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TRANSFUSION

BRIEF REPORT

Anti-human neutrophil antigen-1d specificity is frequently observed in anti-human neutrophil antigen-1b alloantisera

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Abstract

Background: Four amino acids are involved in epitope formation of human neutrophil antigens (HNA)-1 alleles, located at positions 36, 65, 78, and 82. HNA-1a and HNA-1b alloantibody epitopes were recently characterized. The HNA-1b allele also carries the HNA-1d epitope p.78A&p.82N. The current study aimed to identify compound antibody specificities in HNA-1b alloantisera, especially the presence of anti-HNA-1d.

Study Design and Methods: For investigation of binding epitopes for HNA-1b alloantibodies, cells stably expressing different HNA-1 alleles were generated and tested against previously well-characterized HNA-1b antisera ($n = 11$) in an antigen capture assay. Sera with p.82N specificity or p.36S and p.82N specificity were additionally analyzed using adsorption and elution methods.

Results: Three amino acids, p.36S, p.78A, and p.82N, are involved in epitope formation of HNA-1b. The following specificities were identified in 11 HNA-1b alloantisera: p.36S (6/11), p.82N (9/11), and p.78A&p.82N (8/11), of which p.36S was identified as a sole entity in 2/11, whereas 9/11 antisera contained a polyspecific mixture of anti-p.36S, p.82N (1/11), and anti-p.78A&p.82N in combination with anti p.82N (5/11) or compound specificities of anti-p.36S, p.82N, and p.78A&p82N (3/11). In seven of eight antisera with p.82N specificity, anti-p.78A&p.82N was detected.

Discussion: Analysis of HNA-1b antisera indicates compound specificities for HNA-1b alloantibodies with a high variation between HNA-1b immunized individuals. Amino acids p.36S, p.82N, and p.78A&p.82N are necessary for HNA-1b epitope formation. The HNA-1d epitope is recognized by 73% (8/11) of HNA-1b immunized individuals.

Abbreviations: HNA, human neutrophil antigens; MAIGA, monoclonal antibody immobilization of granulocyte antigens; NAIN, neonatal alloimmune neutropenia; PCR-SSP, PCR with sequence-specific primers.

KEYWORDS
CD16b, HNA-1b alloantibodies, HNA-1d epitope

1 | INTRODUCTION

The human neutrophil antigens (HNA)-1 system is the clinically most important antigen systems on neutrophils. Alloantibodies against HNA-1 have been shown to induce neonatal alloimmune neutropenia (NAIN)[1–5] and transfusion-related acute lung injury.[6,7] The HNA-1 polymorphisms are located on CD16b (or FcγRIIIb), a granulocyte-specific glycosylphosphatidlyinositol-anchored low-affinity IgG receptor.[8,9] The protein consists of two extracellular domains whose structure is formed by disulfide bonds,[10,11] HNA-1 polymorphisms are located in the membrane distal domain. The HNA-1 system consists of five alleles, *FCGR3B*01*, *FCGR3B*02*, *FCGR3B*03*, *FCGR3B*04* (*FCGR3B*01 c.316G>A*), and *FCGR3B*05* (*FCGR3B*02 c.244A>G*),[12] encoding the amino acids forming epitopes for HNA-1a (*FCGR3B*01*), HNA-1b (*FCGR3B*02*),[13] HNA-1c (*FCGR3B*03*),[3] and HNA-1d (*FCGR3B*02*).[5] HNA-1a and HNA-1b are the most common alleles in the White European population[14,15] and differ in four amino acid positions: p.R36S, p.N65S, p.D82N, and p.V106I, whereas HNA-1b and HNA-1c differ in one amino acid at p.A78D.[12] Allelic variants of CD16b carry epitopes of HNA-1a, 1b, 1c, and 1d detected by HNA-1 alloantibodies. Recently, the HNA-1 antigenic epitope formation recognized by HNA-1a and HNA-1b alloantibodies was analyzed. In contrast to HNA-1a, where only one amino acid, position p.65N, is involved in the epitope formation, a more complex epitope formation involving several amino acids at position p.36S and p.82N was found in HNA-1b.[16] Importantly, *FCGR3B*02* does not only encode the HNA-1b epitopes but also carries the HNA-1d epitope. This epitope is not characterized by a single amino acid substitution, but consists of two amino acids, p.78A and p.82N.[5] These two amino acids (p.78A&p.82N) are unique to CD16b encoded by *FCRG3B*02* and the motif is not expressed on CD16b encoded by either *FCRG3B*01* or *FCRG3B*03*. Accordingly, if a HNA-1aa individual (genotype *FCGR3B*01*, **01*) is exposed to HNA-1b, the immune system should not only recognize p.36S and p.82N (defining HNA-1b), but also the more complex HNA-1d epitope defined by p.78A&p82.N. The aim of the current study was to analyze the compound antibody composition of HNA-1b alloantisera, especially the presence of antibodies binding the HNA-1d epitope.

2 | MATERIALS AND METHODS

2.1 | Serum samples

Eleven well-characterized HNA-1b alloantisera from women immunized by pregnancy of newborns diagnosed with NAIN were analyzed. Alloantibody specificities were analyzed by a modified monoclonal antibody immobilization of granulocyte antigens (MAIGA) using HNA-1 genotyped neutrophils for evaluation.[17] For antigen immobilization, the monoclonal antibodies LNK16, 3G8, and DJ130c (Santa Cruz Biotechnology, Immunotech) were used.

2.2 | Neutrophil isolation, FCGR3B RNA extraction, obtaining of cDNA, and cloning

Healthy blood donors were *FCGR3B* (HNA-1) genotyped by PCR with sequence-specific primers (PCR-SSP) (BAGene HNA-Type, BAG, Lich, Germany). Neutrophils from freshly collected EDTA whole blood from donors with the genotype HNA-1bb (*FCGR3B*02*) and HNA-1aa (*FCGR3B*01*) were isolated by sedimentation of red blood cells and density gradient separation.[18] Subsequently, RNA was isolated (RNeasy Mini Kit, Qiagen, Germany) and transcribed into cDNA (Ready-To-Go You Prime First Strand Beads, Cytiva) using random hexamer primers (Pharmacia, Piscataway, NJ). To obtain *FCGR3B* cDNA, PCR-SSP was performed.[19] Nucleotides were attached to the *FCGR3B* cDNA fragment ends for blunt/blunt-ligation into the opened vector using Klenow reaction (Klenow-Fragment, Biolabs, Bad Schwalbach, Germany). The cDNA encoding *FCGR3B* was cloned into an ECO RV opened pCDNA3.1 vector with geneticin resistance (Invitrogen, San Diego, CA). By transformation reaction, the constructs created by ligation were transferred into *Escherichia coli* bacteria (Max Efficiency DH5a TMCompetent Cells; GIBCO, Karlsruhe, Germany).

2.3 | Site-directed mutagenesis

The site-directed mutagenesis kit (Stratagene, San Diego, CA) was used for the production of different HNA-1 variants using sequence-specific primers. Primer sequences and reaction details are available upon request.

TABLE 1 Reactivity of human neutrophil antigens (HNA)-1b antisera against seven different variants of CD16b

aa position aa Epitopes	36 65 78 82 106 S S A D V 1b	36 65 78 82 106 R S A N I 1b 1d	36 65 78 82 106 S S A N I 1b 1d	36 65 78 82 106 S S D D V 1b 1c	36 65 78 82 106 S S D N I 1b 1c	36 65 78 82 106 R S D D V 1c	36 65 78 82 106 R S D N I 1b 1c	Specificity Anti-S	Anti-AN or Anti-N
Serum									
Ctrl	neg	neg	neg	neg	neg	neg	neg	—	—
1 Anti-HNA-1b	pos	neg	pos	pos	pos	neg	neg	X	—
2 Anti-HNA-1b	pos	neg	pos	pos	pos	neg	neg	X	—
3 Anti-HNA-1b	neg	pos	pos	neg	pos	neg	pos	—	X
4 Anti-HNA-1b	pos	pos	pos	pos	pos	neg	pos	X	X
5 Anti-HNA-1b	pos	pos	pos	pos	pos	neg	pos	X	X
6 Anti-HNA-1b	neg	pos	pos	neg	pos	neg	pos	—	X
7 Anti-HNA-1b	neg	pos	pos	neg	pos	neg	pos	—	X
8 Anti-HNA-1b	neg	pos	pos	neg	pos	neg	pos	—	X
9 Anti-HNA-1b	pos	pos	pos	pos	pos	neg	pos	X	X
10 Anti-HNA-1b	neg	pos	pos	neg	pos	neg	pos	—	X
11 Anti-HNA-1b	pos	pos	pos	pos	pos	neg	pos	X	X

Note: Red and blue colors indicate S and N specificity, respectively. Underlined indicate AN specificity. Anti-AN and anti-N are indistinguishable in this experiment.
Abbreviations: A, alanine; aa, amino acids; D, aspartic acid; I, isoleucine; N, asparagine; R, arginine; S, serine; V, valine.

TABLE 2 Reactivity of human neutrophil antigens-1b antisera with p.82N specificity before and after adsorption/elution with CD16b SSDNI variant

No.	aa position aa Epitopes	36 65 78 82 106 S S A D V 1b	36 65 78 82 106 R S A N I 1b 1d	36 65 78 82 106 S S A N I 1b 1d	36 65 78 82 106 S S D D V 1b 1c	36 65 78 82 106 S S D N I 1b 1c	36 65 78 82 106 R S D D V 1c	36 65 78 82 106 R S D N I 1b 1c	Identified specificity
3	Serum	−	+++	+++	−	+++	−	+++	Anti-AN, N
	Adsorbate	−	++	++	−	−	−	−	Anti-AN
	Eluate	−	+++	+++	−	+++	−	+++	Anti-N
4	Serum	+++	+++	+++	+++	+++	−	++	Anti-S, AN, N
	Adsorbate	−	+++	+++	−	−	−	−	Anti-AN
	Eluate	+++	+	+++	+++	+++	−	+	Anti-S, N
5	Serum	+++	+++	+++	+++	+++	−	+++	Anti-S, AN, N
	Adsorbate	−	±	++	−	−	−	−	Anti-AN
	Eluate	+++	+++	+++	+++	+++	−	+++	Anti-S, N
6	Serum	−	++	++	−	+++	−	++	Anti-AN, N
	Adsorbate	−	+++	+++	−	−	−	−	Anti-AN
	Eluate	−	+	++	−	+++	−	+++	Anti-N
7	Serum	−	+++	+++	−	+++	−	+++	Anti-AN, N
	Adsorbate	−	+++	+++	−	+	−	++	Anti-AN, N[a]
	Eluate	−	+++	+++	−	+++	−	+++	Anti-N
8	Serum	−	+++	+++	−	+++	−	+++	Anti-AN, N
	Adsorbate	nt	+++	+++	nt	−	−	−	Anti-AN
	Eluate	nt	+++	+++	nt	+++	−	+++	Anti-N
9	Serum	+++	+++	+++	+++	+++	−	+++	Anti-S, AN, N
	Adsorbate	nt	++	++	nt	+	−	+	Anti-AN, N[a]
	Eluate	nt	++	+++	nt	+++	−	+++	Anti-S, N
10	Serum	−	+++	+++	−	+++	−	+++	Anti-AN, N
	Adsorbate	nt	+++	+++	nt	++	−	++	Anti-AN, N[a]
	Eluate	nt	+++	+++	nt	+++	−	+++	Anti-N
11	Serum	+++	+++	+++	+++	+++	−	+++	Anti-S, N
	Adsorbate	nt	−	−	nt	−	−	−	
	Eluate	nt	+	+++	nt	+++	−	+++	Anti-S, N

Note: Red and blue colors indicate S and N specificity, respectively. Underlined characters indicate AN specificity.; −, negative; +, ++, +++, weak, clear, and strong positive.

Abbreviations: A, alanine; aa, amino acids; D, aspartic acid; I, isoleucine; N, asparagine; nt, not tested; R, arginine; S, serine; V, valine.

[a]Anti-N remaining in the adsorbate could be removed by an additional adsorption step in serum no. 10.

2.4 | Expression of recombinant CD16b variants

For expression of HNA-1 allele variants, transfected HEK293-F cells (GIBCO) were used. Transfection of seven different plasmids was performed using Lipofectamine 2000 (GIBCO). The transfected HEK293-F cells were cultured in DMEM high glucose (GIBCO) supplemented with 10% fetal bovine serum (PAA, Pasching, Austria), 1% penicillin–streptomycin (PAA), and 800 µg/ml geniticin (Bioprom AG, Stuttgart, Germany). CD16b expression was confirmed by flow cytometry using PE-conjugated monoclonal antibody LNK16 (Thermo Fisher Scientific, Dreieich, Germany). The presence of CD16b variants was confirmed by sequencing DNA extracted from cultured cells, using sequence-specific primers (Eurofins mwg operon, Ebersberg, Germany) for amplification of *FCGR3B* plasmid cDNA.

2.5 | Characterization of HNA-1b alloantibodies by modified MAIGA

A total of 3×10^5 transfected HEK293-F cells stably expressing CD16b variants were incubated with 50 µl antiserum, then washed and incubated with 10 µl LNK16 (20 µg/ml). After incubation and washing, 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) and proteinase inhibitor cocktail (Sigma–Aldrich, Munich, Germany) were added for cell lysis. The tri-molecular complexes are captured by goat anti-mouse monoclonal antibodies, which are bound to a microtiter plate. The detection of bound IgG was performed with horseradish peroxydase-labeled goat-anti-human IgG (Dianova, Hamburg, Germany). After incubation with the substrate o-phenylenediamine dihydrochloride, the optical density was measured using an absorbance reader (Tecan, Zürich, Switzerland) at 492 and 620 nm.

Additionally, adsorption and elution technique was used to differentiate sera with p.82N or compound p.36S and p.82N specificity to detect the presence of HNA-1d specificity. For adsorption and elution, the variant "CD16b SSDNI," carrying the epitopes of HNA-1b and HNA-1c, but not the HNA-1d epitope "CD16b **AN*," was used. Adsorbate and eluate were analyzed in modified MAIGA using seven transfected HEK293-F cells, each stably expressing CD16b variants. Transfected HEK293-F cells only expressing the HNA-1c epitope "CD16b RSDDV" were used as negative control. Any OD above that obtained with CD16b RSDDV was considered to represent a positive reaction. If the OD was twice as high as the control, this was graded as "clear" reactivity (++), and if the OD was at least fourfold higher than the control, this was graded as "strong" reactivity (+++). Any reaction above the cutoff, but below twice the OD, was graded as a "weak" reaction (+). Adsorption experiments were repeated until all antibodies were specifically removed whenever enough material was available.

3 | RESULTS

The amino acids that differ in five positions (p.36, p.65, p.78, p.82, and p.106) in different CD16b variants are named with the single-letter amino acid code, for example, "CD16b SSANI" to name the cell line, which carries the epitopes of HNA-1b and HNA-1d, but not HNA-1a.

The transfected HEK293-F cells, each expressing different CD16b variants, were tested in modified MAIGA with 11 HNA-1b antisera (Table 1), in which compound antibody specificities binding to different epitopes, were detected. Most HNA-1b antisera (9/11) bound to transfected cells expressing p.82N (CD16b ***N*). Six of 11 HNA-1b antisera showed reactivity with p.36S, of which two bound exclusively to p.36S (CD16b S****).

Whether sera that bind to p.82N also recognize the epitope p.78A in combination with p.82N, the HNA-1d epitope (CD16b **AN*), was investigated by adsorption and elution experiments (Table 2). Results showed that eight of nine sera recognized both p.82N and the p.78A&p.82N epitope and only one serum showed p.82N specificity alone in combination with p.36S (CD16b S**N*). It is possible that antibody specificities in the sera are present in different concentrations, resulting in different reaction strength.

In sera #7, #9, and #10, Anti-N was not only present in the adsorbate, but also in the eluate. When the adsorption was repeated, anti-N could be fully removed from serum #10, indicating that anti-N in high quantity has prevented its full adsorption in standard experiments. We did not have appropriate amounts of sera #7 and #9 and repeated these experiments for these patients.

4 | DISCUSSION

The HNA-1 alleles *FCGR3B*01* and *FCGR3B*02* differ in five nucleotides in positions c.108, c.114, c.194, c.244, and c.316, leading to four amino acid exchanges in position p.36, p.65, p.82, and p.106. Homozygous carriers of *FCGR3B*01* express HNA-1a, but no HNA-1b, -1c, and -1d epitopes. They are capable of forming antibodies against the different epitopes encoded by *FCGR3B*02* (HNA-1b, HNA-1d).[16] From an immunological point of view, individuals with an *FCGR3B*01*, *03* genotype differ in only one amino acid from HNA-1b: at position p.78, characterized by an amino acid exchange p.A78D.

Upon immunization, these individuals can produce HNA-1d alloantibodies recognizing the compound epitope p.78A in combination with p.82N.[5] Due to the differences between *FCGR3B*01* and *FCGR3B*02*, *FCGR3B*01* homozygous individuals shall therefore be able to form alloantibodies against the defined HNA-1b epitopes p.36S, p.82N, and also the compound epitope p.78A&p.82N.

It should be noted that common amino acids, present in CD16b and its homologous CD16a, are unable to lead to immunization if both proteins are expressed in an individual. *FCGR3B*01* differs from *FCGR3A* in two base exchanges, c.194A>G and c.316G>A, resulting in amino acid exchanges p.N65S and p.V106I. Therefore, individuals with a homozygous *FCGR3B*02* genotype and the presence of *FCGR3A* differ in these two amino acids. The amino acid p.65N was described to be the only one recognized by HNA-1a alloantibodies. Position 106 does not seem to be involved in the epitope formation.[16]

Analysis of 11 HNA-1b antisera indicated compound specificities for HNA-1b alloantibodies with a variation between HNA-1b immunized individuals. In 2 of 11 HNA-1b antisera, p.36S was identified as the sole epitope. When p.82N was expressed, most sera became reactive (9/11). Our adsorption and elution technique was capable of differentiating between "p.82N only" and compound "p.78A&p.82N" specificities. However, among these nine positive sera, the combined reactivity to "p.82N only" and "combined p.78A p.82N" was identified as dominant reactivity. Only one serum bound to p.82N in combination with p.36S. These data indicate that p.36S, p.82N, and p.78A&p.82N are all necessary for HNA-1b epitope formation. Interestingly, the HNA-1d epitope is recognized by 73% (8/11) of HNA-1aa genotyped individuals with an anti-HNA-1b immunization, so that the HNA-1d epitope formed by p.78A&p.82N can be considered the most important epitope in HNA-1b epitope formation. We conclude that the majority of anti-HNA-1b sera contains anti-HNA-1d specificities. Our new findings also support our previous observation that p.78A&p82.N are forming a complex epitope: when we defined HNA-1d in 2013, we demonstrated the reactivity of the specific antibody to be dependent on the presence of both amino acids by using different chimeric proteins.[5] Here, we could show that these antibodies can be characterized by adsorption/elution technique and that they can be clearly distinguished from antibodies against p.82N.

Whether immunization against HNA-1d (p78.A&p82.N) plus HNA-1b (p.36S, p.82N) has an influence on the clinical course of alloimmune neutropenia cannot be deduced from our study. This question will have to be addressed in follow-up studies.

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CONFLICT OF INTEREST

The authors have disclosed no conflicts of interest.

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