Primary structure of human neutrophil antigens 1a and 1b

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BACKGROUND: Neutrophil specific Fcγ receptor IIIb (CD16b) is a low-affinity IgG receptor. Its polymorphic variants are associated with human neutrophil antigens (HNA). HNA-1a and HNA-1b differ in four amino acids. Immunization can lead to the production of alloantibodies. The exact contribution of four amino acid exchanges for the formation of HNA-1a, −1b epitopes is currently unknown.

STUDY DESIGN AND METHODS: Permutation of each polymorphic amino acid from wild-type CD16b cDNA constructs was performed and expressed on HEK293 cells. All 16 receptor variants were produced and tested against 19 well-characterized HNA antisera in an antigen capture assay.

RESULTS: Analyzing the reaction pattern revealed that anti-HNA-1a antibodies can bind whenever asparagine (N) is present in position 65, regardless of the three other positions (CD16b *N**). Anti-HNA-1b antibodies can bind when serine (S) is present in position 36 (CD16b S***), when N is present in position 82 (CD16b **N*), or both (CD16b S*N*). CD16b variants with N65 and S36 and/or N82 (such as CD16b SNN*) bind both, anti-HNA-1a and anti-HNA-1b alloantibodies. If these specific amino acids are missing (as in CD16b RSD*), no antibodies will bind.

CONCLUSION: Whereas the primary structure of HNA-1a and HNA-1b usually differs in four amino acids, epitope composition is not "antithetical". N65 alone determines the presence of HNA-1a, and S36 and/or N82 determine the presence of HNA-1b. Amino acid 106 does not participate in epitope formation. Our findings are of specific relevance when a HNA-1 phenotype is predicted from a genotype.

INTRODUCTION

CD16b, also known as FcγRIIIb, is a low-affinity Fcγ receptor exclusively expressed on neutrophils. It has a significant role in the clearance of immune complexes, in phagocytosis, and antibody-dependent cellular cytotoxicity.[1–3] It shares great similarity with CD16a, another low-affinity Fcγ-receptor with broader white blood cell expression. In contrast to CD16a, CD16b is 21 amino acids shorter, lacks a transmembrane domain and represents the only glycosylphosphatidylinositol-anchored Fc receptor.[4] CD16b has numerous polymorphic variants. The most relevant ones are associated with the human neutrophil antigen 1 (HNA-1) system.[5] Alloimmunization against HNA-1 leads to the production of antibodies responsible for immune-mediated neutrophil disorders including, neonatal alloimmune neutropenia (NIN) and transfusion-related acute lung injury (TRALI).

The encoding gene *FCGR3B* is located on chromosome 1q23-24 within a cluster of two families of FcγR genes.[6] Five *FCGR3B* alleles (**01 to *05*) are registered with the ISBT.[5] The most frequent, and clinically most important, alleles are *FCGR3B*01* and *FCGR3B*02*, encoding the HNA-1a and HNA-1b antigens, respectively. Both alleles differ by 5 nucleotide bases in the coding region at positions 108, 114,

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194, 244, and 316. Four of these nucleotide changes result in amino acid differences between HNA-1a and HNA-1b, whereas the nucleotide change at position 114 is silent. Two amino acid exchanges, related to the A194G and G244A mutations, induce additional N-glycosylation sites, resulting in 6 glycosylation sites for HNA-1b and only 4 for HNA-1a, affecting the apparent molecular weight (65 to 80 kDa and 50 to 65 kDa).[7] Despite this detailed knowledge about the genetic background and the biochemistry of variant receptors, it is currently unknown which, if not all, of the four amino acids are in fact involved in the formation of the epitopes recognized by antibodies of anti-HNA-1a or anti-HNA-1b specificity. HNA-1c is encoded by the third allele of *FCGR3B*, *FCGR3B*03*. It only differs from FCGR3B*02 at nucleotide position 233, leading to a single amino acid substitution, p.Ala78Asp.[8] Comparable to many other blood group antigens, this single amino acid exchange alone is sufficient for the expression of epitopes required for the binding of anti-HNA-1c antibodies.[9,10] In routine laboratory testing, the HNA-1 phenotype is now generally predicted from the genotype,[11] with most assays detecting only some, but not all polymorphic nucleotides of *FCGR3B*. Extended testing in some populations such as, Africans and Brazilian Indians, has demonstrated that genetic variability is larger than previously anticipated.[12–18]

Here, we aimed to define the epitopes of HNA-1a and HNA-1b by permutation of all four amino acids and subsequent serological characterization of the variant CD16b proteins.

MATERIALS AND METHODS

Serum samples and antibodies

Human serum samples containing anti-HNA-1a or anti-HNA-1b were obtained from mothers of children with neonatal alloimmune neutropenia. All antibody specificities were characterized by standard monoclonal antibody immobilization of granulocyte specific antigens (MAIGA) assay using HNA genotyped neutrophils, as outlined elsewhere.[16] Only sera with concordant results from two reference laboratories of the International Society of Blood Transfusion (Giessen, Germany and Hagen, Germany) were used. Normal serum collected from a healthy blood donor without white blood cell-specific antibodies was used as control. Moabs 3G8, DJ130c, and LNK16 against different epitopes on CD16b (Immunotech; Santa Cruz Biotechnology) were used in this study. Mouse IgG as control was from Biolegend.

Generation of recombinant CD16b variants

Blood donors were typed for *FCRG3B* by the use of PCR-SSP using BAGene SSP Kit (BAG Health Care). Neutrophils were isolated from donors typed homozygous for *FCRG3B*01* or *FCRG3B*02*. Total RNA was extracted with a RNA isolation kit (RNeasy Mini Kit) and transcribed into cDNA with first-strand beads (Ready-to-Go You-Prime, GE Healthcare) using random hexamer primers (Pharmacia). Full-length cDNA encoding CD16b was amplified by nested PCR (protocol available upon request) and, after Klenow fill-in, ligated into a pCDNA3.1 expression vector (Invitrogen) opened with *Eco*RV (Promega). After chemical transformation into *Escherichia coli*, positive clones were identified by PCR the next day and expanded. After complete sequencing in both directions, new codons for single amino acid exchanges were introduced by site-directed mutagenesis (Stratagene) in order to obtain variants 2 to 15. Successful mutagenesis was determined by complete sequencing in both directions. A detailed protocol for site-directed mutagenesis is available upon request. Finally, 293F cells (GIBCO) kept in Dulbecco's modified Eagle's medium high glucose (GIBCO) supplemented with 10% fetal bovine serum (PAA), 1% penicillin–streptomycin (Paschin), and 1% nonessential amino acids (GIBCO), were transfected with all 16 different plasmids (HNA-1a,HNA-1b, and 14 variants; 4 mg each) and empty vector (mock transfection) by the use of Lipofectamine (GIBCO). After 24 hours, cells were transferred in plates with medium containing 700 mg geneticin/mL (Bioprom). Single clones were collected after 14 to 21 days and expanded on six-well plates. The 293F cells of all 16 types were maintained in culture medium supplemented with geneticin and were used after washings for further testing. The expression of CD16b was determined prior to use by flow cytometry using MoAb LNK16 and Alexa Fluor® 488 conjugated donkey anti-mouse IgG (1:50; Life Technologies, USA).

Mapping of HNA-1a and HNA-1b epitopes by modified MAIGA

The original MAIGA protocol[19] was modified. In brief, 3×10^5 paraformaldehyde-fixed 293F cells were sensitized with 50 μL serum, washed, incubated with 10 μL capture moab (20 μg/mL), and lysed afterwards in 100 μL lysis buffer (2.4 g/L Tris, 8.76 g/L NaCl, 9.5 mL/L Triton X-100, and 1.86 g/L EDTA; pH 7.4) Cell lysates were immobilized onto a microtiter plate precoated with goat anti-mouse IgG. Bound human IgG was then detected with horseradish peroxidase–labeled goat anti-human IgG (Dianova), followed by incubation with the enzyme substrate (*o*-phenylenediamine) and colorimetric determination in an absorbance reader (Tecan) at 492 nm. The cut-off was defined as the twofold absorbance obtained with mock-transfected 293F cells.

STATISTICAL ANALYSIS

Statistics were performed using GraphPad Prism, version 7.03 (Graph Pad Software).

RESULTS

Antigen capture assay

All CD16b variants and their serological characteristics are summarized in Table 1. The single-letter amino acid code

TABLE 1. Amino acid composition and reactivity of CD16b variants [Color table can be viewed at wileyonlinelibrary.com]

Cell line	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
CD16b type aa composition	RNDV	RNDI	RNNV	RSDV	SNDV	RNNI	RSNV	SNDI	SSDV	SNNV	RSDI	SSNV	SSDI	SNNI	RSNI	SSNI
HNA	**1a**	-	-	-	-	-	-	-	-	-	-	-	-	-	-	**1b**
FCGR3B	***1**	***4**	**-**	**-**	-	-	-	-	-	-	-	-	***5**	-	-	***2**
human serum no.																
Anti HNA-1a-1	pos.	pos.	pos.	neg.	pos.	pos.	neg.	pos.	neg.	pos.	neg.	neg.	neg.	neg.	neg.	neg.
Anti HNA-1a-2	pos.	pos.	pos.	neg.	pos.	pos.	neg.	pos.	neg.	pos.	neg.	neg.	neg.	pos.	neg.	neg.
Anti HNA-1a-3	pos.	pos.	pos.	neg.	pos.	pos.	neg.	pos.	neg.	neg.	neg.	neg.	neg.	pos.	neg.	neg.
Anti HNA-1a-4	pos.	pos.	pos.	neg.	pos.	pos.	neg.	pos.	neg.	pos.	neg.	neg.	neg.	pos.	neg.	neg.
Anti HNA-1a-5	pos.	pos.	pos.	neg.	pos.	pos.	neg.	pos.	neg.	pos.	neg.	neg.	neg.	pos.	neg.	neg.
Anti HNA-1a-6	pos.	pos.	pos.	neg.	pos.	pos.	neg.	pos.	neg.	pos.	neg.	neg.	neg.	pos.	neg.	neg.
Anti HNA-1a-7	pos.	pos.	pos.	neg.	pos.	pos.	neg.	pos.	neg.	pos.	neg.	neg.	neg.	pos.	neg.	neg.
Anti HNA-1a-8	pos.	pos.	pos.	neg.	pos.	pos.	neg.	pos.	neg.	pos.	neg.	neg.	neg.	pos.	neg.	neg.
Anti HNA-1b-1	neg.	neg.	pos.	neg.	pos.	pos.	pos.	pos.	pos.	pos.	neg.	pos.	pos.	pos.	pos.	pos.
Anti HNA-1b-2	neg.	neg.	pos.	neg.	pos.	pos.	pos.	pos.	pos.	pos.	neg.	pos.	pos.	pos.	pos.	pos.
Anti HNA-1b-3	neg.	neg.	pos.	neg.	pos.	pos.	pos.	pos.	pos.	pos.	neg.	pos.	pos.	pos.	pos.	pos.
Anti HNA-1b-4	neg.	neg.	pos.	neg.	pos.	pos.	pos.	pos.	pos.	pos.	neg.	pos.	pos.	pos.	pos.	pos.
Anti HNA-1b-5	neg.	neg.	pos.	neg.	pos.	neg.	neg.	pos.	pos.	pos.	neg.	pos.	pos.	pos.	neg.	pos.
Anti HNA-1b-6	neg.	neg.	pos.	neg.	pos.	neg.	pos.	pos.	pos.	pos.	neg.	pos.	pos.	pos.	neg.	pos.
Anti HNA-1b-7	neg.	neg.	pos.	neg.	pos.	pos.	pos.	pos.	pos.	pos.	neg.	pos.	pos.	pos.	pos.	pos.
Anti HNA-1b-8	neg.	neg.	pos.	neg.	pos.	pos.	pos.	pos.	pos.	pos.	neg.	pos.	pos.	pos.	pos.	pos.
Anti HNA-1b-9	neg.	neg.	pos.	neg.	pos.	pos.	pos.	pos.	pos.	pos.	neg.	pos.	neg.	pos.	pos.	pos.
Anti HNA-1b-10	neg.	neg.	neg.	neg.	neg.	neg.	neg.	neg.	neg.	neg.	neg.	pos.	neg.	pos.	neg.	pos.
Anti HNA-1b-11	neg.	neg.	pos.	neg.	pos.	pos.	pos.	pos.	pos.	pos.	neg.	pos.	pos.	neg.	pos.	pos.
present epitopes	***1a***	***1a***	***1a+1b***	***none***	***1a+1b***	***1a+1b***	***1b***	***1a+1b***	***1b***	***1a+1b***	***none***	***1b***	***1b***	***1a+1b***	***1b***	***1b***
absent epitopes	***1b***	***1b***	**-**	***all***	**-**	**-**	***1a***	**-**	***1a***	**-**	***all***	***1a***	***1a***	**-**	***1a***	***1a***

The amino acids at positions 36, 65, 82, and 106 for each variant are given in line 2. Cell line 1 (RNDV) represents HNA-1a and cell line 16 (SSNI) represents HNA-1b. Two of the variant forms 2-15 have been described as naturally occurring (cell line 2, RNDI and cell line 13, SSDI). Note that variant 11 has the same four amino acid substitutions as CD16a and remains non-reactive with all sera tested. The CD16b variants were used in a modified MAIGA and the cut-off values were defined as the twofold absorbance obtained with mock-transfected cells. All experiments were performed in triplicates. A color code is used: red indicates reactivity with anti-HNA-1a (CD16b *N**), blue (CD16b S***), and amber (CD16b **N*) with anti-HNA-1b (either alone or in combination).

for the four positions (36, 65, 82, and 106) in which HNA-1a and HNA-1b differ will be used to address a specific variant throughout the manuscript, i.e., "RNDV" indicates the HNA-1a variant and "SSNI" indicates the HNA-1b variant.

When tested with well-defined sera characterized by two different ISBT reference laboratories for granulocyte serology, 8 of 8 anti-HNA-1a antisera recognized CD16b RNDV, representing HNA-1a, but none of the sera recognized CD16b SSNI, representing HNA-1b. Vice versa, 11 of 11 anti-HNA-1b antisera recognized CD16b SSNI (HNA-1b), but none of the sera recognized CD16b RNDV (HNA-1a). Accordingly, all sera used in the study could be clearly identified as either containing anti-HNA-1a or anti-HNA-1b by the use of recombinant CD16b proteins, the accuracy was 100%. Using OD as a readout, the intra-assay variation was 6.95%, and the inter-assay variation (imprecision) of this test was 12.17%.

Defining HNA-1a epitopes

For better understanding, a color-coding system is used in Table 1 to indicate the determined binding pattern. Anti-HNA-1a antibodies were unable to react with CD16b from cell lines 4, 7, 9, 11, 12, 13, 15, and 16. These cell lines express variants of CD16b with serine at position 65 (CD16b *S**). In contrast, anti-HNA-1a antibodies reacted with cell lines 1, 2, 3, 5, 6, 8, 10, and 14 with asparagine at position 65 (CD16b *N**, in red). We conclude that HNA-1a epitopes are dependent on the presence of asparagine (N) at this position. Interestingly, HNA-1a epitopes are present on all CD16b *N** molecules regardless which other amino acids are adjacent to asparagine 65, with very few exceptions (serum #1 failed to react with CD16b SNNI and serum #3 failed to react with CD16b SNNV). This indicates that no other amino acid is involved in the formation of the HNA-1a epitope(s).

Defining HNA-1b epitopes

HNA-1b epitopes are more complex to define. Only four CD16b variants (expressed in cell lines 1, 2, 4, and 11) gave negative results with anti-HNA-1b sera throughout. Two of these cell lines (1, 2) express CD16b RND*, and the two others (4, 11) express CD16b RSD*. Both CD16b RND* variants (CD16b RNDV = HNA-1a and CD16b RNDI, defined as *FCGR3B*04* previously) are exclusively reactive with anti-HNA-1a. Both CD16b RSD* variants (CD16b RSDV and CD16b RSDI) are non-reactive, i.e., do neither carry HNA-1a nor HNA-1b epitopes. Of note, CD16b RSDI has the same amino acid composition as CD16a (FcγRIIIa). A pattern for HNA-1b epitopes becomes apparent when dissecting the amino acid composition of the 12 reactive variants (3, 5 to 10, and 12 to 16).

First, all variants with serine (S) at position 36 (CD16b S***, in blue) are reactive with anti-HNA-1b (with some exceptions observed for sera #9 and #10). This indicates that serine 36 is one possibility to form HNA-1b epitopes. As one would expect, variants with serine 36 (HNA-1b specific) and asparagine 65 (HNA-1a specific), CD16b SN**, are reactive with almost all antisera, anti-HNA-1a and anti-HNA-1b (cell lines 5, 8, 10, and 14; 4 of 19 sera fail to react with CD16b SN** in 6/76 reactions).

Second, Anti-HNA-1b antibodies are not exclusively reactive with CD16b S***, but the majority does also bind to variants with asparagine (N) at position 82 in the absence of serine 36 (CD16b R*N*, in amber); as present in cell lines 3, 6, 7, and 15. Note that cell line 7 (RSNV) and 15 (RSNI) do not carry any of the amino acids that determine HNA-1a (CD16b *N**) or HNA-1b (CD16b S***) as outlined in the previous paragraph, but do bind anti-HNA-1b antibodies. As reported previously, asparagine 82 in CD16b **N*, when expressed together with alanine 78 (such as in the natural protein encoded by *FCGR3B*02*), forms the HNA-1d epitope.[20] Our findings presented here extend this observation and indicate that the majority of anti-HNA-1b produced by HNA-1aa homozygous individuals contains a mixture of both, "classical" anti-HNA-1b (against CD16b S***), and anti-HNA-1d (against CD16b **N* with Ala78).

Again, as one would expect, variants with asparagine 82 (HNA-1b specific) and asparagine 65 (HNA-1a specific), but not serine 36 (i.e., CD16b RNN*), are reactive with almost all antisera, anti-HNA-1a and anti-HNA-1b (cell lines 3 and 6; 3/19 sera fail to react in 4 of 38 reactions).

Third, three different groups of CD16b variants carry HNA-1b epitopes: CD16b S*D*, CD16b R*N*, and CD16b S*N*. Antibody reactivity for these three variant groups is summarized in Table 2. Statistical comparison of reactivity between the three groups indicates an impaired binding of

TABLE 2. Comparison of different CD16b variants carrying HNA-1b epitopes

	Reactivity with											
	CD16b S*D*			CD16b R*N*					CD16b S*N*			
Cell line Amino acid composition	5 SNDV	8 SNDI	9 SSDV	13 SSDI	3 RNNV	6 RNNI	7 RSNV	15 RSNI	10 SNNV	12 SSNV	14 SNNI	16 SSNI
No. of positive sera	10	10	10	9	10	8	9	8	10	11	10	11
Total no. of positives (%)		39/44 (88.6)				35/44 (79.5)				42/44 (95.5)		

Most CD16b variants (11/16 = 69%) carry HNA-1b epitopes since both CD16 S*** and CD16b **N* either alone or in combination bind Anti-HNA-1b. The additional presence of the HNA-1a defining amino acid impairs the binding of Anti-HNA-1b, as a two-way ANOVA indicates differences between the columns ($p = 0.0041$). Main column effect (Tukey's multiple comparison test) demonstrates no differences between column 1 and column 2 and no differences between column 1 and column 3, but differences between column 2 and 3 ($p = 0.0028$).

TABLE 3. Summary of published natural CD16b variants

First author, year	Described variant†	Nucleotide position 108	194	244	316	Amino acids	Present epitopes	Phenotype discrepant to genetic typing?
Matsuo, 2000[12]	FCGR3B*01 G316A	G	A	G	A	RNDI	a	no
	FCGR3B*02 G194A	C	A	A	A	SNNI	a, b	yes
	FCGR3B*02 A244G	C	G	G	A	SSDI	b	no
	FCGR3B*02 C108G	G	G	A		RSN*	b	no
	FCGR3B*02 G194 T	C	T	A		SSN*	b	no
Flesch, 2002[13]	FCGR3B*01 G244A	G	A	A		RNN*	a, b	yes
	FGGR3B*01 G108C	C	A	G		SND*	a, b	yes
Tong, 2003[14]	FCGR3B*02 A244G	C	G	G		SSD*	b	no
	FCGR3B*02 G194A	C	A	A		SNN*	a, b	yes
	FCGR3B*01 G108C	C	A	G		SND*	a, b	yes
Covas, 2005[15]	FCGR3B*01 A194G	G	G	G		RSD*	none	yes
	FCGR3B*02 A244G	C	G	G		SSD*	b	no
Xu, 2007[16]	FCGR3B*02 A244G	C	G	G		SSD*	b	no
	FCGR3B*02 C108G	G	G	A		RSN*	b	no
Blum, 2009[17]	FCGR3B*02 A244C	C	G	C		SSD*	b	no
	FCGR3B*01 G244A	G	A	A		RNN*	a, b	yes
	FCGR3B*01 G108C	C	A	G		SND*	a, b	yes
	FCGR3B*02 C108T	C	G	A		SSN*	b	no
Terzian, 2012[18]	FCGR3B*01 A194G	G	G	G		RSD*	none	yes

† Numbering of nucleotides according to ISBT nomenclature, which is based on FCGR3B transcript variant 2 (NM_000570.4); the original papers used the previously accepted numbering (108 = 141; 194 = 227, 244 = 277, and 316 = 349). Note that in 9/19 presented variants, the originally predicted phenotype is discrepant to the phenotype deduced from the results of our experimental study.

anti-HNA-1b antibodies to CD16b R*N* when compared to "regular" HNA-1b ($p = 0.0028$).

Finally, we also tested a serum from a mother with a rare genotype (*FCGR3B**01,*05), who gave birth to a neutropenic child. The antibody was initially characterized as Anti-HNA-1b. On the amino acid level, the only difference between these individuals is D82N. Strikingly, the serum reacted with all CD16b **N* variants (3,6,7,10,12,14 and 16), supporting the hypothesis that HNA-1b can be formed by two different epitopes, serine 36 or asparagine 82.

Non-reactive variants

Two out of the 16 recombinant CD16b proteins did react neither with anti-HNA-1a nor with anti-HNA-1b: CD16b RSDV (cell line no. 4) and CD16b RSDI (cell line no. 11). These two variants do not carry any of the following amino acids: serine 36, asparagine 65, asparagine 82; and are thus devoid of the defined HNA-1a/−1b epitope-determining amino acids.

DISCUSSION

Immunization against CD16b is the most relevant cause of neonatal alloimmune neutropenia.[21] The antibody in maternal blood is demonstrated in appropriate immunological methods, which are usually based on isolated donor neutrophils. The phenotype of the cell donor, but also of the mother and her child, is usually predicted from genetic testing for a single base exchange, despite the fact that HNA-1a and HNA-1b differ in four amino acids, of which the individual contribution to the formation of the two allotypes has never been demonstrated conclusively. Here, we were able to demonstrate that the epitope for HNA-1a is defined by the presence of asparagine in position 65. All CD16b *N** variants are reactive with anti-HNA-1a antibodies. Accordingly, to predict a HNA-1a phenotype from genotyping, it is relevant to demonstrate the presence of A194. In contrast, antibodies formed by HNA-1aa individuals against HNA-1b are reactive with CD16b S*** and CD16b **N*. The latter variant carries the HNA-1d epitope defined earlier as an epitope formed by alanine 78 and asparagine 82 together.[20] Since HNA-1d is always expressed on CD16b encoded by *FCGR3B*02* (CD16bSSNI), the regular anti-HNA-1b immune response contains a mixture of anti-HNA-1b and anti-HNA-1d specificities. However, in clinical routine, these fine specificities are undistinguishable. Accordingly, to predict a HNA-1b phenotype from genotyping, it is relevant to demonstrate the presence of 108C and 244A. No evidence was found that V106I affects epitope formation in any way; it is therefore generally unnecessary to study 316G > A to predict HNA-1 phenotypes from genotyping.

For the majority of white Europeans, HNA-1a is represented by CD16b RNDV and HNA-1b is represented by CD16b SSNI. However, some variation in white Europeans, but more so in Africans and Brazilian Indians, has been observed over the last two decades (Table 3). Most of these studies could not predict the phenotypic effect of an observed genetic variation. Matsuo et al. defined a new allele which they termed NA2*06.[12] It could not be clearly identified as either CD16a or CD16b, because serological studies using neutrophils are hampered by the fact that most anti-CD16 monoclonal antibodies react with both receptors.[22] Stroncek did then provide evidence

that NA2*06 (now termed FCGR3B*02-C108G) is a variant of NA2 (now termed HNA-1b); the predicted amino acid composition was CD16b RSN*.[22] Our study confirms this assumption with the corresponding recombinant proteins: all CD16b **N* proteins express the HNA-1b epitope, and all CD16b *S** proteins are devoid of HNA-1a. As a consequence, CD16b RSN* reacts with anti-HNA-1b exclusively. Another previously published, but unexplained observation came from Trounstine et al., who performed some preliminary experiments with recombinant CD16b expressed in COS cells.[23] They observed that one of their variants, CD16b RN**, reacted with anti-HNA-1a and anti-HNA-1b. This can now be explained with our finding that CD16 R*** carries HNA-1a epitopes and CD16 *N** carries HNA-1b epitopes; accordingly, Tronstine's variant must be reactive with both types of antibodies. A naturally occurring CD16b RNN* was also found to be reactive with both types of antibodies.[22]

Based on the published approaches for sequence-specific PCR, we can assume that (at least in white European blood donors) applied genotyping strategies correctly predict the phenotype. Given that the genetic composition of HNA-1 can be complex, some authors have argued that genetic studies in patients or families should involve sequencing.[18] An alternative approach using multiplex PCR was recently published.[24] Laboratories investigating samples from non-European ethnicities should be aware that, depending on their genotyping approach, the patient's phenotype may not be as predicted.

One major restriction applies to the study presented here. We were unable to perform genetic sequencing from all mother/child pairs. In theory, it is possible that some of the sera obtained from these mothers contain antibody specificities which differ from "classical" anti-HNA-1a or anti-HNA-1b antibodies because of the presence of additional mutations in a single family. Few sera gave unexpected reactions with some recombinant proteins (Table 1), i.e., they differed from the majority of sera tested. However, all sera were from white European families. Noteworthy, all sera with atypical reactions (anti-HNA-1a #1, #3, and anti-HNA-1b #5, #6, #10) were taken 10 years or more after the last documented immunizing event, whereas the rest of sera was collected within a period of 12 months; it is therefore also possible that over time, as part of antibody evanescence, some clonal specificities are being lost.

In conclusion, we have defined the composition of HNA-1a and HNA-1b epitopes. Whereas the primary structure of HNA-1a and HNA-1b usually differs in four amino acids, composition of epitopes is not "antithetical." N65 alone determines the presence of HNA-1a, and S36 and/or N82 determine the presence of HNA-1b. Amino acid 106 does not participate in formation of HNA-1a, HNA-1b epitopes.

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CONFLICTS OF INTEREST

None.

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