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Hereditary Human Complement C3 Deficiency Owing to Reduced Levels of C3 mRNA

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An 8-year-old son (L.A.S.) of consanguineous parents, presented recurrent bacterial infections, vasculitis and extremely low levels of serum C3 (0.15 μg/ml). The classical and alternative pathway haemolytic activities and the generation of opsonins and chemotactic factors derived from the activation of the complement system were markedly affected in the proband's serum. An *in vitro* addition of purified C3 restored the classical pathway-dependent haemolytic activity of his serum. Autoradiographs of the proband's lipopolysaccharide (LPS)-stimulated and ^{35}S-labelled fibroblast supernatants after that the SDS-PAGE revealed no C3 α or β chains. The amount of C3 mRNA synthesized by the proband's fibroblasts, as evaluated by reverse transcription–polymerase chain reaction (RT–PCR) assays, was greatly reduced.

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INTRODUCTION

The component C3 of the complement system is one of the most abundant plasma proteins (1–1.5 g/l), playing a major role in all three known activation pathways of the complement system. This protein is synthesized mainly in the liver [1], although its synthesis has also been demonstrated by other cell types [2–5]. Primary and secondary C3 deficiencies are rare. The few reported cases have been associated with severe recurrent infections caused by gram-positive bacteria such as *S. pneumoniae* or gram-negative examples such as *H. influenzae* and *N. meningitidis*. Clinical manifestations associated with these micro-organisms are observed in these patients mainly during childhood and include pneumonia, meningitis, otitis and septicemia (reviewed in [6]). Immune-complex (IC) mediated diseases such as glomerulonephritis and vasculitis are also present in C3-deficient individuals because the clearance of such complexes in the liver is heavily dependent on the C3 deposition [7].

We evaluated some complement dependent immune functions of a C3-deficient Brazilian child, and studied the production of C3 α and β chains and mRNA by the proband's cells.

PATIENT AND METHODS

Case description. L.A.S. is an 8-year-old Brazilian boy from a large family that shows no history of hereditary diseases. His parents were consanguineous (uncle and niece) and before the birth of L.A.S. they had four children who all died before the age of 6 months owing to unspecified acute infections and a cardiopathy (Fig. 1). L.A.S. has one healthy 3-year-old brother. Unfortunately his father died prior to the start of this investigation. The proband was healthy until the age of 2 at which time he suffered a severe adenitis and maxillary sinusitis. He subsequently had another sinus infection, two purulent tonsillitis, bronchopneumonia and a *Giardia lamblia* infestation. A post-infectious purpura distributed on the legs and buttocks and consistently undetectable C3 prompted evaluation for a complement deficiency. A skin biopsy revealed vasculitis without IgA deposition. One particular episode of severe cervical adenitis with a left axillary abscess did not respond to intravenous oxacillin treatment and an infusion of normal human plasma (10 ml/kg) was carried out in order to compensate the proband's serum complement level. Within less than 24 h after the plasma administration the abscess and the adenitis showed an almost complete clinical resolution,

Table 1. C4, factor H and factor I concentrations, classical and alternative pathway dependent haemolytic activities of the proband's and relatives' sera

	Concentration (μg/ml)			Normal(%)*	
Individual	C4	fI	fH	CP	AP
III 5	700	57	735	81	86
III 16	460	83	536	95	86
IV 5	748	66	589	96	88
IV 6	602	55	448	0	0
NHS†	657 ± 108	56 ± 24	668 ± 35	100	100

*Normal values determined for a pool of 10 normal sera. NHS: pool of 46 normal sera. CP: classical pathway; AP: alternative pathway. †Normal values for Brazilian population (Ferreira de Paula and Isaac, unpublished observations). IV6 (proband).

which strongly suggests that the normal C3 present in the plasma successfully controlled the infection. One year after the plasma infusion no anti-C3 immunoglobulin (Ig) was detected in the proband's serum.

Sera specimen collection. Sera were obtained from the proband (with his mother's consent), his relatives and normal donors. Blood samples were kept on ice for 60 min and centrifuged at 600 × ***g*** for 15 min at 4 °C. Aliquoted serum specimens were kept at − 70 °C and thawed immediately prior to use. The normal healthy donors included volunteers of a blood bank from Hospital Universitário, Universidade de São Paulo, Brazil.

Protein concentration determinations. Concentrations of complement proteins, Ig isotypes and IgG subclasses were determined by simple radial immunodiffusion [8]. Goat polyclonal antihuman C4, factor I (fI) and factor H (fH) were purchased from Calbiochem-Novabiochem (La Jolla, CA, USA); rabbit polyclonal antihuman C3c was purchased from Sigma (St. Louis, MO, USA) and sheep polyclonal antihuman IgG, IgM and IgA were acquired from Behringwerke (Marburg, Germany). Competitive solid phase radioimmunoassay (RIA) was performed as previously described [9] using a standard curve (4000–31 ng/ml).

Table 2. Killing of serum treated *C. albicans* by normal human phagocytes

	Percentage of dead *C. albicans* inside the cells Treatment			
Experiment	HBSS	iNHS	NHS	LAS
1st	38.4	57.5	60.9	50.0
2nd	37.3	49.3	59.5	49.9
3rd	48.0	55.2	61.7	53.6

HBSS: Hank's medium; iNHS: inactivated NHS; NHS: pool of 46 normal sera; NHS; LAS: proband's serum. All sera were diluted at 40% in HBSS. Results of LAS are inferior to those of NHS in all three experiments. In the first experiment the results of LAS are inferior to that of iNHS while in the second and the third experiments they are similar, with $\alpha \leq 0.05$. Statistical treatment of the data is described in Patients and Methods.

Haemolytic assays. Classical and alternative haemolytic assays were performed as described by Nilsson and Nilsson [10]. The haemolytic activities were calculated as the percentage of the activity observed for a pool of 10 normal human sera (NHS) (100% lysis).

Chemotactic and phagocytic assays. Leukocyte migratory activity through cellulose ester filters was performed in chemotactic chambers according to methods described previously [11]. Leukocyte migration was determined by the leading front method [12]. *C. albicans* phagocytosis and killing by normal human phagocytes were determined according to methods described previously [11]. The presence of *C. albicans* in the phagocytes and the numbers of live and dead fungi inside the cells were determined under a microscope after staining.

Fibroblast cultures. Skin fragments were taken from the proband (with the family's consent), his mother and two young healthy donors. Fibroblast cell lines were established and maintained according to methods described previously [13].

Metabolic labelling and immunoprecipitation. Approximately 0.5×10^6 fibroblasts were cultured with LPS and labelled with 250 μCi of Tran^{35}S-label (ICN, Costa Mesa, CA, USA). Supernatants were preadsorbed with Protein A Sepharose (4 Fast Flow; Amersham Pharmacia Biotech, Uppsala, Sweden) and immunoprecipitated with rabbit antihuman C3c (Sigma). Goat antihuman factor B (fB; Calbiochem-Novabiochem) was used as control for synthesis and precipitation. Immunoprecipitates were subjected to SDS-PAGE [14] followed by autoradiography using BioMax MR-1 film (Eastman Kodak, Rochester, NY, USA). The amount of radioactivity incorporated in the proteins was estimated by 8% trichloroacetic acid precipitation.

RNA extraction and RT–PCR. Total RNA was extracted from 10^7 LPS-stimulated fibroblasts using the total RNA extraction system (Promega, Madison, WI, USA). RT–PCRs were performed employing the SupersScript One-Step RT–PCR system (Life Technologies, Gaithersburg, MD, USA) according to the manufacturer's protocol. C3-specific primers were used in reactions with 200 ng of proband's RNA or 5, 10, 20 or 200 ng of normal RNA as follows: 35 cycles of 30 s at 94 °C, 30 s at 55 °C and 1 min at 72 °C. Control reactions with fI and glyceraldehyde 3-phosphate dehydrogenase (GAPDH)-specific primers were performed with 200 ng of proband's and normal RNA as follows: (fI) 40 cycles of 30 s at 94 °C, 30 s at 50 °C and 1 min at 72 °C; (GADPH) 18 cycles of 30 s at 94 °C, 30 s at 60 °C and 2 min at 72 °C. C3-specific primers were $^{5'}$GGTCAAGC AGGACTCCTTGT$^{3'}$ and $^{5'}$CCCTTGTTCATGATGAGGTAGG$^{3'}$; fI-specific primers were $^{5'}$GATGACTTCTTTCAGTGT$^{3'}$ and $^{5'}$AGCCAGAAACGATGCATG$^{3'}$ and GAD PH-specific primers

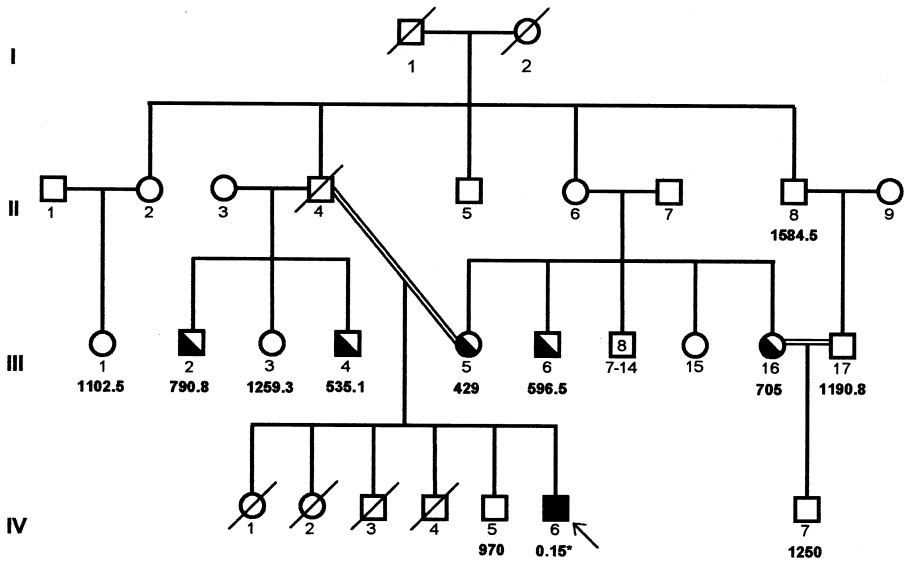

Fig. 1. Heredogram for the proband's family. The arrow indicates the proband. Male (□) and female (○). Inclined bars indicate deceased individuals. C3 concentrations are indicated below the symbols (in μg/ml). *Determined by RIA. Symbols painted by half indicate individuals that were shown to possess half the normal C3 concentrations.

were $^{5'}$ACCACAGTCCATGCCATCAC$^{3'}$ and $^{5'}$TCCACCAC CCTGTTGCTGTA$^{3'}$.

Statistical treatment of the data obtained. ANOVA was performed for the chemotactic assays followed by Tukey's test with a significance level of 95%. In order to analyze the results of phagocytic assays, we compared different proportions using a normal approximation of the chi-square test, with $\alpha \leq 0.05$ [15].

RESULTS

Evaluation of delayed type hypersensitivity responses to tricofitin, levedurin and PPD were all negative in the proband. Anti-HIV and antinuclear factor were not detected in the proband's serum whereas anti-A and anti-B (ABO blood group) titres both were 1/32. Differential leukocyte counting revealed an increase in total eosinophil proportion of 7% of the total leukocyte number. This may indicate the presence of helminths or a previous infection by such parasites. Evaluation of T- and B-lymphocyte populations as well as natural killer (NK) cell numbers were normal in the proband (data not shown). Haematological tests and serum protein electrophoresis were also normal (data not shown).

The proband's serum IgM was significantly elevated in the proband, to 3.88 ± 1.15 mg/ml (in comparison to 1.06 ± 0.66 mg/ml, which is the normal range for Brazilian children [16]). This increase in IgM concentrations may be a consequence of a constant exposure to different microbes. Total IgG, IgA and IgE levels were normal (data not shown). Quantification of IgG subclasses revealed normal values for IgG1, IgG2 and IgG3 (data not shown), while IgG4 was below 0.08 mg/ml, which is the lower limit of detection for radial immunodiffusion (normal range for Brazilian children is 0.08–0.77 mg/ml [16]).

Only minimal antigenic C3, 0.15 μg/ml, was detected by RIA in the proband's serum. No C3 was detected in the proband's cultured leukocyte supernatant by RIA. His mother (III 5) as well as other relatives had significantly diminished C3 concentrations (varying between 35% and 75% of the reference value: 1244 ± 346 μg/ml; Fig. 1). However, the C4, fH and fI concentrations were normal in all the individuals studied (Table 1).

The proband's serum was unable to lyse sheep erythrocytes after activation of the classical or the alternative pathway (Table 1). In order to determine if this failure could be attributed exclusively to the lack of C3, we added human plasma-purified C3 to the proband's serum at a final concentration equivalent to

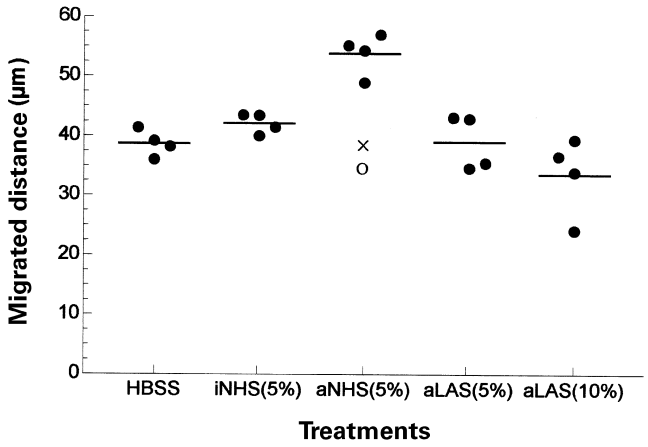

Fig. 2. Leukocyte migration in response to complement derived chemotactic factors of the proband and normal sera. HBSS: Hank's balanced salt solution; NHS: pooled sera from 46 normal adults; iNHS: NHS inactivated by heating to 56°C for 30 min; aNHS: NHS activated with LPS from *E. coli* (serotype: 0111:B4, Sigma); aLAS: activated proband's serum. Sera were diluted at 5% or 10% in HBSS, as indicated. Controls included NHSa in the upper compartment at 5% (X) and 10% (○). Horizontal bars represent the means. Results for HBSS, iNHS (5%), aLAS (5%) and aLAS (10%) are similar whereas the result for aNHS (5%) is different, with $P < 0.05$.

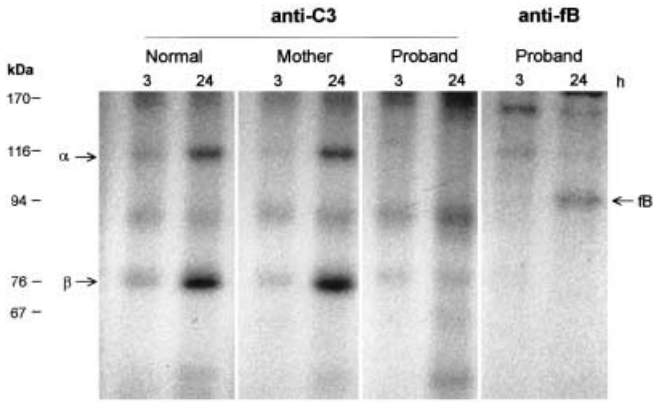

Fig. 3. SDS-PAGE autoradiography of metabolically labelled fibroblast culture supernatants after precipitation with a-C3 and a-fB. Cultures were stimulated with LPS and pulsed with 250 μCi ^{35}S-Met and ^{35}S-Cys and supernatants were collected after 3 and 24 h chasing periods. Sizes of C3 α and β chains (110 and 75 kDa, respectively) and factor B single chain (93 kDa) are indicated by arrows. The film was exposed to the gel for two weeks at −70°C.

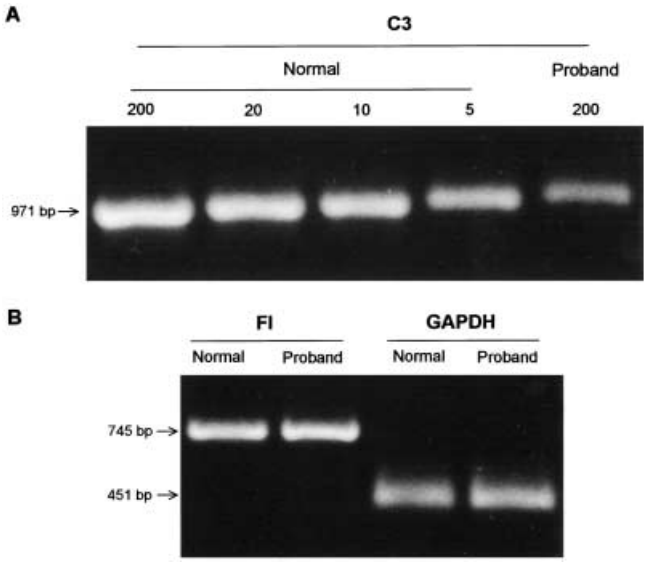

Fig. 4. RT-PCR of the proband's and normal RNA with C3, fI and GAPDH-specific primers. C3 reactions were performed with 200, 20, 10 and 5 ng total RNA from normal LPS-stimulated fibroblasts and 200 ng total RNA from the proband's LPS-stimulated fibroblasts (A). FI and GAPDH control reactions involved 200 ng total RNA from both normal and proband's LPS-stimulated fibroblasts (B). Sizes of the products are shown by arrows. No amplification products were detected in the negative control (no DNA).

that observed in normal serum (1 mg/ml). This addition conferred a classical pathway-dependent haemolytic activity in the proband's serum similar to that observed for NHS (data not shown). Sera from other family members presented normal classical and alternative pathway-dependent haemolytic activities (Table 1).

Leukocyte migration across a nitrocellulose filter in response to the proband's activated serum was very reduced when compared to activated pooled normal sera (aNHS) and comparable to that obtained for Hank's medium (HBSS) and inactivated NHS (iNHS). Even when we employed a greater concentration of the proband's serum (10% dilution in HBSS), we did not observe an increase in leukocyte migration (Fig. 2). The phagocytosis of *C. albicans* opsonized with proband's serum was diminished in comparison to the fungi treated with NHS (data not shown). Intracellular killing of *C. albicans* was also less intense if the fungi had previously been treated with the proband's serum rather than fungi treated with NHS. Instead, the killing of *C. albicans* treated with the proband's serum was comparable to that of fungi treated with iNHS (Table 2).

SDS-PAGE followed by autoradiography of fibroblast culture supernatants immunoprecipitated with anti-C3 revealed the presence of C3 α and β chains with the expected sizes (115 and 70 kDa, respectively) in the mother and one normal control (Fig. 3). The precursor form of C3, Pro-C3 (185 kDa), was not distinguishable from nonspecific precipitated bands with high molecular weight. Furthermore, we were not able to detect C3 α or β chains or any other fragments in the proband's supernatant even after chasing for 24 h. In the control experiments, the stimulated fibroblasts from the proband were shown to synthesize and secrete fB (93 kDa) (Fig. 3).

Amplification of the proband's C3 cDNA with primers that span from position 582–1553 of the structural gene did not reveal differences in the size of the product (971 bp) after electrophoresis in agarose gels. However, there was a significant difference in the amount of the proband's amplified products in relation to the normal control. The product obtained using 200 ng of proband's cDNA was less intense than that obtained using 40 times less normal cDNA (5 ng, Fig. 4A). Amplification with fI and GAPDH-specific primers revealed products of the same size and intensities for both deficient and normal individuals (Fig. 4B). In order to confirm these findings we performed two different RNA extractions of the proband's and normal RNA and carried out three or more amplification reactions for both, obtaining the same results in each experiment.

DISCUSSION

As observed for other C3-deficient individuals reported in the literature, the child described here has a long history of recurrent infections. Although a great proportion of those deficients presented with infections prior to the completion of the first year of life, the proband L.A.S. was healthy until the age of 2. At the age of 4 1/2 he presented purple spots in the legs, diagnozed as vasculitis (similar to the Hennoch–Scholein syndrome) which prompted suspicion of a C3 deficiency. Because this disease occurred a short time after an infectious episode it is therefore possible that either the surface or the secreted bacterial compounds were involved in the pathogenic process.

The proband presented a serum C3 concentration approximately

6000–10 000 times less than normal values (1244 ± 346 μg/ml). In addition, the proband's LPS-stimulated peripheral blood leukocytes failed to secrete C3 as observed by RIA (data not shown), and no C3 α or β chains could be observed in the proband's fibroblast supernatant by SDS-PAGE and autoradiography. The fH and fI concentrations were normal in the proband's serum, indicating that L.A.S. has a primary C3 deficiency. In accordance with this, we observed an impairment in the generation of chemotactic factors and of opsonins, dependent on complement activation. In a previous study of another Brazilian C3-deficient individual, diminished phagocytosis was also observed while the production of chemotactic factors was normal [17].

A nonmeasurable low concentration of IgG4 was detected in the proband's serum while the other IgG subclasses were normal. Lower levels of IgG4 were previously observed in several C1, C2 and C4-deficient individuals and in four C3-deficient individuals, three of whom also had low levels of total IgG2 [18]. This may indicate a defect in the generation of specific B-cell responses to certain antigens, in the absence of C3, although a possible role of classical pathway components in Ig heavy chain class switching can not be discarded.

The molecular defects leading to the primary C3 deficiency are heterogeneous. In previously investigated cases, these included partial deletions [19], point mutations [20–22] and unidentified defects leading to diminished C3 production [23] or secretion [24,25]. Southern blot analysis of genomic DNA from the proband, his mother and normal individuals digested with restriction endonucleases and hybridized with a full length C3 cDNA probe, confirmed that this deficiency did not arise from large genomic deletions, insertions or rearrangements in the C3 gene (data not shown). The fact that the proband's fibroblasts produced significantly less C3 mRNA, as observed by RT–PCR, suggests that the deficiency could be attributed to a diminished production of C3 mRNA or a greater instability of this mRNA.

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REFERENCES

1 Alper CA, Johnson AM, Birtch AG, Moore FD. Human C′3: evidence for the liver as the primary site of synthesis. Science 1969;163:286–8.

2 Einstein LP, Hansen PJ, Ballow M *et al.* Biosynthesis of the third component of complement (C3) *in vitro* by monocytes from both normal and homozygous C3-deficient humans. J Clin Invest 1977;60:963–9.

3 Botto M, Lissandrini D, Sorio C, Walport MJ. Biosynthesis and secretion of complement component (C3) by activated human polymorphonuclear leukocytes. J Immunol 1992;149:1348–55.

4 Senger DR, Hynes RO. C3 component of complement secreted by established cell lines. Cell 1978;15:375–84.

5 Naughton MA, Botto M, Carter MJ, Alexander GJ, Goldman JM, Walport MJ. Extrahepatic secreted complement C3 contributes to circulating C3 levels in humans. J Immunol 1996;156:3051–6.

6 Singer L, Colten HR, Wetsel RA. Complement C3 deficiency: human, animal and experimental models. Pathobiol 1994;62:14–28.

7 Davies KA, Schifferli JA, Walport MJ. Complement Deficiency and Immune Complex Disease. Springer Semin Immunopathol 1994:15:397–416.

8 Mancini G, Carbonara AO, Heremans JF. Immunochemical quantitation of antigens by single radial immunodiffusion. Immunochemistry 1965;2:235–54.

9 Isaac L, Isenman D. Structural requirements for thioester bond formation in human complement component C3. J Biol Chem 1992;67:10062–9.

10 Nilsson UR, Nilsson B. Simplified assays of hemolytic activity of the classical and alternative complement pathways. J Immunol Meth 1984;72:49–59.

11 Naked GM, Florido MP, Ferreira de Paula P, Vinet AM, Inostroza JS, Isaac L. Deficiency of human complement factor I associated with lowered factor H. Clin Immunol 2000;96:162–7.

12 Zigmond SH, Hirsch JG. Leukocyte locomotion and chemotaxis: New method for the evaluation and demonstration of cell-derived chemotactic factor. J Exp Med 1973;137:387–410.

13 Vyse TJ, Morley BJ, Bartók I *et al.* The molecular basis of hereditary complement factor I deficiency. J Clin Invest 1996;97:925–33.

14 Sambrook J, Fritsch EF, Maniatis T. Molecular Cloning. A Laboratory Manual. 2nd edn. New York: Cold Spring Harbor Laboratory Press, 1989;18:60–70.

15 Zar JH. Biostatistical Analysis. 4th edn. New Jersey: Prentice Hall, 1999:195–257.

16 Carneiro-Sampaio MM, Grumach AS. Alergia e Imunologia em Pediatria. São Paulo: Sarvier, 1992.

17 Grumach AS, Vilela MM, Gonzalez CH *et al.* Inherited C3 deficiency of the complement system. Braz J Med Biol Res 1988;21:247–57.

18 Bird P, Lachmann PJ. The regulation of IgG subclass production in man: low serum IgG4 in inherited deficiencies of the classical pathway of C3 activation. Eur J Immunol 1988;18:1217–22.

19 Botto M, Fong KY, So AK *et al.* Homozygous hereditary C3 deficiency due to a partial gene deletion. Proc Natl Acad Sci USA 1992;89:4957–61.

20 Botto M, Fong KY, So AK, Rudge A, Walport MJ. Molecular basis of hereditary C3 deficiency. J Clin Invest 1990;86:1158–63.

21 Huang JL, Lin CY. A hereditary C3 deficiency due to aberrant splicing of exon 10. Clin Immunol Immunopathol 1994;73:267–73.

22 Singer L, Whitehead WT, Akama H, Katz Y, Fishelson Z, Wetsel RA. Inherited human complement C3 deficiency. An amino acid substitution in the β-chain (Asp_{549} to Asn) impairs C3 secretion. J Biol Chem 1994;269:28494–9.

23 Singer L, Van Hee M, Lokki ML, Kramer J, Borzy MS, Wetsel RA. Inherited complement C3 deficiency: reduced C3 mRNA and protein levels in a Laotian kindred. Clin Immunol Immunopathol 1996;81:244–52.

24 Katz Y, Wetsel RA, Schlesinger M, Fishelson Z. Compound heterozygous complement C3 deficiency. Immunology 1995;94: 5–7.

25 Fishelson Z, Kozer E, Sirhan S, Katz Y. Distinction between processing of normal and mutant complement C3 within human skin fibroblasts. Eur J Immunol 1999;29:845–55.