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Advances in Brief

Reduced Risk of AIDS Lymphoma in Individuals Heterozygous for the *CCR5-Δ32* Mutation[1]

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Abstract

Non-Hodgkin's lymphoma (NHL) has been increasing in frequency in the industrialized world, but the environmental and genetic factors that contribute to susceptibility are not known. B-cell lymphomas represent a major cause of morbidity and mortality in HIV-infected individuals. The identification of a deletion in the *CCR5* chemokine receptor gene that alters the risk for infection and progression to AIDS led us to examine a potential role of this gene in AIDS lymphoma. A matched case-control analysis was performed using all eligible NHL cases in the Multicenter AIDS Cohort Study. Patients were matched for age, study center, time AIDS-free, and slope of the CD4+ T-cell decline. The *CCR5-Δ32* allele was found to be associated with a 3-fold lower risk of NHL among individuals after controlling for time of infection and progression toward AIDS. The *CCR5* gene was not associated with a difference in risk for Kaposi's sarcoma, another common malignancy in AIDS patients, or opportunistic infections. Costimulation of normal phorbol 12-myristate 13-acetate-treated B cells with the CCR5 ligand RANTES induced a proliferative response, indicating that RANTES is a mitogen for B cells. Taken together, these findings suggest that the *CCR5* gene plays a role in the risk of NHL in HIV-infected patients, perhaps through a mechanism involving a decreased response of B cells to the mitogenic activity of RANTES.

Introduction

The chemokine receptor 5 gene (*CCR5*) encodes a cell surface receptor for the chemokines MIP1α, MIP1β, MCP2, and RANTES on lymphocytes and macrophages, and it also serves as a coreceptor for HIV-1 (1, 2). A 32-bp deletion in this gene, termed *CCR5-Δ32*, is common in Caucasians and prevents the expression of the receptor on cell surfaces (3–5). The presence of this allele confers a high level of protection against HIV-1 infection in homozygous individuals and a delayed progression to AIDS in heterozygotes (3–9).

B-cell NHLs[3] are highly elevated in patients infected with HIV and account for a significant fraction of HIV-related morbidity and mortality (10). These lymphomas are particularly resistant to treatment, and the average survival of patients is less than half a year after NHL diagnosis (11). NHL is also elevated in non-HIV-infected patients with immune suppression, suggesting that immune surveillance plays an important role in lymphomagenesis (12).

Materials and Methods

Details of the study design and the methodology of follow-up have been published previously (13). Clinical conditions diagnostic of AIDS (14) were ascertained and confirmed by medical records on a continuous basis. Individuals with fewer than 200 CD4+ T cells/ml who did not have another AIDS-defining illness were not considered AIDS positive for this analysis. HIV-1 seropositivity was determined by positive ELISA and confirmed by Western blot. T lymphocyte subset determinations were performed using standard flow cytometric methodology (15, 16). Viral load measurements were performed retrospectively on the repository specimens using a bDNA assay (Chiron Corp., Emeryville, CA) and then a reverse transcription-PCR assay (Roche Molecular Biochemicals). All viral load measurements were standardized to the reverse transcription-PCR assay (17).

Selection of Cases and Controls. Cases and controls consisted of Caucasian MACS participants with known HIV-1 serostatus. Of the 169 total NHL cases that have occurred in the MACS as of January 1997, 140 met these study criteria; exclusions included those who were non-Caucasian ($n = 25$), had insufficient repository samples for analysis ($n = 2$), and insufficient information to determine the time of seroconversion because they became infected after study withdrawal ($n = 2$). Controls were matched to cases by indicators of HIV-1 progression and study center at enrollment. Matching criteria depended on whether NHL was the initial AIDS diagnosis for the case and on the time from seroconversion to AIDS. Participants who developed lymphoma subsequent to another AIDS diagnosis were matched to controls by initial AIDS outcome and year of initial AIDS diagnosis (within 1 year). In addition, the control had to be lymphoma free for at least the same time as the case. Controls for HIV-1 seroconverters who presented with NHL as their initial AIDS diagnosis were required to be HIV-1 seroconverters and free of NHL after seroconversion for at least as long as the cases. HIV-1 seroprevalent participants who presented with NHL as their initial AIDS diagnosis were matched with controls by time from study entry to AIDS (at least as long as the case) and CD4+ cell count (within 50 cells/μl) at the third study visit. This latter requirement was to account for the time from infection to study entry because CD4+ cell counts in treatment-naive participants were shown to be a good marker for time since seroconversion in this cohort (18). One control was randomly selected from the matched risk set for each case whenever possible. This matching process resulted in 95 pairs.

To assess the specificity of the *CCR5* relationship with NHL, similar case-control analyses were performed using different AIDS outcomes: (*a*) KS; (*b*) CMV; (*c*) MAC; and (*d*) PCP. In brief, the same criteria used for NHL were used to select cases and controls for the other comparisons with two additions. For MAC, cases and controls were restricted to those who developed AIDS before January 1993, the time when MAC prophylaxis became widely available. For PCP, cases and controls were restricted to those who developed AIDS

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[3] The abbreviations used are: NHL, non-Hodgkin's lymphoma; MACS, Multicenter AIDS Cohort Study; PMA, phorbol 12-myristate 13-acetate; KS, Kaposi's sarcoma; PCP, *Pneumocystis carinii* pneumonia; MAC, *Mycobacterium avium* complex; OR, odds ratio; CNS, central nervous system; CI, confidence interval; CMV, cytomegalovirus.

before January 1990, the time when PCP prophylaxis became widely used in the cohort.

Statistical Analyses. Data analysis was performed using SAS statistical software (SAS Institute, Cary, NC). Matched ORs (an estimate of relative risk) and their 95% CIs were obtained from conditional logistic regression models using the discrete logistic model of the PHREG procedure in SAS. The effects of covariates such as CD4 slope and *CCR2* and *CCR5* promoter polymorphisms were also investigated in multivariate conditional logistic models. CD4 slope was calculated as the change in the number of cells/6 months using all available CD4 measurements since the third visit for seroprevalent men and since the third positive visit for seroconverters. All reported *P*s are two-sided.

B-Cell Isolation and Stimulation. B cells were purified from the peripheral blood mononuclear cells of normal leukaphoresis donors under NIH guidelines. B cells were isolated using the Milteny Biotec (Auburn, CA) B-cell isolation kit. This negative selection procedure was used to limit prior stimulation of the isolated cells. The purified cells were 95% CD19 positive by fluorescence-activated cell sorting. The cells were cultured in RPMI 1640 containing penicillin-streptomycin, 10% fetal bovine serum, and L-glutamine at 1×10^5 cells/well in a 96-well plate. The concentration of human RANTES (Peprotech) and PMA (Sigma) was varied, with each condition being performed in triplicate. After 72 h of culture in a humidified CO_2 incubator, [^{3}H]thymidine was added, and the culture was continued for 4 additional h. The cells were harvested, and the incorporated radiolabel was measured by scintillation counting.

Table 1 *Demographic and clinical characteristics of the cases and controls*

Characteristic	Cases (n = 95)	Controls (n = 95)
Average age (yrs) at study entry	34.1	31.3
Center at enrollment		
Baltimore	24	23
Chicago	22	22
Pittsburgh	6	6
Los Angeles	43	44
Median time (yrs) free from AIDS	6.9	7.5
Median CD4 slope (cells/μl/semester)	−39.9	−31.7
Median date of first AIDS diagnosis	90.2	90.4
Presenting AIDS diagnosis		
NHL	38	0
KS	14	19
PCP	26	33
CMV disease	4	5
Esophageal candidiasis	5	5
Other opportunistic infection	1	6
HIV dementia	0	3
Wasting syndrome	1	4
Multiple diagnoses[a]	4	3

[a] The conditions occurring concomitantly among the cases were KS and esophageal candidiasis, histoplamosis and PCP, wasting syndrome and PCP, and CMV disease and PCP. Two controls presented with PCP and KS, and one control presented with candidiasis and PCP.

Results

To ascertain whether the *CCR5*-Δ*32* allele differentially affects specific AIDS outcomes, we determined the frequency of the *CCR5*-+/Δ32 genotype in Caucasian AIDS patients from our original study (5) according to AIDS-defining illness. The overall frequency of *CCR5*-+/Δ32 heterozygotes was 17% in 647 Caucasian AIDS patients, and this frequency was nearly identical in subsets diagnosed with KS, pneumocystis pneumonia, or other opportunistic infections. However, only 8.5% of 52 lymphoma patients were heterozygous for *CCR5*-Δ32 (Fig. 1).

A case-control study nested within the MACS cohort was designed to estimate the effect of the *CCR5*-+/Δ32 genotype on the risk of NHL. MACS is a prospective study of the natural history of HIV-1 infection among homosexual and bisexual men in the United States (13, 19). From April 1984 to March 1985, 4954 men were enrolled in four metropolitan areas (Baltimore, Chicago, Pittsburgh, and Los Angeles). An additional 668 men were later recruited to increase minority enrollment and the number of seroconverters in the study. Of the 5622 participants, 2195 were HIV-1 seropositive at entry into the study (seroprevalent), and 556 seroconverted to HIV-1 (seroconverters) during study follow-up. Men younger than 18 years and those with a prior AIDS diagnosis were ineligible. The men were followed by semiannual visits with interviews, physical examinations, and collection of specimens for concomitant laboratory testing and storage in local and national repositories. More frequent contact with AIDS patients and passive follow-up methods were used to capture clinical events comprehensively. A review of medical records was used to confirm reports.

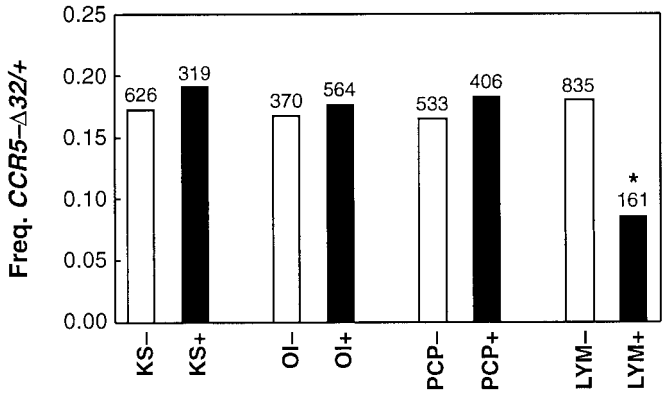

Fig. 1. Frequency of the *CCR5*Δ*32*/+ genotype among different AIDS outcomes. The frequency of the *CCR5*Δ*32*/+ genotype is shown for Caucasian AIDS patients in the MACS, Multicenter Hemophilia Cohort Study, Hemophilia Growth and Development Study, San Francisco City Cohort, and AIDS Link to Intravenous Experience cohorts who have (+) or do not have (−) specific AIDS outcomes. Descriptions of these cohorts have been published previously (5). *OI*, opportunistic infection; *LYM*, lymphoma. *Numbers* on *top* of *bars* represent sample numbers, and the *asterisk* refers to a statistically significant value (χ^2 = 7.3; P = 0.007).

Characteristics of the NHL cases and matched controls are given in Table 1. The two groups were similar with respect to age and marker for disease progression. In addition, the number of CD4+ T cells (460 and 446 for cases and controls, respectively) at visit 3 were similar for case-control pairs that matched this criteria. It has previously been shown that neither sexual activity nor history of infection, other than EBV, is associated with lymphoma in this cohort (13). Because *CCR5*-Δ32 is virtually absent in non-Caucasians, only Caucasians were included in the analysis. Of 140 eligible NHL cases, 95 were eligible to be matched by indicators of HIV-1 progression and study center. These included 2 seronegative subjects, 85 seroprevalent subjects (HIV-1 seropositive at entry), and 8 HIV-1 seroconverter subjects.

The frequencies of *CCR5* heterozygosity (+/Δ32) were 9.5% and 23.2% in the MACS matched cases and controls, respectively. Of the 25 case-control pairs discordant for *CCR5* genotype, 19 consisted of discordant *CCR5*-+/+ NHL cases and *CCR5*-+/Δ32 controls. Conditional logistic regression analysis showed an OR of 0.32 (95% CI, 0.13–0.79; P = 0.01), indicating that *CCR5* heterozygosity is associated with approximately a 3-fold reduced risk of lymphoma in this cohort.

The lymphoma cases were divided into two groups based on lymphoma type (disseminated or brain/CNS) to test whether the effect of *CCR5* was type specific. The OR indicated that the *CCR5*-+/Δ*32* genotype was protective for both the 61 disseminated lymphoma cases (OR = 0.27; 95% CI, 0.08–0.98; P = 0.05) and the 32 brain/CNS cases (OR = 0.38; 95% CI, 0.10–1.41; P = 0.15), although statistical significance was reached only in the former. There were two NHL cases for whom anatomical site at the time of diagnosis was unavailable. These data reveal no significant difference in the effect by

lymphoma subtype, although the brain/CNS lymphoma type should be re-evaluated with a larger sample size.

CD4 slope and visit 3 HIV-1 RNA level ($\log_{10}$ transformed) were added as covariates in the conditional logistic regression analysis to further test and control for any residual confounding by HIV-1 disease progression. CD4 slope was not found to be a significant variable (OR = 1.00; $P = 0.42$), but RNA level was significantly associated with NHL (OR = 3.01; $P = 0.02$). The adjusted OR for the *CCR5*+/Δ*32* genotype was similar to that derived from the unadjusted analysis (OR = 0.30; 95% CI, 0.10–0.93; $P = 0.04$). The frequency of *CCR5* heterozygosity in the unmatched cases was similar to that of the matched cases (11.1% *versus* 9.5%, respectively). The lack of significant difference between these groups indicates that the matched cases are representative of all of the NHL cases in the MACS; therefore, the results are generalizable to this population.

The significance of the *CCR5*-Δ*32* effect on AIDS lymphoma is underscored by its specificity. Using similar case-control approaches, this allele had no apparent effect on the development of other common AIDS outcomes including KS (OR = 0.97; 95% CI, 0.60–1.57), MAC infection (OR = 1.22; 95% CI, 0.51–2.95), CMV infection (OR = 1.14; 95% CI, 0.64–2.01), and PCP (OR = 0.90; 95% CI, 0.37–2.22).

An allele of the chemokine receptor 2 gene characterized by a single missense mutation, *CCR2–64I*, is associated with delayed progression to AIDS in HIV-positive individuals at a level similar to that of *CCR5*-Δ*32* (20, 21). Additionally, a specific genotype of the *CCR5* promoter region is associated with accelerated progression to AIDS (22). These variants were typed in the lymphoma cases and controls, and after controlling for *CCR5*-Δ*32* genotype, the ORs of 0.93 (95% CI, 0.43–2.01) for *CCR2–64I* and 1.75 (95% CI, 0.89–3.42) for the promoter genotype were determined, indicating no effect of these loci on the development of lymphoma. The effect of the *CCR5*-+/Δ*32* genotype on NHL remained significant after the inclusion of these other genotypes.

NHL is a B-cell lymphoma, but HIV predominantly targets T cells and cells of the monocyte/macrophage lineage. Because CCR5 is expressed on B cells, we investigated the response to the CCR5 ligands as a first step in understanding how CCR5 might regulate NHL induction. Whereas RANTES alone had little effect on B-cell proliferation, the inclusion of 10^{-9} M PMA and 100 ng/ml RANTES led to a 5-fold increase in B-cell proliferation compared to the inclusion of PMA alone (Fig. 2). The inclusion of 1000 ng/ml RANTES, which is known to induce T-cell proliferation, did not have a costimulatory effect on the PMA response of B cells (23). This synergistic response to RANTES and PMA in B cells is distinctive from the direct T-cell mitogenic effect of RANTES. No costimulatory effect was observed with MIP1α or MIP1β. MCP-2 and lower concentrations of RANTES were not tested. SDF-1 also did not stimulate the proliferation of these cells, and pokeweed, a B-cell mitogen, provided a modest stimulation, consistent with the fact that these are mature B cells. We conclude that stimulation through the CCR5 receptor can play a role in the growth control of B lymphocytes.

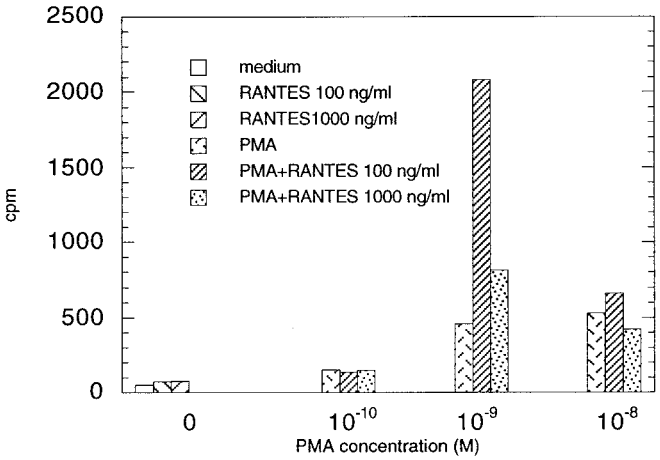

Fig. 2. B-cell proliferation induced by PMA and RANTES. Purified human peripheral B cells were stimulated with RANTES and/or PMA. The induced increase in proliferation was measured by [^{3}H]thymidine incorporation. Each point was repeated in triplicate with less than a 5% SE/data point. This is a representative graph with some variation observed between donors (data not shown).

Discussion

Results presented in this study indicate that individuals having a single copy of the *CCR5*-Δ*32* allele are protected against AIDS-related lymphoma. The *CCR5*-Δ*32* allele encodes a truncated product that is not expressed on the cell surface (3) but remains in the endoplasmic reticulum. CCR5-Δ32 molecules are capable of forming heterocomplexes with normal CCR5 in heterozygous individuals (*CCR5*-Δ*32*/+), retaining normal CCR5 molecules in the endoplasmic reticulum and reducing cell surface expression of CCR5 (24). Thus, the mechanism of *CCR5*-Δ*32* protection against lymphoma is likely to involve reduced cell surface expression of CCR5.

It is well documented that progression toward AIDS in *CCR5*-Δ*32* heterozygous individuals is delayed, and it follows that development of HIV-related immune suppression may also be retarded in these individuals. Immune suppression is associated with lymphoma in HIV-negative individuals, suggesting that defective immune surveillance plays a role in the development of NHL (12, 25). We controlled for severity of AIDS in the case and control selection criteria, but the extent and persistence of immune surveillance are difficult to quantify and hence to control. Thus, it will be important to repeat our analysis in a larger sample of lymphoma subjects who are HIV negative and immune competent to determine whether *CCR5*-Δ*32* is also protective in the absence of HIV infection and immune suppression.

CCR5 is expressed on B cells and plays a role in recruiting leukocytes to regions of inflammation and tissue damage (26, 27). Muller *et al.* (28) reported that RANTES serum levels are increased in HIV-1-infected individuals. In this study, we observed that RANTES augments the proliferation of B cells. A reduced response to mitogenic stimulation by RANTES could provide a basis for the lower frequency of lymphoma cases in individuals heterozygous for the *CCR5*-Δ*32* allele. Additional studies to determine the level of response to RANTES by cells from individuals heterozygous for *CCR5*-Δ*32*/+ may address this possibility.

Cancers are complex diseases involving both environmental agents and host genetic factors. Tumor incidence in AIDS NHL is associated with clonal integration of the EBV genome (10). Among HIV-1-infected individuals, NHL is considerably more common in males and is also more frequent in Caucasians than in other racial groups, underscoring the importance of genetic and environmental factors in this disease (25). If the relationship between CCR5 and NHL is confirmed, then antiretroviral therapies directed toward CCR5 that are currently being developed (29) could have applications toward NHL as well. In a similar vein, genetic variants affecting the expression of CCR5 chemokine ligands should also be examined in the context of lymphomagenesis.

Note Added in Proof

Rabkin *et al*. recently published (Blood, *93:* 1838–1842, 1999) that the *CCR5*-Δ*32* allele is protective for AIDS lymphoma, supporting the findings presented here.

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