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Discriminating between allelic and interlocus differences among human immunoglobulin V_H4 sequences by analyzing single spermatozoa

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Abstract To address the challenging issue of distinguishing allelic and interlocus differences among repetitive sequences, human immunoglobulin V_H4 loci in the parental haplotypes of 13 donors were determined by analyzing single spermatozoa. V_H4 sequences detected among these donors were assigned to their corresponding loci based on the fact that allelic sequences usually segregate into different gametes. Four out of the ten V_H4 loci were shown to contain null alleles that are undetectable with diploid materials. The distribution of the allelic variation within the analyzed regions at the V_H4 loci is highly biased.

Introduction

Although a large portion of the human genome consists of repetitive sequences (Schmid and Deininger 1975; Houck et al. 1979), little is known about the detailed physical structure of the chromosomal regions containing these sequences. One of the difficult issues is how to distinguish allelic and interlocus differences for these sequences because the differences among the sequences from different loci are usually small and indistinguishable from allelic differences. In this report, this issue is addressed by a genetic approach using the human immunoglobulin V_H4 gene family as an experimental system. The human V_H segments are subdivided into seven families (Lee et al. 1987; Schroeder et al. 1987; Berman et al. 1988; Buluwela and Rabbitts 1988; Humphries et al. 1988; Tomlinson et al. 1992; van Dijk et al. 1993). Segments in each family share > 80% sequence identity. The V_H sequences are also highly polymorphic. In a Southern analysis with a short oligonucleotide probe flanking the 5′ end of the V_H3 gene segments, Souroujon et al. (1989) observed 11 bands, of which 7 (64%) were polymorphic in a presence/absence fashion. Of the 22 bands detected with a V_H3 oligonucleotide probe by van Dijk et al. (1989), 9 (42%) were presence/absence polymorphisms. Sasso et al. (1990) showed that three V_H3 gene segments, 56p1, T5M10, and 1.9III, had a prevalence of 62%, 35%, and 92%, respectively. In the study by van Dijk et al. (1991), 15 restriction fragments were detected by a V_H4 oligonucleotide probe, 12 were polymorphic and the prevalence of these fragments ranged from 1% to 97%. Similar results were obtained by Rubinstein et al. (1993) with oligonucleotide probes for the V_H2 and V_H4 gene families. With two-dimensional gel electrophoresis, Walter et al. (1990) showed that 9 of 12 characterized V_H gene polymorphisms were insertion/deletion polymorphisms. The high degree of sequence identity shared by the large number of V_H segments and the presence of extensive polymorphisms has hindered understanding of the detailed physical structure of the V_H region. For a long while, estimates of the number of V_H segments in each haploid genome varied widely (Berman et al. 1988; Walter and Cox 1988; Souroujon et al. 1989).

Recently, we have developed an efficient method for V_H sequence identification (X. Cui and H. Li, submitted). With this method, the sequences of a V_H family are amplified by family-specific PCR primers. The amplified products from different loci are of the same or similar lengths and are resolved by denaturation gradient gel electrophoresis (DGGE), capable of resolving DNA fragments differing by as few as 1 bp (Fischer and Lerman 1980, 1983; Myers et al. 1985 a, b; Sheffield et al. 1989). By testing different primer combinations, we maximized the inclusion of the V_H4 sequences. With the optimized primer pair set, sequences from all distinct V_H4 loci could be amplified. Eighteen bands were detected from a panel of 41 human subjects. These bands were named in numbers with the prefix 4, based on their positions on the gel from top to bottom. The same nomenclature system is used in the current study. Unlike the traditional Southern analysis, the DGGE method allowed us to determine the nucleotide sequences of the fragments resolved by DGGE

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after excising these bands from the gel and reamplifying the fragments by PCR. In the current study, we applied the PCR-DGGE method to determine the V_H4 segments in the parental haplotypes of the donors by analyzing single spermatozoa. Other than the very rare alleles (each of which was detected from only one haplotype), all detected V_H4 alleles could be assigned to the corresponding loci without ambiguity.

Materials and methods

Single sperm sample preparation

Semen samples used in this study were collected from 13 unrelated healthy donors. Of these donors, eight lived in the Los Angeles area and five in the Philadelphia area. Eleven were Caucasian, one Hispanic, and one African-American. Single sperm samples were prepared by the method described by Lien et al. (1993) with modifications. Briefly, the purified sperm were mixed with 0.5% low melting point agarose in H_2O. The mixture was plated on a glass slide to form a thin layer. After air drying for 10 min, single sperm were scraped up with a syringe needle under a microscope, placed into microtubes, and lysed according to Cui et al. (1989).

PCR

The V_H4 segments in each sperm were amplified by a two-round PCR protocol with the previously optimized primer set mentioned in the Introduction. In the first round, two family-specific primers, V4M1BC (a 2:1 mixture of two sequences of 5′ CTCACATGGGAAATACTTTCTGAGA 3′ and 5′ CTCACATGGGAAGTGCTTTCTGAGA 3′) and V4M4 (5′ cgccgcccccgccccGAAGGCTTCACCAGTCCTG 3′), were used. PCR was performed in a DNA thermal cycler 480 (Perkin-Elmer). Each reaction (50 µl) contained 1 × PCR buffer (50 mM KCl, 100 mM TRIS-HCl, pH 8.3, 1.5 mM $MgCl_2$, 0.1 mg/ml gelatin), four dNTPs (each at 100 µM), primers (each at 0.2 µM), and 1 U *Taq* DNA polymerase (Perkin-Elmer). For each PCR cycle, 95°C for 30 s was used for denaturation and 72°C for 30 s for extension. The annealing step was at 50°C for 5 min for the first three cycles, 55°C for 3 min for the following seven cycles, and 60°C for 2 min for the last 30 cycles. In the second round, a 2-µl aliquot from each PCR product was reamplified in a final volume of 50 µl with primer V4M2 (5′ GAACATGAAACACCTGTGGTTCT 3′) which was used to replace V4M1BC and was internal (nested) with respect to V4M1BC and the primer GC tail (5′ cgcccgccgcgcccgcgcccgtcccgccgcccccgcccc 3′) containing a 25-base GC-rich domain at it 5′-end and a 15-base domain at its 3′-end. The latter was identical to the GC-rich non-genomic sequence at the 5′-end of V4M4. The region flanked by the primers V4M2 and V4M4 was ~ 145 bp. The PCR thermal profiles for the first and second steps were 95°C for 30 s for denaturation and 72°C for 30 s for extension. The annealing step was 55°C for 1 min for the first three cycles and 60°C for 1 min for the following 25 cycles. In the third step, 10 µl of PCR mix containing the primers (each at 2 µM) and 1 U *Taq* polymerase was added to each reaction. One PCR cycle was performed with 95°C for 2 min for denaturation, 60°C for 1 min for annealing, and 72°C for 10 min for extension to minimize the amount of DNA heteroduplexes which may form additional DGGE bands.

DGGE

The DGGE gels (10% polyacrylamide) were prepared according to the manufacturer of the DGGE apparatus (C.B.S. Scientific, California). The denaturing gradient was from 55% to 73%. From each PCR product, 15 µl was used for resolving the V_H4 sequences by electrophoresis at 60°C and 113 V for 15 h.

Results

Determination of the V_H4 segments in individual haplotypes

After lysis, the V_H4 gene segments in single sperm were amplified by the two-round PCR protocol described in Materials and Methods. The final PCR products from different V_H4 loci were of the same or similar lengths and were resolved by DGGE. To ensure that the parental haplotypes were correctly determined, 20-40 single sperm from each donor were analyzed. Based on the banding patterns, the majority of sperm from each donor could be divided into two groups. The two parental haplotypes (I and II) of each donor were deduced, based on the banding patterns of these groups. A few sperm from some donors were either poorly lysed, poorly amplified or, apparently,

Table 1 Banding patterns for the single sperm from donor L1. (Note: because only one copy of each target sequence was used at the start of the PCR, some bands were not detected in a small fraction of sperm, for example band 3 in samples 53-16 and 53-18. The cause for this may be mainly because of insufficient PCR amplification at the corresponding loci.)

Sperm number	Band											
	1	2	3	4	5	7	9	10	12	14	17	18
Haplotype I	±	±	±		±	±	±	±	±			±
53–12	+	+			+	+	+	+	+			+
53–16	+	+	+			+	+	+	+			+
53–17	+	+	+		+	+	+	+	+			+
53–18	+	+			+	+	+	+	+			+
53–20	+	+	+		+	+	+	+	+			+
54–01	+	+	+		+	+	+	+	+			+
54–05	+	+	+		+	+	+	+	+			+
54–12	+	+	+		+	+	+	+	+			+
54–17	+	+				+		+	+			
54–23	+	+	+		+	+	+	+	+			+
54–24	+	+	+		+	+	+	+	+			+
54–25	+	+	+		+	+	+	+	+			+
54–26	+	+			+	+	+	+	+			+
Haplotype II	±	±	±	±	±		±	±	±	±	±	
53–11	+	+	+	+	+		+	+	+	+	+	
53–13	+	+	+	+	+		+	+	+		+	
53–19	+	+	+	+	+		+	+	+	+	+	
54–02	+	+	+	+	+		+	+	+	+	+	
54–03	+	+	+	+	+		+	+	+	+	+	
54–04	+	+	+	+	+		+	+	+	+	+	
54–06	+	+	+	+	+		+	+	+	+	+	
54–07	+	+	+	+	+		+	+	+	+		
54–08				+								
54–11	+	+		+	+		+	+	+	+	+	
54–15	+	+	+	+	+		+	+	+	+	+	
54–16	+	+	+	+	+		+	+	+	+	+	
54–18	+	+			+		+	+	+			
54–19	+	+	+	+	+		+	+	+	+	+	
54–20	+	+	+	+	+		+	+	+	+	+	
54–21			+	+				+	+	+	+	
Others												
53–15	+	+	+	+	+	+	+	+	+	+		
54–14							+					

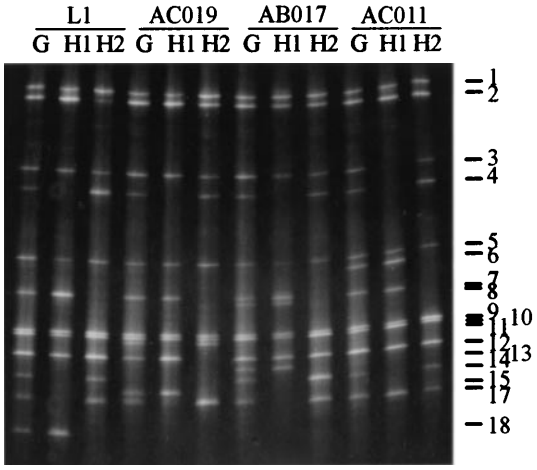

Fig. 1 Denaturation gradient gel electrophoretic banding patterns for individual sperm with different haplotypes (*H1* and *H2*) and for the genomic DNA *(G)* from the corresponding donor

recombinants. Therefore, these sperm were placed in the third (or Others) group. The results from the sperm of donor L1 are shown in Table 1. It is clear that haplotype I, deduced from the banding patterns of the 13 single sperm contains the V_H4 sequences represented by the nine bands 1, 2, 3, 5, 7, 9, 10, 12, and 18, while haplotype II determined by the banding patterns of the 16 single sperm contains the V_H4 sequences represented by the ten DGGE bands 1, 2, 3, 4, 5, 9, 10, 12, 14, and 17. It is obvious that haplotype II contains one more V_H4 sequence than haplotype I. The two haplotypes differ at the V_H4 loci represented by bands 4, 7, 14, 17, and 18. One sperm, 54-16, in the Others group is apparently abnormal because bands 4 and 7 were codetected from this sperm but not from any others. The haplotype of another sperm, 54-14, in this group remains unclear because only one band which was detected from both haplotypes was observed. By the same approach, the haplotypes in the additional 12 individuals were determined. The banding patterns of sperm with different haplotypes (lanes H1 and H2) from four donors are shwon in Fig. 1. The banding patterns from all 26 haplotypes are listed in Table 2.

V_H4 loci and their alleles determined by the banding patterns of the haplotypes

Because alleles at each locus usually segregate into the gametes with different parental haplotypes, they should not be codetected from all single sperm samples other than those subjected to certain unequal crossovers during meiosis. However, such genetic events should be rare and the resulting haplotypes should not be dominant among the meiotic products. Based on this principle, the bands representing corresponding loci are grouped and the results are listed in Table 2. Ten bands, 1, 2, 3, 4, 5, 9, 10, 12, 14, and 17, were codetected from five haplotypes (L1-2, L16-1, AB017-2, AC011-2, and WL77-2; Table 2). In

Table 2 V_H4 sequences detected in individual haplotypes. The two haplotypes of each sample are distinguished by -1 and -2 after the sample names. The bands with the highest frequencies are listed in the table heading with + indicating that the corresponding band was detected and - not detected. Bands with lower frequencies are listed with the assigned loci from which the alleles with highest frequencies were not detected in the corresponding haplotypes

Haplotype	Segment number	Band									
		1	2	3	4	5	9	10	12	14	17
L1-1	9	+	+	+	7	+	+	+	+	–	18
L1-2	10	+	+	+	+	+	+	+	+	+	+
L9-1	10	+	+	+	+	+	+	+	+	11	+
L9-2	10	+	+	+	+	+	+	+	+	+	16
L15-1	10	+	+	+	+	+	+	+	+	+	16
L15-2	10	+	+	+	+	+	+	+	+	+	16
L16-1	10	+	+	+	+	+	+	+	+	+	+
L16-2	9	+	+	+	+	–	+	+	+	+	16
L18-1	10	+	+	+	+	+	+	+	+	+	16
L18-2	9	+	+	+	7	+	+	+	+	–	+
WL29-1	10	+	+	+	+	+	+	+	+	+	16
WL29-2	8	+	+	+	7	–	+	+	+	–	+
AB002-1	8	+	+	+	7	+	–	+	+	–	+
AB002-2	10	+	+	+	+	+	+	+	+	+	16
AB017-1	10	+	+	+	7	+	+	+	+	8	13
AB017-2	10	+	+	+	+	+	+	+	+	+	+
AC011-1	9	+	+	6	7	+	+	+	+	–	+
AC011-2	10	+	+	+	+	+	+	+	+	+	+
AC019-1	9	+	+	+	7	+	+	+	+	–	15
AC019-2	9	+	+	+	+	+	+	+	–	11	+
AC016-1	10	+	+	+	+	+	+	+	+	11	+
AC016-2	9	+	+	+	+	+	–	+	+	+	16
WL44-1	10	+	+	+	+	+	+	+	+	11	16
WL44-2	10	+	+	+	+	+	+	+	+	+	16
WL77-1	10	+	+	+	+	+	+	+	+	11	+
WL77-2	10	+	+	+	+	+	+	+	+	+	+

many other haplotypes, eight or nine of these ten bands were codetected. Therefore, these bands should represent distinct V_H4 loci. Three bands, 7, 11, and 16, were detected from at least five haplotypes from which bands 4, 14, and 17 were not detected, respectively. It is likely that these two groups of bands represent the alleles from three distinct loci.

Five bands, 6, 8, 13, 15, and 18, were each detected from only one haplotype. Normally, these bands should be readily assigned to the corresponding loci without ambiguity. However, bands 8 and 13 were detected from the same haplotype and null alleles were detected from more than one locus in the haplotypes from which bands 6, 15, and 18 were detected. Given these complications, these bands cannot be precisely assigned based on the sperm analysis. However, it is clear that only two possible loci can be considered for assigning each of these bands. The

locus represented by band 14 is one of the candidate loci for all these bands. These bands were finally assigned as shown in Table 2, based on our previous sequence data (X. Cui and H. Li, submitted). The locus represented by band 14 was eliminated for assigning bands 6, 15, and 18 because the sequences from these rare bands are 16, 6, and 6 bp different from the sequences from band 14, respectively, while only 1 bp different from the common alleles of the corresponding assigned loci. Bands 13 and 8 were detected from the same haplotype. Because the sequence from band 13 is only 1 bp different from that of band 16 and 4 bp from that of band 14, band 13 was assigned to the locus represented by band 16. The locus represented by band 14 is the only possibility left for band 8. Therefore, the PCR-DGGE method resolved 18 bands from ten V_H4 loci represented by bands 1, 2, 3 (and 6), 4 (and 7), 5, 9, 10, 12, 14 (8 and 11), 17 (13, 15, 16, and 18). Results from sequence analysis indicate that these bands were amplified from the previously reported V_H4 loci, 4-34, DP-69, 4-80, 4-59, 4-39, 4-31, 4-55, 4-30.2, 4-61, and 4-4 (Matsuda et al. 1993; Cook et al. 1994; Tomlinson et al. 1994), respectively.

The V_H4 region diversity at the V_H4 loci

As shown in Table 2, the V_H region is diversified at both gene and nucleotide sequence levels. At the gene level, null alleles were detected at four (4-5, 4-9, 4-12, and 4-14) of the ten loci. The frequencies for these null alleles range from 3.8% to 23.1%. Because each of these null alleles was detected from only one of the two parental haplotypes of the corresponding donor, they would be undetectable if diploid materials were used. On the other hand, because of the null alleles, the numbers of V_H4 gene segments in nine (34.6%) haplotypes are less than the ten detected in the other 17 haplotypes (Table 2). Three of the ten V_H4 loci differ in a presence/absence fashion between the four pairs of haplotypes, WL29-2 and AC016-2, WL29-2 and AC019-2, AB002-1 and AC019-2, and AB002-1 and L16-2.

Because of the assignment of the V_H4 sequences to the corresponding loci, the V region diversification at the nucleotide sequence level becomes clear. Each of the four loci, 4-3, 4-4, 4-14 and 4-17, has more than one plus allele. Based on previous sequence data (X. Cui and H. Li, submitted), the allelic differences in the 145-bp amplified regions are: 1 bp at 4-3, 1 bp at 4-4, 6 bp at 4-14, and 4 bp at 4-17.

Discussion

Directly examining the nature of the differences among the V_H sequences requires comparison of the organization of these sequences in corresponding haplotypes. If two V_H sequences in different haplotypes are shown at the same position, they are clearly alleles. Otherwise, they are sequences of different loci. However, determining the V_H segment organization in individual haplotypes is a challenging issue because of the human genome diploidy, the large number of V_H segments sharing high degrees of sequence identity, and the presence of extensive polymorphisms in this region. Although family analysis can be used to obtain some information, it is limited by the facts that all human subjects are diploid, many loci in each family are not informative, and families are informative at different loci. In a previous study (X. Cui and H. Li, submitted), we analyzed a panel of unrelated human subjects with the PCR-DGGE method. The frequency of each allele at a V_H locus was estimated, based on the rates of the DGGE band detection, by solving the equation:

$$\text{Band frequency} = x^2 + 2x(1-x)$$

where x is the estimated allele frequency. Because the sum of the frequencies for any two alleles at a genetic locus should be equal to or less than 100%, the allelic and interlocus differences among certain V_H sequences could be discriminated. However, this method is limited by its incapapacity to discriminate between the sequences from the V_H loci with multiple alleles. When each locus has several alleles and more than one locus is involved in the analysis, it is difficult to determine to which locus an allele belongs. In the current study, the parental haplotypes of 13 sperm donors were determined by analyzing the V_H4 sequences in single sperm with the PCR-DGGE approach. Other than the rare alleles, each of which was detected from only one haplotype, all alleles were assigned to the V_H4 loci without ambiguity. For the rare alleles, the number of possible candidate loci was limited to only two for each. With the sequence data, these alleles were assigned to the V_H4 loci with very limited ambiguity.

Successfully assigning the alleles to the corresponding V_H4 loci allowed us to reveal the null alleles at four out of the ten V_H4 loci. It is likely that the detection of most, if not all, of these null alleles was not because of insufficient PCR amplification caused by mismatches between the primers and the templates. First, under the PCR conditions used, a target sequence may not be amplified well only if the mismatches are located either actually at, or only 1 or 2 bp away from, the 3′ ends of the primers used in the first round. To address this problem, we have tested several primer combinations with additional primers, and the same results were obtained. In addition, because only one plus allele was detected from each of the three loci (4–5, 4–9, and 4–12) among the four loci with null alleles, allelic variation caused by base changes in the amplified regions from these loci seems very rare. Interestingly, although the null alleles were identified in nine (34.6%) out of the 26 haplotypes, they were identified from different loci. Therefore, these null alleles are all cryptic in the analyzed donors, i.e., they would not be detectable if diploid materials were used. Therefore, most carriers of these null alleles in the human population may have at least one copy of the corresponding V_H4 segments at each locus. It is important to learn whether homozygosity at one or more of these loci for the null alleles is associated with susceptibility to certain immune defects, or whether some immune defects are caused by such a homozygosity.

Precisely assigning the alleles to the corresponding loci also allowed us to analyze the distribution of the allelic base substitutions among the V_H4 loci. Of the 14 base substitutions, 12 are located within the sequences amplified from 4-14 and 4-17. It is of biological significance to know whether the regions immediately adjacent to these amplified regions are also hypervariable and whether this hypervariability is restricted to some V_H4 regions.

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