Virus and host determinants of HIV-1 infection and AIDS pathogenesis

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Chapter 5

"CCR5 cell-surface expression in relation to CCR5 genotype and the clinical course of HIV-1 infection"

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Chapter 2

CCR5 cell-surface expression in relation to CCR5 polymorphisms and the clinical course of HIV-1 infection


Clinical and experimental studies of HIV-1 infection
CC chemokine receptor 5 cell-surface expression in relation to CC chemokine receptor 5 genotype and the clinical course of HIV-1 infection

CCR5 cell-surface expression was studied in relation to CCR5 genotype and clinical course of HIV-1 infection. HIV-1-infected CCR5 +/+ individuals had higher percentages of CCR5-expressing CD4+ T cells as compared with HIV-1-infected CCR5 Δ32/+ individuals. For both genotypic groups, the percentages of CCR5-expressing cells were higher than for the uninfected counterparts (CCR5 -/-, HIV+ 28% and HIV 15% [P < 0.0001]; CCR5 Δ32/+, HIV+ 21% and HIV 10% [P = 0.001], respectively). In HIV-1-infected individuals, high percentages of CCR5-expressing cells were associated with low CD4+ T cell numbers (P = 0.001), high viral RNA load in serum (P = 0.046), and low T cell function (P = 0.054). As compared with nonprogressors with similar CD4+ T cell numbers, individuals who did progress to AIDS had a higher percentage of CCR5-expressing CD4+ T cells (32% vs 21% [P = 0.002]). Longitudinal analysis of CCR5 +/- individuals revealed slight, although not statistically significant, increases in CCR5-expressing CD4+ T cells and CD4+ T cell subsets characterized by the expression of CD45 isoforms, during the course of HIV-1 infection. Pre-seroconversion, the percentage of CCR5-expressing CD4+ T cells was higher in individuals who subsequently developed AIDS (28%) than in those who did not show disease progression within a similar time frame (20%; P = 0.059). Our data indicate that CCR5 expression increases with progression of disease, possibly as a consequence of continuous immune activation associated with HIV-1 infection. In turn, CCR5 expression may influence the clinical course of infection.

Specific G-protein-coupled seven transmembrane spanning chemokine receptors have been found to function as coreceptors for human immunodeficiency virus type 1 (HIV-1)450. Non-syncytium-inducing (NSI) HIV-1, including macrophage-tropic variants that initiate HIV-1 infection1192,1193 use the CC chemokine receptor 5 (CCR5)451. The CXC chemokine receptor 4 (CXCR4) was described as entry cofactor for T-cell-line-adapted (TCLA) and primary syncytium-inducing (SI) HIV-11190,1197,1199,1202,1203. In vitro, some SI variants were able to use CCR2b and/ or CCR31197,1202 but the relevance of these coreceptors for in vivo infection remains unclear.

Both CXCR4 and CCR5 are expressed on peripheral blood lymphocytes (PBL)119,1205,1206, but to different extents on different T cell subsets. CXCR4 is predominantly found on resting, naive (HLA-DR- and CD26hi, CD45RA+) T cells whereas CCR5 is expressed on activated, memory (HLA-DR+ and CD26hi, CD45RO+) T cells119,120,1205.

Healthy individuals who are heterozygous for a 32-bp deletion in the CCR5 gene (CCR5 Δ32/) showed decreased numbers of CCR5-expressing PBL and decreased mean CCR5 expression levels on PBL as compared with individuals with a CCR5 wild-type genotype (CCR5 +/+). Nevertheless, considerable overlap between CCR5 expression levels of both groups of individuals was observed. The mean AIDS-free survival period of HIV-1-infected CCR5 Δ32/+ heterozygotes was shown to be prolonged as compared with the AIDS-free survival period of individuals with the CCR5 +/+ genotype292,293,294,295,394.111. This might be explained by lower CCR5 expression resulting in reduced spread of the virus. Although the mean AIDS-free survival period of HIV-1-infected CCR5 Δ32+/+ individuals is prolonged, rapid disease progression can be observed for some CCR5 Δ32/+ individuals. Recently, we have demonstrated that this is not due to the occurrence of HIV-1 variants able to use other coreceptors, because even CCR5 Δ32/+ heterozygotes can develop AIDS in the sole presence of CCR5-restricted NSI HIV-1 variants1404.

Considering the large variation in CCR5 expression among CCR5 Δ32/+ individuals, we analyzed whether CCR5 expression correlated with the clinical course of HIV-1 infection, both in CCR5 +/+ individuals and in CCR5 Δ32/+ individuals.
MATERIALS AND METHODS

Study Subjects. Cross-sectional analysis was performed on peripheral blood mononuclear cells (PBMC) derived from 30 healthy labworkers (mean age at time of analysis, 30.8 years) and 55 HIV-1 seropositive participants of the Amsterdam Cohort Studies on AIDS (mean age at time of analysis, 42.3 years). The latter included 31 participants who entered the study while still seronegative for HIV-1 antibodies. For these individuals, the seroconversion date was estimated to be the midpoint between the last seronegative and the first seropositive visit. The remaining 24 HIV-1-infected persons were seropositive at their first visit and the seroconversion date for these individuals was estimated to be 18 months before entry in the study. Follow-up visits of the HIV-1 positive men occurred every 3 months. At these visits, blood was collected for the determination of CD4 T cell numbers, T cell function and virus phenotype, and PBMC were cryopreserved. These frozen PBMC were used for analysis of CCR5, CXCR4, CD4, CD45RA, and CD45RO expression. Twenty-five of the 55 HIV-1 seropositive individuals developed AIDS during the study period after a mean follow-up of 98 (26-172) months after seroconversion (mean age at time of analysis, 42.9 years). Thirty HIV-1 seropositive individuals did not develop AIDS during a mean follow-up period of 136 (78-179) months after seroconversion (mean age at time of analysis, 41.9 years). In these cases, the end-point of the study was March 1998 or the start of anti-retroviral therapy with three or more drugs.

The longitudinal analysis included 24 HIV-1 seropositive individuals of the Amsterdam Cohorts Studies on AIDS of whom 17 participants were HIV-1 seronegative and seven were HIV-1 seropositive at their first visit. During the study period, 10 individuals developed AIDS (mean age at first moment of analysis, 39.2 years), who were analyzed on average at -25 (-33 to -11), +29 (+11 to +50), and +97 (+66 to +149) months relative to time of seroconversion. AIDS diagnosis was on average 94 (43 to 141) months after seroconversion. The 14 HIV-1-infected individuals who did not develop AIDS during the study period (mean age at first moment of analysis, 37.3 years) were analyzed on average at -38 (-69 to -11), +26 (+7 to +50), and +107 (+74 to +181) months relative to time of seroconversion. Follow-up period of individuals who did not develop AIDS during the study period was on average 121 (70 to 179) months after seroconversion.

Analysis of CD4 T cell counts, T cell function, virus phenotype and RNA load in serum. Routine analysis of CD4 T cell numbers was conducted by flow cytometry. PBMC were stained with CD4 monoclonal antibodies (mAbs) according to standard procedures for FACS analysis. T cell reactivity in response to stimulation with CD3 mAbs in vitro was determined in whole blood cultures. The proliferative response was measured after 4 days of culture by means of [3H]-thymidine incorporation. The SI HIV-1 phenotype was determined by cocultivation with MT2 cells. RNA levels were analyzed in cryopreserved serum samples derived from the same (or at most 3 months apart) visit as that from which the PBMC samples were obtained by use of a nucleic acid based amplification assay (HIV-1 RNA QT; Organon Teknika, Boxtel, The Netherlands).

CCR5 genotyping. Genomic DNA was isolated from cryopreserved PBMC (Qiagen blood kit; Chatsworth, CA). CCR5 genotyping was performed by PCR analysis using primers flanking the 32-bp deletion in CCR5.

mAbs, immunofluorescent staining, and FACS analysis. The CCR5 mAb 2D7 was kindly provided by Dr. C. Mackay. Mouse IgG2a was produced at the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service (CLB; Amsterdam, the Netherlands). PE-conjugated CXCR4 (12G5) was purchased from Pharmingen (La Jolla, CA), FITC-labeled CD45RO mAb (UCHL-1) from DAKO (Glostrup, Denmark) and PE-conjugated CD45RA mAb (2H4-RD1) was obtained from Coulter Immunology (Hialeah, FL). PERCP-conjugated CD4 and PE- and FITC-labeled goat anti-mouse IgG2a were purchased from Becton Dickinson (San Jose, CA).

Cryopreserved patient PBMC were thawed, washed once with PBS, and resuspended in PBS containing 0.5% BS A (staining buffer). Cells, 2 x 10⁶, were incubated with saturating amounts of directly labeled mAbs. When unconjugated mAbs were used, 5 x 10⁵ cells were incubated with goat anti-mouse Ig-FITC. Subsequently, cells were incubated with normal mouse serum (CLB; Amsterdam, the Netherlands) to block aspecific staining during incubation with additional mAbs. All incubation steps were performed at 4°C for 20 minutes. After each step, cells were washed twice with staining buffer and finally 10⁶ cells were analyzed on a FACScan (Becton Dickinson).

Statistical analysis. In the cross-sectional analysis, one randomly selected post-seroconversion sample per individual was used. The Student's t test was used to compare differences between 2 groups (i.e., HIV* vs HIV', CCR5 +/- vs CCR5 A32/+, and progressors vs non-progressors). The Pearson's correlation coefficient (r) was calculated to analyze correlations between CCR5 expression, progression markers, and CD45RO expression.

In the longitudinal study, differences between the three time points were analyzed either with an ANOVA (i.e., CCR5, CXCR4, CD45RA, and CD45RO expression on CD4 T cells) or a Kruskal-Wallis test (i.e., CCR5 and CXCR4 expression on CD4 T cell subsets). The Student's t test was used to compare differences between 2 groups (i.e., progressors vs non-progressors).

Normality of groups was tested by use of Normal plots and the Shapiro-Wilk W test for normality. SPSS for Windows (version 7.5.2) was used to perform all statistical analyses.
RESULTS

CCR5 genotype and CCR5 expression on CD4* T cells and CD4* T cell subsets. The relationship between CCR5 genotype and CCR5 expression on CD4* T cells was examined cross-sectionally in 30 HIV-1 seronegative individuals of whom eight were CCR5 Δ32/+ heterozygotes, and 55 HIV-1 seropositive individuals of whom 17 were CCR5 Δ32/+ heterozygotes. The remaining HIV-1 seronegative and seropositive individuals had the CCR5 +/- genotype. In the HIV-1-uninfected individuals, higher numbers of CCR5-expressing CD4* T cells were found in the individuals with the CCR5 +/- genotype compared with the CCR5 Δ32/+ individuals, 15% (7-23%) versus 10% (6-14%), respectively (P = 0.003; Fig. 1a). The same was found for HIV-1-infected individuals with 28% (11-59%) CCR5-expressing CD4* T cells for the individuals with the CCR5 +/- genotype as compared with 21% (7-38%) for the CCR5 Δ32/+ individuals (P = 0.02). Comparison of CCR5 expression between HIV-1-infected and uninfected individuals showed that within both the CCR5 +/+ (P < 0.0001) and the CCR5 Δ32/+ (P = 0.001) genotypic groups the HIV-1 seropositive individuals had higher percentages of CCR5-expressing CD4* T cells.

Because CCR5 is expressed mainly on CD45RO* CD4* T cells, differences in CCR5 expression on this memory T cell subset between CCR5 +/- and CCR5 Δ32/+ individuals were analyzed. Both in the HIV-1 seropositive and seronegative individuals statistically significant higher numbers of CCR5-expressing CD45RO* CD4* T cells were found in the CCR5 +/- individuals than in the CCR5 Δ32/+ heterozygotes (Fig. 1b). The mean percentages of CCR5* CD45RO* T cells in the HIV-1 seronegative persons were 30% and 19% for CCR5 +/- and CCR5 Δ32/+ individuals, respectively (P < 0.0001), whereas these respective percentages were 39% (CCR5 +/-) and 27% (CCR5 Δ32/+) for the HIV-1 seropositive persons (P = 0.022). Both for CCR5 +/- and CCR5 Δ32/+ individuals, higher percentages of CCR5-expressing CD45RO* T cells were observed for HIV-1 seropositive as compared with HIV-1 seronegative individuals (CCR5 +/-: P = 0.001; CCR5 Δ32/: P = 0.065). Considerable variation and overlap in percentages CCR5-expressing cells could be observed on both CD4* and CD45RO* T cells of HIV-1 seropositive CCR5 +/- and CCR5 Δ32/+ individuals.

CCR5 expression and disease stage. To determine whether CCR5 expression is correlated with HIV-1 disease stage, CCR5 expression was analyzed in relation to CD4* T cell counts, T cell function, and viral RNA load in serum. As shown in Figure 2a, the proportion of CCR5-expressing CD4* T cells and the total number of CD4* T cells were inversely correlated (r = -0.432, P = 0.001). This association could be observed both for individuals solely carrying NS1 variants and for individuals carrying both SI and NSI HIV-1 variants.

In agreement, it was found that low T cell function as measured by αCD3 reactivity was associated with a high percentage of CCR5-expressing CD4* T cells (r = -0.292; P = 0.054; not shown). Individuals with high viral RNA load had a relatively high percentage of CCR5-expressing cells.
CD4+ T cells as compared with individuals with a low viral RNA load ($r_s = 0.330, P = 0.046$; not shown).

In agreement with the fact that CCR5 is mainly expressed on CD45RO+ CD4+ T cells, correlation analysis showed a higher proportion of CCR5+ CD4+ T cells at higher percentages of memory cells (Fig. 2b; $r_s = 0.508, P = 0.003$). In addition, as was previously described by Roederer et al. [455], the percentage of CD45RO+ T cells increased with decreasing CD4+ T cell numbers (Fig. 2c; $r_s = -0.546, P = 0.001$).

**CCR5 expression in relation to disease progression.** Although the cross-sectional analysis showed a clear correlation between advanced disease stage and increased CCR5 expression, it remained unclear whether the increased CCR5 expression was a cause or a consequence of clinical progression. To determine the effect of increased CCR5+ CD4+ T cell counts on progression, we first compared CCR5 expression in individuals who did or did not develop AIDS within a similar study period (average: progressors, 98 months; non-progressors, 136 months). At the moment of analysis, both groups had similar CD4+ T cell counts (progressors 453 CD4+ T cells/μl; non-progressors 553 CD4+ T cells/μl; $p = 0.145$; not shown). The groups were also similar with respect to the genotypic distribution, with approximately one-third of the individuals having the CCR5 A32/+ genotype (progressors and non-progressors 32% and 29%, respectively; Fig. 3), thereby excluding a possible biasing effect of genotype on the CCR5 expression in both groups.

HIV-1 seropositive individuals who developed AIDS had, on average, 32% CCR5-expressing CD4+ T cells, whereas HIV-1 seropositive individuals who did not develop AIDS had 21% CCR5-expressing CD4+ T cells ($P = 0.002$; Fig. 3). The higher percentage of CCR5-expressing cells in the CD4+ T cell population was mirrored by a higher percentage of CCR5+ cells in the CD45RO+ CD4+ T cell subset in the individuals who developed AIDS (44%) as compared with the non-progressors (32%; $P = 0.009$; not shown). The same was observed for the CD45RA+ T cell subset (progressors, 24%; non-progressors, 10%; $P = 0.012$; not shown) and the

![Fig. 2. CD4+ T cell numbers and CCR5 and CD45RO cell-surface expression. Correlation between percentage of CCR5-expressing CD4+ T cells and CD4+ T cell numbers (a), percentage of CCR5-expressing CD4+ T cells and percentage of CD45RO+ CD4+ T cells (b), and percentage of CD45RO+ CD4+ T cells and CD4+ T cell numbers (c) of individuals carrying NSI variants only (•) or carriers of both NSI and SI variants (o).](image-url)
CD45 double-dull T cell subset (progressors, 41%; nonprogressors, 20%; \(P = 0.001\); not shown). Because analysis was performed at similar CD4* T cell counts, these data suggest that higher CCR5 expression is not solely a consequence of the stage of disease but that CCR5 expression may influence disease progression.

To further unravel whether increased CCR5 expression was caused by and/or induced disease progression, longitudinal analysis of 24 HIV-1 seropositives with the CCR5 +/- genotype was performed. In this study group, 14 individuals progressed to AIDS and 10 individuals did not develop AIDS during the study period. At three different time points, percentages of CCR5-expressing T cells were determined: before seroconversion (average: progressors, -25 and non-progressors, -38 months), relatively early (average: 29 and 26 months, respectively) and relatively late (average: 94 and 107 months, respectively) in HIV-1 infection. Relatively late in infection, the two groups showed a clear difference in CD4* T cell numbers (Fig. 4a). Proportional changes in CD4* T cell subsets over time were similar in both groups (Fig. 4a, insert). In the progressors, the percentages of CCR5-expressing CD4* T cells increased slightly over time from 28% (21-40%) to 31% (19-59%) to 35% (19-56%) (Fig. 4b). The 14 individuals who did not show progression to AIDS within the study period also showed a slight increase in percentage of CCR5-expressing T cells from 20% (13-28%) to 25% (11-38%) to 28% (11-51%) (Fig. 4b).
points was not significant. As shown in Figure 4b (insert), analysis of CD4+ T cell subsets showed that the percentage of CCR5-expressing CD45RO+, CD45RA+, and CD45 double-dull cells within the CD4+ T cell population all increased slightly. The increases in CCR5-expressing cells of different CD4+ T cell subsets in both groups of individuals are summarized in Table 1.

The percentage of CCR5+ CD4+ T cells analyzed at the pre-seroconversion time point was higher in those individuals who did develop AIDS (28%) than in those who did not show progression to AIDS within the study period (20%; P = 0.059; Fig. 4b). Similarly, early and late in infection progressors had more CCR5-expressing CD4+ T cells (31% and 35%, respectively) as compared with those individuals who did not show progression to AIDS during the study period (early, 25%, P = 0.266; late, 28%, P = 0.296; Fig. 4b). The different pre-seroconversion set-points suggest that numbers of CCR5-expressing cells may influence disease progression.

**CXCR4 expression in relation to disease progression.** Increased percentages of CD45RO+ T cells and CCR5+ T cells over time possibly reflect increased immune activation. Since the coreceptor of SI variants, CXCR4, is mainly expressed on naive resting cells, we analyzed whether the expression of CXCR4 decreases during HIV-1 infection. Indeed, both non-progressors as well as progressors showed significantly decreasing percentages of CXCR4-expressing CD4+ T cells over time as shown in Figure 4c (nonprogressors, pre-seroconversion 82%, to early 66%, to 54% late in infection, P = 0.001; progressors, pre-seroconversion 76%, to early 65%, to 52% late in infection, P = 0.029). In analogy with increased CCR5 expression on each CD4+ T cell subset, CXCR4 expression decreased on each subset (Fig 4c, insert). The decreases in CXCR4-expressing cells of different CD4+ T cell subsets in both groups of individuals are summarized in Table 1. Unlike CCR5 expression, no differences in pre-seroconversion CXCR4 expression could be observed between individuals who did or did not show disease progression.

**DISCUSSION**

Several factors may be responsible for the variable clinical course of HIV-1 infection among different individuals. With the identification of CCR5 as the principle coreceptor for primary NSI HIV-1, differential functioning of this coreceptor has been hypothesized to be such factor. This was substantiated by the observation that individuals heterozygous for a 32-bp deletion in CCR5 showed a delayed progression to AIDS. However, also among individuals with a wild-type CCR5 genotype, long-term nonprogressors could be identified, whereas some CCR5 heterozygotes showed rapid disease progression. Here, we observed that on CD4+ T cells, and also on the memory CD4+ T cell subset, the percentage of CCR5-expressing cells was indeed lower in CCR5 Δ32/+ heterozygotes as compared with CCR5+/+ individuals. The large range in the percentage of CCR5-expressing cells supported the idea that a differential expression of CCR5 could contribute to the variable clinical course of HIV-1 infection.
At a time point in the clinical course of infection when CD4 T cell numbers were similar in the two groups, progressors indeed had higher percentages of CCR5-expressing cells as compared with individuals who remained asymptomatic. Moreover, in longitudinal analyses, the percentage of CCR5-expressing cells in PBMC samples obtained before seroconversion was higher in individuals who developed AIDS within the study period as compared with individuals who remained asymptomatic during follow-up. It can be hypothesized that higher levels of CCR5 expression allow higher levels of HIV-1 replication, resulting in higher viral RNA load in plasma and consequently accelerated disease progression as compared with individuals with low CCR5 expression. A higher proportion of CCR5-expressing cells just before seroconversion may also be associated with a more severe primary infection. Of interest in this respect is our observation that CCR5 +/+ individuals as compared with CCR5 Δ32/+ heterozygotes continuously had a lower absolute CD4 T cell number of ≈100 cells/mm³ directly after the recovery from their primary infection onwards, when CD4 T cell numbers had returned to subnormal levels[^96]. This impaired set-point could indeed explain how CCR5 expression might influence the clinical course of infection.

We also obtained evidence for an increased percentage of CCR5-expressing cells as a consequence of disease progression. In a cross-sectional analysis, we could demonstrate a correlation between high CCR5 expression and low CD4 T cell numbers, low T cell function, and high viral RNA load in serum. In agreement, we observed an increase in the percentages of CCR5-expressing CD4 T cells with progression of disease. This could be explained by increased percentages of CCR5-expressing CD45RO*, CD45RA*, and CD45 double-dull CD4 T cells. In addition, an increase in the number of CD45RO-expressing cells was observed, which could also account for the increase in the proportion of CCR5-expressing CD4 T cells.

Numbers of CXCR4-expressing CD4 T cells decreased over time as did CXCR4 expression on CD4 T cell subsets. The decrease in percentage of CD45RA T cells reciprocated the increase in CD45RO T cells, while the decrease in CXCR4 cells in the CD4 T cell population was much greater than the increase in CCR5 cells (Table 1). This might be explained by the relatively high decrease in CXCR4-expressing CD45RO* cells, compared with an only slight increase in CCR5-expressing CD45RO T cells. Secondly, because about 80% of the CD45RA T cells express CXCR4 and only 30% of the CD45RO T cell express CCR5, the equal loss of CD45RA T cells and gain of CD45RO T cells would result in a higher loss of CXCR4 than gain of CCR5. The increase in CCR5 and CD45RO expression may reflect immune activation due to HIV-1 infection. In agreement, Ostrowski et al. showed that CCR5 expression is associated with HLA-DR expression and that expression of both surface markers increases with ongoing HIV-1 infection[^22].

We observed an increasing percentage of CCR5 and a decreasing percentage of CXCR4-expressing CD4 T cells with progressive disease, both in individuals solely carrying NSI variants and in individuals carrying both SI and NSI HIV-1 variants. This indicates that despite the increasingly favorable conditions for NSI variants, SI variants can emerge and compete with the NSI variants. It may be that sufficient SI-specific target cells are left throughout infection since the percentage of CXCR4-expressing cells, although decreasing, always remained above that of CCR5-expressing cells. Additionally, elevated percentages of CCR5-expressing CD4 T cells may actually accelerate the appearance of specific SI mutations through increased NSI HIV-1 replication and therefore enhanced mutation frequency. In agreement, we previously demonstrated that SI conversion tended to be more rapid, but not more frequent, in CCR5 +/+ individuals who generally have higher CCR5 expression levels than CCR5 Δ32/+ individuals[^96]. Thus, higher CCR5 expression does not appear to prevent and may even accelerate the emergence of SI variants.

The genotype-related differences in CCR5 expression were independent of HIV-1 serostatus. However, the range in percentage of CCR5-expressing cells was much larger in HIV-1-infected individuals as compared with the uninfected subjects, confirming previous studies[^22,405]. The large variability in CCR5-expressing cells is in good agreement with the variability in CD4 T cell numbers in this group of
HIV-infected individuals in combination with the here described inverse correlation between percentages of CD4-expressing and CCR5-expressing cells. Alternatively or in addition, highly variable percentages of CCR5-expressing cells may reflect differences in levels of immune activation associated with HIV-1 infection.

Interestingly, the percentage of CCR5-expressing cells in individuals that ultimately became infected with HIV-1 was higher than the percentage of CCR5-expressing cells in HIV-1 negative control subjects. This higher CCR5 expression may be explained by the notion that individuals with high-risk sexual behavior more frequently encounter other pathogenic microorganisms that may cause immune activation and consequently enhance CCR5 expression. The higher CCR5 expression may determine the host susceptibility for HIV-1 infection. Whether individuals with a relative resistance to HIV-1 infection indeed have lower percentages of CCR5-expressing cells remains to be established.

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