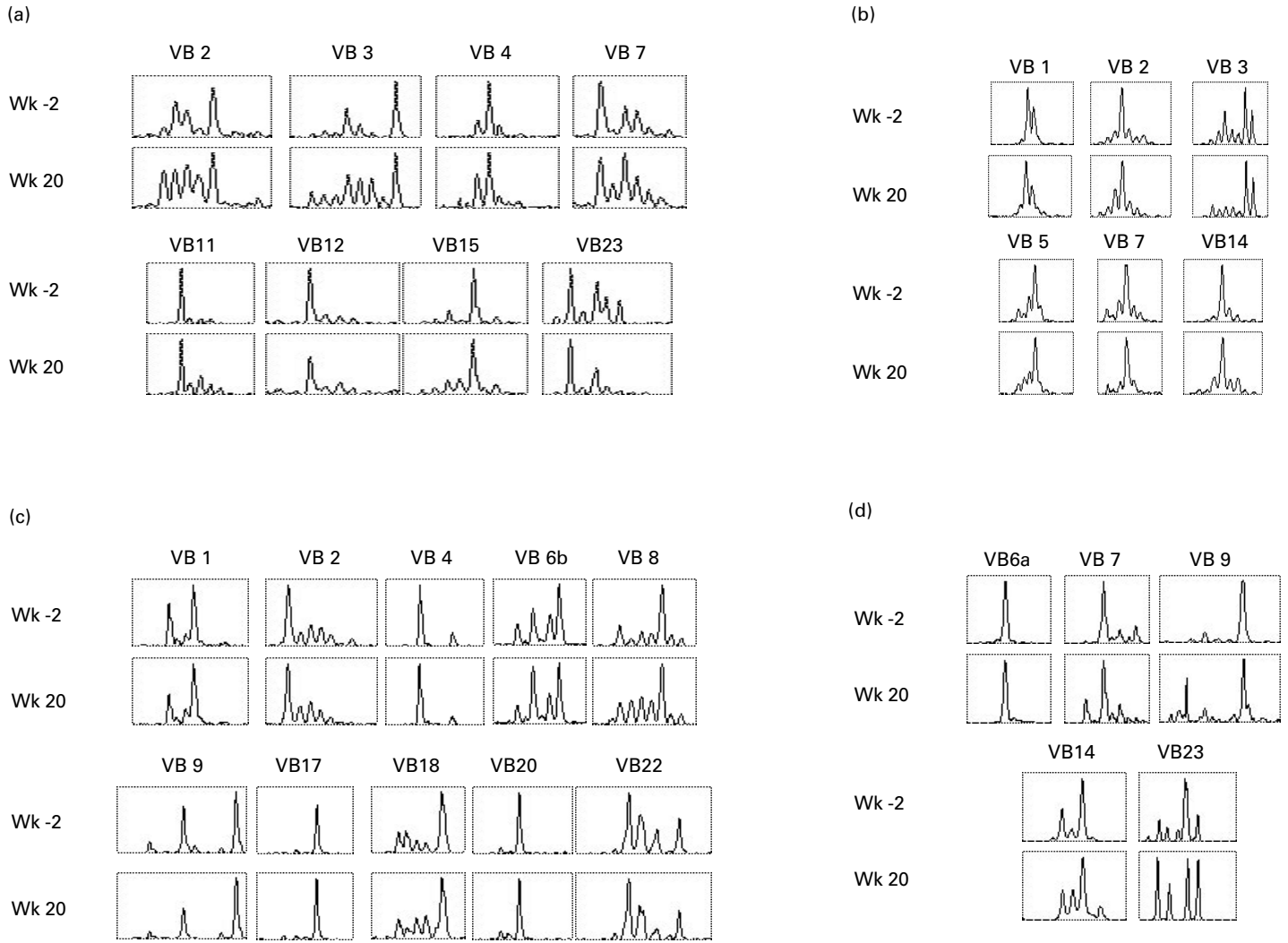


Fig. 2. Maintenance of clonal restriction. Dominant CDR3 lengths seen prior to the initiation of therapy are still in evidence 20 weeks into therapy, despite a prompt and sustained drop in viremia and an increase in $CD4^+$ T-cell count. (a) Subject P5/1; (b) P5/2; (c) P5/3; (d) P5/4.

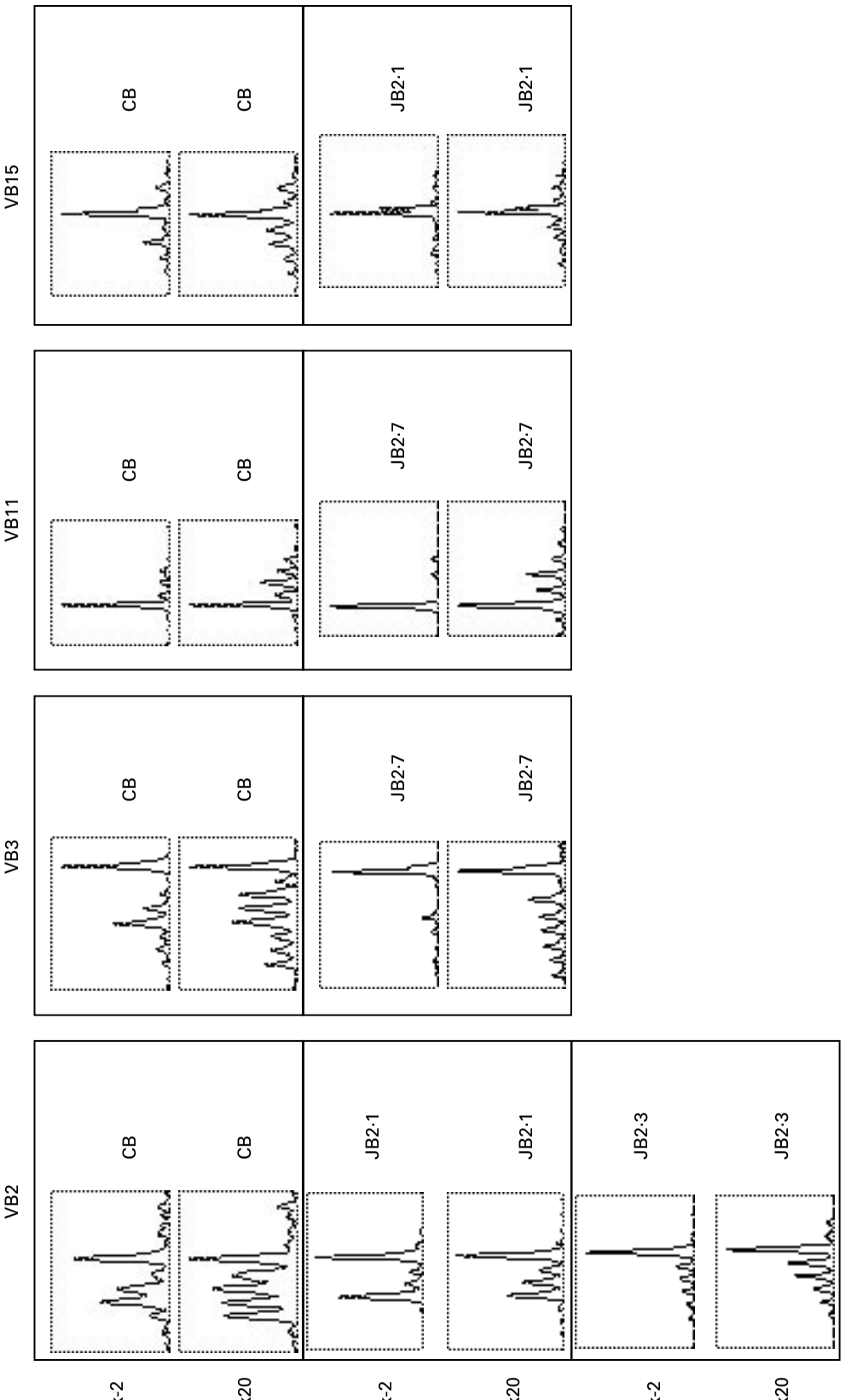


Fig. 3. Conserved TCR expansions are created by expansions within a restricted number of JB families. CDR3 spectratypes dissected out into their JB elements reveal that the over-represented CDR3 lengths originate in a restricted number of JB elements, strongly supporting their clonality. Other JB elements (not shown) in the given Vbs failed to show an over-represented CDR3 of the appropriate length.

would occur rapidly following the suppression of viremia [11,22], though the result of this would persist, allowing detection at the 20 week time point. Any of the aforementioned scenarios could increase the apparent diversity within the circulating T-cell compartment, although the production of new, naive cells would arguably be the most beneficial to the subject, representing true immune reconstitution.

These results must be interpreted with caution because of small numbers. However, there is strong evidence that initially over-represented TCRVβ CDR3 expansions are maintained over a period of 5 months on HAART (Figs 1, 2 and 3) in each of the children. Such data suggest that specific cells are, at the very least, maintaining their place within the periphery, if not actively proliferating to re-populate the circulating cellular compartment. There are several possible explanations of the persistence of these dominant CDR3 lengths. They may represent cells responding to on-going antigenic stimuli other than the HIV-derived antigens; influx of new cells could be insufficient to displace these over-represented cells; and/or the level of residual HIV antigens [23] may be sufficient to maintain a clonal HIV-specific response. The phenomenon of mature $CD4^+$ T cells proliferating after successful HAART intervention in HIV-infected adults has already been observed [12], and peripheral expansion of T cells has been shown in animal models [24–26]. Recently, the observation that the size of the $CD8^+$ $CD28^-$ T-cell population continues to be significantly elevated in HIV-infected children up 12 months after the initiation of HAART [27] (compared with uninfected children) would suggest that these cells could be clonally restricted.

Recent thymic emigrants were seen in all of the children both before and after therapy, despite low CD4 counts at baseline, demonstrating the relatively high thymic function in children. In agreement with Zhang *et al*. [13], we failed to detect an increase in TREC relative abundance (Table 1) after 5 months of therapy; this does not exclude a contribution to the periphery by RTEs but suggests rather that their relative level does not increase. Whether this is due to factors such as activation and proliferation, rapid turn-over, or the over-abundance of the detected clonal expansions is unclear. Others, such as Douek [20], have reported an increase in RTE levels post therapy.

Bohler *et al*. [28], monitoring the efficacy of HAART in children, noted a decrease in expression of the apoptotic marker CD95 following therapeutic intervention. This may suggest a down-regulation of the activation-induced naive to memory phenotype ''switch', and a subsequent decrease in activation-induced apoptosis, which would certainly agree with our observations.

Our findings imply that in successful paediatric therapy, with a decrease in viral load and an increase in $CD4^+$ T-cell numbers, the reconstitution of the peripheral T-cell compartment is a composite of redistribution of cells from other sites and the proliferation of pre-existing T cells, possibly mirroring data which have been reported in adults [9], despite the differences in thymic function in adults and children.

In children, those cells which are present at the initiation of therapy would appear to play a significant role in the subsequent composition of the T-cell repertoire. Though this may be diluted out over a greater period of time, after 5 months of HAART intervention, the continued clonal restriction is very apparent and could limit the breadth of antigens that the 'pool' would recognize.

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