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Linkage of the CCR5Δ32 Mutation with a Functional Polymorphism of CD45RA

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A 32-bp deletion in CCR5 (CCR5Δ32) confers PBMC resistance to HIV-1 isolates that use CCR5 as a coreceptor. To study this mutation in T cell development, we have screened 571 human thymus tissues for the mutation. We identified 72 thymuses (12.6%) that were heterozygous and 2 (0.35%) that were homozygous for the CCR5Δ32 mutation. We found that thymocyte development was normal in both CCR5Δ32 heterozygous and homozygous thymuses. In 3% of thymuses we identified a functional polymorphism of CD45RA, in which cortical and medullary thymocytes failed to down-regulate the 200- and 220-kDa CD45RA isoforms during T cell development. Moreover, we found an association of this CD45 functional polymorphism in thymuses with the CCR5Δ32 mutation (p = 0.00258). In vitro HIV-1 infection assays with CCR5-using primary isolates demonstrated that thymocytes with the heterozygous CCR5Δ32 mutation produced less p24 than did CCR5 wild-type thymocytes. However, the functional CD45RA polymorphism did not alter the susceptibility of thymocytes to HIV-1 infection. Taken together, these data demonstrate association of the CCR5Δ32 mutation with a polymorphism in an as yet unknown gene that is responsible for the ability to down-regulate the expression of high m.w. CD45RA isoforms. Although the presence of the CCR5Δ32 mutation down-regulates HIV-1 infection of thymocytes, the functional CD45RA polymorphism does not alter the susceptibility of thymocytes to HIV-1 infection in vitro.

Materials and Methods

Thymus tissue

Human thymuses were obtained from the Department of Pathology at Duke Medical Center as discarded tissues taken in the course of corrective cardiovascular surgery or therapeutic thymectomy for myasthenia gravis using a Duke institutional review board-approved protocol. No tissue was removed that was not clinically indicated by the surgical procedure being performed. Fresh thymus tissue was either teased with forceps and scissors, and thymocyte suspensions were prepared, or a portion (~0.5 x 0.5 cm) was placed in RPMI 1640 medium supplemented with 7.5% DMSO and 15% FCS and snap-frozen in liquid nitrogen.

Monoclonal Abs

Mouse anti-human CD45 mAb F10-89-4 and CD45RA mAb F8-11-13 were provided by Rosemarie Dalchau (London, U.K.) (17). Anti-CD45RO mAb UCHL-1 was provided by P. C. L. Beverley (London, U.K.) (18). Anti-CD45RB mAb N-L162 and anti-CD45RC mAb N-L121 were obtained through the Fifth International Workshop on Human Leukocyte Differentiation (19). P3×63 IgG1 paraprotein (P3) was produced by the P3×63 Ag8.652 myeloma cell line (20) and used as a control Ab. PE-conjugated anti-CD4-PE and Cy5-conjugated CD8 were purchased from PharMingen (San Diego, CA).

Cells and tissue culture conditions

Frozen thymocytes were thawed by incubation at 37°C for 1 h in RPMI 1640 medium containing 10% FCS and 10 μg/ml of DNase I (Sigma, St. Louis, MO). After thawing, thymocytes were passed through a Ficoll-Hypaque gradient by centrifugation at 1500 rpm for 30 min and washed twice with RPMI 1640 containing 10% FCS. Thymocytes were cultured in RPMI 1640 medium supplemented with 10% FCS and 10 ng/ml of IL-2 and were maintained at 37°C in a humidified 5% CO2 incubator. Human PBMCs were prepared from buffy coats of healthy, HIV-1-seronegative individuals obtained through the laboratory services of the American Red Cross, Carolina region (Charlotte, NC). PBMCs were isolated by Ficoll-Hypaque gradient centrifugation and were used as positive control cells for in vitro HIV-1 infection assays. PBMC were washed twice in RPMI 1640 medium containing 20% heat-inactivated FCS, resuspended at a density of 2.5 x 10^6 cells/ml in the same medium containing 10% DMSO, and frozen in 1-ml aliquots in liquid nitrogen. PBMC were prescreened for the ability to support the replication of syncytium-inducing (SI) and non-SI (NSI)
primary isolates of HIV-1 to confirm the expression of appropriate coreceptors, including CCR5.

**HIV-1 virus stocks**

Seven different strains of HIV-1 belonging to the genetic clade B subtype were used to infect thymocytes and PBMC in vitro. The IIB strain is an SI, T cell line-adapted strain that uses CXCR4 as its major coreceptor (3, 21, 22). 89.6 is an SI primary isolate that infects macrophages and CD4+ lymphocytes (25) and is capable of using multiple coreceptors (22), although CXCR4 usage dominates (24). We used the uncloned stock of 89.6 that had been passaged minimally in PBMC and was expanded in IL-2 for use in this study. V67970 is an SI primary isolate that uses both CXCR4 and CCR5 (24, 25) and was expanded once in PBMC from the original coculture supernatant. Ba-L is an NSI HIV primary isolate that infects macrophages and CD4+ lymphocytes (26) and uses CCR5 as its sole coreceptor (22). P15 and P46 are NSI primary isolates obtained during early seroconversion (27) and use CCR5 as their sole coreceptor (D. Montefiori, unpublished observations). These latter two HIV-1 isolates were used after a single expansion in PBMC of original coculture supernatants. JR-FL is a CCR5-using NSI primary isolate obtained from frontal lobe brain tissue of a patient with AIDS dementia (28). To be consistent with current nomenclature (29), HIV-1 viruses that use CCR5 as their major coreceptor will hereafter be referred to as R5 strains (Ba-L, P15, P46, and JR-FL), viruses that use CXCR4 will be termed X4 strains (IIB), and viruses that use both coreceptors will be termed R5X4 strains (89.6 and V67970). Ba-L and JR-FL were obtained from the National Institutes of Health AIDS Research and Reference Reagent Program (Rockville, MD).

**Infection of thymocytes**

Calcofluor-stained thymocytes (2 × 10^6 cells/ml/vial) and PBMC were thawed at 37°C and suspended in 20 ml of stimulation medium consisting of RPMI 1640 containing heat-inactivated FBS (20%), gentamicin (50 μg/ml), and 10% of the IL-2. Advanced Biotecnology Corporation (D. Montefiori, unpublished observations). These latter two HIV-1 isolates were used after a single expansion in PBMC of original coculture supernatants. JR-FL is a CCR5-using NSI primary isolate obtained from frontal lobe brain tissue of a patient with AIDS dementia (28). To be consistent with current nomenclature (29), HIV-1 viruses that use CCR5 as their major coreceptor will hereafter be referred to as R5 strains (Ba-L, P15, P46, and JR-FL), viruses that use CXCR4 will be termed X4 strains (IIB), and viruses that use both coreceptors will be termed R5X4 strains (89.6 and V67970). Ba-L and JR-FL were obtained from the National Institutes of Health AIDS Research and Reference Reagent Program (Rockville, MD).

**Immunoprecipitation and SDS-PAGE**

Thymocytes (0.5 × 10^6 cells) were labeled with [125I] (NEN Life Science Products, Boston, MA) (30) and lysed in 500 μl of lysis buffer (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 2 μg/ml aprotinin, 2 μg/ml leupeptin, and 1% of Nonidet P-40). Immunoprecipitation were conducted as previously described (31). Briefly, cell lysates were first precleared by incubation with control mAb P3 followed by protein A/G-agarose (Sigma). Pre-cleared cell lysates were the incubated with CD45, CD45RA, or control mAbs for 4 h followed by incubation with protein A/G-agarose (Sigma). Immune complexes were washed five times with buffer (10 mM Tris-HCl (pH 8.0), 140 mM NaCl, and 0.025% NaN3), resuspended in SDS-PAGE sample buffer, boiled for 5 min, and subjected to SDS-PAGE on 7% polyacrylamide gels.

**Indirect immunofluorescence staining and flow cytometry**

Serial frozen 5-μm sections of thymus were cut and incubated with saturating amounts of mAbs against CD3, CD4, CD8, CD45RA, CD45RO, CD45RB, and CD45RC or control mAb P3 in PBS containing sodium azide for 30 min at room temperature, washed twice with PBS, and then incubated with saturating amounts of goat anti-mouse IgG-FITC for 30 min at room temperature. These sections were washed three times with PBS, and then viewed under fluorescence microscopy. Thymocytes in suspension (0.5 × 10^6 cells/tube) before and after activation with PHA were incubated for 30 min at 4°C with various mAbs against the cell surface molecules, including CD4, CD8, CD45RA, CD45RO, CD45RB, and CD45RC, and CXCR4 and control mAb P3 in 100 μl of PBS containing 0.2% BSA and 0.1% sodium azide. Cells were washed twice with PBS with BSA/sodium azide, incubated with goat anti-mouse IgG-FITC for 30 min at 4°C followed by washing twice with PBS with BSA/sodium azide. In some experiments in which three-color flow cytometric analysis was performed, thymocytes were incubated with PE-conjugated mouse anti-CD4, and Cy5-conjugated mouse anti-CD8 before incubation with the goat anti-mouse IgG-FITC. After staining, thymocytes were washed, fixed in 4% paraformaldehyde in PBS, and protected from light at 4°C until analysis by a flow cytometer. Three-color flow cytometry was performed using a FACScan flow cytometer (Becton Dickinson, San Jose, CA). Data acquisition and analysis were conducted with CellQuest software. For each cell sample, a total of 10^4 cell events were analyzed. Data are expressed as the mean fluorescence channel (MFC), an indication of the intensity of cells stained with specific mAbs.

**Statistical analysis**

The Fisher exact test was used to analyze association of the heterozygous or the homozygous CCR5Δ32 mutation with the presence of the abnormality of CD45RA expression in thymocytes.

**Results**

Detection of the CCR5Δ32 mutation in a bank of human thymus tissues

The Human Thymus Bank at Duke University contains 571 normal or myasthenia gravis thymus. These thymuses were from patients with congenital heart disease (age range, 1 day to 17 years) and patients with myasthenia gravis (age range, 3–80 years). We screened these 571 thymus tissues for the CCR5Δ32 mutation using PCR (Table I and Fig. 1). Seventy-two thymuses (12.6%) were heterozygous for the CCR5Δ32 deletion mutation (CCR5Δ32+/−), and two thymuses (0.35%) were homozygous for CCR5Δ32 deletion mutation (CCR5Δ32−/−). Although both thymuses that were homozygous for CCR5Δ32 were from patients with congenital heart disease, there was no statistical difference in the frequency of CCR5Δ32 in patients with myasthenia gravis compared with that in children with congenital cardiovascular disease (not significant). Sequence analysis of thymus CCR5Δ32−/− DNA revealed the same CCR5Δ32 deletion mutation sequence as that previously reported in PBMC (data not shown) (7, 8). Because thymuses were collected as randomly acquired discarded tissues, no data were
kept for patients regarding race or ethnic background, and therefore, no relationship of the frequency of CCR5Δ32 mutation with race was studied. However, the overall distribution of CCR5 genotype in the thymus tissue bank was similar to the distribution of CCR5 genotypes as reported for a randomly selected population (7, 8).

Abnormal expression of CD45RA in thymocytes

To characterize thymocytes with the heterozygous and homozygous CCR5Δ32 mutations, serial frozen thymus tissue sections were reacted with mAbs against cell surface molecules, including CD3, CD4, CD8, CD45RA, CD45RO, CD45RB, and CD45RC. Normal distribution of CD3, CD4, and CD8 cell populations was found in thymuses with heterozygous and homozygous CCR5Δ32 mutations compared with that in thymuses without the CCR5Δ32 mutation (data not shown).

Thymocyte maturation was normal in CCR5Δ32+/− and CCR5Δ32+/− thymuses compared with that in CCR5 wild-type thymuses, in that phenotypic analysis of CCR5 genotyping. The single 326-bp band indicates the normal CCR5 genotype. The single 326-bp band indicates thymus tissue homozygous for the CCR5Δ32 mutation, and two bands with sizes of 326 and 294 bp indicate thymus tissue heterozygous for the CCR5Δ32 heterozygous mutation.

Characterization of CD45RA abnormal thymocytes

The CD45RA abnormal phenotype in thymus was investigated by flow cytometric analysis of purified thymocytes using CD45 mAbs. The mean fluorescence intensity of CD45RA abnormal thymocytes was ∼10-fold higher with CD45RA mAb staining (MFC 664) than that of thymocytes isolated from thymocytes with the normal CD45RA staining pattern (MFC 75; Fig. 3). In contrast, there were no differences in the level of expression of CD45RO, CD45RB, and CD45RC between thymocytes isolated from thymus tissues with the CD45RA normal vs the abnormal phenotype (Fig. 3).

CD45 is comprised of at least five different isoforms, ABC, AB, BC, B, and O, with molecular masses of 220, 200 (AB and BC), 190, and 180 kDa, respectively. On the protein level, the heterogeneity of different CD45 isoforms can be resolved by mAbs that react with restricted CD45 epitopes (19). In immunoprecipitation assays, protein bands immunoprecipitated by pan-CD45 Abs are 220, 200, 190, and 180 kDa, and those immunoprecipitated by CD45RA Abs are 220 and 200 kDa. Immunoprecipitation assays were conducted to characterize the CD45 isoforms using cell lysates of thymocytes from CD45RA normal and abnormal phenotype individuals. CD45 molecules from cell lysates of thymocytes were first immunoprecipitated using the pan-CD45 mAb F10-89-4 that is directed against a common epitope shared by all CD45 isoforms. We found that CD45 mAb F10-89-4 immunoprecipitated protein bands of 200 and 180 kDa in the CD45RA normal thymocytes, whereas the pan-CD45 mAb immunoprecipitated a large 220-kDa band in addition to 200- and 180-kDa bands in CD45RA abnormal thymocytes (Fig. 4). When normal thymocytes were analyzed with the CD45RA mAb F8-11-13, there were no major protein bands identified in normal control thymocytes (because only ∼7% of normal thymocytes were CD45RA+; Fig. 4), indicating that the 200-kDa band seen by the pan-CD45 mAb in normal thymocytes was either the 200-kDa CD45BC isofm or the 190-kDa CD45B isoform. However, in cell lysates of thymocytes of CD45RA abnormal individuals, in which ∼90% of thymocytes are CD45RA+, CD45RA mAb F8-11-13 immunoprecipitated two

Table II. Thymocyte development in CCR5Δ32+/−, CCR5Δ32+/− and CCR5 wild-type human thymocytes

<table>
<thead>
<tr>
<th>Thymus Genotype</th>
<th>CD4+ “CD8−” DN thymocytes</th>
<th>CD4+ “CD8−” DP thymocytes</th>
<th>CD4+ “CD8+” SP thymocytes</th>
<th>CD4− “CD8+” SP thymocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCR5 wild type</td>
<td>10.7 ± 1.3</td>
<td>42.6 ± 14.7</td>
<td>31.3 ± 13.5</td>
<td>15.6 ± 1.3</td>
</tr>
<tr>
<td>CCR5Δ32+/−</td>
<td>11.3 ± 3.1</td>
<td>46.9 ± 14.1</td>
<td>26.9 ± 13.8</td>
<td>15.1 ± 1.8</td>
</tr>
<tr>
<td>CCR5Δ32+/−</td>
<td>8.8 ± 3.3</td>
<td>31.7 ± 18.8</td>
<td>37.1 ± 10.9</td>
<td>22.4 ± 1.2</td>
</tr>
</tbody>
</table>

* There were no significant differences in percentage of positive cells in each subset of CD4+ “CD8−”, CD4+ “CD8−”, CD4+ “CD8−”, and CD4− “CD8+” thymocytes among groups of CCR5 wild-type thymocytes and the CCR5Δ32+/− (p > 0.05) thymocytes, or among the groups of CCR5 wild-type thymocytes and the combination of the CCR5Δ32+/− or the CCR5Δ32+/− (p > 0.05).
The cortex (c) and medulla (m) in A have the normal CD45RA mAb staining pattern characterized by negative staining of lymphocytes in the cortex and few positive cells in the medulla of thymus T-264. In contrast, there is positive CD45RA mAb staining in both cortex and medulla of thymus T-325 with the CD45RA abnormal phenotype in B. The staining patterns of CD45RO (C and D) and CD45RC (E and F) in these two thymuses were similar, with CD45RO mAb reactive with thymocytes in both cortex and medulla (B and D) and CD45RC mAb reactive with thymocytes in medulla (E and F). Data are representative of 12 CD45RA abnormal thymuses studied (all panels, ×400).

FIGURE 2. Immunofluorescence staining of human thymus with CD45RA and CD45RO mAbs. Shown are immunofluorescence photomicrographs of serial frozen sections of thymuses T-264 (CD45RA normal phenotype; A, C, and E) and T-325 (CD45RA abnormal phenotype; B, D, and F). Major isoforms of 220 and 200 kDa, demonstrating that the CD45RA reactivity in the CD45RA abnormal phenotype thymuses was due to the lack of normal down-regulation of 200- and 220-kDa CD45RA isoforms during thymocyte development.

To analyze the subsets of thymocytes that expressed the CD45RA abnormal phenotype, three-color flow cytometry was performed. As previously noted in Table II, CD45RA abnormal thymuses had normal percentages of CD4⁺/CD8⁻ DN, CD4⁺/CD8⁻ DP, CD4⁺/CD8⁻ SP, and CD4⁺/CD8⁺ SP populations (Fig. 5A). In CD45RA normal thymuses, about 7% of all thymocytes were CD45RA⁺; CD45RA⁺ thymocytes were primarily present in DN CD4⁺/CD8⁻ (28% of DN thymocytes were CD45RA⁺) and SP CD4⁺/CD8⁻ (31% CD45RA⁺) subsets. In the SP CD4⁺/CD8⁺ population, only 9% of thymocytes were CD45RA⁺ (Fig. 5B).

In contrast, in those thymuses found to express the CD45RA abnormal phenotype, all subsets of thymocytes expressed CD45RA, including DN CD4⁺/CD8⁻, DP CD4⁺/CD8⁺, SP CD4⁺/CD8⁺, and SP CD4⁺/CD8⁻ thymocyte populations (Fig. 5). Thus, ~90% of all CD45RA abnormal thymocytes were CD45RA⁺.

Striking differences in CD45RA expression were observed between the CD45RA normal and abnormal thymuses in all thymocyte populations. For example, while 90% of DP CD4⁺/CD8⁻ cells and 98% of CD4⁺/CD8⁺ cells were CD45RA positive in the CD45RA abnormal phenotype thymuses, only 0.2% of DP CD4⁺/CD8⁻ and 9% of CD4⁺/CD8⁺ thymocytes were CD45RA positive in CD45RA normal thymocytes (Fig. 5B).

Relationship of CD45RA abnormal phenotype in thymocytes with a previously described genetically determined lack of CD45RA-negative lymphocytes in PBMC

It has been previously reported that a genetically determined lack of CD45RA-negative PB T cells is present in 8% of healthy individuals (35). No loss of CD45RA expression in PB T cells of these individuals was observed after in vitro activation with PHA due to selective lack of down-regulation of the 200-kDa CD45RA isoform (35, 36). To address whether the CD45RA abnormal phenotype identified in thymus in our study was similar to the genetically determined lack of CD45RA-negative T cells found in peripheral blood lymphocytes (35, 36), CD45RA normal and abnormal thymocytes were activated in vitro with 1 μg/ml of PHA, and analyzed for surface expression of CD45RA and CD45RO.

As shown in Fig. 6A, most unactivated CD45RA normal thymocytes were CD45RAlow or negative with an MFC of 101, and CD45RO⁺ with an MFC of 224. In contrast, most thymocytes from the CD45RA abnormal thymus were CD45RA⁺ with an MFC of 409. Activation of thymocytes in vitro with PHA down-regulated CD45RA expression in thymocytes with the CD45RA normal phenotype (as reflected by a lower MFC of 50 in Fig. 6B). However, CD45RA expression in thymocytes with the CD45RA abnormal phenotype was not down-regulated after PHA activation, but, rather, was increased by almost 200% to an MFC of 832 (Fig. 6B). After PHA activation, expression of CD45RO was up-regulated in both CD45RA normal and CD45RA abnormal phenotype thymocytes, and no differences in CD45RO expression after in
vitro activation between CD45RA normal phenotype and CD45RA abnormal phenotype thymocytes was seen.

Because PB lymphocytes were not available from any subjects whose thymus tissue was studied, we could not directly confirm that the abnormality described in our study of lack of CD45RA down-regulation in thymocytes was the same as that previously described in PBMC. However, given the identical lack of down-regulation of CD45RA mAb reactivity with T cell activation, the two conditions probably reflect the same functional polymorphism.

Analysis of linkage of abnormal thymocyte expression of CD45RA with the CCR5Δ32 mutation

Four hundred and six of the 571 original thymus tissues were available to screen for the CD45RA abnormal phenotype. We found statistically significant higher numbers of the CD45RA abnormal phenotype thymocytes (T-592) compared with 15% in those not expressing it. Using two-tailed Fisher’s exact test to test for association between CD45RA abnormality and CCR5Δ32 mutation, a statistically significant association between CD45RA abnormality and CCR5Δ32 mutation was demonstrated (p = 0.00258). We next performed three pairwise comparisons of the CCR5Δ32 genotypes. Pairwise comparison of the homozygous and heterozygous CCR5Δ32 genotypes with the wild-type CCR5 genotype detected statistically significant association of both the homozygous and heterozygous CCR5Δ32 genotypes with the CD45RA abnormal phenotype (Table IV).

Susceptibility of human CCR5Δ32+/− and CD45RA abnormal thymocytes to HIV infection in vitro

Differences in HIV-1 infectivity have been previously suggested in normal PBMC CD45RA+ naive vs CD45RO+ memory CD4+ PB T cells (37), and the functional capacity of CD45RO+ CD4+ T cells is affected by HIV-1 more than that of CD45RA+ CD4+ T cells (37). However, to date, CCR5Δ32+/− thymocytes have not been studied regarding HIV-1 infectivity. Considering the importance of CCR5 in HIV-1 infection (38) taken together with the association between the CD45RA abnormal phenotype and CCR5Δ32 genotype in thymocytes described here, it was also of interest to determine whether the CD45RA abnormal phenotype could influence the infectability of thymocytes with different CCR5 genetic backgrounds. Individuals who are homozygous for the CCR5Δ32 allele are highly resistant to infection by R5 strains of HIV-1, and their PBMC cannot be infected with R5 strains in vitro (8, 39). Individuals who are heterozygous for the CCR5Δ32 allele, on the other hand, are susceptible to infection with R5 strains, but progress to AIDS at a slower rate relative to infected individuals who have wild-type CCR5 alleles (12, 13, 40). Likewise, PBMC from heterozygous CCR5Δ32 individuals are infectable by R5 strains, although not always to the same extent as PBMC with normal CCR5 alleles (8, 41). Lower levels of infection...
in heterozygous CCR5Δ32 PBMC might in some cases be due to decreased amounts of CCR5 on the cell surface (41, 42).

Sufficient cell numbers were not available to test thymocytes homozygous for the CCR5Δ32 mutation in HIV-1 infectivity assays. However, thymocytes with the CCR5Δ32 mutation were tested in HIV-1 infectivity assays in combination with either wild-type CCR5 alleles or alleles that were heterozygous for the CCR5Δ32 mutation. Cells in each group were tested for infectability with five primary R5 isolates, two primary R5X4 isolates, and one X4 T cell line-adapted strain of HIV-1. Infection was compared with the infectability of PBMC from a healthy, HIV-1-negative individual who had normal wild-type CCR5 alleles. Equal numbers of viable cells of each cell type were used so that direct comparisons of levels of infection could be made. Viral replication was measured by p24 production on days 4 and 8 of infection.

Thymocytes in each group were infectable by all strains of HIV-1 tested, although infection proceeded at a slow rate in all thymocyte samples compared with PBMC (Table 5). For example, HIV-1 p24 production in PBMC increased from 6.1 to 496 ng of p24/ml on day 4 of incubation, whereas infection in thymocytes was lower (0.2–2.0 ng of p24/ml) or undetectable (<0.1 ng of p24/ml) on day 4. The medium was replaced on day 4, and p24 again was again measured on day 8. Higher levels of p24 were seen in the thymocyte cultures at this time, in some cases approaching the levels detected in PBMC (Table V). Although the results varied among cell types and viruses, at least two general observations were made. First, R5 primary isolates were capable of producing a productive infection regardless of the CCR5 genotype and CD45RA phenotype. The level of infection was lower in thymocytes that were heterozygous for the CCR5Δ32 mutation compared with CCR5 wild-type thymuses (p < 0.01). Second, the level of HIV-1 infection of thymocytes was independent of the CD45RA expression phenotype. We saw no difference in the level of p24 production on day 4 or 8 in CD45RA normal vs CD45RA abnormal thymocytes (p > 0.5). R5 viruses replicated in thymus as well as, if not better than, X4 and R5X4 viruses in most cases. One possible exception was thymocytes from thymus T-690 that was CCR5Δ321/2 and CD45RA normal phenotype. Here, no infection with R5 strains was detectable on days 4 and 8 of incubation. However, infection with X4 and X4R5 strains in these cells was also low, suggesting that in this case the lack of detectable infection with R5 strains may be a quantitative issue related to slow replication rather than a qualitative aspect of the infectability of the thymocytes.

**Discussion**

In this study we have demonstrated the association of the CCR5Δ32 mutation with a functional polymorphism of CD45RA..
expression in thymocytes. Moreover, we have shown that thymocytes, like PB T cells, when heterozygous for the CCR5Δ32 mutation, produced lower HIV p24 in vitro after infection with HIV. However, we could determine no effect of the CD45RA polymorphism on in vitro HIV infectivity of thymocytes.

A number of mutations in HIV-1 coreceptor genes have been found that modify HIV-1 infectivity in vitro, modify the clinical course HIV-1 infection, or both. These include CCR2 (43), stem cell-derived factor-1 (44), and CXCR4 (45) mutations as well as the CCR5Δ32 mutation (7–16). The identification of a functional polymorphism of CD45RA expression in association with the CCR5Δ32 mutation is of potentially great interest. The presumption is that whatever evolutionary advantage conferred on humans by the CCR5Δ32 mutation may have been potentially impacted in an as yet unknown way by linkage to the CD45RA abnormal phenotype.

The family of human CD45 leukocyte common Ags is comprised of five glycoprotein members with Mr of 180 kDa (O), 190 kDa (B), 200 kDa (AB and BC), and 220 kDa (ABC) that are derived from alternative splicing of a single gene. The heterogeneity of CD45 isoforms can be identified by mAbs that specifically react with epitopes of the distinct isoforms. The expression of CD45RA and CD45RO isoforms has, in general, defined complementary subsets of T cells that differ in naive and memory functional properties (46, 47).

Because CD45RA+ PB CD4+ T cells when infected with HIV-1 produce lower levels of p24 (37), we postulated that abnormal expression of CD45RA normal thymocytes down-regulates the expression of CD45RA, PHA activation of CD45RA abnormal thymocytes resulted in further up-regulation of CD45RA. Data are representative of two separate experiments performed.

Table III. CD45RA expression abnormality in human thymuses of the CCR5 wild type, and in thymuses heterozygous and homozygous for the CCR5Δ32 mutation

<table>
<thead>
<tr>
<th>CCR5Δ32 Status</th>
<th>No. with abnormal CD45RA expression</th>
<th>No. with normal CD45RA expression</th>
<th>% with abnormal CD45RA expression</th>
</tr>
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<tr>
<td>Homozygote (−/−)</td>
<td>1</td>
<td>1</td>
<td>50</td>
</tr>
<tr>
<td>Heterozygote (+/−)</td>
<td>5</td>
<td>59</td>
<td>7.8</td>
</tr>
<tr>
<td>Wild type (+/+)</td>
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<td>12</td>
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as yet unknown way that may be unrelated to modulating host resistance to HIV-1 infection. Functionally, CCR5Δ32 PB T cells are normal in in vitro T cell proliferation assays (8). We have found CCR5Δ32−/− and CCR5Δ32+/− thymocytes undergo normal T cell maturation (Table II and Fig. 5A) and proliferate normally in vitro in response to TCR-mediated triggering (unpublished observations). Moreover, signaling in CCR5Δ32−/− thymocytes via chemokines other than CCR5 ligand is normal, while signaling with CCR5 ligands is, as expected, absent (H.-X. Liao and B. F. Haynes, unpublished observations). Thus, as yet, no physiologically relevant effect of the CCR5Δ32 mutation has been found on PB T cell or thymocyte function.

It has been shown that CD4+ T cells with the heterozygous CCR5Δ32 mutation are partially resistant to HIV infection with R5 HIV-1 strains (7–9). One explanation for the severe lymphopenia seen in thymuses from patients with end-stage AIDS is infection and destruction of thymocytes by HIV-1 (48); however, in situ hybridization for HIV-1 in these thymuses did not show a large thymus burden of HIV-1 (48). Previous studies have shown that human thymocytes are susceptible in vitro to HIV-1 infection (49–51). We have found that thymocyte cultures were less sensitive to HIV-1 infection in vitro compared with PBMC cultures. Thymocytes with the heterozygous CCR5Δ32 mutation infected with R5 strains produced lower levels of p24 than did CCR5 wild-type thymocytes infected with the same HIV-1 isolates. These results suggested that thymocytes with the heterozygous CCR5Δ32 mutation are partially resistant to R5 HIV-1. In contrast, thymocytes with the heterozygous CCR5Δ32 mutation and thymocytes with wild-type CCR5 had similar susceptibility to X4 and R5X4 strains. Although CXCR4 is expressed on fresh thymocytes and up-regulated in activated thymocytes, the levels of p24 produced in thymocyte cultures infected with the X4 strain, IIIB, and the R5X4 strain, 89.6, were lower than those in thymocyte cultures infected with R5 HIV-1 strains. Thus, as postulated by others there may be thymotropic strains of HIV-1 that are more destructive to thymuses than other less thymotropic HIV-1 strains (52).

Given the similarity of the defect of activation-induced CD45RA isofrom down-regulation in thymocytes described in this report to that of the previously described lack of CD45RA down-regulation in PB T cells (35), we suggest that the defects are identical, and that we have now observed the consequence of the previously described PB T cell CD45RA abnormality in thymocytes (35, 36). However, this question remains unresolved until PB and thymocyte specimens are available from the same person with the CD45RA abnormality for study.

It is of interest that in the CD45RA abnormal phenotype described here, there is lack of down-regulation of both the 220- and 200-kDa CD45RA isofroms during thymocyte development. In contrast, in the previously described polymorphism of persistent CD45RA expression on memory PB T cells (34), in the transition from naive to memory T cells only the 200-kDa CD45RA isofrom was not down-regulated. Therefore, if the two polymorphisms of CD45 isoform regulation are the same, they are expressed differently in thymocyte maturation (lack of down-regulation of both the 220- and 200-kDa CD45RA isoform) vs that in naive to memory

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**Table IV.** The association between abnormal CD45RA expression and the CCR5Δ32 mutation

<table>
<thead>
<tr>
<th>CCR5 Genotype Pairwise Comparison</th>
<th>Frequency of Abnormal CD45RA Expression</th>
<th>p Valueb</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homozygous vs wild type</td>
<td>CCR5Δ32: 1/2, CCR5 WT: 6/340</td>
<td>0.041</td>
</tr>
<tr>
<td>Heterozygous vs wild type</td>
<td>CCR5Δ32: 5/64, CCR5 WT: 6/340</td>
<td>0.018</td>
</tr>
<tr>
<td>Combined homozygous and heterozygous vs wild type</td>
<td>CCR5Δ32: 6/66, CCR5 WT: 6/340</td>
<td>0.006</td>
</tr>
</tbody>
</table>

a WT, wild type; +/+ for CCR5 gene expression; CCR5Δ32, cells carry either one (−/−) heterozygous or two (−/−, homozygous) CCR5 alleles with the CCR5Δ32 mutation.

b Statistical significance determined by the Fisher exact test.

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**Table V.** Assay on day 8 for p24 to determine HIV-1 infection in human thymuses and PBMC by various strains of HIV-1

<table>
<thead>
<tr>
<th>CCR5+/− , CD45RA NL</th>
<th>p24 Production by Virus (phenotype)b</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cells</td>
<td>IIIB (X4)</td>
</tr>
<tr>
<td>T-324</td>
<td>2.0 ± 1.0</td>
</tr>
<tr>
<td>T-310</td>
<td>2.7 ± 1.8</td>
</tr>
<tr>
<td>T-471</td>
<td>1.1 ± 0.3</td>
</tr>
<tr>
<td>CCR5+/− , CD45RA NL</td>
<td>T-684</td>
</tr>
<tr>
<td>T-698</td>
<td>2.1 ± 1.1</td>
</tr>
<tr>
<td>T-690</td>
<td>1.4 ± 0.7</td>
</tr>
<tr>
<td>CCR5+/− , CD45RA ABNL</td>
<td>T-205</td>
</tr>
<tr>
<td>T-592</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td>T-668</td>
<td>1.0 ± 0.4</td>
</tr>
<tr>
<td>CCR5+/− , CD45RA ABNL</td>
<td>T-223</td>
</tr>
<tr>
<td>T242</td>
<td>0.9 ± 0.1</td>
</tr>
<tr>
<td>PBMC</td>
<td>111.8 ± 29.4</td>
</tr>
</tbody>
</table>

a CCR5+/−, wild-type alleles; CCR5+/−, heterozygous for the CCR5Δ32 allele; CD45RA NL, normal CD45RA phenotype; CD45RA ABNL, CD45RA abnormal phenotype.

b Values are the average concentration of p24 (ng/ml) ± SD (n = 4 wells).
PB T cell conversion (lack of down-regulation of only the 200-kDa isofrom).

Thus, we have shown a new association of the CCR5Δ32 mutation with an as yet uncharacterized gene mutation responsible for the inability to appropriately down-regulate CD45RA isoforms during thymocyte development and activation. Because CD45 is encoded on chromosome 1 (53, 54), and CCR5 is encoded on chromosome 3 (12), this association must be mediated by an unidentified gene product that modifies CD45 processing and is located on chromosome 3 at a distance from CCR5. It will be of interest to determine in population studies whether the presence of the CD45RA abnormal polymorphism in association with the CCR5Δ32 mutation has any effect on the clinical outcome of HIV-1 infection.

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References


