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Neutrophil FcγRIIIb allelic polymorphism in anti-neutrophil cytoplasmic antibody (ANCA)-positive systemic vasculitis

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SUMMARY

Neutrophils constitutively express FcγRIIa and FcγRIIIb receptors. Both receptors exhibit allelic variants which have different quantitative functional capacities: the biallelic FcγRIIa-R131 and -H131 alleles, and the neutrophil antigen (NA) NA1/NA2 alleles. ANCA activation of neutrophils requires ligation of FcγRIIa receptor, but recent data have shown that ANCA can also bind FcγRIIIb receptor. The aim of this study was to determine whether the FcγRIIIb polymorphism was a risk factor for the development of ANCA-associated systemic vasculitis, or the associated nephritis. FcγRIIIb receptor genotyping was determined by allele-specific polymerase chain reaction. Genomic DNA was extracted from 101 Caucasian patients with $ANCA^+$ vasculitis (of whom 84 had renal disease) and 100 ethnically matched controls. Of the patients with $ANCA^+$ systemic vasculitis, 71 had ANCA with specificity for proteinase 3 and 30 with specificity for myeloperoxidase (MPO). Overall no significant difference in genotype distribution or allele frequencies was found between patients and controls, or between patients with renal disease and controls. However, there was a trend for an increase in homozygosity for the NA1 allele in patients with a vasculitis and this was significant in patients who had anti-MPO antibodies. The FcγRIIIb receptor polymorphism is not a major factor predisposing to the development of $ANCA^+$ systemic vasculitis or the associated nephritis. The over-representation of the FcγRIIIb homozygous NA1 allele in patients with anti-MPO antibodies may have implications for disease susceptibility.

Keywords FcγRIIIb receptor polymorphism ANCA systemic vasculitis

INTRODUCTION

Neutrophils are involved in the vascular injury seen in the ANCA-associated vasculitides. This is clearly indicated by histological observation of neutrophils in early glomerular lesions [1,2], the strong correlation between the extent of renal involvement and the number of neutrophils present in renal biopsies [3], and the apparent neutrophil trapping in renal capillaries [4]. Further, ANCA bind to ANCA antigens expressed on the surface of primed neutrophils, engage Fcγ receptors and activate neutrophils leading to an oxidative burst [3,5–8].

Neutrophils constitutively express two classes of Fcγ receptors: FcγRIIa and FcγRIIIb which have allelic variants with differing functional capacities [9–13]. Previous studies have shown that ANCA-induced neutrophil activation involved the FcγRIIa receptor [6–8,14]. However, incomplete blocking of ANCA-mediated respiratory burst despite saturating doses of anti-FcγRIIa Fab [6–8,14] and evidence of FcγRIIIb receptor engagement by ANCA [15], raise the possibility that FcγRIIIb receptor may have a role in neutrophil activation. We hypothesize that FcγRIIa and FcγRIIIb receptor polymorphisms may influence disease susceptibility or disease expression in the ANCA-associated systemic vasculitis, secondary to differential IgG binding and neutrophil activation. Already, we and others have shown no association between FcγRIIa receptor polymorphism and ANCA-associated vasculitis [16,17]. The biallelic polymorphism of FcγRIIIb receptor is designated neutrophil antigen 1 and 2 (NA1 and NA2). The isoforms of the FcγRIIIb receptor differ by four amino acids, with changes at amino acid positions 65 and 82 resulting in two extra glycosylation sites (six instead of four) in the FcγRIIIb-NA2 allotypic form [18,19]. Although the two isoforms have similar IgG subclass binding properties, the NA1 isoform facilitates a more robust FcγR-mediated phagocytosis, respiratory burst and degranulation responses compared with the NA2 isoform [11,12]. To test the hypothesis that FcγRIIIb alleles might influence susceptibility to the development of $ANCA^+$ systemic vasculitis, the distribution of the FcγRIIIb genes in Caucasian patients with $ANCA^+$ vasculitis was compared with that of healthy Caucasian controls. Specifically, the relationship between FcγRIIIb genotype and nephritis was also examined.

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PATIENTS AND METHODS

Subjects for FcγRIIIb genotyping

Peripheral blood was collected into tubes containing 1/10 volume of 0·5 M EDTA (pH 8·0) from 100 disease-free Caucasian individuals who attended hospital for minor surgery, and 101 consecutive Caucasian patients who presented with an ANCA$^+$ systemic vasculitis. Patients were classified using the Chapel Hill Consensus conference definitions [20]. Renal involvement was defined as a plasma creatinine of >150 μmol/*l*, creatinine clearance of <100 ml/min, the presence of haematuria, proteinuria $>0·5$ g daily, or biopsy-proven histology showing necrotizing vasculitis.

Determination of FcγRIIIb genotypes by allele-specific polymerase chain reaction

Genomic DNA was extracted using a nucleic acid extraction kit (ORCA Research Inc., Bothwell, WA) from peripheral blood obtained from subjects. For amplification of the FcγRIIIb-NA1 DNA, the following gene-specific primers were used: sense primer (5′-CAG TGG TTT CAC AAT GTG AA-3′) and anti-sense primer (5′-CAT GGA CTT CTA GCT GCA CCG-3′). The reaction mixture contained 10 μl of DNA (1/4 of the isolated DNA), 0·8 μM sense and anti-sense primers, 2·75 mM magnesium chloride, 5 μl of 10× reaction buffer (Promega, Southampton, UK), 200 μM deoxynucleotide triphosphates and 0·1 U Taq DNA polymerase (Promega) in a final volume of 50 μl. The cycle programme was set to the following parameters: 95°C for 5 min, 60°C for 1·5 min, 72°C for 2·5 min (one cycle); 95°C for 1 min, 60°C for 1·5 min, 72°C for 2·5 min (10 cycles); 95°C for 1 min, 57°C for 1 min, 72°C for 1 min (25 cycles); 72°C for a 10-min extension. After polymerase chain reaction (PCR) amplification, a 141-bp FcγRIIIb gene was amplified. The following gene-specific primers were used for amplification of the FcγRIIIb-NA2 DNA: sense primer (5′-CTC AAT GGT ACA GCG TGC TT-3′) and anti-sense primer (5′-CTG TAC TCT CCA CTG TCG TT-3′). The PCR reactions were performed in a total volume of 50 μl containing 10 μl of DNA (1/4 of the isolated DNA), 0·1 μM sense and anti-sense primers, 2·75 mM magnesium chloride, 5 μl of 10× reaction buffer (Promega), 200 μM deoxynucleotide triphosphates and 0·1 U Taq DNA polymerase (Promega). The cycle programme was set to the following parameters: 95°C for 5 min, 64°C for 1·5 min, 72°C for 2·5 min (one cycle); 95°C for 1 min, 64°C for 1·5 min, 72°C for 2·5 min (35 cycles); 72°C for a 10-min extension. The FcγRIIIb-NA2-specific primers amplify a 169-bp product. The PCR products were visualized by ethidium bromide staining in a 1% agarose gel dissolved in TAE buffer. Positive controls for the NA1 and NA2 PCR were included in each round of the PCR, and comprised DNA of known FcγRIIIb genotype kindly provided by Dr Van de Winkel, Utrecht, The Netherlands.

ANCA testing

Indirect immunofluorescence. ANCA activity of samples was determined by indirect immunofluorescence on ethanol-fixed neutrophils using standard techniques [21] and by antigen-specific ELISA [16].

Statistical analysis

FcγRIIIb genotype distribution (NA1/NA1, NA1/NA2, NA2/NA2) and allele frequencies (NA1, NA2) were analysed by applying the χ^2 test. To reject the null hypothesis, a probability of 0·05 (two-tailed) was used. Comparisons were made between ANCA$^+$ vasculitis patients and controls.

RESULTS

Patient demographics

All patients were ANCA$^+$ as determined by indirect immunofluorescence and antigen-specific ELISA. Of the 101 ANCA$^+$ vasculitis patients who had FcγRIIIb genotyping performed, 45 had Wegener's granulomatosis (16 had the limited form), 52 patients had microscopic polyangiitis, three had classical polyarteritis nodosa and one patient had Churg–Strauss syndrome. Seventy-one patients had proteinase 3 (PR3)-ANCA and 30 had myeloperoxidase (MPO)-ANCA.

FcγRIIIb genotype frequency

The FcγRIIIb genotype distribution and allele frequency in all patients are shown in Table 1. No skewing was observed in the overall genotype distribution ($\chi^2 = 1·9397$, $P = 0·379$) or allele frequency ($\chi^2 = 0·7852$, $P = 0·376$) between ANCA$^+$ vasculitis patients and healthy control subjects. To see whether FcγRIIIb alleles were risk factors for the development of nephritis, the genotype frequency of patients with and without renal disease was examined. Altogether, 84 patients had evidence of renal involvement (as defined above) and 17 patients did not have renal involvement (Table 1). Again, no skewing was observed in the genotype distribution ($\chi^2 = 0·8173$, $P = 0·665$) or allele frequency ($\chi^2 = 0·1209$, $P = 0·728$) between those vasculitis patients with renal disease and healthy control subjects.

Of the patients who had FcγRIIIb genotyping performed, 45 patients had Wegener's granulomatosis (WG) and 52 patients had microscopic polyangiitis (Table 2). No skewing was observed in the overall genotype distribution ($\chi^2 = 2·2889$, $P = 0·3184$) or allele frequency ($\chi^2 = 1·6118$, $P = 0·2042$) between patients with WG and healthy control subjects. Similarly, no skewing was observed in the overall genotype distribution ($\chi^2 = 2·1482$, $P = 0·3416$) or allele frequency ($\chi^2 = 0·068$, $P = 0·7942$) between patients with microscopic polyangiitis and healthy control subjects.

The genotype distribution and ANCA status are shown in Table 3. No skewing was observed in the overall genotype distribution ($\chi^2 = 0·4559$, $P = 0·796$) or allele frequency ($\chi^2 = 0·0645$, $P = 0·800$) between PR3-ANCA$^+$ vasculitis patients and healthy control subjects. Similarly, no skewing was observed in the overall genotype distribution ($\chi^2 = 4·4257$,

Table 1. Distribution of FcγRIIIb genotypes and the allele frequencies in controls and vasculitis patients

	Controls, $n = 100$	Vasculitis patients, $n = 101$	Vasculitis patients with renal disease, $n = 84$
Genotype distribution			
Subject numbers (%)			
NA1/NA1	7 (7)	13 (13)	9 (11)
NA1/NA2	47 (47)	45 (44)	37 (44)
NA2/NA2	46 (46)	43 (43)	38 (45)
Allelic frequency (%)			
NA1	(30·5)	(35)	(33)
NA2	(69·5)	(65)	(67)

Table 2. Distribution of FcγRIIIb genotypes and the allele frequencies in controls and patients with Wegener's granulomatosis (WG) and microscopic polyangiitis

	Controls, $n = 100$	Patients with WG, $n = 45$	Patients with microscopic polyangiitis, $n = 52$
Genotype distribution			
Subject numbers (%)			
NA1/NA1	7 (7)	6 (13)	7 (13)
NA1/NA2	47 (47)	23 (51)	20 (39)
NA2/NA2	46 (46)	16 (36)	25 (48)
Allelic frequency (%)			
NA1	(30·5)	(39)	(33)
NA2	(69·5)	(61)	(67)

Table 3. Distribution of FcγRIIIb genotypes and the allele frequencies in controls and ANCA$^+$ patients

	Controls, $n = 100$	Vasculitis patients with PR3-ANCA, $n = 71$	Vasculitis patients with MPO-ANCA, $n = 30$
Genotype distribution			
Subject numbers (%)			
NA1/NA1	7 (7)	7 (10)	6 (20)
NA1/NA2	47 (47)	32 (45)	13 (43)
NA2/NA2	46 (46)	32 (45)	11 (37)
Allelic frequency (%)			
NA1	(30·5)	(32)	(42)
NA2	(69·5)	(68)	(58)

Table 4. Association between NA1 homozygosity and disease

	Odds ratio (90% confidence intervals)	χ^2	P
Vasculitis *versus* controls	1·9 (0·8–4·3)	1·93	0·16
PR3-ANCA *versus* controls	1·4 (0·6–3·6)	0·45	0·50
MPO-ANCA *versus* controls	3·3 (1·3–8·7)	4·33	0·037

$P = 0{\cdot}109$) or allele frequency ($\chi^2 = 2{\cdot}1199$, $P = 0{\cdot}145$) between MPO-ANCA$^+$ vasculitis and healthy control subjects. Overall, there was a trend for an increase in NA1 homozygosity in patients with a vasculitis and this was significant in patients with MPO-ANCA (odds ratio 3·3, 90% confidence limits 1·3–8·7; $\chi^2 = 4{\cdot}33$, $P = 0{\cdot}037$) (Table 4).

DISCUSSION

The clear segregation of quantitative neutrophil activation by FcγIIIb genotype *in vitro* [11–13] and the ability of ANCA to bind to FcγIIIb receptor [15], raised the possibility that FcγIIIb receptor polymorphism might be a genetic risk factor for the development or expression of disease in ANCA-associated systemic vasculitis. The present study found no overall skewing of FcγRIIIb alleles in vasculitis. Further, no association was found between the functionally more active NA1 allele and renal disease. However, a significant increase of NA1 homozygosity in patients with an anti-MPO$^+$ vasculitis was found. This observation must however be interpreted with caution because of the multiplicity of comparisons. There is only one other study which examined FcγRIIIb receptor polymorphism in systemic vasculitis [22]. Kimberly *et al*. characterized the distribution of FcγRIIIb alleles using allele-specific PCR in 145 patients with WG [22]. The functionally more active NA1 allele was significantly enriched in patients with renal disease.

FcγRIIIb receptor is linked to the plasma membrane by a glycosylphosphatidyl inositol anchor [23,24] and is released from the cell surface through cleavage by serine and/or metalloproteases upon cell activation [25,26]. Engagement of FcγRIIIb receptor can lead to degranulation [27,28] and release of reactive oxygen species [29–33]; mechanisms which could lead to vascular injury in vasculitis. Mulder *et al*. examined the contribution of FcγRIIa and FcγRIIIb receptors to the ANCA-mediated neutrophil respiratory burst [6]. Blockade of FcγRIIa receptors reduced the ANCA-induced respiratory burst by almost 50%, whereas blockade of FcγRIIIb receptor by MoAb 3G8 had no inhibitory effect. However, FcγRIIIb receptor shedding was not inhibited in this study, and surface re-expression of this receptor could have offset any inhibitory effect. Indeed, ANCA engagement of FcγRIIIb receptor was clearly shown by flow cytometry in a later study which had blocked receptor shedding [15]. Nonetheless, clear evidence that ANCA induce neutrophil respiratory burst through FcγRIIIb receptor engagement remains to be seen. *In vitro*, co-operation between FcγRIIa and FcγRIIIb receptors has been described in a number of functional studies, including: the production of the respiratory burst [30], FcγRIIa-mediated phagocytosis [34], immune complex-induced neutrophil actin assembly [35] and the release of hydrolytic enzymes induced by IgM anti-FcγR autoantibodies [36]. *In vivo*, Fijen *et al*. found that individuals with a deficiency of a component of the terminal complement pathway (C6 or C8) in combination with both the FcγRIIa-R/R131 and the FcγRIIIb-NA2/NA2 allotypes had experienced more meningococcal infections than C6- or C8-deficient family members with other combinations of these FcγR allotypes [37]. These observations support an interaction between the magnitude of humoral response and Fcγ receptor allotypes.

In conclusion, a major role for FcγRIIIb polymorphism in determining disease susceptibility in ANCA-associated vasculitis could not be shown in this study. The clinical relevance of the over-representation of the FcγRIIIb homozygous NA1 allele in patients with anti-MPO antibodies remains to be determined. Lastly, the possibility of a type II error cannot be excluded in the present study, since in order to show a 15% difference in the hypothesized at risk allele NA1 with an α value of 0·05 (two-sided) with 90% power, 230 patients and 230 controls would need to be recruited. Given the rare incidence of ANCA-associated vasculitis, a sample size of this magnitude would be difficult to recruit in a single centre. It remains possible that FcγRIIIb receptor polymorphism, perhaps in combination with FcγRIIa and other non-Fcγ polymorphisms, may determine disease susceptibility or severity in vasculitis.

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