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Michael J. Atkinson · Morton J. Cowan · Ann J. Feeney

New alleles of *IGKV* genes *A2* and *A18* suggest significant human *IGKV* locus polymorphism

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Abstract The human kappa light chain consists of approximately 35 potentially functional *IGKV* genes. However, an estimation of the diversity in the *IGKV* repertoire of an individual will be affected by the extent of polymorphisms for the different *IGKV* genes and their patterns of inheritance. To date, little information is available to indicate the extent of allelic variation of the *IGKV* genes. We examined the extent of allelism for one *IGKV* gene pair, the distal region *A2* gene and its closely related proximal region duplicate *A18*. We found two new alleles for *A2* and one new allele for *A18*, and sequenced ~1 kilobase flanking each gene. The new *A18* allele, unlike the originally described allele, appears to be functional. All these alleles were found at relatively high frequencies in the four ethnic populations studied, with the exception of the defective *A2b* allele which was highly represented only in Navajos. The originally described *A2a* allele encodes for the predominant protective antibody against *Haemophilus influenzae*. Therefore, the patterns of allelic inheritance described for this *IGKV* gene pair indicate that allelism in the *IGKV* locus is likely to have a significant impact on immune responses.

Introduction

The human immunoglobulin (*Ig*) kappa light chain locus consists of one *IGKC*, 76 *IGKV*, and five *IGKJ* gene segments (Schäble and Zachau 1993). These *IGKV* gene segments are organized into two regions, a J-C proximal region consisting of 40 *IGKV* gene segments and a distal region consisting of 36 *IGKV* gene segments. The proximal (p) and distal (d) *IGKV* regions are separated by an 800 kilobase (kb) stretch of DNA that appears to be free of *IGKV* gene segments (Schäble and Zachau 1993). The entire *IGKV* locus spans 1800 kb. The distal region appears to have arisen as a duplication of most of the proximal locus. Consequently, 36 of the 40 proximal *IGKV* genes have similar or identical gene segments present in a transcriptionally inverted orientation in a much more *IGKJ*-distal location.

Zachau and colleagues mapped, cloned, and sequenced all *IGKV*-like gene segments in human DNA (Schäble and Zachau 1993). *IGKV* genes were categorized into those which, according to sequence analysis, are potentially functional, those which have minor defects, and those which are pseudogenes (Zachau 1989). There are 35 potentially functional genes, 16 genes with minor defects, and 25 pseudogenes. Minor defects include one or two base pair (bp) changes that create stop codons, or alter splice donor/acceptor sequences, recombination signal sequences (RSS), or conserved promoter elements. Additional defects may alter conserved amino acids in framework regions. Consequently, these minor changes result in non-functional genes. However, they are classified as minor defects, since it is possible that a change in the few aberrant nucleotides could create a functional allele of the gene.

IGKV gene polymorphism could have significant effects on diversity of the germline repertoire and immune responses. Polymorphisms may result in the disruption of an *IGKV* gene segment resulting in a non-functional allele. Other polymorphisms may result in alleles with significant changes in the coding region of functional *IGKV* genes, thereby changing the antigen specificity. Another possibility is the appearance of functional alleles of genes that have been described as having minor defects. To date, three *IGKV* genes have been identified that have both functional and non-functional alleles (Liu et al. 1989; Pargent et al. 1991a; Pech et al. 1984). Therefore, the estimation of polymorphism within the *IGKV* locus and the potential repertoire of unique antigen binding specificities will increase as more germline sequences from a diverse array of individuals are analyzed. Here we report *A2/A18* gene pair polymorphism.

M. J. Atkinson · A.J. Feeney (✉)
The Scripps Research Institute, Department of Immunology, IMM-22, 10666 North Torrey Pines Road, La Jolla, CA 92037, USA

M. J. Cowan
University of California, San Francisco, CA 94143 USA

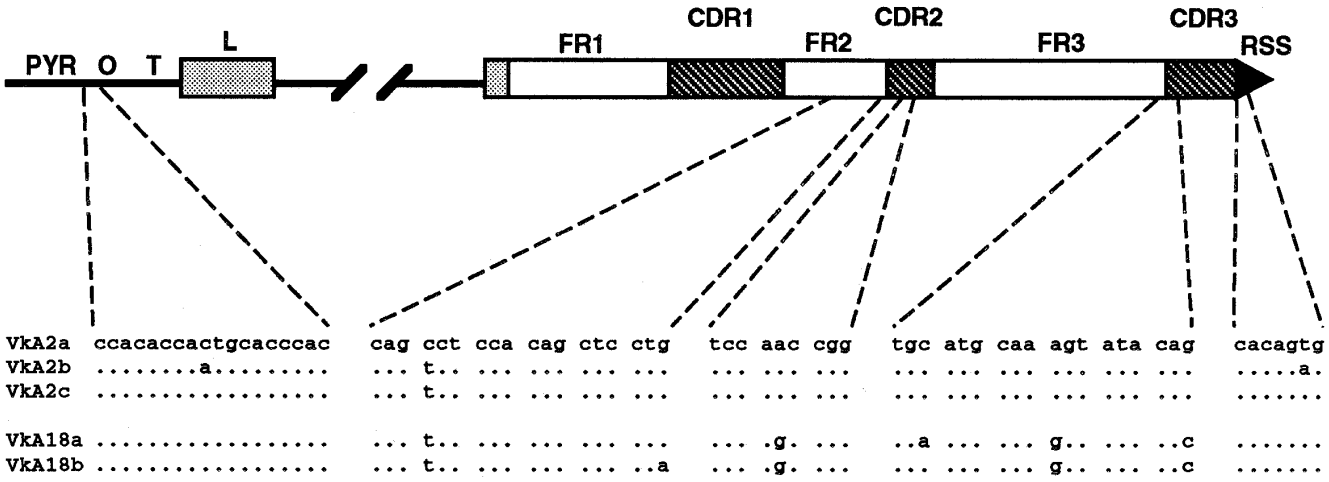

Fig. 1 Alleles of *A2* and *A18*. A diagram of the *IGKV* gene spanning 1.1 kb of DNA is illustrated here to show the regions where polymorphic residues of the indicated alleles are located and their composition. *PYR* Pyrimidine rich sequence; *O* Octamer/decamer box; *T* TATA box; *L* Leader peptide sequence (*lightly shaded boxes*); *FR1, FR2, FR3* Framework region sequences (*open boxes*); *CDR1, CDR2, CDR3* Complementarity determining regions (*darkly shaded boxes*); *RSS* Recombination signal sequence (*solid triangle*). Not illustrated here are a further eight nucleotide differences between *A2a* and *A18a* summarized previously (Schäble and Zachau 1993). The complete sequences of *A2b, A2c*, and *A18b* can be obtained from GenBank (accession numbers U41643, U41644 and U41645)

Materials and methods

DNA preparation

DNA was prepared according to standard procedures (Maniatis et al. 1982) from buffy coat white blood cells of healthy unrelated Navajos, collected as part of an ongoing gene linkage study in Athabascan-speaking Native Americans. All other peripheral blood samples were obtained from healthy donors at the Scripps General Clinical Research Center and peripheral blood mononuclear cells (PBMC) were enriched on Ficoll-Hypaque gradients. DNA was prepared as previously described (Chukwuocha et al. 1995).

PCR analysis

The polymerase chain reaction (PCR) was performed with a two-layer reagent system separated by paraffin wax. The 25 µl lower layer contained 300 ng of each oligonucleotide primer, 4.5 mM $MgCl_2$, 1 × PCR buffer (Promega, Madison, WI), and 0.5 mM dNTP. The 50 µl upper layer contained 1.4 × PCR buffer, 1.5 units of *Taq* polymerase (Promega), 0.04 units of PFU polymerase (Stratagene, La Jolla, CA), and 100–200 ng of DNA. The primers used for PCR amplification of germline genes were: 5′ *A2/A18*: AF76-ATGGCTCGAGCCACAC-CACTGCA and AF100-GCTCCTCGAGAAGACATATCTACC, 3′ *A2/A18*: AF68-CTGAGCGGCCGCCCAGACAAGCAGTGCAAG; and for rearranged genes, *A2/A18* intron: AF90-CATTAAGCTTTCA-CATAACCTTGCAC and *IGKJ*: AF80-ACGTTTGAATTC-CACCTTGGTCCC. PCR reactions were performed for one cycle at 94 °C for 4 min, 25–30 cycles of [(94 °C for 1 min) (55 °C for 1 min) (72 °C for 2 min)] and one cycle at 72 °C for 5 min.

Cloning and sequencing

The PCR primers used in this study were designed to incorporate a *Xho* I restriction site in the 5′ primer and a *Not* I restriction site in the 3′ primer. Following PCR amplification the DNA was digested with *Xho* I and *Not* I. Bands were size selected on 1.5% agarose gels, excised, and purified by the Qiaex gel extraction kit (Qiagen, Studio City, CA). The purified *Xho* I-*Not* I fragment was ligated into precut pBluescript (Stratagene), and electroporated into XL1 Blue electrocompetent bacteria (Stratagene). Mini-preps and sequencing were done as previously described (Feeney and Thuerauf 1989).

Statistics

A chi-square analysis was performed to determine whether there was random association between the observed alleles at the *A2* and *A18* loci. Haplotypes could not be unambiguously assigned for much of the data generated by the PCR analysis of alleles. Therefore, we tested for random association for all possible allele combinations versus the allele combinations actually seen in our data set. For both control and Navajo populations, a 6 × 3 contingency table was generated testing all possible combinations of *A2* and *A18* alleles. The statistic was calculated using Fisher's exact test with 10 degrees of freedom.

Results

Germline DNA sequence analysis

In order to determine the germline sequence of the *IGKV* genes, PBMC DNA was amplified with primers 5′ of the coding region of the gene and 3′ of the RSS. The distal region *A2* and its proximal region partner *A18* have only 15 nucleotide differences in 1242 bp of total reported sequence including 5′ and 3′ flanking DNA (Lautner-Rieske et al. 1992; Scott et al. 1989). The PCR primers selected for this study were designed to recognize conserved sequences flanking both *A2* and *A18* and therefore should amplify these genes with similar efficiency. Indeed, of the 866 clones analyzed, 49.3% were *A2* alleles and 50.7% were *A18* alleles. Initially, the AF76 and AF68 primer pair was used to amplify *A2* and *A18* genes. The PCR product for this reaction was 955 bp, and it spanned all of the *V* region coding sequence, intron, leader peptide, and some promoter sequence. Full sequence analysis of PCR products from several individuals revealed three new alleles of the *A2/A18*

gene pair. Since much of the promoter region was not included in this PCR product, longer PCR reactions were performed using the AF100/AF68 primer pairs and the entire PCR product was sequenced. This additional promoter region was fully sequenced for at least two independent samples for each new allele.

Seven polymorphic residues are clustered toward the 3′ end of the gene (Fig. 1) and allowed for easy screening using a single sequencing reaction for each clone. Typically, ten clones for each PCR reaction were sequenced and allotyped. There were many instances where genotypes could not be unambiguously assigned to an individual after analysis of one PCR reaction (e.g., instances where only one allele was identified for either *IGKV* gene). In these cases two or more sets of PCR amplification, cloning, and sequencing were performed.

Alleles of A2

A2 (designated here *A2a*) was originally described by Scott and co-workers (1989) as the functional distal region gene segment of the *A2/A18* pair. Sequence analysis of *A2* clones in this study revealed two new alleles for this gene, designated *A2b* and *A2c* (Fig. 1). *A2c* has only one nucleotide difference in framework region 2 (FR2), resulting in a serine to proline change.

A2b has three differences compared with the *A2a* allele (Feeney et al. 1996). The first change is C to A and occurs in the promoter region near a pyrimidine-rich sequence 5′ of the octamer. The second change is from proline to serine in FR2, identical to the change seen in the *A2c* allele. The third change is a T to A change in the heptamer sequence of the RSS flanking the coding region. This results in a change from the consensus CACAGTG to CACAGAG. This deviation from the consensus RSS has a significant impact on the recombination efficiency of this allele (Feeney et al. 1996).

New functional A18 allele

The reported sequence for *A18* (Lautner-Rieske et al. 1992) is that of a non-functional gene, since it has a termination codon instead of a conserved cysteine in FR3. We designated this allele *A18a*. A second allele for *A18* was identified in this study and designated *A18b* (Fig. 1). *A18b* has two bp differences from *A18a*: one is a G to A change in FR2 and is silent; the second is an A to C change in FR3. This second change results in the loss of the stop codon and restoration of the cysteine seen in all functional kappa light chains.

To determine whether *A18b* was a functional gene, we analyzed *V-J* rearrangements involving *A18b*. *A2/A18* rearrangements were amplified from several individuals and 15 rearrangements utilizing *A18b* were obtained. Of these *A18b* rearrangements, 12 were in frame indicating that cellular selection was operating on B cells using this gene product. Therefore, *A18b* can be considered a functional allele. This *A18b* allele differs from the functional *A2a* allele by four amino acids, three of which are located in complementarity determining regions (CDR). Therefore, the expressed A18b protein may have a germline antigen specificity different from A2a.

Table 1 Distribution of *A2* and *A18* alleles in individuals from different populations

A Caucasian, Hispanic, and Asian populations

		A2a	*A2b*	*A2c*	*A18a*	*A18b*
H1	Caucasian	+/+			+/+	
H5	Caucasian	+/+			+/+	
H6	Caucasian	+/+			+/+	
H10	Caucasian	+/+			+/+	
H12	Caucasian	+/+			+/+	
H7	Caucasian	+/+			+	+
H4	Caucasian			+/+	+	+
H15	Caucasian	+		+	+	+
Frequency		.81	.00	.19	.81	.19
H8	Hispanic	+/+			+/+	
H9	Hispanic		+	+	+	+
H11	Hispanic	+		+	+	+
H13	Hispanic	+		+	+	+
H2	Asian	+		+	+	+
H3	Asian	+		+	+	+
H14	Asian			+/+	+	+
Frequency		.43	.07	.50	.57	.43
Total frequency		.63	.03	.34	.70	.30

b Navajo population

	A2a	*A2b*	*A2c*	*A18a*	*A18b*
LC	+/+			+/+	
RC	+/+			+/+	
EB	+/+			+/+	
N14	+/+			+/+	
N16	+/+			+/+	
N2			+/+		+/+
N5			+/+		+/+
N17		+/+		+/+	
N20		+/+		+	+
HC	+	+		+/+	
KB	+	+		+/+	
N9	+	+		+/+	
N21	+	+		+/+	
N23	+	+		+/+	
N18	+	+		+/+	
N19	+		+	+/+	
N4		+	+		+/+
N12		+	+		+/+
N27		+	+		+/+
N22	+	+		+	+
N3		+	+	+	+
N15		+	+	+	+
N11		+		+	+
N1	+		+	+	+
N8	+		+	+	+
N10	+		+	+	+
N24	+		+	+	+
N26	+		+	+	+
Total Frequency	.41	.32	.27	.64	.36

Distribution of A2 and A18 alleles

Non-Navajo populations

As this study was initially undertaken to analyze possible *A2* polymorphisms in Navajos, we have a larger database of Navajo genotypes than of other ethnic groups. Our randomly sampled control population includes eight Caucasians, three Asians, and four Hispanics (Table 1). For the *A2* gene, the most frequent allele was *A2a* occurring with a frequency of 0.63, followed by *A2c* with a frequency of 0.34. The *A2b* allele was found only on a single individual and occurred within this population with a frequency of 0.03. This individual is a Hispanic originally from Mexico. The occurrence of homozygous individuals agrees fairly well with these figures. There were five *A2a* homozygotes (expected is $0.63^2 \times 15 = 6.0$), and two *A2c* homozygotes (expected is 1.7). The *A18a* allele occurred with a frequency of 0.70 and the *A18b* allele with a frequency of 0.30. There were five *A18a* homozygotes (expected is 7.4) and no *A18b* (expected is 1.4). Thus, the distribution of individuals homozygous for these alleles follows a classical Mendelian pattern of inheritance.

The largest control group studied were Caucasians and we determined the distribution of alleles (Table 1). This group had the highest frequency of the *A2a* and *A18b* alleles (0.81 for both alleles).

Navajo population

We examined DNA samples from a total of 28 Navajos (Table 1). For the *A2* gene, the *A2a* allele occurred with a frequency of 0.41 and the *A2c* allele with a frequency of 0.27. Thus, these two alleles were represented at a slightly lower frequency in the Navajo compared with control populations. The striking difference was in the frequency of the defective *A2b* allele, which was represented in the Navajo population with a frequency of 0.32. There were six *A18a* homozygotes (expected is $0.41^2 \times 28 = 4.7$) and no *A2c* homozygotes (expected is 2.0). There were two *A2b* homozygotes (expected is 2.9), with one individual, N11, potentially hemizygous for the *A2* gene. From three different PCR reactions for N11, we obtained 24 *A18* clones and five *A2* clones. Individuals have been described who are missing the distal half of the *IGKV* locus containing *A2* (Pargent et al. 1991b; Scott et al. 1991; Weichhold et al. 1993), but the small amount of DNA available precluded Southern analysis to confirm this speculation.

The expected frequency of *A2b* homozygotes in the Navajos is approximately 100 times greater (0.09 vs 0.001) than that expected for the control population. The *A18a* allele occurred with a frequency of 0.64 and the *A18b* allele with a frequency of 0.36, essentially the same as the control population. There were 16 *A18a* homozygotes (expected is 11.5) and five *A18b* homozygotes (expected is 3.6).

Linkage of A2 and A18 alleles

The association between *A2* and *A18* alleles, or linkage disequilibrium, was determined for Navajo and control populations. Both showed very significant disequilibrium (control population $P < 0.0004$, Navajo population $P < 0.001$, chi-square analysis). Examination of the allotype data (Table 1) indicates the sources of disequilibrium between these two loci. The *A2a* allele is almost always seen in association with the *A18a* allele and the *A2c* allele is very often seen in association with the *A18b* allele. In contrast, *A2b* can be associated with either *A18a* or *A18b*, although more commonly with *A18a*.

Discussion

To date, the extent of allelic variation of individual kappa genes has not been extensively studied. We have identified two new alleles for *A2* and one new allele for *A18* in a survey of 43 individuals. This brings the total number of known alleles for these genes to three alleles for *A2* (*A2a, b, c*; Fig. 1) and two alleles for *A18* (*A18a, b*; Fig. 1).

One new allele for *A2, A2c*, has only one nucleotide change compared with the previously described *A2a* allele. This change in FR2 results in a proline to serine change. Other expressed *IGKV-II* family genes have serine in this position and therefore this change is not deleterious to the production of a functional light chain protein. Furthermore, this change is in a region distant from CDR2, and as such probably will not have a direct effect on interaction between antigen and the CDRs. However, this change is in a region associated with heavy and light chain interaction and therefore it is possible that it may have an indirect effect on antigen binding.

The second allele identified for *A2, A2b*, has three nucleotide sequence changes from *A2a* (Feeney et al. 1996). *A2b* has the same proline to serine change in FR2 as in *A2c*. However, the other two changes could dramatically affect rearrangement and/or expression of *A2b*. One change in *A2b* is in the heptamer of the RSS from the consensus CACAGTG to CACAGAG. We have recently shown that this allele is defective in its ability to undergo *V(D)J* recombination, being reduced to approximately one-tenth the rearrangement frequency of the *A2a* allele (Feeney et al. 1996). The third change identified in the *A2b* gene is C to A in the promoter region, and it occurs 16 bp 5′ of the octamer sequence. The sequence elements required for efficient transcription of either germline and/or rearranged *IGKV* genes have not been thoroughly explored. However, this region contains at least two different sequence elements 5′ of the octamer implicated in transcriptional regulation: CCAAT and CACCC. There are three CACCC sites in this region. Mocikat and co-workers (1988) described unidentified proteins from B cells binding to ACCC sequences upstream of the octamer/decamer box. It is not known what effect these elements have on human *IGKV* gene expression. However, a group of three CACCC elements has been

identified in the 5′ region of the mouse germline *IGHCG1* region (Xu and Stavnezer 1992). Disruption of any of these elements by mutation results in dramatically depressed levels of germline transcription of this region, which is a necessary prerequisite for a switch-recombination mediated rearrangement to the *IGHCG1* gene. The third CACCC element in the human kappa promoter is in the immediate vicinity of the *A2b* change at position 201. This change creates a second CCAAT box adjacent to the CACCC binding site. If this CACCC site is important for germline transcription of the *IGKV* gene prior to its rearrangement, then occupation of this region by any of the many CCAAT box binding proteins may have an inhibitory effect and thus render this gene non-functional. Thus, *A2b* has nucleotide substitutions which reduce the ability of this gene to rearrange and possibly also to be expressed.

The new *A18* allele identified here, *A18b*, has two changes from the *A18a* allele. One change is a silent mutation in FR2, and the second change is in FR3, resulting in the reversion from the termination codon seen in *A18a* to the conserved cysteine seen in all functional light chain genes. Therefore, this new allele is predicted to encode a functional protein. Indeed, when rearrangements utilizing this gene are analyzed, 80% were found to be in frame. Thus, these rearrangements had undergone the cellular selection and clonal expansion characteristic of functional *Ig* genes. There is one published sequence of a rearranged *VJ* gene from a translocation in a Burkitts lymphoma cell line that may have used an *A18b* allele. This gene was highly mutated but did contain both changes in FR2 and FR3 characteristic of the *A18b* allele (Kato et al. 1991). Another report of a rearrangement using an *A18* gene with cysteine 88 has been made (Klein et al. 1993).

These additional alleles demonstrate that the *IGKV* locus may be more polymorphic than previously appreciated. In addition, these data may have direct biological consequences on the composition of the antibody response against *Haemophilus influenzae* type b (Hib) in individuals carrying various alleles of this gene pair. The antibody response to Hib is pauciclonal and is dominated by antibodies using the *A2* light chain (Lucas et al. 1991). These *A2* antibodies have high avidity for Hib, and can be unmutated, as opposed to all other antibodies against Hib sequenced to date (Adderson et al. 1992; Adderson et al. 1993; Pinchuk et al. 1995; Scott et al. 1989). Since Navajos and other genetically related Native Americans have a five-to-tenfold increased incidence of Hib disease as compared with Caucasian North Americans, we searched for a polymorphism in Navajos which could potentially contribute to disease susceptibility. We found such a polymorphism in *A2b* (Feeney et al. 1996). However, we also found two other alleles of *A2/A18* which could potentially play a role in the repertoire of Hib-specific antibodies. Of most importance is the change observed in *A18b*. It has been observed that the distal region *IGKV* genes are significantly underrepresented in the peripheral expressed *IGKV* repertoire (Cox et al. 1994; Klein et al. 1993; unpublished observations). Thus, a functional *A18* gene located only 450 kb from the *IGKJ* cluster is likely to be a significant contributor to the primary antibody repertoire. *A18b* has four amino acid differences from *A2a*, one in CDR2 and two in CDR3. It is not clear a priori whether *A18b* can encode for effective Hib-specific antibodies. To date, no *A18* allele have been seen in the sequenced Hib-specific antibodies. This either indicates that the CDR changes in *A18b* significantly preclude Hib binding, or that the existing sequences were derived from individuals homozygous for the non-functional *A18a*. The *A2c* allele has only one change in FR2, and thus is predicted to encode an Hib-binding antibody. In fact, among the sequenced Hib-specific light chains, one apparently uses *A2c* (Adderson et al. 1993).

The previously described *A2a* was the most common *A2* allele seen in both control and Navajo groups (64% and 41%, respectively). The new potentially functional alleles, *A2c* and *A18b*, were seen in both Navajo and non-Navajo populations with similar frequencies. Previously reported sequence studies of *IGKV* genes were largely derived from individuals of European descent (Cox et al. 1994; Klein et al. 1993; Schaible et al. 1993). Additionally, the largest database for the control population in the study reported here was derived from Caucasians. For these reasons we examined the distribution of these alleles in Caucasians compared with non-Caucasians (Table 1). There was a higher frequency of the *A2a* allele and the *A18a* allele in Caucasians compared with either Navajos or with non-Caucasians in the control group. In contrast, a significant difference was seen in the distribution of the *A2b* allele. Only one individual in the control population, a Hispanic originally from Mexico, had this allele. It is not known whether there is Navajo or any other genetically related Native American ancestry in her background.

Similarities in the distribution of alleles between ethnic groups has been observed for other *Ig* genes. A study of *IGHV* region polymorphisms identified a several allele pairs with relatively similar distribution frequencies between Caucasians and Asians (Walter and Cox 1991). Thus, the appearance and distribution of many *Ig* alleles, including *A2a*, *A2c*, *A18a*, and *A18b*, predates the ethnic diversification of Asians and Caucasians, some 30 000 years ago (Guidon and Delrais 1986). Navajos, Apaches, and some Alaskan Eskimos are genetically related, and are descendants of the Na-dene who migrated from Asia 12 000 years ago (Torroni et al. 1993; Williams et al. 1985). In contrast to the other *A2/A18* alleles, the *A2b* allele was found almost exclusively in the Navajo subjects whom we studied, thus suggesting a more recent origin of this allele.

Statistical analysis of the frequency of the *A2* and *A18* alleles indicates significant linkage disequilibrium. *A2a* is often associated with *A18a* and *A2c* is often associated with *A18b*. This linkage disequilibrium is somewhat surprising considering the distance separating these two genes ($\sim$1100 kb). The nature of the selective pressures maintaining these associations is unknown.

In conclusion, we found new alleles for two *IGKV* genes, *A2* and its proximal region partner *A18*, which occur at significant frequencies. One new *IGKV* gene is a functional allele of a previously described non-functional *IGKV* gene, while another *IGKV* gene is a defective allele

of a previously described functional *IGKV* gene. Therefore, depending on the patterns of inheritance, some individuals will have a more restricted *IGKV* repertoire and some will have a more diverse *IGKV* repertoire attributable to these new alleles. Such *IGKV* gene polymorphisms are predicted to result in genetic differences in the ability of individuals to mount specific immune responses.

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