CCR5 deficiency increases risk of symptomatic West Nile virus infection

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West Nile virus (WNV) is a reemerging pathogen that causes fatal encephalitis in several species, including mouse and human. Recently, we showed that the chemokine receptor CCR5 is critical for survival of mice infected with WNV, acting at the level of leukocyte trafficking to the brain. To test whether this receptor is also protective in man, we determined the frequency of *CCR5Δ32*, a defective *CCR5* allele found predominantly in Caucasians, in two independent cohorts of patients, one from Arizona and the other from Colorado, who had laboratory-confirmed, symptomatic WNV infection. The distribution of *CCR5Δ32* in a control population of healthy United States Caucasian random blood donors was in Hardy-Weinberg equilibrium and *CCR5Δ32* homozygotes represented 1.0% of the total group ($n = 1,318$). In contrast, *CCR5Δ32* homozygotes represented 4.2% of Caucasians in the Arizona cohort (odds ratios [OR] = 4.4 [95% confidence interval [CI], 1.6–11.8], P = 0.0013) and 8.3% of Caucasians in the Colorado cohort (OR = 9.1 [95% CI, 3.4–24.8], P < 0.0001). *CCR5Δ32* homozygosity was significantly associated with fatal outcome in the Arizona cohort (OR = 13.2 [95% CI, 1.9–89.9], P = 0.03). We conclude that CCR5 mediates resistance to symptomatic WNV infection. Because CCR5 is also the major HIV coreceptor, these findings have important implications for the safety of CCR5-blocking agents under development for HIV/AIDS.

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West Nile virus (WNV), a member of the flavivirus family, is a reemerging pathogen maintained primarily between mosquitoes and birds, with humans representing one of several incidental hosts. First isolated in Uganda in 1937, WNV outbreaks have subsequently been reported in the Middle East, Africa, Western Asia, Australia, and, most recently, in the US, where it has rapidly spread westward and into Latin America and the Caribbean since arriving in New York City in 1999 (1–4).

Clinical manifestations of WNV infection can be quite variable. Epidemiologic studies indicate that although ∼80% of infections remain subclinical, ∼20% progress to a febrile illness (3). Of these, >30% of cases in the US progress to neuroinvasive disease, including meningitis, encephalitis, and/or flaccid paralysis. Survivors of

severe disease typically require prolonged rehabilitation for chronic neurologic sequelae (5). To date, no specific treatments or vaccines for WNV disease have been approved by the U.S. Food and Drug Administration.

In the US, there were 16,577 laboratory-confirmed cases of WNV reported to the Centers for Disease Control and Prevention from 2001 to 2004, of which 648 were fatal (3.9%; references 6–9). Elderly and immunocompromised individuals have increased susceptibility to WNV (2, 6, 10, 11), but genetic risk factors and immunologic control mechanisms have not been fully delineated. We have recently identified the monocyte and T lymphocyte chemokine receptor CCR5 as a key factor in viral clearance and survival in a mouse model of WNV encephalitis, in which the mechanism involved trafficking of leukocytes into the infected brain (12). Consistent with this, WNV induces expression of CCR5 ligands in WNV-infected mouse brain (12, 13).

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Table I. Characteristics of study subjects

Characteristics	All racial groups				Caucasians only		
	Arizona $n = 247$	Colorado $n = 148$	Combined $n = 395$	Ariz WNV− $n = 145$	Arizona $n = 143$	Colorado $n = 72$	Combined $n = 72$
Mean age (y)	53 ± 19	50 ± 18	52 ± 19	45 ± 20	57 ± 18	50 ± 18	54 ± 18
Male sex (%)	55.6	48.6	52.8	44.3	53.8	41.7	49.8
Caucasian (%)	57.9	48.6	54.4	13.1	100	100	100
Hispanic (%)	10.1	11.5	10.6	2.8	N/A	N/A	N/A
Asian/Pacific Islander (%)	2.0	0.0	1.3	0.7	N/A	N/A	N/A
Native American (%)	2.4	0.0	1.5	2.1	N/A	N/A	N/A
Black (%)	0.0	0.7	0.3	0.7	N/A	N/A	N/A
Not specified (%)	27.5	39.2	31.9	80.7	N/A	N/A	N/A
Fever (%)[a]	26.7	24.3	25.8	N/A	34.3	22.2	30.2
Meningitis (%)[a]	25.9	39.9	31.1	N/A	30.8	43.1	34.9
Encephalitis (%)[a]	24.7	35.8	28.9	N/A	31.5	34.7	32.6
Not specified (%)[a]	22.7	0.0	14.2	N/A	3.5	0	2.3
% Mortality (n)	3.2 (8)	7.4 (11)	4.8 (19)	N/A	4.9 (7)	1.4 (1)	3.7 (8)

Arizona and Colorado refer to cohorts of patients with symptomatic seropositive WNV infection. Ariz WNV− refers to a second Arizona cohort of symptomatic patients in whom WNV was considered as the diagnosis but ruled out. Data pooled from the WNV+ Arizona and Colorado cohorts are also shown (Combined).
[a]Patients were classified into one of the four indicated disease categories by physician interview.
N/A, not available or applicable.

In the present study, we attempted to translate this finding to man by analyzing the distribution of the defective *CCR5* allele *CCR5Δ32* in two independent cohorts of symptomatic WNV-seropositive individuals from the US. *CCR5Δ32* contains a 32-bp deletion within the *CCR5* coding sequence and encodes a truncated protein that fails to traffic to the plasma membrane (14). *CCR5Δ32* originated in Northern Europe and is found in homozygous form in ∼1% of Caucasians in the US (15, 16). These individuals completely lack CCR5 function, but appear healthy, suggesting that any important homeostatic functions are compensated for by other leukocyte chemoattractant receptors. In addition to regulating monocyte and T cell trafficking, CCR5 functions pathologically as a major HIV coreceptor (16). Accordingly, *CCR5Δ32* homozygosity is strongly associated with resistance to HIV—one of the strongest genetic susceptibility factors known in infectious disease (15, 17). Similarly, the hypothesis that CCR5 plays an important role in WNV pathogenesis in man predicts that the distribution of *CCR5Δ32* among symptomatic patients may be strongly skewed relative to its distribution in the general population. Here, we test this prediction.

RESULTS AND DISCUSSION

Three cohorts of patients, the Colorado WNV-seropositive cohort, the Arizona WNV-seropositive cohort, and the Arizona WNV-seronegative cohort, were identified that had symptomatic illness in which WNV was considered in the differential diagnosis. Inclusion criteria for patients in these cohorts are defined in the Materials and methods section and the clinical characteristics are given in Table I. Genotypes were defined for 247 of the 261 WNV-seropositive Arizona cases (94.6%); for 148 of the 155 WNV-seropositive Colorado cases (95.5%); for 143 of 152 and 72 of 74 of the self-reporting Caucasian subsets of the Arizona and Colorado WNV-seropositive cohorts, respectively; for all WNV-seronegative random blood donors ($n = 1,318$); and for 145 of 154 WNV-seronegative subjects from Arizona (94.2%).

Table II. Association of the *CCR5Δ32* allele with symptomatic WNV infection in Arizona and Colorado

Cohort	*CCR5Δ32* Genotype, n (%)					
	All racial groups			Self-reporting Caucasians		
	+/+	+/Δ32	Δ32/Δ32	+/+	+/Δ32	Δ32/Δ32
Arizona WNV+	205 (83.0)	31 (12.6)	11 (4.5)	115 (80.4)	22 (15.4)	6 (4.2)
Colorado WNV+	111 (75.0)	31 (20.9)	6 (4.1)	49 (68.1)	17 (23.6)	6 (8.3)
Combined WNV+	316 (80.0)	62 (15.7)	17 (4.3)	164 (76.3)	39 (18.1)	12 (5.6)
Arizona WNV−	125 (86.2)	19 (13.1)	1 (0.7)	16 (84.2)	3 (15.8)	0 (0)
RBD	N/A	N/A	N/A	1102 (83.6)	203 (15.4)	13 (1.0)

All patients from Arizona and Colorado had symptoms for which WNV infection was considered as the underlying cause. WNV+, WNV seropositive; WNV−, WNV seronegative; +, wild-type *CCR5* allele; *Δ32, CCR5Δ32* allele; RBD, random blood donors; N/A, not applicable.

Table III. *CCR5Δ32* homozygotes have increased risk of symptomatic WNV infection

Cohort	All racial groups			Self-reporting Caucasians		
	OR	95% CI	P	OR	95% CI	P
Arizona	4.7	2.1–10.6	<0.0001	4.4	1.6–11.8	0.0013
Colorado	4.2	1.6–11.3	0.0018	9.1	3.4–24.8	<0.0001
Combined	4.5	2.2–9.4	<0.0001	5.9	2.7–13.2	<0.0001

Values were calculated in comparison to the *CCR5Δ32* homozygous genotype frequency in 1,318 North American Caucasian random blood donors.

The genotype distribution observed for the combined Arizona and Colorado WNV-seropositive cohorts deviated strongly from Hardy-Weinberg equilibrium (P = 0.013), mainly because of increased frequency of *CCR5Δ32* homozygotes. 17 (4.3%) of the 395 informative cases in the combined Arizona and Colorado seropositive cohorts unstratified by race were *CCR5Δ32* homozygotes (Table II). This value greatly exceeds the percentage of *CCR5Δ32* homozygotes reported for the general US Caucasian population (0.8–1.1%; reference 18). To quantitate the significance of this genotype–disease association, we have used as a reference the group of 1,318 healthy Caucasian random blood donors from the US, in which 1.0% were *CCR5Δ32* homozygotes. Compared with this value, the increased frequency of *CCR5Δ32* homozygotes in both the Arizona and Colorado WNV-seropositive cohorts was highly statistically significant (Table III). When all genotyped WNV-seropositive cases unstratified by race were compared with the US random blood donors, the odds ratios (OR) for this genetic factor were 4.7 for the Arizona cohort (95% confidence interval [CI], 2.1–10.6, P < 0.0001) and 4.2 for the Colorado cohort (95% CI, 1.6–11.3, P = 0.0018). It is important to note that this is a conservative analysis because the frequency of *CCR5Δ32* homozygotes is certain to be lower in non-Caucasian racial groups from these cohorts (Table I).

Consistent with this, when the data were stratified to include only self-reporting Caucasians in both the WNV-seropositive cohorts (n = 215), the strength of the association increased by 30% (12 *CCR5Δ32* homozygotes, 5.6%). When the self-reporting Caucasian portions of the two WNV-seropositive cohorts were compared separately to the random blood donor benchmark, the OR was 4.4 in the Arizona cohort (95% CI, 1.6–11.8, P = 0.0013), and 9.1 in the Colorado cohort (95% CI, 3.4–24.8, P < 0.0001).

Although unlikely, it is possible that the large increase in frequency of *CCR5Δ32* homozygotes in these cohorts may be caused by an atypical geographic distribution of this allele in the general population of Arizona and Colorado. To directly address this possibility, we analyzed the distribution of *CCR5Δ32* in a symptomatic Arizona WNV-seronegative cohort (n = 145). Because the *CCR5Δ32* allele frequency in this cohort (7.2%) is only slightly lower than in the Arizona WNV-seropositive cohort (10.7%) and the US Caucasian random blood donors (8.7%), it is likely that this cohort contains only a slightly smaller percentage of Caucasians than the other two. Thus, given the size of the Arizona WNV-seronegative group, there is sufficient power at this

allele frequency to meaningfully analyze the distribution of genotypes. Only one *CCR5Δ32* homozygote was identified in the cohort (0.7%). Moreover, unlike the WNV-seropositive cohort, the observed *CCR5Δ32* genotypic frequencies in the WNV-seronegative cohort did not deviate from Hardy-Weinberg equilibrium (P > 0.99). This strongly suggests that the general population in Arizona does not have an anomalous genetic structure with regard to the *CCR5* locus, with a skewed increase in *CCR5Δ32* homozygotes that could account for the high frequencies we observed in the WNV-seropositive cohorts. When the *CCR5Δ32* homozygote genotype frequency was compared between the race-unstratified Arizona WNV-seronegative and -seropositive cohorts, an OR of 6.7 was determined (95% CI, 0.86–52.6, P = 0.07).

With regard to the distribution of *CCR5* genotypes in symptomatic WNV-seropositive cases as a function of clinical outcome, no significant differences were observed compared with the distribution in the overall combined group (Table IV) or in the Arizona or Colorado WNV-seropositive cohorts considered separately (not depicted), with the exception of death (Fig. 1). A total of 19 of 395 individuals in the two cohorts died from WNV neuroinvasive disease (4.8%), which is similar to the national average of mortality reported for WNV cases from the past three years: 2002 (4,156 cases, 284 deaths, 6.8%); 2003 (9,862 cases, 264 deaths, 2.7%); and 2004 (2,539
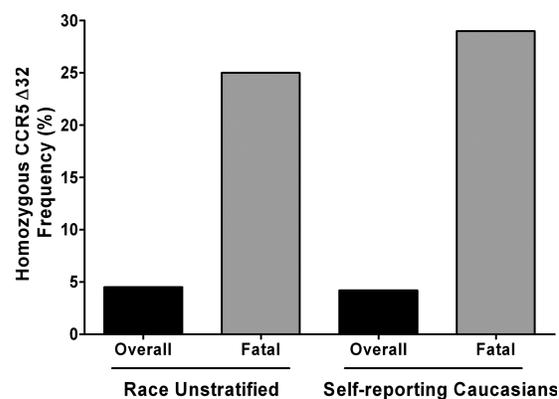

Figure 1. **CCR5 deficiency is associated with increased risk of death from WNV infection.** Data presented are from the Arizona WNV-seropositive cohort and demonstrate that *CCR5Δ32* homozygosity is associated with fatal outcome in both the race-unstratified (OR = 8.5 [95% CI, 1.5–48.2], P = 0.04) and self-reporting Caucasian (OR = 13.2 [95% CI, 1.9–89.9], P = 0.03) groups.

Table IV. Distribution of *CCR5Δ32* allele as a function of clinical outcome in WNV-seropositive patients

	Genotype, *n* (%)					
	All racial groups			**Self-reporting Caucasians**		
Clinical outcome	**+/+**	**+/Δ32**	**Δ32/Δ32**	**+/+**	**+/Δ32**	**Δ32/Δ32**
Fever[a]	85 (83.3)	12 (11.8)	5 (4.9)	52 (80.0)	9 (13.8)	4 (6.2)
Meningitis[a]	100 (81.3)	19 (15.4)	4 (3.3)	59 (78.7)	13 (17.3)	3 (4.0)
Encephalitis[a]	82 (71.9)	27 (23.7)	5 (4.4)	48 (68.6)	17 (24.3)	5 (7.1)
Not Specified[a]	49 (87.5)	4 (7.1)	3 (5.4)	5 (100.0)	0 (0)	0 (0)
Death	13 (68.4)	4 (21.1)	2 (10.5)	6 (75.0)	0 (0)	2 (25.0)
Total	316 (80.0)	62 (15.7)	17 (4.3)	164 (76.3)	39 (18.1)	12 (5.6)

Data were pooled from the Arizona and Colorado cohorts.

[a]Patients were classified into one of the four indicated disease categories by interview with the referring physician. +, wild-type *CCR5* allele; *Δ32*, *CCR5Δ32* allele.

cases, 100 deaths, 3.9%). The combined total for this time interval in the US is 16,577 reported cases with 648 deaths (3.9%; references 7–9). The Arizona WNV-seropositive cohort accounted for 8 of the 19 total deaths, 7 in Caucasians and 1 in a Hispanic (Table I). The Colorado WNV-seropositive cohort accounted for 11 deaths, 1 in a Caucasian. Two of the 19 fatalities (10.5%) in the combined cohorts were *CCR5Δ32* homozygotes, both self-reporting Caucasians from the Arizona WNV-seropositive cohort. The ages of the two *CCR5Δ32* homozygotes who died were 70 and 74, similar to the average age of the other 17 fatal cases (74 yr). The two *CCR5Δ32* homozygote fatalities represent 25 and 29% of the race-unstratified (*n* = 8) and Caucasian (*n* = 7) Arizona WNV-seropositive cases who died, respectively. These values exceed the expected values based on the frequency of *CCR5Δ32* homozygotes among the race unstratified and Caucasian groups in the Arizona cohort (4.5 and 4.2%, respectively), and the differences are statistically significant: OR = 8.5 (95% CI, 1.5–48.2), P = 0.04, and OR = 13.2 (95% CI, 1.9–89.9), P = 0.03, respectively (Fig. 1). This association was not observed in the Colorado cohort. When Caucasians from both cohorts were analyzed together, the association test gave an OR of 6.6 (95% CI, 1.2–37), P = 0.07.

The present study provides the first evidence that the defective *CCR5* allele *CCR5Δ32* is a risk factor for symptomatic WNV infection, the first genetic risk factor identified for this disease. The association was restricted to *CCR5Δ32* homozygotes and was very strong, similar in magnitude to the association we and others have previously reported for *CCR5Δ32* homozygosity with the exposed, uninfected HIV resistance phenotype (e.g., OR = 6.04 [95% CI, 1.42–25.7], P = 0.02, in reference 15). Although our results are based on retrospective analysis, they are unlikely to be because of chance for six reasons. First, the results are statistically strong (an OR greater than four). Second, the results were obtained in two independent as well as geographically and temporally distinct cohorts. Third, *CCR5Δ32* is a complete loss-of-function mutation and therefore homozygous individuals completely lack functional CCR5. Fourth, WNV infection is associated with infiltration of T cells and macrophages (cell types known to express CCR5) into brains of patients infected with WNV, suggesting biologically plausible in-

volvement of CCR5 in regulating leukocyte migration to the brains of infected patients (19, 20). Fifth, WNV infection of mice induces expression of the CCR5 ligand CCL5 and accumulation of CCR5+ leukocytes in the brain (12, 13). Sixth, WNV infection in CCR5$^{-/-}$ mice is uniformly fatal (12).

Together these results imply that wild-type CCR5 functions as a host defense factor in WNV infection in man. CCR5 could potentially restrict WNV infection at the level of initial infection. This is not addressed by our retrospective study design and may not be feasible to resolve prospectively by measuring the association of *CCR5Δ32* homozygosity with asymptomatic WNV infection because this genotype and WNV infection are both uncommon in the general population. More likely, CCR5 restricts disease progression after initial infection so that its absence results in an increased likelihood of an infected patient coming to medical attention as a symptomatic case. Our data are consistent with this interpretation, and even suggest that a fatal outcome is more likely in the absence of CCR5 (Fig. 1). Additional work will be needed to more critically address the latter point because the positive association we observed between CCR5 deficiency and death in the Arizona cohort, although statistically significant, was based on only two cases, and could not be detected in the Colorado cohort, possibly because of its smaller size.

Another limitation of our study was the incomplete information about the racial background of cases (Table I), which is needed to more precisely quantitate risk because *CCR5Δ32* is primarily found in Caucasians. We have addressed this by consistently applying a conservative analysis of the data that has enabled us to test boundary conditions for quantitating the strength of the genotype–disease association. Larger prospective studies will be needed to more precisely quantitate risk as well as to further define mechanisms in man.

Our data indicate that immunocompromised patients could be particularly susceptible to symptomatic WNV infection if CCR5 function is missing or blocked. Additional studies will be needed to address this issue, which should include close monitoring of AIDS patients treated with antagonists now in advanced stages of development targeting the HIV coreceptor activity of CCR5. Such agents are intended to imitate the homozygous *CCR5Δ32* genetic defect and

could render AIDS patients particularly vulnerable to severe and possibly fatal WNV infection.

In summary, we have established that homozygous *CCR5Δ32* is a strong host genetic risk factor for symptomatic laboratory-confirmed WNV infection, the first one identified for this disease. In contrast, the homozygous *CCR5Δ32* genotype has previously been strongly associated with resistance to HIV. These genetic data imply that CCR5 plays opposite roles in HIV and WNV infection, facilitating the former and restricting the latter. Our results have important implications regarding the potential safety of CCR5-blocking agents now under development for the treatment of HIV/AIDS. Clinical care of individuals taking these medicines while residing in WNV-endemic areas may mandate strict measures to limit mosquito exposure and a high index of suspicion for symptoms consistent with WNV.

MATERIALS AND METHODS

Study populations. The study was approved by the Office of Human Subjects Research of the US National Institutes of Health. Four patient groups were defined: (a) healthy North American Caucasian random blood donors from the National Institutes of Health Department of Transfusion Medicine ($n = 1,318$) previously collected under an IRB approved protocol; (b) the Arizona WNV-seropositive cohort ($n = 261$), defined as patients presenting with acute illness in Arizona who tested positive for WNV but negative for St. Louis encephalitis virus (SLE) by specific IgM ELISA of serum or cerebrospinal fluid (CSF) at the Arizona Department of Health Services between 5/26/04 and 10/2/04, the dates for receipt by the Department of the first and last patient samples during mosquito season in the state of Arizona in 2004; (c) the Colorado WNV-seropositive cohort ($n = 155$), defined as patients presenting with acute illness to a physician in Colorado who tested positive for WNV but negative for SLE by specific IgM ELISA of serum or CSF at the Colorado Department of Public Health and Environment between 7/20/03 and 9/30/03, the dates for receipt by the Department of the first and last patient samples for 2003; (d) the Arizona WNV-seronegative cohort ($n = 154$), defined as patients presenting with acute illness to a physician in Arizona in whom WNV and SLE had been considered in the differential diagnosis but ruled out by serological testing at the Arizona Department of Health Services between 5/26/04 and 10/2/04. In the Arizona WNV-seropositive cohort, 89% of the samples collected were available and all of these were analyzed. In the Colorado WNV-seropositive cohort, only 164 samples remained that had a sufficient volume for purification of DNA and all of these samples were analyzed. This represented 14% of all Colorado samples originally collected. The following information was requested from the medical provider during case investigation by local health department communicable disease staff: age, sex, self-reported racial group, and clinical presentation based on the Centers for Disease Control–defined clinical parameters of disease; i.e., fever, meningitis, encephalitis, and death (Table I). At the time of data collection each symptomatic patient was classified into one of the three disease categories: fever, meningitis, or encephalitis. Follow-up data on disease course and the maximal severity of symptomatic disease in each patient were not available. All fatal cases were caused by complications of WNV neuroinvasive disease. Study investigators were blinded to unique patient identifiers.

DNA isolation. 100 μl of serum or CSF was thawed for genomic DNA purification using a QiaAmp 96 DNA Blood kit according to the manufacturer's instructions (QIAGEN). Purified DNA was eluted into 100 μl of the recommended buffer and stored at 4°C until further use. DNA from random blood donors was isolated as previously described (15).

CCR5Δ32 genotyping. Genotyping was performed by standard methods described previously (21). In brief, 2 μl of patient DNA was amplified by

PCR using primers that flank the site of the 32-bp deletion: 5′-GTCTT-CATTACACCTGCAGCTCTC-3′ and 5′-GTCCAACCTGTTAGAG-CTACTGC-3′. PCR products were analyzed by electrophoresis on a 3.0% agarose/TBE gel with known wild-type *CCR5* and *CCR5Δ32* amplicon controls (233 and 201 bp, respectively) and visualized with Gelstar (Cambrex) DNA staining. Each sample was tested in two independent PCR reactions, and results were concordant, as determined by two independent investigators.

Statistical analysis. OR and 95% confidence limits were calculated using a recessive genetic model (i.e., *CCR5Δ32* homozygotes compared with the combination of wild-type *CCR5* and *CCR5Δ32* heterozygotes) by cross-tabulation. With the exception of the death data, significance was determined by χ^2 tests using a two-sided P value and Yates' continuity correction, and 95% CIs were estimated using the approximation of Woolf as implemented in the Prism statistics program version 4 (GraphPad Software). Association of *CCR5Δ32* homozygosity with death, due to small numbers, was analyzed by Fisher's exact test. Tests of Hardy-Weinberg equilibrium were performed using a χ^2 test and two degrees of freedom after using the Hardy-Weinberg equation ($p^2 + 2pq + q^2 = 1$, where p and q represent the frequency of the two alleles) to calculate expected frequencies of each of the three genotypes.

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