BRIEF COMMUNICATION

Detection of the CCR5-Δ32 HIV resistance gene in Bronze Age skeletons

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A mutant allele of the chemokine receptor CCR5 gene (CCR5-Δ32), which confers resistance to HIV-1 infection, is believed to have originated from a single mutation event in historic times, and rapidly expanded in Caucasian populations, owing to an unknown selective advantage. Among other candidates, the plague bacillus *Yersinia pestis* was implicated as a potential source of strong selective pressure on European populations during medieval times. Here, we report amplifications of the CCR5-Δ32 DNA sequence from up to 2900-year-old skeletal remains from different burial sites in central Germany and southern Italy. Furthermore, the allele frequency of CCR5-Δ32 in victims of the 14th century plague pandemic in Lübeck/northern Germany was not different from a historic control group. Our findings indicate that this mutation was prevalent already among prehistoric Europeans. The results also argue against the possibility of plague representing a major selective force that caused rapid increase in CCR5-Δ32 gene frequencies within these populations.

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The human chemokine receptor CCR5 serves, together with CD4, as the principal coreceptor for macrophage-tropic (R5) human immunodeficiency virus type 1 (HIV-1) strains.¹ A large number of genetic variants in the coding or the promotor region of the CCR5 gene have been identified in different ethnic groups.^{2,3} Several of these naturally occurring mutations result in alterations of the receptor's amino-acid sequence and affect the ability of CCR5 to act as a chemokine receptor or HIV coreceptor.^{4,5} The finding that nonfunctional receptor alleles are relatively frequent among different human populations has led to the hypothesis that a selective advantage might be associated with the loss of CCR5 function.

The CCR5-Δ32 mutant, which is characterized by a 32-bp deletion in the gene segment encoding the second extracellular loop of the receptor, provides an instructive example of how host genetic variation may contribute to HIV-related pathology. Individuals homozygous for CCR5-Δ32 are almost completely protected against HIV-1 infection due to the absence of functional receptors from the cell surface.^{6–8} Population surveys revealed a north to south gradient of CCR5-Δ32 allele frequencies of 16 to 3% across Europe and its absence in individuals of non-Caucasian descent.^{9–11} Determination of the intrahaplotypic variation of flanking microsatellite loci in strong

linkage disequilibrium with CCR5-Δ32 allowed calculation of the approximate age of the CCR5-Δ32 containing ancestral haplotype. Two different studies that analyzed separate microsatellite markers concluded that the CCR5-Δ32 allele originated from a single mutation event that took place in historic times approximately 700 years (95% confidence interval (CI): 275–1800)¹¹ to 3500 years (CI 400–13 000)¹⁰ ago in northeastern Europe. Both studies assumed a heterozygote advantage of the CCR5-Δ32 mutant. The European locale and timing of the bubonic plague of the 14th century coincide with the calculated date when the CCR5-Δ32 allele started to increase in frequency. Moreover, the plague bacillus *Yersinia pestis* infects macrophages, which carry CCR5. Therefore, the 'Black Death' has been implicated among other possible causes as a potential source of CCR5-Δ32 selection.¹¹

We set out to test directly the hypothesis of the relatively recent origin of the CCR5-Δ32 mutation by analyzing ancient DNA (aDNA) that was recovered from historic and prehistoric skeletal material from the same geographic area in central Germany. The study also included a modern reference group ($n = 346$) consisting of healthy blood donors and staff members from the University of Göttingen. To compare the geographic distribution of CCR5-Δ32 allele frequencies in different parts of Europe in historic times, skeletal material from 19 individuals who were buried during a period dating from 1750 to 1810 in a churchyard cemetery in Goslar (central Germany) was analyzed along with bones from 19 inhabitants of the village of Alia (Sicily) who died during an outbreak of cholera at about the same time (1837). The question of dating the time when CCR5-Δ32

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started to increase in frequency was addressed by analyzing the bones of 17 individuals who were recovered from the Lichtenstein Cave in the Harz mountains (central Germany), dating back to 900 BC. In order to test the hypothesis that plague might have represented a major selective force for the expansion of the CCR5-Δ32 mutant during the Middle Age, two separate mass graves from the town of Lübeck, northern Germany, were investigated. The first sample consisted of the bones of 14 individuals who died during the plague epidemic in 1350. The second (control) sample comprised 20 individuals who were victims of the famine of 1316 in the same town.

Ancient DNA studies are prone to a number of artifacts, which, among other factors, may include the contamination of preserved remains with modern human cellular material.¹² We therefore employed a strategy that enables to assign an *a priori* nonindividual specific sequence, as is given by the CCR5 locus, to a certain individual. To this end, a set of newly designed CCR5 primers were incorporated into a multiplex PCR

assay, which simultaneously acquires an autosomal short tandem repeat (STR)-based genetic fingerprint of the sample. This genetic fingerprint is in all likelihood (matching probability 10^{-18} – 10^{-5}) unique for a certain individual and, at the same time, allows identifying admixtures of contaminating DNA.¹² Additionally, the coamplified microsatellite profiles from historic and prehistoric samples were compared with the profiles from all staff who handled the material as well as with the results from within this study. We thereby controlled for possible crosscontaminations between the samples and amplification product carryover. A representative electropherogram of an STR multiplex amplification from a prehistoric CCR5-Δ32 gene carrier is shown in Figure 1, and the results of STR-based DNA typing of all individuals who were analyzed within this study are documented in Supplemental Table 2a–e. Furthermore, we adhered to standardized and commonly accepted procedures for sample preparation, aDNA extraction, amplification and analysis, as described before.^{13,14} This protocol included the removal of all sample surfaces

Figure 1 Electropherogram of a genetic profile of the Bronze Age sample DO 1103. The solid peaks represent the amplified alleles of the sample while the light gray peaks originate from the allelic ladder, which was superimposed in order to simplify the allele determination. Product sizes are given on the X-axis in base pairs. The sample DO 1103 reproducibly showed the CCR5 wild-type (130 bp) as well as the mutant CCR5Δ32 allele (98 bp). The STR-based genetic fingerprint of DO 1103 is 6/9.3 for TH01, 14/15 for VWA, 9/11 for TPOX and 11/12 for D5S818; the sex is female, indicated through a single peak at 106 bp, which represents the amelogenine locus on the X-chromosome.

Table 1 Distribution of CCR5-Δ32 in modern, historic and prehistoric European populations^a

Population (location, date)	No. studied	CCR5-Δ32			Allele frequency (%)
		wt/wt	wt/mut	mut/mut	
Göttingen, central Germany, 2000	346	287	54	5	9.2
Goslar, central Germany, 1750–1810	19	12	7	—	18.4
Alia, Sicily, 1837	19	18	1	—	2.6
Lübeck, northern Germany, Black Death mass grave, 1350	14	10	4	—	14.2
Lübeck, northern Germany, famine mass grave, 1316	20	15	5	—	12.5
Lichtenstein cave, central Germany, 900 BC	17	13	4	—	11.8

^aMethods: The skeletons of the burial sites of Goslar, Alia and the Lichtenstein cave had previously been genetically analyzed in studies that aimed at the reconstruction of kinship on an individual and population level^{13,19–21} or that focused on the ΔF508 mutation at the cystic fibrosis locus.²² In all cases, the DNA was preserved in excellent condition. The DNA was extracted from aliquots of 0.3 g of pulverized bone material through a phenol–chloroform-based method,²³ which was previously found to be particularly suitable for DNA extraction from long-term buried skeletal material containing humic acids.¹³ The sequence in question on chromosome 3 at p21.3 was amplified by PCR using primers that were designed to yield a 130 bp product for CCR5 wild type and a 98 bp fragment for the CCR5-Δ32 mutation. The newly designed primers were integrated to a multiplex PCR consisting of microsatellite loci and the sex chromosomal amelogenine locus in order to ensure the authenticity of the aDNA results.¹² The reaction mixes consisted of 50 mM KCl, 10 mM Tris-HCl, 2.5 mM MgCl₂ (1.5 mM MgCl₂ for the Lübeck samples), 200 μM of each dNTP, 0.28 μM of each primer CCR5, 0.2 μM of each primer TH01, 0.25 μM of each primer amelogenine, 0.2 μM of each primer VWA, 0.24 μM of each primer TPOX, 0.22 μM of each primer D5S818, 1.5–2.5 U AmpliTaqGold™ (Applied Biosystems), 2–10 μl ancient DNA and sterile water (Ampuwa™, Fresenius) in a total volume of 25 μl. One primer at each locus was labelled with a fluorescent dye. The primer sequences for the multiplex amplification system and the amplification parameters are given in Supplemental Table 1. Cycling parameters were 94°C for 11 min (initial activation of Taq DNA polymerase), followed by 35 cycles at 94°C for 1 min, 54°C for 1 min, 72°C for 1 min and a final delay at 60°C for 30 min (TC1 thermal cycler; Perkin-Elmer Applied Biosystems and Master Cycler Gradient, Eppendorf). In some of the samples, primarily from the Goslar and the Lübeck series, an additional 5–10 cycles were carried out in order to obtain sufficient amplification product for the analysis. To be typed unequivocally, at least four amplifications from two different extractions with reproducible results were required. PCR products were analyzed on denaturing 6.75% polyacrylamide gels by using the automated fluorescence fragment length detection system GeneScan 672 on an ABI Prism 373A stretch DNA sequencer (Perkin-Elmer Applied Biosystems) employing GeneScan™ Collection and GeneScan™-Analysis software. For allele determination, allelic ladders for each amplified locus and the internal lane standard GS 500 ROX (Applied Biosystems) were used.

prior to aDNA extraction, the use of protective clothing throughout all processing steps and the strict separation of pre- and post-PCR handling of the samples. Each skeleton was sampled twice in order to enable an independent reproduction of the results. From each of the independently processed extracts, at least two amplifications were performed, that is, each result was based on at least four analyses.

The analysis revealed that seven out of the 19 individuals from the early modern Goslar series, but only one out of 19 individuals from Alia, were heterozygous carriers of the CCR5-Δ32 allele (Table 1). Random sampling of nonrelated individuals is *a priori* not possible in burial site populations of both historic and modern origin. The exclusion of first-degree relatives, which were identified by STR typing, Y-STR typing and mitochondrial haplotyping of hypervariable regions, did not affect the general outcome of the study (not shown). Thus, our data suggest that differences in CCR5-Δ32 gene frequencies across Europe known from modern studies^{10,11} were also present in historic populations.

We also reliably detected the CCR5-Δ32 allele in four out of 17 individuals from the Bronze Age Lichtenstein burial site by PCR. Sequence identity with the modern wild-type and mutant reference alleles (GenBank accession nos. U95626 and AF052244) was confirmed in two heterozygous individuals by direct DNA sequencing. This result indicates that the CCR5-Δ32 allele was prevalent among prehistoric populations in central Europe at a point in time (900 BC) at which this mutation

was previously thought to just have started to increase in frequency in northeastern Europe.^{10,11} Most importantly, our data provide direct evidence for the existence of CCR5-Δ32 in (pre-)historic populations with gene frequencies that up to now were only estimated using various mathematical models.

Finally, the CCR5-Δ32 allele frequency (14.2%) in skeletal remains from a well-documented medieval plague mass grave of Lübeck in northern Germany did not significantly differ from a control sample (12.5%), which matches for time and location. If the causative pathogen(s) of the 'Black Death' represented a strong selective force, the CCR5-Δ32 allele frequency would have been much lower in the plague mass grave than in the famine control sample. Within the limits of the statistical analysis, these data refute the notion that carriers of the mutant form of CCR5 had a relative plague mortality risk of less than 0.063 (94% protection; $P = 0.05$; χ^2 statistics; Monte-Carlo simulation) compared to wild-type homozygotes. If the mutant allele is assumed to be fully dominant, that is, heterozygotes were protected to the same degree as CCR5-Δ32 homozygotes, the relative risk of carriers of the mutant allele was no less than 0.35 (65% protection; $P = 0.05$).

The result of this study has implications for the natural history of the CCR5-Δ32 HIV resistance gene. Previously, it was hypothesized that a strong selection advantage may have been conferred to carriers of the nonfunctional CCR5 allele, which drove its frequency rapidly upward in ancestral Caucasian populations. Resistance to an

unknown infectious agent that caused a widespread fatal epidemic in a manner similar to, but clearly different from, HIV provides an attractive explanation for the selective expansion of polymorphic CCR5 variants.^{10,11,15} This hypothesis draws further support from the finding that members of the poxvirus family exploit chemokine receptors, including CCR5, for the infection of migratory leukocytes¹⁶ and this was also speculated to be relevant for infections with *Y. pestis*.¹¹ The results from our studies as well as two other recent studies^{17,18} suggest that bubonic plague most probably did not exert major selective pressure on this mutation. For a disease to exert strong selective pressure resulting in the specific geographic distribution of CCR5-Δ32 as is found in modern European populations, it would have to have a significant effect on morbidity and mortality before reproductive age. Moreover, this disease would have to exert these effects in this particular locale over extended periods of time. Smallpox is a good candidate,¹⁷ but other (non-)infectious diseases may also have accounted for this effect.

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