Analysis at the DNA Level of Human FcγRII Isoforms in Relation to the Polymorphic Binding of Murine IgG2b to Human B Cells

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Three classes of human leucocyte Fcγ receptors (hFcγR) have been identified so far: hFcγRI, hFcγRII, and hFcγRIII. Previous studies have demonstrated that genetically determined differences between individuals exist both with respect to the binding of murine IgGl (mIgGl) to hFcγ receptors, and with respect to the binding of murine IgG2b (mIgG2b). The polymorphism in binding of mIgGl could be ascribed to hFcγRIIA, an isoform of hFcγRII. The authors have now investigated whether one of the isoforms of hFcγRII is also responsible for the polymorphism in binding of mIgG2b. In these studies the authors used EBV-transformed human B cells that demonstrated either binding or no binding of mIgG2b in EA-rosetting assays. mRNA obtained from these cells was amplified by reverse transcriptase and polymerase chain reaction (RT-PCR). Hybridization experiments with the RT-PCR products revealed that the hFcγRIIB but not the hFcγRIIA isoform was present in these cells. DNA sequencing further demonstrated that the nucleotide sequence of both the extracellular part and the cytoplasmic moiety of hFcγRIIB was identical for all individuals tested, regardless of their ability to bind mIgG2b. These findings indicate that the polymorphic binding of mIgG2b cannot be ascribed to one of the isoforms of hFcγRII. Since hFcγRI and hFcγRIII are not present on the cell surface of these cells, the authors conclude that an Fc receptor different from the known hFcγ receptors must be responsible for the polymorphic binding of mIgG2b. These data further expand the complexity of hFcγR.

INTRODUCTION

When murine monoclonal antibodies (MoAb) are used for immunotherapy, their biological effects and clinical side-effects are strongly influenced by their interaction (or lack of it) with human Fcγ receptors (hFcγR). This has been demonstrated both in tumour therapy [1, 2] and in immunosuppressive therapy after organ transplantation [3, 4]. Three classes of human leucocyte FcγR have been identified at the molecular level: hFcγRI, hFcγRII, and hFcγRIII [5, 6]. Murine IgG2a can bind to hFcγRII, mIgG1 can bind to an isoform of hFcγRII, but this binding is polymorphic: 70% of Caucasians are mIgG1 ‘high-responder’ (HR) whereas 30% are mIgG1 low-responder (LR) [7, 8].

We have described a similar polymorphism with respect to the binding of mIgG2b to human mononuclear cells. T cell proliferation was induced by mIgG2b anti-CD3 MoAb only when accessory cells were present that could bind mIgG2b. In this way mIgG2b-HR and mIgG2b-LR individuals could be defined [9]. The percentage of mIgG2b-HR is much lower (3%) than the percentage of mIgG1-HR (70%). It is important to note that mIgG2b anti-CD3 MoAb (in contrast to mIgG1) was still mitogenic after the complete removal of monocytes from the mononuclear cells of mIgG2b-HR individuals [9], indicating that other cells than monocytes (presumably B cells) can also provide accessory function. The mIgG2b polymorphism could also be demonstrated in EBV-transformed B cells [9-11]. The hFcγR responsible for the
polymorphic binding of mIgG2b has not yet been identified. Since hFcγRI and hFcγRIII are not present on EBV-transformed B cells that can bind mIgG2b [9], hFcγRII appeared to be the most likely candidate, as was also suggested by studies with polymeric mIgG2b [12].

hFcγRII occurs in several isoforms, that are encoded by three genes: hFcγRIIA, hFcγRIIB, and hFcγRIIC. The transcripts from hFcγRIIA (hFcγRIIa) and hFcγRIIB (hFcγRIIb) are highly homologous in the extracellular and transmembrane regions, but differ in the cytoplasmic region [13]. The transcript of FcγRIIC (hFcγRIIc) is identical to hFcγRIIb in its extracellular and transmembrane region, whereas in the cytoplasmic region it is identical (except for a single amino acid) to hFcγRIIa [14]. Three different isoforms of hFcγRIIb have been described, that result from alternative splicing. hFcγRIIib2 and hFcγRIIib3 differ only in the signal sequence, whereas hFcγRIIib1 differs from hFcγRIIib2 by a 19-amino acid insertion in the cytoplasmic tail [13]. hFcγRIIa occurs in two allelic forms, and is responsible for the polymorphism in binding of mIgG1 to human monocytes [5, 6].

We have now employed EBV-transformed B cells obtained from mIgG2b-HR or mIgG2b-LR to analyse, at the DNA level, whether hFcγRIIa or another isoform of hFcγRII is responsible for the polymorphic binding of mIgG2b. We have previously reported that EBV-transformed cells from mIgG2b-HR can provide, just like monocytes, accessory cell function in the mitogenic response to mIgG2b anti-CD3 MoAb [9]. Whereas monocytes express both hFcγRII and hFcγRI (and sometimes also hFcγRIII), EBV-transformed cells do not express hFcγRII or hFcγRIII and therefore provide an excellent model to study the role of hFcγRII with respect to the mIgG2b polymorphism. Furthermore, it was difficult to obtain sufficient numbers of highly purified monocytes or B cells from the few mIgG2b-HR available. We therefore decided to use only EBV-transformed B cells in the present study.

### MATERIALS AND METHODS

**Cells.** Leucocytes were obtained by cytapheresis from healthy donors, and peripheral blood mononuclear cells (PBMC) were isolated by centrifugation on Ficoll–Hypaque (Pharmacia Biotech, Uppsal, Sweden). Monocytes were purified from PBMC by counterflow centrifugation [15] and were > 90% pure, as judged by staining with May–Grünwald–Giemsa and nonspecific esterase. They were cryopreserved [16] until use. We have demonstrated before that cryopreserved mononuclear cells or monocytes still display the mIgG2b polymorphism [9]. EBV-transformed B-cells were obtained from mIgG2b-HR or mIgG2b-LR individuals [9], and were cultured in RPMI-1640 medium containing both Hepes and sodium bicarbonate (ICN Biomedicals, Costa Mesa, CA, USA), supplemented with 10% heat inactivated FCS (Hyclone, Logan, UT, USA), 2 mM glutamine, 1 mM sodium pyruvate, 50 μg ml⁻¹ streptomycin, and 50 IU ml⁻¹ penicillin.

**Oligonucleotides.** All oligonucleotides were selected from the nucleotide sequences as published by Brooks et al. [13] (hFcγRIIb: Genbank M31935; hFcγRIIa: Genbank M31932), and nucleotides are numbered according to this reference. With respect to hFcγRIIb,
several other groups [17–20] have published a virtually identical sequence with one exception: in contrast to Brooks et al. they all report the sequence G-C (not C-G) at nucleotide positions 980–981, and therefore we have assumed that this is the correct sequence at these positions.

The sequences for all oligonucleotides are given in Table 1. In order to identify the hFcγRII isomorph distribution of EBV-B cell lines, a partial sequence of hFcγRII was amplified using two primers that do not distinguish between the different isoforms of hFcγRII: P63 [21], that anneals in the extracellular region, and BECFII-3 annealing in the transmembrane region. Subsequent hybridization of amplified DNA on Southern blots was then performed either with the hFcγRII-specific oligonucleotide probe P52 [21], or with BEC-3int that anneals to the extracellular part of all isoforms of hFcγRII.

For amplification of RNA coding for the complete extracellular part of hFcγRII, we used a primer set consisting of BECFII-5 annealing at the upstream end of the extracellular domain, and BECFII-3 (described above). These primers do not discriminate between the different isoforms of hFcγRII. For subsequent sequencing, two internal oligonucleotides were selected: BCP-5int, and BEC-3int (described above).

The cytoplasmic region of hFcγRIIB was amplified using a sense primer BCPII-5 (annealing in the transmembrane region of hFcγRIIB), and an antisense primer BCPII-3 annealing in the untranslated part of the cytoplasmic region. For the sequencing of this comparatively short stretch of nucleotides, one internal oligonucleotide was sufficient: BCP-5int (corresponding to a sequence in the transmembrane region of hFcγRIIB).

All oligonucleotides were purified by reversed phase HPLC. Optimal annealing temperatures were empirically determined; amplification primer set BECFII-5/BECFI I-3: 60°C; primer set BECFII-5/BCPII-3: 52°C; primer set P63/BECFII-3: 56°C; all sequencing primers (BCF-5int, BCP-3int, and BCP-Sint): 60°C; hybridization probe P52: 53°C; and hybridization probe BCP-Sint: 63°C.

Oligonucleotides were radio labelled by phosphorylation in a 20 μl reaction volume containing 25–50 pmol oligonucleotide, 2 μl 10X kinase buffer (500 mM Tris-HCl pH 7.5, 100 mM MgCl2, 50 mM DTT, 1 mM spermidine), 5–10 μl 32P-P-y-ATP (3000 Ci/mmol, Amersham International, Amersham, UK) and 1 μl T4 polynucleotide kinase (10 U μl–1, Pharmacia Biotech, Uppsala, Sweden) for 45 min at 37°C and 10 min at 68°C.

Labelled probes were purified by spin column centrifugation (G-25 superfine DNA grade, Pharmacia Biotech, Uppsala, Sweden; 1 ml equilibrated in 0.1x STE) using four elution volumes of 100 μl 0.1X STE.

**RNA isolation and PCR.** Total cellular RNA was isolated from EBV-cells, monocytes, or cell lines U937, K562, Daudi and Raji, using the guanidine thiocyanate method [22], concentrated by ethanol precipitation, and stored at −70°C until use.

cDNA of the intended region was obtained by incubation of 1 μg total RNA for 10 min at 20°C, 45 min at 42°C and 10 min at 95°C in a reaction volume of 20 μl, containing 30 pmol of the related downstream primer, 0.63 mM dNTP (Boehringer Mannheim, Mannheim, Germany), 10 mM DTT, 20 U RNasin (Promega, Madison, WI, USA), 4 μl 5X ‘first strand buffer’ (250 mM Tris-HCl pH 8.3, 375 mM KCl, 15 mM MgCl2) and 200 U M-MLV Reverse Transcriptase (Life Technologies Inc., Gaitersburg, MD, USA).

Subsequent amplification of acquired cDNA was performed in a thermal cycler (Perkin Elmer, Norwalk, CT, USA) after adding 80 μl of a PCR mixture, containing 30 pmol upstream primer, 10 μl 10X PCR buffer (500 mM KCl, 200 mM Tris-HCl pH 8.4, 15 mM MgCl2, 1 mg ml−1 BSA) and 1.5 U Taq polymerase (Life Technologies Inc., Gaitersburg, MD, USA) using an amplification protocol consisting of 5 min template denaturation at 94°C, 30 cycles for 1 min at 94°C, 1 min at the primerset optimal annealing temperature and 1.5 min at 72°C, followed by a terminal extension step for 10 min at 72°C.

For additional reamplification, 1 μl of PCR product was added to 99 μl PCR mixture, containing 30 pmol of each primer, 0.125 mM dNTP, 10 μl 10X PCR buffer, 1.5 U Taq polymerase and (when radiolabelled products were required) supplemented with 32P-dCTP (3000 Ci/mmol, Amersham International, Amersham, UK). Reamplification was performed using the amplification protocol described above.

**Single strand conformation polymorphism.** Radio labelled PCR products diluted 1:5 in SSC loading buffer (20 mM EDTA pH 8.0, 20 mM NaOH, 0.05% xylene cyanol, 0.05% bromophenol blue in 96% formamide) were heated for 3 min at 85°C and kept at 0°C for 10 min. Two μl treated samples were loaded on a polyacrylamide gel (6%, 49:1, optional 0–10% glycerol, in 1X TBE), separated at constant power (2 W) and gels were exposed to Kodak X-OMAT S film. In some experiments the PCR products were cleaved into two smaller fragments by adding 1 μl restriction enzyme BglI (Life Technologies Inc., Gaitersburg, MD, USA) and 2 μl 10X reaction buffer (500 mM Tris-HCl pH 8.0, 100 mM MgCl2, 500 mM NaCl) and incubating for 60 min at 37°C.

**Non-denaturing polyacrylamide gel electrophoresis.** In order to separate the two hFcγRIIB cytoplasmic region isoforms (b1 and b2), 5 μl radio labelled PCR products diluted 1:5 in TBE loading buffer (15% ficoll, 0.25% xylene cyanol, 0.25% bromophenol blue in 1X TBE) were loaded on low-crosslinker polyacrylamid gel (6%, 49:1 in 1X TBE buffer). The gel was analysed by autoradiography and segments containing DNA bands of interest were cut out. dsDNA was extracted by adding 100 μl Tris-HCl pH 8.0, 1 mM EDTA incubating for 15 min at 80°C and 16 h at 4°C.

**Hybridization analysis of PCR products.** PCR products were analysed by separation on 2% (wt/vol) agarose and stained with ethidium bromide. Southern blotting was performed by transferring DNA to Hybond-N + (Amersham International, Amersham, UK) in 0.4M NaOH, 25 mM EDTA. Membranes were washed (2X SSC) and incubated in 30 μl Standard Hybridisation mix [21] (5X SSC, 0.1% laurylsarcosine, 0.02% SDS 1% Blocking Reagent (Boehringer–Mannheim, Mannheim, Germany) for 1.5 h at the optimal annealing temperature. After adding 25 pmol hybridization probe incubation was continued for 2 h at the same temperature followed by a washing procedure at stringency of 2X SSC, 0.1% SDS, twice at room temperature (20°C) and twice at the hybridization temperature. Blots were analysed after autoradiography using intensifying screens.

**Sequence analysis.** Prior to their use as templates in sequence analysis, PCR products were purified by the Wizard PCR Preps DNA Purification System (Promega Corporation, Madison, WI, USA) according to the manufacturer’s description. They were then applied as template in the Sanger dideoxyribonucleotide termination sequencing method [23]. In brief, a 7.5 μl aliquot of a mixture, containing 10 μl purified template, 11 μl sequencing PCR buffer (30 mM Tris-HCl pH 9.0, 5 μM MgCl2, 30 mM KCl, 0.025% NP-40, 0.025% Tween-20), 10 μl 32P labelled sequencing oligonucleotide (2.5 pmol in 0.1X STE) and 0.5 μl Taq polymerase (5 U μl−1) was added to 2.5 μl of the four termination mixes individually containing 160 μM ddGTP, 80 μM dNTP, 1.28 μM ddATP, 40 μM dNTP, 1.92 μM ddCTP, 40 μM dNTP, 640 μM ddCTP, 40 μM dNTP all
diluted in sequencing PCR buffer and overlaid with 20 µl mineral oil. Sequencing reaction was performed according to the amplification protocol described above for 15 cycles. Reaction products were dispensed in 10 µl sequencing loading buffer (20 µM EDTA pH 8.0, 0.05% xylene cyanol, 0.05% bromophenol blue, 91% formamide), heated for 4 min at 94°C before loading 3 µl on polyacrylamide gel (7%, optimal 6%, 24:1, 7 µm urea in 0.5× TBE). After separation and autoradiography the sequences were analysed.

RESULTS

hFcγRIIB but not hFcγRIIA is present in EBV-transformed B cells

RNA was isolated from EBV-transformed B cells of either mIgG2b-HR or mIgG2b-LR individuals, and RNA coding for the extracellular part of hFcγRII was amplified by RT-PCR using primers that do not discriminate between the different isoforms. The RT-PCR products from the EBV-transformed cells hybridized with a probe recognizing both the hFcγRIIA and hFcγRIIB isoforms, but did not hybridize with an hFcγRIIA-specific probe (Fig. 1). Hybridization with the hFcγRIIA-specific probe was observed with RT-PCR products obtained from monocytes, from cell lines U937 and K562 and, very weakly, B cell line Daudi, and was not detectable with B cell line Raji. We conclude that hFcγRIIB but not hFcγRIIA is present in the EBV-transformed cells (regardless whether they originate from mIgG2b-HR or LR individuals).

Single strand conformation polymorphism (SSCP) analysis of hFcγRIIB

We then focused on a more detailed analysis of hFcγRIIB obtained from the EBV-transformed cells, and investigated whether there was a polymorphism detectable at the DNA level. Our first approach was SSCP analysis, which may detect allelic forms of DNA. We found no indications for allelism of hFcγRIIB, not even when (after cleavage by restriction enzymes) shorter stretches of DNA were analysed.

Sequencing of hFcγRIIB

Since SSCP analysis can reveal allelic differences but the absence of detectable differences is not conclusive, we decided to sequence the extracellular part of hFcγRIIB. As shown in Table 2, this sequence was completely identical for mIgG2b-HR and mIgG2b-LR individuals. The same sequence was found in a total of four high-responders and three low-responders.

Whereas the extracellular part of the receptor is responsible for ligand binding, the cytoplasmic tail is involved in signal transduction (as discussed below), and therefore it was conceivable that a difference in the sequence of the intracellular part of the receptor would affect its biological effects. Therefore we also sequenced the intracellular part of hFcγRIIB. We found that both the hFcγRIIB1 and the hFcγRIIB2 isoform were present (b1 containing a 57-nucleotide insert when compared with b2). Again, we found no sequence differences when RT-PCR products from four mIgG2b-HR and three mIgG2b-LR were analysed (data not shown).

DISCUSSION

We have previously reported that mIgG2b can bind in a polymorphic way to human mononuclear cells. The presence of an Fc receptor that can interact with mIgG2b could also be demonstrated on EBV-transformed cells from mIgG2b-HR [9]. These EBV-transformed cells had the phenotype of B cells, and they did not express hFcγRI or hFcγRIII. In immunofluorescence studies, staining was seen with MoAb KB61 but not MoAb IV.3 [9]. KB61 recognizes the hFcγRIIB1 isomorphism present on B cells, whereas IV.3 primarily binds to hFcγRIIa that is expressed on monocytes and granulocytes [24].

An isoform of hFcγRII is responsible for the polymorphic binding of mIgG1, and it has been suggested that hFcγRII is responsible for binding of mIgG2b [12]. In functional studies, however, we found no evidence for an involvement of hFcγRII in the polymorphic binding of mIgG2b. MoAbs against hFcγRII (including KB61) did not inhibit mIgG2b anti-CD3 induced T cell proliferation [9]. Furthermore, proteolytic enzymes strongly reduced the expression of hFcγRII on EBV-transformed cells, whereas they caused an increased binding of mIgG2b in EA-rosetting [11]. Although these findings argue against the hypothesis that the polymorphic
binding of mIgG2b is caused by a polymorphic isoform of hFcγRII, we felt that only an analysis at the DNA level (by sequencing) could yield definitive conclusions. Furthermore, by performing RT-PCR with primers that correspond to hFc binding of mIgG2b is caused by a polymorphic isoform of isoforms at RNA level. Sarmay et al. have studied isoform expression in resting and activated B cells. In resting B cells, hFcγRIIIB2 was the predominant isoform, although hFcγRIIIB1 and (at a low level) hFcγRIIa were also present. Upon activation the expression of hFcγRIIIB1 increased, whereas hFcγRIIIB2 and hFcγRIIa decreased [25]. As we reported before, the EBV-transformed cells have a high expression of HLA-DR and transferrin receptor, and in this respect they resemble activated rather than resting B cells [11]. With respect to hFcγRIIA, we found abundant expression in monocytes and the cell lines U937 and K562. B cell line Daudi expressed hFcγRIIA at a very low level, but in B cell line Raji it was not detectable. These results with cell lines are very similar to results obtained after Northern analysis [13], although others have reported expression of hFcγRIIA in several B cell lines including Raji [25, 26].
We could not detect allelic differences in hFcγRIIB between mIgG2b-HR and mIgG2b-LR by the SSCP analysis. To ascertain that there are indeed no differences in sequence, we determined the nucleotide sequence in a total of seven EBV-transformed cell lines. The sequence for the extracellular part (Table 2) is exactly the same as reported by Brooks et al. [13], except for an A instead of a T at position 564. Two other groups have also reported an A at the corresponding position [19, 20]. More importantly, the same nucleotide was found in both mIgG2b-HR and mIgG2b-LR. Since the primers used for sequencing the extracellular part of the receptor do not discriminate between hFcγRIIb and hFcγRIIc, it is obvious that even in the case that hFcγRIIc would be expressed in these cells the nucleotide sequence would be identical for mIgG2b-HR and mIgG2b-LR.

Finally, we sequenced the intracellular region of hFcγRIIB. Studies on the corresponding murine Fc receptor have revealed that the cytoplasmic domain of the receptor determines its ability to endocytose immune complexes [27]. Similarly, the association of different isoforms of hFcγRIIb with intracellular molecules and their biological function is determined by the cytoplasmic domain [28]. It was therefore conceivable that differences in EA-rosetting between HR and LR were caused by differences in the intracellular part of hFcγRIIb. We found that both hFcγRIIb1 and hFcγRIIb2 were present in the EBV-transformed cells, but there were no differences in sequence between mIgG2b-HR and mIgG2b-LR. The sequence we found was precisely the same as reported by Brooks et al. [13].

Identity of the nucleotide sequences predicts that the polypeptide chain of hFcγRIIb is identical for mIgG2b-HR and mIgG2b-LR. Our data cannot exclude the possibility that a sequence difference between mIgG2b-HR and mIgG2b-LR. The sequence we found was precisely the same as reported by Brooks et al. [13].

Recently, a polymorphism of the hFcγRIIIA gene was identified [29]. Although in immunofluorescence experiments no evidence was found for expression of this receptor on EBV-transformed cells [9], mononuclear cells from five mIgG2b-HR were genotyped for this hFcγRIIIA polymorphism. However, no association with a particular hFcγRIIIA allele was found (M. de Haas, personal communication).

In conclusion, we found that both hFcγRIIb1 and hFcγRIIb2 but not hFcγRIIa are detectable in EBV-transformed B cells that bind mIgG2b. The hFcγRIIb isoforms do not display any sequence difference between mIgG2b-HR and mIgG2b-LR cells. Therefore we conclude that the polymorphic binding of mIgG2b to human B cells cannot be ascribed to one of the isoforms of hFcγRII. This conclusion is also supported by our previous studies on the effect of proteolytic enzymes on Fc receptor expression and function [11]. Furthermore, since there is no evidence that hFcγRII or hFcγRIII can be expressed on EBV-transformed B cells [9], we must conclude that apparently a yet unidentified hFcγR different from hFcγRI, hFcγRII, or hFcγRIII is responsible for the polymorphic binding of mIgG2b to B cells. Although we did not perform a similar analysis with monocytes, it should be stressed that accessory cell function in mIgG2b anti-CD3 induced T cell proliferation can be provided by EBV-transformed B cells from mIgG2b-HR as adequately as by HR monocytes [9]. A recent study on the effects of human IgG2 on human B cells also concluded that these effects were mediated by a hFcγR different from the three receptors identified so far [30].

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REFERENCES

3 Tax WJM, Frenken LAM, Glaudemans CA, Tamboor WP, Koene RAP. Polymorphism of Fc receptor (FcγRII) is reflected in cytokine release and adverse effects of mIgG1 anti-CD3/TCR antibody during rejection treatment after renal transplantation. Transplant Proc 1995;27:867-8.
12 Jones DH, Looney RJ, Anderson CL. Two distinct classes of IgG


17 Stuart SG, Simister NE, Clarkson SB, Kacinski BM, Shapiro M, Mellman I. Human IgG Fc receptor (hFcRII; CD32) exists as multiple isoforms in macrophages, lymphocytes and IgG-transporting placental epithelium. EMBO J 1989;8:3657–66.


23 Innis MA, Myambo KB, Gelfand DH, Brow MA. DNA sequencing with Thermus aquaticus DNA polymerase and direct sequencing of polymerase chain reaction-amplified DNA. Proc Natl Acad Sci USA 1988;85:9436–40.


