METHODS

Simultaneous Genotyping for All Three Known Structural Mutations in the Human Mannose-Binding Lectin Gene

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We describe a rapid and simple method for genotyping the three known structural mutations within exon 1 of the mannan-binding lectin (MBL) gene. A PCR-amplifiable synthetic DNA (Universal Heteroduplex Generator) was annealed to genomic PCR product from exon 1 to generate unique DNA heteroduplexes for each mutation. Heteroduplexes were then resolved by non-denaturing polyacrylamide gel electrophoresis. The technique was initially validated with previously typed samples and then applied to previously untyped samples with the results confirmed by DNA sequencing. Hum Mutat 9:41–46, 1997. © 1997 Wiley-Liss, Inc.

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INTRODUCTION

Mannose-binding lectin (MBL) [synonyms: mannose binding lectin (MBL), mannose binding protein (MBP), mannann-binding protein (MBP)] is an acute-phase protein involved in activation of the classical complement pathway (Ikeda et al., 1987; Ohta et al., 1990; Matsushita et al., 1992); low levels of the protein have been associated with an opsonic deficiency (Super et al., 1989). In serum the protein exists as a mixture of oligomers of between 2 and 6 MBL subunits, each made up of three identical 32-kDa peptide chains covalently linked at the N-terminus. Each peptide chain consists of an N-terminal collagenous region joined via a neck region to a carbohydrate recognition domain at the C-terminus (Drickamer and McCleary, 1987; Sastry et al., 1989; Taylor et al., 1989).

MBL serum levels are profoundly reduced by mutations in the coding region of the MBL gene, which is located on chromosome 10 at q11.2–q21 (Sastry et al., 1989). The gene comprises four exons (Sastry et al., 1989; Taylor et al., 1989) and all structural mutations so far described are point mutations occurring in exon 1 (Sumiya et al., 1991; Lipscombe et al., 1992; Madsen et al., 1994), which encodes the N-terminal part of the collagenous region (Sastry et al., 1989). These result in amino acid substitutions and appear to interfere with the ability of the protein to form multimers (Sumiya et al., 1991). To date three mutations have been described in exon 1 at codons 52 (CGT → TGT), 54 (GGC → GAC) and 57 (GGA → GAA), and these differ considerably in their frequencies in different populations (Lipscombe et al., 1992; Madsen et al., 1994; Lipscombe et al., 1996).

Given the possible impact on neonatal health, a rapid and cost-effective method for screening MBL mutations would be desirable. Current methods for genotyping individuals for MBL mutations are based on the polymerase chain reaction (PCR) and include restriction enzyme analysis of PCR products (Lipscombe et al., 1992; Madsen et al., 1994), probing with radiolabelled allele specific oligonucleotides (ASO) (Madsen et al., 1994), or the amplification refractory mutation system (ARMS) (Davies et al., 1995). As currently formatted these systems require either multiple steps or reactions post-PCR, or multiple PCRs to screen for all mutations thereby increasing the time and cost of each procedure.

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Here we describe a rapid and effective genotyping method based on the generation of DNA heteroduplexes using a PCR-amplifiable synthetic DNA (Universal Heteroduplex Generator, or UHG) (Bidwell et al., 1993). In this procedure a section of exon 1 covering the site of the three mutations is PCR amplified while at the same time in a separate tube the UHG DNA is also amplified. The UHG is based on exon 1 but contains base deletions and insertions contiguous with the mutation sites. When allowed to anneal with the PCR product from genomic DNA, heteroduplexes form which are unique for the allele present, and these can be resolved by non-denaturing polyacrylamide gel electrophoresis. This technology has been successful for genotyping sickle-cell anaemia mutations (Wood et al., 1993a), and phenylketonuria mutations (Wood et al., 1993b), and has proved equally effective in the case of MBL using either previously typed samples, or previously untyped samples with the UHG results confirmed by DNA sequencing.

**MATERIALS AND METHODS**

**Nomenclature**

This paper uses the following system of nomenclature. Homozygous wild type is identified by the notation WT, but in all other cases the status of both alleles is shown with WT designating one wild-type allele, and R52C, G54D, and G57E, indicating an allele with a mutation at codon 52, 54, or 57, respectively. For example, WT/G54D would indicate a heterozygous individual with one wild-type allele and one allele with the codon 54 mutation.

**Construction of the MBL-Universal Heteroduplex Generator (MBL-UHG)**

To identify the three point mutations in exon 1, a specific MBL-UHG was constructed containing identifiers for the three mutations (Wood and Bidwell, 1996). These consisted of a two base (TI) insertion and a three base deletion which permitted detection of all three mutations (Fig. 1).

To avoid successive synthetic G residues, the noncoding sequence of the UHG was synthesised as previously described (Wood et al., 1993b). The oligonucleotide longer was PCR-amplified in a 100-μl reaction containing 10 μl of DNA, 1 μl each of 50 μM LMBL (5’-CTGTGACCTGTGAGGATGC-3’) and RMBL (5’-CCAAACGTACCTGGTTCC-3’) primers, 10 μl dNTP mix [dATP, dCTP, dGTP, dTTP] (Promega, Southampton, UK), each at 2 mM, 10 μl 10 × NH₄ PCR buffer (Bioline, London, UK), 3 μl 50 mM MgCl₂, 63 μl MilliQ water, and 2 μl VentDNA polymerase (New England BioLabs, Hitchin, UK) at 0.5 U/μl. Reactions were overlaid with 50 μl light mineral oil (Sigma, Poole, UK). The resulting amplicon was purified to remove nonincorporated primers, dNTPs, and “spent” enzyme, using a Chroma-spin 100 column (Clone tech, supplied by Cambridge Bio-Sciences UK, Cambridge, UK) according to the protocol supplied by the manufacturer.

Aliquots of purified material were cloned using the pCR-ScriptSK( + ) system (Stratagene, Cambridge, UK). White colonies were selected and cultured overnight in nutrient broth containing 50 mg/L ampicillin. Plasmid DNA was isolated from these cultures by the Holmes and Quigley boiling lysis method (Sambrook et al., 1989), and samples were PCR-amplified in 100-μl reactions as described above, but using 2 μl Taq polymerase (Bioline, London, UK) at 0.5 U/μl in place of Vent polymerase.

Enzyme was added at 94°C to hot start the reaction, followed by 32 rounds of thermal cycling at 94°C for 1 min, 56°C for 1 min, and 72°C for 1 min using a Hybaid Thermal Reactor 1 (Hybaid, Teddington, UK). Cycling was followed by a 10-min extension step at 72°C.

Amplicons were accurately sized by agarose electrophoresis (Metaphor, E.M.C., supplied by Flowgen, Sittingbourne, UK). This was necessary because the cloning system included repair and expansion steps that could result in the ligation of incomplete sequences. DNA from the clone that generated the correct size fragment was amplified, and the products were gel purified as previously described (Wood et al., 1993b) and the DNA eluted in an Atto electroelution device (Atto, Japan supplied by G.R.I., Dunmow, UK). This provided the primary stock of MBL-UHG for subsequent heteroduplex analysis.

The sequence of the MBL-UHG was verified by manual sequencing using the Promega Silver Sequence system.

**Heteroduplex Analysis of DNA**

Genomic DNA was prepared from fresh whole blood using standard protocols. Separate 100 μl PCR amplifications were performed for MBL-UHG and genomic DNA. PCRs were performed as described above with 10 μl genomic DNA (0.5–1 μg) or 10 μl MBL-UHG serially diluted to 1 in 100,000.

Heteroduplex generation was achieved by mixing 15 μl of PCR-amplified genomic DNA and 15 μl of PCR-amplified MBL-UHG DNA and heating to 94°C for 3 min and allowing to cool to 37°C at a rate of 45 sec/°C to allow heteroduplexes to form. A single 100 μl UHG PCR was sufficient for six genomic typings.
Next, 3.5 ml of standard sucrose gel loading buffer were added to the heteroduplex mix and the entire mixture loaded onto a 20% nondenaturing polyacrylamide minigel (Protogel, National Diagnostics, Hull, UK) containing 2 × 90 mM Trisborate 1 mM EDTA (TBE). The outer wells of the gel were not loaded to avoid distortion effects. The gels were run for 3 hours at 200 V using 1 × TBE as running buffer, after which heteroduplexes were visualised by staining for 20 min in 1 × TBE containing 0.5 mg·ml–1 ethidium bromide, and viewing on a UV transilluminator.

**RESULTS**

Using samples previously typed by alternative methods, the MBL-UHG produced unique heteroduplex patterns for the heterozygous or homozygous states of the known MBL exon 1 mutations (Fig. 2). The use of 2 × TBE as the gel buffer was necessary to resolve the bands with similar apparent molecular sizes, particularly the 302-bp and 295-bp bands. Each allele should generate two different DNA heteroduplexes corresponding to the two DNA strands from each allele. In theory homozygotes should generate four heteroduplex bands. All the heterozygote samples did generate four bands, except for WT/R52C individuals, where only three bands were observed. We believe that this is due to the co-migration of two heteroduplexes as a single

**Automated DNA Sequencing**

The PCR products generated in the heteroduplex typing protocol were sequenced using a solidphase approach, essentially as described by Hultman et al. (1987). PCR reactions were performed as before but using an equal mixture of 5´-biotinylated and nonlabeled LMBL or RMBL primer. After capture on streptavidin coated magnetic beads (Dynabeads M-280 Streptavidin, Dynal, New Ferry, UK) and conversion to single-stranded DNA using 0.1 M NaOH, the PCR fragments were sequenced using an automated sequencing kit (Autoread kit, Pharmacia Biotech, Uppsala, Sweden) with the opposite primer using either a 5´-fluorescein labelled primer, or fluorescein 15*dATP (Pharmacia Biotech, Uppsala, Sweden) for internal labelling of the dideoxy products according to the manufacturer’s recommendations.

Sequencing reaction products were run on an ALF DNA sequencer (Pharmacia Biotech, Uppsala, Sweden) with a 6% denaturing polyacrylamide gel (Sequegel-6, National Diagnostics, Hull, UK) with sequence data generated using ALF manager software (Pharmacia Biotech, Uppsala, Sweden).

**FIGURE 1.** Nucleotide sequence (5´ to 3´) of exon 1 of the human mannose binding lectin gene and the flanking promoter and first intron regions. The amino acid sequence encoded by wild-type exon 1 is shown above the nucleotide sequence.

The sequences of the exon 1 point mutations and the MBL-UHG are shown aligned to the wild-type sequence, with (–) indicating identity and (*) indicating a deletion. The locations of LMBL and RMBL primers used in the PCR are underlined.
upper band arising from one strand of the R52C allele and one strand of the WT allele. Additional evidence for this comes from analysing the gel with a GlykoFACE digital imager to measure the relative band fluorescence of the heteroduplexes. For all samples except WT/R52C, the heteroduplex bands were of approximately equal intensities, but for WT/R52C the intensity of the upper band (apparent size 551 bp) was twice that of the lower two bands (302 and 295 bp) (Wood and Bidwell, 1996). This failure of resolution did not, however, impair the interpretation of the pattern.

We have been able to identify a small number of individuals as heterozygous for two different MBL mutations. The clarity of results from these individuals led us to predict a pattern for R52C/G57E heterozygotes, which we have not yet observed naturally.

The prediction was tested by mixing equal amounts of DNA from a R52C/R52C individual and a G57E/G57E individual. The band pattern observed (Fig. 2a) was exactly as predicted.

To test the validity of the technique, whole blood was obtained from 22 healthy laboratory staff volunteers and their genotypes tested by heteroduplex analysis. Of the 22 volunteers, 12 were found to be WT, 5 were WT/G54E, and 5 were WT/R52C. The PCR products obtained from these DNA samples were also sequenced using an automated sequencer as described in the Materials and Methods. All heteroduplex typings were found to be in complete concordance with the DNA sequencing (data not shown).

A point for consideration with any genotyping technique is whether it is able to type DNA same
pies prepared by suboptimal methods. In order to investigate this aspect, samples which had been prepared from blood following long term storage in heparin were compared to samples prepared from fresh EDTA blood samples. The primers were able to amplify the DNA without previous incubation with heparinase, and sufficient PCR product was obtained to give unambiguous typings for all these samples (data not shown).

DISCUSSION

As with the previously described UHG procedures for classifying HLA class II alleles (Bidwell et al., 1993), sickle cell (Wood et al., 1993a) and phenylketonuria (Wood et al., 1993b) mutations and other heteroduplex techniques (for a recent review, see Bidwell et al., 1994), the present procedure generates DNA heteroduplexes differing in conformation and migrating with distinctive electrophoretic mobilities. The proximity of the three MBL mutations (within six codons in exon 1) suggested that UHG-based technology should be particularly appropriate for MBL, and the technique has proved to be a rapid, convenient method for nonisotopic screening of all three mutations. Since the procedure uses only a one tube PCR for the sample DNA and only one-step post-PCR this represents an advantage over multiple tube PCR systems such as ARMS, and multiple step post-PCR techniques such as ASO probing or restriction enzyme typing. Another potential advantage of this technique is that since the sites of the primers used for PCR are not fixed at the site of the mutations the primers used can be fully optimised with no siting restrictions.

The test was robust, giving clear and definitive results that were readily interpreted even with samples that had been difficult to amplify. It was particularly important that successful typing was possible with DNA extracted from blood samples stored in heparin for at least two years, since the presence of heparin has been associated with PCR failure (Satsangi et al., 1994) due to direct inhibition of Taq polymerase (Koller and Kohli, 1993).

The application of the UHG procedure to the 22 unknown samples proved to be interesting because 5/22 samples typed as WT/R52C which corresponds to an R52C allele frequency of 0.11, whereas that expected for this mutation in North European populations is 0.05 (Madsen et al., 1994). However, all the UHG typings were confirmed by automated sequencing and the high frequency of WT/R52C samples must presumably be explained by the analysis of a relatively small sample group.

As with other specialised genotyping methods, the UHG would not necessarily detect novel structural mutations in exon 1. However, any additional mutations close to the identifier regions of the UHG should generate heteroduplexes that are unlikely to be identical to those of either the wild type or the three known mutations. If two heteroduplex bands did co-migrate, as for the WT/R52C samples in this study, a clear dosage effect would be observed by eye or by digital imaging.

In a routine analytical setting interpretation of the band patterns could be performed manually (as here), possibly with the aid of a reference grid overlaid on the gel, since the migration distances of the bands were relatively uniform. Alternatively, a digital imager such as the GlykoFACE system already mentioned, linked to appropriate software, would permit automated analysis. The reliability and practical simplicity of this genotyping method should permit large scale investigations into the effect of mannos-binding lectin deficiency in child health.

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