Historic Exposure to Plague and Present-day Frequency of CCR5del32 in Two Isolated Island Communities of Dalmatia, Croatia

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Aim To assess the frequency of deletion of 32 base pairs in a CCR5 gene, shown to confer resistance to HIV infection, in two isolated island communities of Dalmatia, Croatia, with different histories of exposure to "plague" during the medieval period and beyond.

Methods Random samples of 100 individuals from highly isolated communities of Lopar (island of Rab) and Komiža (island of Vis) were selected in 2002 and their DNA was extracted. An extremely high level of 3-generational endogamy was found in both communities (98% and 91%, respectively), indicating very limited gene flow, which was confirmed by available historic records. The two settlements also differed in their historic exposure to plague: between 1449 and 1456, Lopar was decimated by plague, while Komiža remained unaffected. Genotyping of the CCR5 polymorphism was performed using the polymerase chain reaction (PCR) method with primers flanking the region containing 32-bp deletion.

Results The frequency of CCR5del32 in Lopar was 6.0% and in Komiža 1.5% ($P = 0.037$). A previous study in 303 random Croatian blood donors showed a frequency of CCR5 32bp deletion of 7.1%.

Conclusion This study does not rule out the possible role of plague in positive selection at CCR5del32. However, analyses of further neighboring isolated island communities need to be made in order to provide more substantial support for this hypothesis.

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The chemokine receptor CCR5 was shown to be a co-receptor for the macrophage-tropic strains of HIV-1 (1,2). A mutant form of this gene with a 32bp deletion, in its homozygous form, was shown to confer resistance to infection by HIV (1,2). In European populations, this mutant allele is fairly common, with a clear north to south gradient of frequency, ranging from 16% in Mordvinia, Russian Federation, to 4% in Sardinia (3). Slavic populations of central Europe, with the estimated frequency of 10.9% for the Poles (4), 10.7% for the Czechs (5), 8.7% for the Slovenes (6), and 7.1% for the Croatians (7), are placed in the middle of the European gradient, which is expected according to their geographic location. An analysis of flanking microsatellites in multiple populations showed strong linkage disequilibrium between specific microsatellite alleles and the 32-bp deletion (8). This data inferred that most 32-bp deletions originated from a single mutation event, which probably took place in northeastern Europe (8).

It was initially thought that the high frequency of the 32-bp deletion allele could not be explained simply by random genetic drift and was probably associated with some selection advantage conferred by either the heterozygous or homozygous mutant allele (8,9). Lucotte et al (10) undertook a comparative analysis of this variant in 40 populations from Europe, the Middle East, and North Africa, and confirmed the clear north-south divide. They concluded that the CCR5 32bp deletion was probably disseminated by the Vikings and that it had a protective effect against smallpox in the period between the 8th and 10th century. However, others hypothesized that the mutation was more recent in origin and under much stronger selection, and that the *Yersinia pestis* may have had strong selective pressure on European populations during medieval times and beyond, especially between 1347 and 1670 (3,11,12).

A number of scientists have recently conducted research in order to provide further support for the "plague hypothesis" or to challenge it. The problem is that the term "plague" is ill defined in this context, as for a long period of human history it was associated with any epidemic that was causing high mortality, and the microbial causes could have been entirely different in each episode (2,9). The bubonic plague, caused by *Yersinia Pestis*, is only one of several possible examples (9). CCR5 32-bp deletion was identified in 2900-year-old skeletons from the Bronze Age burials in central Germany at the same frequency as in victims of the 14th century pandemic in Lubeck in northern Germany (13,14). Experiments showed no difference in susceptibility to infection and death between CCR5 deficient and normal mice after infection with *Yersinia pestis* (15), but there may still be a difference in the pathogenesis of *Yersinia pestis* between mice and men. Furthermore, in vitro experiments of macrophage uptake of *Yersinia pestis* showed some 30-fold reduction in homozygous mutant mice (16). Some epidemiological models based on highly complex sets of assumptions supported the role of plague (9), the others supported the role of smallpox (17), while some found no evidence of positive selection at all (18). All these studies indicate that the role of the 32-bp deletion in relationship to *Yersinia pestis* infection is still inconclusive.

In this study, we aimed to contribute further evidence to this debate. Due to massive migrations that occurred in Europe since the medieval period, there are very few European populations, located within the same gradient of CCR5del32 frequency, that remained isolated after very differing histories of exposure to plague. However, in Dalmatia, Croatia, some of the island isolates may be unique in exhibiting precisely this set of conditions that may allow testing the hypothesis of plague as a positive selective force on CCR5del32.

Subjects and methods

Choice of population

We analyzed the CCR5 32bp deletion in 2 carefully chosen Dalmatian island communities, the village of Lopar on the island of Rab and the village of Komiža on the island of Vis. An earlier study showed that these two island isolates were genetically different from the neighboring populations (19,20).

Historic records showed that a plague epidemic affected the island of Rab 1449 and 1456, and that between 60 and 95% of the inhabitants of all the settlements (including Lopar) died or were forced to take refuge (21-32). It should be noted that this plague may not have been related to *Yersinia pestis*, as this period (1449-1456) does not overlap with the second pandemic in Europe during the period of "Black Death" (1347-1361) or with the third and final pandemic in Europe (1665-1670) (9). As opposed to Rab, the villages on the islands of Vis (including Komiža) were spared from all major epidemics associated with high mortality during medieval period and beyond (21,22,29,30).

If the form of medieval plague in Lopar contributed to the positive selection at CCR5del32, then its frequency in present-day population that evolved subsequent to this bottleneck effect would be increased in comparison with the contemporary populations of neighboring isolates not affected by plague.

Sample selection, genotyping, and statistical analysis

In both settlements, a random sample of 100 examinees was selected in order to study the frequency of CCR5del32 mutation. Methods of sampling of the examinees, field work activities, and procedures for obtaining the material, their storage, and transport to the Human Genetics Unit of the Medical Research Council (HGU MRC) in Edinburgh, UK were described in detail by Vitart et al (20). Genotyping the CCR5 polymorphism was performed at the HGU MRC using the polymerase chain reaction (PCR) method with the following primers flanking the region containing 32-bp deletion: forward primer – ACCAGATCT-CAAAAAGAAGGTCT, reverse primer – CATGATGGTGAAGATAAGCCTCACA.

The PCR products were analyzed by 2% agarose gel electrophoresis. The normal allele was detected as a 225bp fragment and the CCR5 32-bp deletion allele was detected as a 193bp fragment. These are standard methods to detect CCR5del32 mutation in humans and they have been described in greater detail elsewhere (3,8,18).

The differences in allele frequency of CCR5del32 between the two settlements, and between them and the general Croatian population, were determined by χ^2 test for independent samples (33). The analyses were performed in Microsoft Excel, using the program's statistical functions and χ-distribution.

Results

A total of 98 samples from Lopar and all 100 samples from Komiža were successfully genotyped (Table 1). Allele frequency of CCR5del32 was 6.0% in Lopar and 1.5% in Komiža. This gave the frequency of the wild-type allele CCR5

Table 1. Observed frequency of genotypes at CCR5 locus in Lopar and Komiža villages and differences in allele frequencies of del32 mutation between Lopar, Komiža, and general Croatian population*

	Setting		
Parameter	Lopar	Komiža	Croatia
Sample (No. subjects)	98	100	303
Number (%) of observed genotypes:			
CCR5WT/WT	87 (88.7%)	97 (97.0%)	263 (86.8%)
CCR5WT/del32	10 (10.2%)	3 (3.0%)	37 (12.2%)
CCR5del32/del32	1 (1.1%)	0 (0.0%)	3 (1%)
CCR5 del32 allele frequency	6.0%	1.5%	7.1%
P	0.651†	0.014‡	0.037§

*Data on general Croatian population from ref. 21.
†Lopar vs Croatian general population.
‡Komiža vs Croatian general population.
§Lopar vs Komiža.

of 94.0% in Lopar and 98.5% in Komiža. Given the observed allele frequencies, the expected genotype frequencies under Hardy-Weinberg equilibrium for wild type homozygotes (wt/wt) in Lopar and Komiža were equal to 88.4% and 97.0%; for CCR5del32 homozygotes (del32/del32) 0.4% and 0.0%; and for the heterozygotes 11.2% and 3.0%, respectively. When these expected genotype frequencies were compared with the observed genotype frequencies, it became apparent that they closely followed the expectations and that both populations were in Hardy-Weinberg equilibrium at CCR5 locus (Table 1).

According to our hypothesis of plague as the selective force responsible for the increase in the frequency of CCR5del32 among the Europeans, it was predicted that the frequency of CCR5del32 in Komiža would be significantly lower and significantly higher in Lopar than the general population average. The frequency in general Croatian population was 7.1%, based on a study of 303 blood donors (7). The analysis showed that the frequency in Komiža was indeed significantly lower than in both the general population ($P=0.014$) and in Lopar ($P=0.037$) (Table 1). However, the frequency in Lopar was not significantly different from the general population average.

Discussion

The fact that the frequency of CCR5del32 was significantly decreased in Komiža but not significantly increased in Lopar in comparison with the general population, has several possible interpretations. First, this does not rule out the possible role of medieval plague in the positive selection at CCR5del32. If this hypothesis was correct, then the low frequency of CCR5del32 in Komiža could be interpreted as the consequence of avoiding the epidemics during the Middle Ages. Under this hypothesis, the lack of increased frequency in Lopar could also be explained. During the medieval period, many different diseases associated with high mortality were termed "plague," some of them of different microbial etiology. Furthermore, the period of epidemic in Lopar (1449-1456) overlapped neither with the second pandemic (1347-1361) nor with the third pandemic in Europe (1665-1670). Therefore, if the CCR5del32-selective plague was caused by a different etiological agent than the "plague" that struck Lopar, then no difference would be expected between Lopar and the general population, as observed. In addition, perhaps the historical and cultural position of Rab island, that was not as isolated from the rest of the land as Vis island, contributed to this finding. Rab was hardly spared from the events taking place on the mainland, including epidemics, which could also be responsible for almost the same frequency of CCR5 del32 as in the general population.

However, an alternative hypothesis is also possible, in which "plague" does not represent a selective force behind the increase in CCR5del32 mutation. Under this hypothesis, Lopar frequency would again be the same as in general population, as observed. The decreased frequency in Komiža could then be explained in several ways: 1) as an expected consequence of the European north-south cline, although, as Lopar is situated in northern and Komiža in southern Adriatic Sea, which makes the expected difference due to the geographic cline unlikely to be substantial, the founder effect and subsequent genetic drift could have acted together to separate their frequencies further apart; 2) as a consequence of genetic drift toward the extinction of the allele in an isolated island community; 3) CCR5del32 positive selection was driven by another infectious disease (not plague), which was also absent in Komiža due to geographic isolation.

To resolve this uncertainty, further analyses of neighboring isolated island communities will be needed and the consistency of findings among the northern Adriatic and southern Adriatic villages may eventually provide more substantial support for the "plague" hypothesis or perhaps

rule it out. Whatever the reason for this allelic variation in different populations, it is still an important observation that homozygosity of the CCR5 32bp deletion protects individuals from HIV infection today and may have had an important role in the protection against some infectious agent in the past, which justifies the efforts to achieve greater understanding of this observation.

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References

1 Quillent C, Oberlin E, Braun J, Rousset D, Gonzalez-Canali G, Metais P, et al. HIV-1-resistance phenotype conferred by combination of two separate inherited mutations of CCR5 gene. Lancet. 1998;351:14-8. Medline:9433423

2 Schliekelman P, Garner C, Slatkin M. Natural selection and resistance to HIV. Nature. 2001;411:545-6. Medline:11385558

3 Libert F, Cochaux P, Beckman G, Samson M, Aksenova M, Cao A, et al. The deltaCCR5 mutation conferring protection against HIV-1 in Caucasian populations has a single and recent origin in Northeastern Europe. Hum Mol Genet. 1998;7:399-406. Medline:9466996

4 Jagodzinski PP, Lecybyl R, Ignacak M, Juszczyk J, Trzeciak WH. Distribution of del32 allele of the CCR5 gene in the population of Poland. J Hum Genet. 2000;45:271-4. Medline:11043507

5 Drabek J, Petrek M. 32 bp deletion in CCR-5 gene and human immunodeficiency virus epidemic in the Czech Republic. Acta Virol. 1998;42:121-2. Medline:9770081

6 Poljak M, Tomazic J, Seme K, Maticic M, Vidmar L. Prevalence of mutant CCR5 allele in Slovenian HIV-1-infected and non-infected individuals. Acta Virol. 1998; 42:23-6. Medline:9645239

7 Ristic S, Starcevic Cizmarevic N, Brajenovic-Milic B, Crnic-Martinovic M, Kapovic M. Frequency of CCR5 gene 32-basepair deletion in Croatian normal population. Croat Med J. 2005;46:693-4. Medline:16100775

8 Martinson JJ, Chapman NH, Rees DC, Liu YT, Clegg JB. Global distribution of the CCR5 gene 32-basepair deletion. Nat Genet. 1997;16:100-3. Medline:9140404

9 Duncan SR, Scott S, Duncan CJ. Reappraisal of the historical selective pressures for the CCR5-Delta32 mutation. J Med Genet. 2005;42:205-8. Medline:15744032

10 Lucotte G. Frequencies of 32 base pair deletion of the (Delta 32) allele of the CCR5 HIV-1 co-receptor gene in Caucasians: a comparative analysis. Infect Genet Evol. 2002;1:201-5. Medline:12798016

11 Stephens JC, Reich DE, Goldstein DB, Shin HD, Smith MW, Carrington M, et al. Dating the origin of the CCR5-Delta32 AIDS-resistance allele by the coalescence of haplotypes. Am J Hum Genet. 1998;62:1507-15. Medline:9585595

12 Bamshad MJ, Mummidi S, Gonzalez E, Ahuja SS, Dunn DM, Watkins WS, et al. A strong signature of balancing selection in the 5′ cis-regulatory region of CCR5. Proc Natl Acad Sci U S A. 2002;99:10539-44. Medline:12149450

13 Hummel S, Schmidt D, Kremeyer B, Herrmann B, Oppermann M. Detection of the CCR5-Delta32 HIV resistance gene in Bronze Age skeletons. Genes Immun. 2005;6:371-4. Medline:15815693

14 Kremeyer B, Hummel S, Herrmann B. Frequency analysis of the delta32ccr5 HIV resistance allele in a medieval plague mass grave. Anthropol Anz. 2005;63:13-22. Medline:15830584

15 Mecsas J, Franklin G, Kuziel WA, Brubaker RR, Falkow S, Mosier DE. Evolutionary genetics: CCR5 mutation and plague protection. Nature. 2004;427:606. Medline:14961112

16 Elvin SJ, Williamson ED, Scott JC, Smith JN, Perez De Lema G, Chilla S, et al. Evolutionary genetics: ambiguous role of CCR5 in Y. pestis infection. Nature. 2004;430:417. Medline:15272490

17 Galvani AP, Slatkin M. Evaluating plague and smallpox as historical selective pressures for the CCR5-Delta 32 HIV-resistance allele. Proc Natl Acad Sci U S A. 2003;100:15276-9. Medline:14645720

18 Sabeti PC, Walsh E, Schaffner SF, Varilly P, Fry B, Hutcheson HB, et al. The case for selection at CCR5-Delta32. PLoS Biol. 2005;3:e378. Medline:16248677

19 Rudan I, Campbell H, Rudan P. Genetic epidemiological studies of eastern Adriatic island isolates, Croatia: objectives and strategies. Coll Antropol. 1999;23:531-46. Medline:10646227

20 Vitart V, Biloglav Z, Hayward C, Janicijevic B, Smolej-Narancic N, Barac L, et al. 3000 years of solitude: extreme differentiation in the island isolates of Dalmatia, Croatia. Eur J Hum Genet. 2006;14:478-87. Medline:16493443

21 Burton RF. A visit to Lissa and Pelagosa. J Royal Geographic Society. 1879;49:151-90.

22 Regensburg F. Lissa [In German]. Stuttgart: Thieme; 1907.

23 Stanich S. Historical and critical appraisal on the island and the anthic city of Vis (Issa) [in Italian]. Roma: Archivio storico per la Dalmazia. 1926

24 Politeo D. Rab [in Croatian]. Vienac. 1895;28:1-40.

25 Azais P. The island Rab and the surrounding isolates [in Italian]. Milano: Archivio storico; 1922.

26 Praga G. The story on Rab island in a recent monography [in Italian]. Zara: Atti e memorie della Soc dalmata; 1926.

27 Buklijas T. Plague: forming the disease identity [in Croatian]. Hrvatska Revija. 2002;2:1-26.

28 Markovic M. Croatian Adriatic islands [in Croatian].

Zagreb: Naklada Jesenski i Turk; 2004.

29 Novak G. The island of Vis in the Middle Ages [in Croatian]. Zagreb: Starohrvatska prosvjeta; svezak 3; 1954.

30 Novak G. Vis. Volume one. Since 6th century BC to 1941 [in Croatian]. Zagreb: JAZU; 1961.

31 Skreblin L, Simicic L, Sujoldzic A. Ethnohistorical processes, demographic structure and linguistic determinants of the Island of Vis. Coll Antropol. 2002;26:333-50. Medline: 12137318

32 Smoljanovic M, Smoljanovic A, Nejasmic I. Population of Croatian islands [in Croatian]. Split: Zavod za javno zdravstvo Županije Splitsko-Dalmatinske; 1999.

33 Kirkwood BR, Sterne JA. Essentials of medical statistics. London: Blackwell Publishers; 2005.

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