IgG1 heavy chain-coding gene polymorphism (G1m allotypes) and development of antibodies-to-infliximab

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Objective **The chimeric anti-tumor necrosis factor-α antibody infliximab is known to induce antibodies-to-infliximab (ATI) in some treated patients. Immunogenicity in murine variable domains is expected; however, constant domains of its human heavy γ1 chain may also be implicated as it expresses G1m1 and G1m17 allotypes. This allelic form may be immunogenic in patients that are homozygous for the G1m3 allotype commonly expressed in Caucasoid populations.**

Methods **As G1m allotypic divergence may explain the presence of ATI or may influence their concentration, a genotyping method was developed and validated to determine antithetical (i.e. mutually exclusive) G1m3 and G1m17 allotypes (amino acid 120 of CH1 according to the international ImMunoGeneTics information system unique numbering) at the IGHG1 gene level (CH1 359g/a nucleotide polymorphism). Two hundred forty-five blood donors and 118 previously described patients suffering from Crohn's disease, treated with infliximab, and having developed ATI in 73 of them, were genotyped.**

Results **The IGHG1 CH1 359g/a polymorphism does not depart from the Hardy–Weinberg equilibrium in the control population, and allele frequencies were similar in controls and patients. No association was found between the patient G1m allotypes and the presence of ATI or their concentration. It remains possible that anti-Gm1 antibodies are not well detected by the enzyme-linked immunosorbent assays used for ATI detection and/or that the G1m allotypes are minor antigens on IgG1.**

Conclusion **The IGHG1 polymorphism does not seem to play a major role in the induction of ATI. Further analyses will be required to determine whether it is also the case for humanized or fully human antibodies bearing the same G1m allotypes.** ***Pharmacogenetics and Genomics*** **00:000–000 © 2009 Wolters Kluwer Health | Lippincott Williams & Wilkins.**

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Introduction

The impact of infliximab immunogenicity in the form of increased risk of infusion reactions and reduced duration of therapeutic response was first demonstrated by some of us in 2003 [1]. In this cohort of Crohn's disease (CD) patients, antibodies-to-infliximab (ATI) were detected in about 61% of them after the fifth injection. Other studies showed that 13 [2] to 36% [3,4] of CD patients and up to 47% of rheumatoid arthritis patients developed ATI [5–7]. It is therefore obvious that only a proportion of treated patients developed an immune response to infliximab.

Infliximab immunogenicity probably results from the presence of murine variable domains within this chimeric anti-tumor necrosis factor-α IgG1κ antibody [8]. However, in early animal experiments, it was noticed that polymorphisms in the constant regions (allotypes) also contributed to immunogenicity of chimeric antibodies [9]. Allotypes of heavy γ1 chains are defined as G1m allotypes (Table 1 and Fig. 1) and those of the light κ chains as Km allotypes [14]. From its published sequences [15], it can be deduced that infliximab γ1 chains probably bear the G1m1, 17 and Km3 allotypes [14]. Although the Km3 allotype is very common in Caucasoid populations (0.90) [14], the G1m1, 17 allotype is much less frequent (0.15–0.35) than G1m3 (0.65–0.85) (Table 1) [14,16]. As a consequence, it is expected that, in a Caucasoid population, 42–72% of the patients are homozygous for the G1m3 allotype and are administered with a therapeutic IgG1 antibody bearing the antithetical G1m1, 17 allotype.

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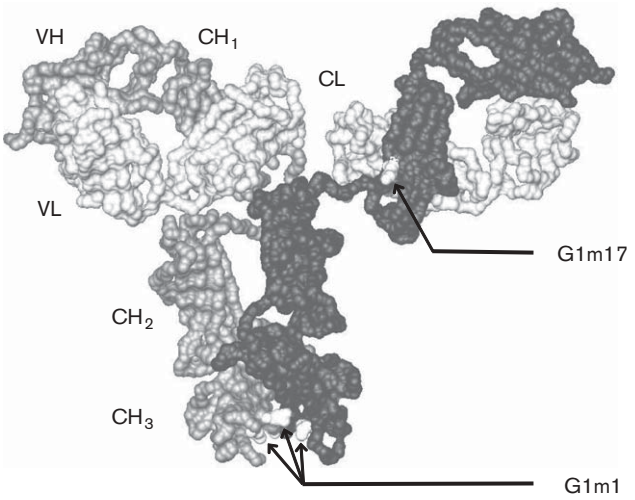
Table 1 Human IG γ1 chain (G1m) allotypes and corresponding IGHG1 alleles

G1m allotypes	IGHG1 allele names	Amino acid positions[a]		
		CH1[b]	CH3[b]	
		120	**12**	**14**
		(97)	(16)	(18)
		214	*356*	*358*
G1m1, 17	IGHG1*01	Lys K	Asp D	Leu L
	IGHG1*02	a**a**a	gat	ctg
G1m3	IGHG1*03	Arg R	Glu E	Met M
		a**g**a	gag	atg

[a]Amino acid numbering in bold is according to international ImMunoGeneTics information system unique numbering for C-domain [10] (IMGT Repertoire, http://www.imgt.org), between parentheses: exon numbering [11] and in italics: EU numbering [11].
[b]CH1 Lys 120 determines the G1m17 allotype, whereas CH3 Asp 12 and Leu 14 determine the G1m1 allotype (present on G1m1, 17 γ1 chains). CH1 Arg 120 determines the G1m3 allotype (present on G1m3 γ1 chains) [9]. Nucleotides highlighted in bold in codon 120 are those characterized by the IGHG1 CH1 359g/a genotyping method used in this study.

Fig. 1



Localization of the amino acids corresponding to the G1m1 and G1m17 allotypes on three-dimensional structure of a human IgG1κ. Space-filling representation of the monoclonal antibody b12 directed against the HIV envelope glycoprotein gp210 [12] (Protein Data Bank and international ImMunoGeneTics information system (IMGT)/3Dstructure-DB, *http://www.imgt.org* 1 hzh). Figure generated with VMD software [13]. The two identical heavy γ1 chains, indicated in grey and black have one variable domain VH and three constant domains CH1, CH2, and CH3, and the two identical light κ chains in pale-grey have one variable domain VL and one constant domain CL. Heavy and light chains are linked together with disulfide bonds. The analysis of the sequence suggests that the heavy γ1 chains of this IgG1κ bear G1m1, 17 allotypes. The corresponding amino acids are colored in white: the lysine at position 120 of CH1 according to the IMGT unique numbering [10] corresponds to the G1m17 allotype [9] (Table 1). The asparagine and the leucine at positions 12 and 14, respectively of CH3 according to the IMGT unique numbering [10] correspond to the G1m1 allotype [9] (Table 1).

We therefore hypothesized that the risk to develop ATI could be dependent on G1m allotypes. To avoid interferences with circulating infliximab, which would affect the patients G1m serological determination, we first developed a genotyping method for the determination of the G1m3 and G1m17 allotypes. The method is based on the IGHG1 CH1 nucleotide (nt) polymorphism at position 359g/a (Table 1 and Fig. 1). We then analysed the patients G1m allotypes in relation with the presence of ATI or their concentration. An in-house enzyme-linked immunosorbent assays (ELISA) developed for ATI determination was also evaluated for its ability to detect anti-G1m allotypes.

Methods

Patients and controls

One hundred eighteen CD patients from the cohort already described by Baert *et al.* [1] were genotyped. Two hundred forty-five blood donors (from the Etablissement Français du Sang Centre-Atlantique, Tours, France) were also included in this study. All of them were genotyped and the sera of 44 of them were subjected to G1m allotyping by serological methods. Six control sera containing anti-G1m allotype antibodies were obtained from the Centre d'Anthropologie in Toulouse (France): four displayed anti-G1m1 specificity and low-to-high hemagglutination titers, and two others displayed anti-G1m17 specificity and low titers.

G1m allotype serological determination

G1m allotype serological determination of therapeutic IgG1 monoclonal antibodies

Infliximab and alemtuzumab (Schering-Plough, Levallois-Perret, France), basiliximab (Novartis, Huningue, France), daclizumab, rituximab, trastuzumab (Roche, Neuilly, France), and cetuximab (Merck, Lyon, France) were subjected to allotype determination by inhibition of hemagglutination (HAI). Briefly, sheep red blood cells (srbcs) were coated with a purified preparation of IgG1 myeloma protein of either G1m3 or G1m1, 17 allotype using chromium chloride [17]. The monoclonal antibodies HP 6027 anti-G1m3 (The Medical School, University of Birmingham, Birmingham, UK) [18], HP 6184 anti-G1m1, and HP 6189 anti-G1m17 (Central Laboratory of The Netherlands Red Cross Blood Transfusion Service, Amsterdam, The Netherlands) [19] were titrated against the coated srbcs and the end point was determined as the highest dilution where agglutination was still obvious, or minimum hemagglutinating dose. The therapeutic antibodies were each titrated from 50 μg/ml in a series of 11 doubling dilutions. Twice the concentration of monoclonal antibodies (2 minimum hemagglutinating dose) and the diluted therapeutic antibodies or control were added in each well before srbcs. The plates were then allowed to settle at room temperature. If HAI had occurred, the srbcs would fall into a button; no inhibition would resemble the control of a carpet of agglutinated cells over the bottom of the well. The results were very obvious with either total agglutination or tight buttons.

G1m allotype serological determination on blood donor sera

Forty-four sera samples among the 245 obtained from blood donors were tested for G1m allotypes at the Centre d'Anthropologie in Toulouse, France, using the standard method of inhibition of agglutination [20].

IGHG1 CH1 359g/a genotyping method

In this multiplex allele-specific PCR, the 20 µl reaction mixture contained the following: 50 ng genomic DNA, 1 mmol/l $MgCl_2$ (Eurobio, Les Ulis, France), 200 µmol/l of each deoxyribonucleotide triphosphate (MBI Fermentas, Mundolsheim, France), 20 pmol of sense primer 20-mer, 5′-CCCCTGGCACCCTCCTCCAA-3′ (IGHG1 CH1 codons 6–12, nt 16–35 according to the international ImMunoGeneTics information system unique numbering [10]) (Proligo, Paris, France); 4 pmol of 359g-antisense primer 19-mer, 5′-GCTGGCCTCTCACCAACT**C**-3′ (nt 359 in bold) (Proligo); 10 pmol of 359a-antisense primer 37-mer, 5′-*TAATAAAATACTTTTTATA*CTGGCCTCTCACCAACT**T**-3′ (non-IG sequence added for primer length extension, in italics) (Proligo), 0.5 U of EurobioTaq DNA polymerase (Eurobio) in its buffer (67 mmol/l Tris–HCl, pH 8.8; 16 mmol/l $(NH_4)_2SO_4$; 0.01% Tween 20). The PCR amplification profile was as follows: initial denaturation at 94°C for 5 min, followed by 40 cycles of denaturation at 94°C for 1 min, annealing at 65.6°C for 1 min and extension at 72°C for 1 min. This amplification was performed on Bio-Rad thermocycleur (Bio-Rad, Marnes-La-Coquette, France). The PCR products were separated in an 8% precast polyacrylamide gel (Invitrogen, Cergy Pontoise, France) and visualized after ethidium bromide staining. The observed PCR products of 281 and 299 base pairs long correspond to G1m3 (amplified with the 359g-antisense primer) and to G1m17 (amplified with the 359a-antisense primer), respectively (Table 1).

Enzyme-linked immunosorbent assays

Detection and quantification of antibodies-to-infliximab

Quantification of ATI in serum was based on a 'double antigen format' ELISA. As described earlier [1], ATI in patients sera were detected by an ELISA from Prometheus Laboratories (San Diego, USA) calibrated with affinity-purified polyclonal rabbit anti-mouse IgG $F(ab)'_2$. The cut-off value was determined at 1.69 µg/ml.

An in-house double antigen format ELISA was also developed. Briefly, microtiter plates (Nunc-Immuno Plate Maxisorp surface, Brand Products, Illkirch, France) were coated with infliximab, and the presence of ATI in tested sera was revealed by horseradish peroxydase-labeled infliximab (labeling by Protéogénix, Oberhausbergen, France). Calibration was based on a mouse anti-human IgG all subclasses monoclonal antibody (provided by M.G.). Ortho-phenylene diamine (Sigma-Aldrich, Lyon, France), substrate of horseradish peroxydase is added and the microplate is read at 492 and 620 nm.

Evaluation of the detection of anti-G1m1 and anti-G1m17 antibodies

To evaluate whether the in-house ELISA developed for ATI was detecting anti-G1m1 and anti-G1m17 antibodies, which may arise as part of the ATI in patients with Gm3 allotypes, control sera with anti-G1m1 or anti-G1m17 specificities (provided by JMD) were tested in the ELISA assay.

Statistical methods

Exact tests (GENEPOP software [21]) were used to test departure at the Hardy–Weinberg equilibrium, genic and genotypic differentiation of various populations and subpopulations studied. Significance threshold of P value was fixed at 0.05.

Results

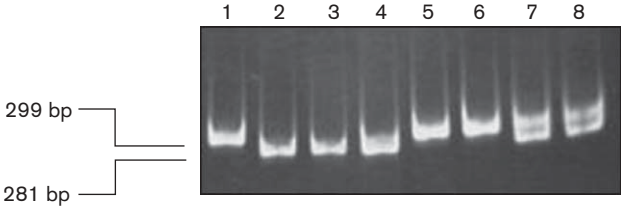
The G1m allotypes, G1m1, G1m3, and G1m17, were determined for seven IgG1 therapeutic antibodies by HAI (Table 2). Four of them bear G1m1, 17 allotypes (alemtuzumab, daclizumab, infliximab, and rituximab), two are G1m3 positive (basiliximab and cetuximab). Trastuzumab has only the G1m17 allotype, in agreement with the G1m1 allotype being engineered out [22].

Determination of G1m allotypes in sera from 44 blood donors by HAI was 100% concordant with IGHG1 CH1 359g/a genotyping from the same blood donors. IGHG1 CH1 359g/a genotyping therefore performed in 201 other blood donors (245 total) (Fig. 2 and Table 3) showed that the expected frequencies of each genotype, 50.7, 41.0,

Table 2 Serological G1m allotype determination of therapeutic IgG1 monoclonal antibodies

	HP 6027 anti-G1m3	HP 6184 anti-G1m1	HP 6189 anti-G1m17
Therapeutic antibodies			
Alemtuzumab	−	+	+
Basiliximab	+	−	−
Cetuximab	+	−	−
Daclizumab	−	+	+
Infliximab	−	+	+
Rituximab	−	+	+
Trastuzumab	−	−	+
Positive controls			
IgG1m3	+	−	−
IgG1m1, 17	−	+	+

Fig. 2



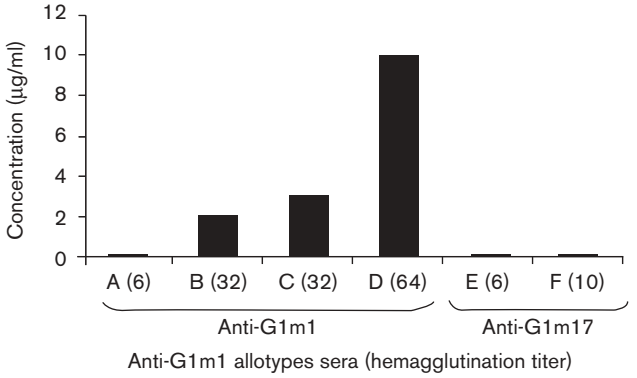
IGHG1 CH1 359g/a genotyping results for some healthy blood donors. Polyacrylamide gel (8%) electrophoresis of PCR products from seven healthy blood donors ($n=44$) with annealing at 65.6°C and 1 mmol/l of $MgCl_2$. Blood donors in lanes 1, 5, and 6 are homozygous IGHG1 CH1 359a/a, in lanes 2, 3, and 4 are homozygous IGHG1 CH1 359g/g, and in lanes 7 and 8 are heterozygous IGHG1 CH1 359g/a, expressing G1m17 or G1m3 or G1m3 and G1m17 on their IgG1, respectively.

Table 3 IGHG1 CH1 359g/a genotyping in 118 Crohn's disease patients treated with infliximab

		IGHG1 CH1-359 genotype (G1m phenotype)				Alleles frequencies		
Patients	HWE[a]	g/g (3/3) (%)	g/a (3/17) (%)	a/a (17/17) (%)	p[b]	g (%)	a (%)	p[b]
Blood donors ($n=245$)	NS	127 (51.8)	95 (38.8)	23 (9.4)	0.675	71.2	28.8	0.659
Crohn's patients ($n=118$)	NS	60 (50.8)	44 (37.3)	14 (11.9)		69.5	30.5	
ATI negative ($n=45$)	NS	25 (55.5)	16 (35.5)	4 (8.9)	0.416	73.3	26.7	0.378
ATI positive ($n=73$)	NS	35 (47.9)	28 (38.3)	10 (13.7)		67.1	32.9	
ATI <8 mg/ml ($n=32$)	NS	16 (50.0)	12 (37.5)	4 (12.5)	0.744	68.8	31.2	0.725
ATI >8 mg/ml ($n=41$)	NS	19 (46.3)	16 (39)	6 (14.6)		65.9	34.1	

ATI, antibodies-to-infliximab; HWE, Hardy–Weinberg equilibrium; NS, Non significant.
[a]Exact test for the Hardy–Weinberg equilibrium.
[b]Exact test for populations differentiation.

Fig. 3



Evaluation of the detection of anti-G1m1 and anti-G1m17 antibodies from control sera. Six control sera with anti-G1m antibodies were tested in the in-house enzyme-linked immunosorbent assays developed for antibodies-to-infliximab: four contained anti-G1m1 antibodies (A, B, C, and D) and two anti-G1m17 antibodies (E and F). The capture of anti-G1m antibodies on coated infliximab and horseradish peroxydase-labeled infliximab in a 'double antigen format' assay was observed for three anti-G1m1 sera with high hemagglutination titers (serums B, C, and D). Sera with low hemagglutination titers (one anti-G1m1 and the two anti-G1m17) were undetectable (serums A, E, and F).

and 8.3% for IGHG1 CH1 359g/g, 359g/a, and 359a/a, respectively, were very similar to the observed frequencies (Table 3), indicating that this population was in the Hardy–Weinberg equilibrium for these genotypes. Very similar genotype and allele frequencies were also observed in the cohort of 118 CD patients. This cohort was then divided into two groups, based on the presence or absence of ATI [1], and the ATI positive patients were further divided into two subgroups based on the ATI concentration (with a threshold of 8 µg/ml, defined as clinically relevant [1]). As shown in Table 3, no difference in IGHG1 genotype frequency was observed whatever the presence of ATI. There was also no difference when the ATI concentration was considered. The frequency of ATI and their mean concentrations were also similar whether patients were IGHG1 CH1 359g/g homozygotes (that is bearing the G1m3 allotype, antithetical to the infliximab G1m17 allotype) or IGHG1 CH1 359a carriers (not shown).

To evaluate whether the in-house ELISA developed for ATI was detecting anti-G1m1 and anti-Gm17 antibodies which may arise as part of the ATI in patients with Gm3 allotypes, six control sera, four containing anti-G1m1 antibodies, and two containing anti-G1m17 antibodies were tested in the ELISA assay (Fig. 3). The three sera, which gave a significant signal in ELISA were those characterized by an anti-G1m1 specificity but also by high hemagglutination titers ($\geq 1:32$). The two anti-G1m17 sera did not provide any signal.

Discussion

IGHG1 appears as a good candidate gene influencing infliximab immunogenicity because this drug bears the G1m1, 17 allotypes, which are present at low frequency in Caucasoid populations (Table 1). We studied 245 healthy blood donors among whom 44 allowed us to validate the G1m3/17 (IGHG1 CH1 359g/a) genotyping method and then a cohort of 118 patients suffering from CD previously studied for their ATI status [1]. None of the considered groups departs from the Hardy–Weinberg equilibrium. The comparison of 'healthy blood donors' versus the whole cohort of treated patients, 'ATI negative' versus 'ATI positive', 'ATI of less than 8 µg/ml' versus 'ATI of greater than 8 µg/ml', with an exact test for population differentiation does not show a significant difference in genotype distribution or alleles frequencies (Table 3). The statistical tests adapted to the limited numbers in each group or subgroup did not reveal any tendency to an association between IGHG1 polymorphism and ATI production suggesting that enlarging the cohort would not modify the results. The first explanation to the fact that no association was found between the presence of ATI or their concentrations and G1m allotypes could be the inability of the ATI double-antigen ELISA to detect anti-allotype antibodies [1]. The evaluation of the in-house ELISA for the detection of anti-G1m1 and anti-G1m17 antibodies shows that control sera containing anti-G1m1 antibodies gave positive results, provided that they were at high hemagglutination titers. In contrast, three control sera, one anti-G1m1 and two anti-Gm17, which had low hemagglutination titers gave negative results. Although these negative results may be explained

by the low titers, they might also be because of epitope masking or to steric hindrance owing, for example to the localization of the Gm17 allotype on CH1.

Another explanation for the lack of association between the presence of ATI or their concentrations and G1m allotypes could be that the G1m17 allotype, which corresponds to one amino acid change in the IGHG1 CH1 domain (Table 1, Fig. 1 and [9]) behaves as a minor antigenic determinant when compared with the infliximab variable domains which likely dominate the ATI response. In contrast, it has been suggested that the two amino acid changes in the CH3 domain that characterize the G1m1 allotype is a possible helper T cell epitope [23] which could be hypothesized to favor the development of antibodies directed against any part of the therapeutic antibody, comprising in the variable domains. This study shows that it is probably not the case. However, allotypes remain potential immunogenic B cell and T cell epitopes, particularly for humanized or human therapeutic antibodies. In this context, it has to be noted that trastuzumab has been engineered with two amino acid changes IGHG1 CH3 D12 > E, L14 > M (Tables 1 and 2) to convert the G1m1 allotype to the iso-allotype nG1m1, the resulting γ1 chain being Gm17, nG1m1, in an attempt to reduce the risk of anti-G1m1 antibodies interfering with therapy [22]. Whether IGHG1 polymorphism is a genetic factor influencing immunogenicity of anti-tumor necrosis factor-α antibodies and other therapeutic antibodies needs therefore to be carefully studied in other cohorts of patients.

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