Heavy transfusions and presence of an anti-protein 4.2 antibody in 4.2(–) hereditary spherocytosis (949delG)

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ABSTRACT

Background and Objectives. A patient with hereditary spherocytosis (HS) was found not to have red cell membrane protein 4.2. This rare form of HS, or 4.2(–) HS, stems from mutations within the ELB42 or the EPB3 genes. The patient had long suffered from a gastric ulcer and impaired liver function. He had had several dramatic episodes of gastrointestinal tract bleeding and had received numerous transfusions. An antibody against a high frequency, undefined antigen was found, creating a transfusional deadlock. We elucidated the responsible mutation and searched for an anti-protein 4.2 antibody.

Design and Methods. Red cell membranes were analyzed by SDS-PAGE and by Western blotting. Nucleotide sequencing was performed after reverse transcriptase-polymerase chain reaction (RT-PCR) and nested PCR.

Results. The not previously described mutation was a single base deletion: 949delG (CGC → CC, exon 7, codon 317) in the homozygous state. It was called protein 4.2 Nancy. The deletion placed a nonsense codon shortly downstream so that no viable polypeptide could be synthesized. The patient carried a strong antibody against protein 4.2 as shown by Western blotting.

Interpretation and Conclusions. The manifestations resulting from the mutation described were compared with the picture of HS stemming from other ELB42 gene mutations. We discuss the mechanism through which the anti-protein 4.2 antibody developed. There was no way to establish or to rule out whether the antibody participated in the transfusional deadlock found in our patient.

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Key words: anemia, hereditary spherocytosis, protein 4.2 Nancy, transfusion

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Red cells and Iron

Protein 4.2 is an important protein of the red cell membrane.1,2 It binds to the cytoplasmic domain of band 3, and is linked to the lipid bilayer inner leaflet through myristoyl3 and palmitoyl residues.4,5 Its corresponding gene, the ELB42 gene, maps to 15q15-q21,6 encompassing about 20 kb, and contains 13 exons.7 Protein 4.2 is formed of 721 amino acids when all the exons of its gene are expressed (74 kD).1,2,7 The major spliceoform (72 kD) lacks the last 90 nucleotides of exon 1 (out of 99 nucleotides). Protein 4.2 is widely distributed in tissues. The absence of protein 4.2 causes an atypical form of hereditary spherocytosis [4.2(–)HS]. In detail, the osmotic resistance of the red cells is moderately decreased and splenectomy is of limited therapeutic use. The inheritance pattern of 4.2(–)HS is strictly recessive.8-11 Transfusions are needed occasionally.

We found a novel mutation in our patient in a homozygous state: 949delG (CGC → CC, exon 7, codon 317), causing premature termination of protein 4.2 synthesis. Unlike any other patient with primary deficiency of protein 4.2 so far reported on, our patient had a history of recurrent gastrointestinal tract (GIT) hemorrhages, requiring surgery and massive transfusions. We found, by Western blotting, that his serum contained a strong antibody against protein 4.2. The patient developed a transfusional deadlock along with an antibody against a high frequency, undefined antigen. It was not understood what the relationship was between the latter antibody and the anti-protein 4.2 antibody, nor whether the anti-protein 4.2 antibody contributed to the transfusional deadlock.

Design and Methods

Case report

Our proband, a Gipsy, was born in 1931 from first cousin parents. The family history was marked by the demise of two premature twin sisters presenting with icterus a few days after birth, and the mother’s death 10 days after delivery. We have no information regarding the patient until the age of 24. He had a...
spleenectomy for a hemolytic anemia identified as hereditary spherocytosis (HS). The operation was, however, of limited use and the patient kept on presenting with episodes of jaundice, soon associated with cholelithiasis and pancreatitis. A gastric ulcer led to repeated GIT surgery. All operations were complicated by considerable bleeding. Repeated transfusions resulted in an allo-immunization. In 1968, prior to an operation, the patient was found to have anti-D (anti Rh) and anti-S (anti-MNS3) antibodies. Transfusions of packed red cells (unknown number) were administered until an episode of intravascular hemolysis occurred, associated with the appearance of an allo-anti-Fy2 (anti-Fyβ) antibody.

At the end of 1995, the patient was admitted to hospital because of a hernia. Before surgery, the aforementioned allo-antibodies were still present. Following surgery, the patient had yet another GIT hemorrhage, requiring the administration of five units of packed red cells, from day 1 to day 6. On day 9, he had icterus, hemoglobinuria, increased hemoglobinemia (599 g/L) and a fall in haptoglobin (0.06 g/L). The direct antiglobulin test was strongly positive whereas it had been weak prior to surgery. A search for unusual antibodies was positive using all erythrocytes devoid of D, S, Fyb antigens which were tested. On the day of intravascular hemolysis, elution of circulating erythrocytes revealed antibodies directed against a high frequency, but undefined antigen. The direct antiglobulin test became negative again, but the antibody against the unknown antigen remained. A transfusional deadlock had been reached. On examination in April 1997, the red cell indices were as follows: red blood count: 4.85 × 10¹²/L; hemoglobin 144 g/L; mean cell volume: 87.1 fL; reticulocyte count: 1.25%. Blood smears showed acanthocytes, echinoctyes, and spherocytes (not shown). Osmotic gradient ektacytometry showed no impairment of maximum deformability, but a moderate reduction in the surface/volume ratio corresponding to a shift of the curve by 15 mOsm towards the right (not shown).

### Table 1. Primers used.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>(ACTTACCTT)/GTGTTACCCGGGTGTTG*</td>
<td>263-284 nt</td>
</tr>
<tr>
<td>B</td>
<td>GG(TACC)/GTAGCAACTGGACACCTC*</td>
<td>83-103 nt</td>
</tr>
<tr>
<td>C</td>
<td>gc(paaccttc)↓105agtcaaggtgtctacagg↓155*</td>
<td>5' Τ</td>
</tr>
<tr>
<td>D</td>
<td>GG(GAATTC)↑122↓CCCTCTTGACTGGCTTGACC↑191*</td>
<td>exon 9</td>
</tr>
<tr>
<td>E</td>
<td>gc(paaccttc)↓9aagtgataaagagccgg↓9*</td>
<td>5' Τ</td>
</tr>
<tr>
<td>F</td>
<td>GG(GAATTC)↓355CACCTGATGATGGACATCTC↓375*</td>
<td>exon 4</td>
</tr>
<tr>
<td>G</td>
<td>GG(GAATTC)↓350ACCATCTTGTGCTTGACCTC↓354*</td>
<td>exon 2</td>
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<tr>
<td>H</td>
<td>GG(GAATTC)↓350GTGGTTCAGGTGACCTGGCTC↓354*</td>
<td>exon 7</td>
</tr>
<tr>
<td>N</td>
<td>88ATGGCCAGGCGTGGTGG*</td>
<td>exon 6</td>
</tr>
</tbody>
</table>

* Sense primers; °antisense primers. Annealing temperatures are provided. Primers A and B were designed after Lux et al.,12 primers C to H after Sung et al.,12 and primer N after Korsgren et al.14

### Protein analysis

Preparation of red cell membranes, polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate (SDS-PAGE) of red cell membrane proteins, Western blotting of the gels, extraction of DNA, and preparation of reticulocyte RNA were carried out as described and quoted elsewhere.13,14

### Nucleic acid analysis

cDNA was obtained by reverse transcription (RT) carried out using random primers Pd(N)6 (Pharmacia Biotech, Uppsala, Sweden). Northern blotting was performed according to standard procedures,15 using the 531 bp product of nested PCR 1 as a probe (see below). Polymerase chain reaction (PCR) following RT was performed using primers C and D (Table 1; Figure 1), encompassing a segment extending from the 5'UT to exon 9 (Figure 1). Nested PCR 1 and 2 were carried out on the above RT-PCR products using primers E and F, and G and H, respectively. The fragments were resolved on agarose gel, and stained with ethidium bromide. Direct sequencing from PCR products was performed with the Thermo sequenase radiolabeled terminator cycle sequencing kit (Amersham, Buckinghamshire, UK) using [α-33P]dNTPs. The reaction mixture contained dNTPs, [α-33P]dNTPs, 50 to 500 ng of PCR products, the reaction buffer and the polymerase. Extension was obtained by an amplification process using single primer H. Part of exon 7 was amplified at the gene level, using primers N and H. The amplified fragments were digested using endonuclease Mvnl, because the mutation cancels a Mvnl site (CG↓CG→CCG), and monitored on an agarose gel. Direct sequencing of PCR products was carried out as described above. PstI polymorphism in the EPB3 gene,12,13 PCR was assessed by using primers A and B (Table 1; Figure 1), designed according to procedure used by Lux et al.14

### Immunochemistry

Blood from the patient (collected in July 1996), from another 4.2(−) patient detailed below, and from controls was collected in EDTA. Erythrocytes were digested using endonuclease Mvnl, because the mutation cancels a Mvnl site (CG↓CG→CCG), and monitored on an agarose gel. Direct sequencing of PCR products was carried out as described above. PstI polymorphism in the EPB3 gene,12,13 PCR was assessed by using primers A and B (Table 1; Figure 1), designed according to procedure used by Lux et al.14
separated from white cells essentially using the technique described by Beutler et al. with modifications. Membranes from the washed cells were prepared by hypotonic lysis and inactivated by serine proteases as already outlined. Membranes, kept frozen in 1% SDS and 5 mM N-ethylmaleimide, were thawed and mixed with electrophoresis buffer containing, when indicated, 40 mM diethiothreitol as a reducing agent. Samples were denatured for 3 minutes in a boiling water bath. All samples were alkylated with an excess of N-ethylmaleimide, electrophoresed on SDS-PAGE containing 8% total acrylamide. Gels were blotted onto polyvinylidene fluoride membranes (Immobilon P) (Millipore, Bedford, MA, USA). Blots were incubated overnight as described elsewhere with 2% serum. Serum samples from the patients and controls were thawed and prepared with 3 mM diisopropylfluorophosphate. Bound IgG antibodies were visualized by incubation for 4 hours with 4×10^5 cpm/mL of 125I-iodinated recombinant protein G (Sigma, St. Louis, MO, USA) having a specific activity of 26×10^6 cpm/µg. Blots were washed and dried. Autoradiographs were obtained with a Phosphorimager, using a linear grey tone scale (Molecular Dynamics, Sunnyvale, CA, USA).

Results
Protein 4.2 was undetectable by SDS-PAGE (Figure 2). Western blotting confirmed the absence of this protein and no trace amount of the major 72 KD isoform was visible in the membranes from the patient (see below).

The PstI polymorphic site of the EPB3 gene encoding protein AE1 was present in both alleles. As a consequence, we could not rule out that the EPB3 gene was the mutated gene in the homozygous state. However, if it had been so, the (secondary) reduction of protein 4.2 would not have been total, as exemplified by band 3 Fukuoka (~45%). The total absence of protein 4.2 and the fact that protein 4.2 mRNA was not detected by Northern blotting (not shown) made it more likely that the ELB42 gene was involved. We, therefore, investigated this gene.

Nested PCR 1 (5'UT to exon 4) yielded fragments of 531, 400 (weak), and 297 bp respectively, in both the patient and the control (not shown). An additional 111 bp fragment (weak) was recorded in the control only. Nucleotide sequencing indicated that the 531 bp fragment contained all exons, and that the 297 and 111 bp fragments lacked exons 3, and exons 2+3, respectively (not shown). Nested PCR 2 (exon 2 to exon 7) yielded fragments of 829, 595, 490 and 402 bp in the patient and controls. Nucleotide sequencing showed that the 829 bp fragment contained all exons, and that the 595, 490 and 402 fragments lacked exon 3, exon 3+5, and exons 4+5+6, respectively (not shown).

Nucleotide sequencing indicated that the patient’s spliceoforms from nested PCR 2 carried the deletion of a G in the first position of codon 317 (949delG (CGC→CC), exon 7) (Figure 3). This deletion introduced a frameshift and placed in phase a non-sense codon two triplets downstream: 319 and 320 (GTG A CC). It defined allele 4.2 Nancy. We confirmed the deletion of G at position 1 of codon 317 using nucleotide sequencing and MvnI restriction mapping (Figure 3).

Anti-protein 4.2 antibody
The patient’s serum, as well as a rabbit serum raised against purified human protein 4.2, were tested on membranes from a normal control, from our 4.2(–) patient and, finally, from a 4.2(–) patient carrying protein 4.2 Lisboa. Allele Lisboa, like allele Nancy, has a frameshift mutation (one base deletion in codon 88 or

Figure 1. Positioning of the various primers used. Boxes represent exons.

Figure 2. SDS-PAGE (left) of red cell membrane proteins. C, control; N, protein 4.2 Nancy.
as such, in both instances, the carrier’s immune system never recognizes protein 4.2 as a component of self. The patient with allele Lisboa had received one transfusion.

The patient’s serum, as well as the serum from the immunized rabbit, reacted strongly with protein 4.2 from a normal membrane (Figure 4). The patterns obtained were superimposable. As expected, the two anti-sera elicited no reaction with protein 4.2-free membrane from the patients with alleles Nancy and Lisboa. These data indicated that the serum of the patient with mutation Nancy contained an anti-protein 4.2 antibody. In contrast, the serum of the patient with mutation Lisboa failed to elicit any immune reaction against protein 4.2 and was considered free of any anti-protein 4.2 antibody.

Presence of other antibodies in the sera of 4.2(−) patients

The serum from the patient carrying allele Nancy reacted with spectrin and band 3. It also reacted with bands that we did not identify but which should correspond to blood group (glyco)proteins. Most of them stood below protein 4.2. One was above band 3 (Figure 4).

The serum from the patient with allele Lisboa reacted with spectrin, band 3, and still the same band above band 3. Incidentally, the rabbit serum cross-reacted with human spectrin.

Discussion

Mutations in protein 4.2

Protein 4.2 Nancy is the eighth known variant of protein 4.2 producing HS, associated with a complete or, in some cases (allele Nippon), a near-complete absence of protein 4.2, as reviewed by Gallagher and Forget. It is the third mutant discovered in Europe and North Africa, compared with the five found in Japan. Several protein 4.2 mutants have been found in

![Figure 3: cDNA and genomic DNA analysis. A: nucleotide sequencing of cDNA following RT-PCR and nested-PCR around codon 317 (primer H). B: restriction analysis using the MveI endonuclease; the one-base deletion cancelled a MveI site. C: control; N: patient with protein 4.2 Nancy (1: undigested; 2: digested).](image)

![Figure 4: Anti-protein 4.2 antibodies. Red cell membranes were run on SDS-PAGE and blotted. Blots were either stained (*) or incubated with sera. The origins of the antigens (red cell membrane) are indicated above the blots: C, controls; N and L: homozygotes for 4.2(−) alleles Nancy and Lisboa, respectively. The origins of the sera are indicated below the blots (N and L: as above). The sera from the patient with allele 4.2 Nancy and from an immunized rabbit provided superimposable patterns regarding their reactivity with protein 4.2. The serum from the patient with allele 4.2 Lisboa did not contain any detectable anti-protein 4.2 antibody. Spectrin and band 3 were, presumably, recognized by naturally occurring auto-antibodies. The reactivity of the serum from the patient studied with additional bands may correspond to anti-blood group antibodies which the patient developed as a result of massive transfusions.](image)
the homozygous state. Others have been discovered in the compound heterozygous state along with allele 4.2 Nippon. The last allele appears with some frequency among Japanese, increases the occurrence of compound heterozygosity and facilitates the discovery of other variants.

Four mutations lead to a premature termination of translation: mutation Fukuoka, mutation Lisboa, mutation Notame, and mutation Nancy, as identified in this study. Patients with protein 4.2 Lisboa and protein 4.2 Nancy have a very small amount of protein 4.2 mRNA, consistent with the fact that mRNAs with premature non-sense codons are degraded and/or undergo disturbed exon skipping. The four other variants, Nippon, Tozeur, Komatsu, and Shiga carry a single amino acid substitution. It should be mentioned that mutation Nancy (non-sense) occurs in the same codon as mutation Shiga (missense), and that the latter is the farthest downstream mutation known to cause the non-attachment of protein 4.2 to band 3.

We now have enough background information to appreciate that the clinical, hematologic and ektac- tometric phenotypes are basically homogeneous. No aggravating, nor attenuating genetic factors have so far been observed.

Antibodies against other proteins

The natural occurrence of autoantibodies against band 3 and spectrin has been documented. In our investigation, heavy bands under protein 4.2 presumably represented blood group components which were stained by allo-antibodies present in the patient’s serum. The staining of the unidentified component migrating above band 3 is puzzling since it occurred in the sera of both patients (carrying alleles 4.2 Nancy and Lisboa), yet to variable amounts.

To conclude, we have characterized a new variant of protein 4.2. Homozygosity for this variant yielded a form of hereditary spherocytosis associated with the absence of protein 4.2. Our patient carried a strong anti-protein 4.2 antibody, elicited by massive transfusions (not related to hereditary spherocytosis). It is unclear how anti-protein 4.2 allo-antibodies developed, but massive transfusions were plausibly critical to their generation. Nor is it clear whether the anti-protein 4.2 allo-antibody was related to the antibody against a high frequency, as yet undefined antigen, or whether it played a role in the transfusion deadlock presented by the patient.

This study emphasizes the clinical relevance of molecular studies in patients with hereditary spherocytosis, a subject recently considered in this journal.

Contributions and Acknowledgments

AB-N, LM and VB-C: protein and molecular biology experiments. TC: ektacometry. HUL and PS: immunological experiments. OA and RP-K: recruitment of the case, transfusional management. AL and SP: gift of samples for several control experiments. GT and JD: supervision of the investigation and writing of the manuscript.

We thank the patient for his kind cooperation, Dr. Henri Cohen for providing us with cord blood, and Mrs. Muriel Bozon for her skilful technical assistance.

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Disclosure

Conflict of interest: none.

Redundant publications: this is the third mutation in the EPB42 gene which we describe for the first time (Hayette et al. Br J Haematol 1995; 89:762-70; Blood 1995; 85:250-6; this manuscript). There is an inevitable overlap as it happens that the clinical and biological presentations, and the inheritance pattern are quite alike. On the other hand, the present case has, as none before, an immunological component due to the presence of an anti-protein 4.2 antibody. Altogether, we would assess that the overlap is much less than 50%.

References


