PIEDAD MATILDE AGUDELO FLÓREZ

ESTUDO GENÉTICO-MOLECULAR DA DOENÇA GRANULOMATOSA CRÔNICA

Este exemplar corresponde à versão final da Tese de Doutorado apresentada à Faculdade de Ciências Médicas da Universidade Estadual de Campinas, para obtenção do título de Doutor em Saúde da Criança e do Adolescente.

Campinas, 04 de agosto de 2004.

Prof. Dr. Antonio Condino Neto
Orientador

CAMPINAS

2004

PIEDAD MATILDE AGUDELO FLÓREZ

ESTUDO GENÉTICO-MOLECULAR DA DOENÇA GRANULOMATOSA CRÔNICA

Tese de Doutorado apresentada à Pós-Graduação da Faculdade de Ciências Médicas, da Universidade Estadual de Campinas, para obtenção do Título de Doutor em Saúde da Criança e do Adolescente, área de Saúde da Criança e do Adolescente.

Orientador: *Prof. Dr. Antonio Condino Neto*

CAMPINAS

2004

FICHA CATALOGRÁFICA ELABORADA PELA BIBLIOTECA DA FACULDADE DE CIÊNCIAS MÉDICAS UNICAMP

Ag92e

Agudelo Flórez, Piedad Matilde
Estudo genético-molecular da doença granulomatosa crônica / Piedad Matilde Agudelo Flórez. Campinas, SP : [s.n.], 2004.

Orientador : Antonio Condino Neto
Tese (Doutorado) Universidade Estadual de Campinas. Faculdade de Ciências Médicas.

1. Mutação. 2. Fagocitose. I. Antonio Condino Neto. II. Universidade Estadual de Campinas. Faculdade de Ciências Médicas. III. Título.

Banca Examinadora da Tese de Doutorado

Orientador:

Prof. Dr. Antonio Condino Neto

Membros:

1. **Prof. Dr. Antonio Condino Neto**
2. **Prof. Dr. José Dirceu Ribeiro**
3. **Profa. Dra. Carmen Silvia Bertuzzo**
4. **Prof. Dr. Luiz Vicente Rizzo**
5. **Profa. Dra. Beatriz Tavares Costa Carvalho**

Curso de Pós-graduação em Saúde da Criança e do Adolescente da Faculdade de Ciências Médicas da Universidade Estadual de Campinas.

Data: 2004

DEDICATÓRIA

À Sofia... O melhor trabalho de teses que nunca imaginei poderia fazer.

Ao Juan, meu amigo, complice e parceiro de sonhos e realizações.

AGRADECIMENTOS

Ao meu orientador, Prof. Dr Antonio Condino, pelas oportunidades recebidas, sua orientação e por acreditar em mim.

Aos Drs. José Dirceu Ribeiro, Marcos Nelasco, Beatriz Costa Carvalho, Luiz Vicente Rizzo, Carmen Bertuzzo, Magda Carneiro Sampaio, Pérsio Roxo Jr, Marluce dos Santos Vilela, Anette Grumach, Mônica Cornejo, Lílian de Souza Li e Pablo Patino, pelo apoio, orientação e colaboração em diferentes momentos.

Às biólogas Jussara Redher e Dulcineia Alburquerque pelo seu apoio técnico.

À secretária da pós-graduação, Simone Cristina Ferreira, pela ajuda, amizade e dedicação.

Aos amigos Carolina e André pelo apoio e amizade oferecidos em tudo momento.

Aos amigos do laboratório de Biologia Molecular do Centro de Investigação em Pediatria, pela acolhida e apoio.

Aos pacientes e seus pais, pela cooperação para a realização deste trabalho.

À minha família, pelo apoio e compreensão ao me perdoar meus pecados de ausência.

A FAPESP e CAPES, pelo apoio financeiro.

SUMÀRIO

RESUMO

Doença Granulomatosa Crônica (DGC) é uma imunodeficiência caracterizada por infecções recorrentes graves. Os defeitos moleculares que levan a DGC são geralmente devidos a mau funcionamiento, ausência o baixa expressão de um dos componentes do sistema NADPH oxidase.

Este trabalho analisou o uso de RT-PCR para a triagem de defeitos moleculares responsáveis por DGC ligada ao X em 8 pacientes. RNA total foi preparado de linfócitos B trasformados com vírus de Epstein-Barr e transcrição reversa com hexâmeros randomicos. O cDNA resultante foi amplificado por PCR com oligonucleotídeos específicos para 3 regiões exônicas abrangendo toda a extensão do gene. Com esta estratégia foi possível a detecção da expressão defeituosa de gp91-*phox* em 7 pacientes. Concluímos que a análise por meio de RT-PCR, um método alternativo menos complexo, rápido e econômico foi apropriado para detecção inicial de defeitos moleculares em 7 de 8 pacientes com DGC ligada ao X.

Posteriormente investigamos em detalhe os defeitos genetico-moleculares de 7 crianças não relacionadas com DGC ligada ao X. Todos os pacientes foram procedentes do Chile e Brasil. Encontramos uma inserção c.1267_1268insA no paciente JY no exon 10 levando a uma mutação tipo "frameshift". Esta mutação é um novo registro na literatura. Detectamos duas substituições "nonsense", uma no paciente PT, c.95 G>A no exon 2 que leva a um códon de parada W28X e outra no paciente MF, c.229 C>T no exon 3 que leva a um códon de parada R73X. Em 4 casos, nos pacientes IC, Vin, RS, GG, diferentes erros de "splicing" foram encontrados. Dois pacientes apresentaram uma subtituição c.264 G>A ao final do exon 3. Os dois restantes apresentaram uma subtituição c.1326 + 1 G>A no intron 10 e outra subtituição c.1164 – 2 A>G no intron 9. Esta última mutação também é um novo registro na literatura.

As mutações identificadas na proteína gp91-*phox* confirmam um alto grau de heterogeneidade molecular como é relatado em outros grupos étnicos e a importância de investigar os defeitos moleculares em diferentes populações. Contrastando com esta heterogeneidade, a DGC associada com defeitos na proteína p47-*phox*, apresentam pouca variabilidade. Neste estudo, os pacientes analisados, dois irmãos, mostram uma deleção homozigota GT (△GT) no começo do exon 2.

Também é analisado o caso de um paciente com infecções recorrentes que inicalmente recebeu o diagnóstico de deficiência de G6PD. Estudos moleculares mostraram que a deficiência de G6PD foi devida a uma mutação 202 G→A, variante Africana. O paciente também mostrou uma reduzida atividade da explosão respiratória como observado em DGC ligada ao X. A análise do gDNA mostrou uma subtituição 264 G→A na região do splicing do exon 3 da proteína gp91-*phox*. A seqüência do cDNA detectou uma deleção do exon 3, levando a uma mutante inestável ou não funcional da proteina gp91-*phox* e resultando no fenótipo de DGC ligada ao X. Propomos que frente a um paciente com deficiência de G6PD com episódios de infecções graves considerem a possibilidade de um defeito na atividade fagocítica e uma eventual associação com DGC.

Palavras chave: Doença Granulomatosa Crônica, Imunodeficiência Primaria, *CYBB*, mutações, neutrofilos,fagócitos

ABSTRACT

Chronic granulomatous disease (CGD) is a rare primary immunodeficiency characterized by early onset recurrent severe infections. The molecular defects causing CGD are generally due to the absence, low expression or malfunctioning of one of the NADPH oxidase components. This work analyzed the potential use of reverse transcription (RT)-PCR for screening molecular defects responsible for X-linked CGD in 8 Brazilian patients. Total RNA was prepared from EBV-transformed B-lymphocytes and reverse transcribed using random hexamers. The resultant cDNA was PCR-amplified by specific and overlapping pairs of primers regarding 3 exonic regions of gp91-*phox* gene. This strategy made possible the detection of defective gp91-*phox* expression in 7 patients. We conclude that RT-PCR analysis, a less complex, more economic and faster alternative method, was appropriate for screening molecular defects in 7 out 8 X-linked CGD patients.

We further investigated the molecular genetic defects in 7 unrelated patients with X-linked CGD, from Chile and Brazil. We found an insertion c.1267_1268insA in exon 10 leading to a frameshift mutation. This mutation is a novel report. We detected two single base-pair substitutions that lead to nonsense mutations. The first was a c.95 G>A substitution in the exon 2 which predicts a stop codon W28X and the second was a c.229 C>T substitution in the exon 3 which predicts a stop codon R73X. We also identified different splice site mutations in 4 cases. Two patients presented a c.264 G> A substitution at the end of exon 3. The remaining two patients presented either a c.1326 + 1 G>A substitution in intron 10 or a c.1164 – 2 A>G substitution in intron 9. This last mutation is also novel. The gp91-*phox* mutations identified in these patients show a high degree of molecular heterogeneity as reported in other ethnic groups and the importance to investigate molecular genetic defects in different populations. Contrasting with the heterogeneity of mutations observed in X-linked CGD, the disease associated with defect in p47-phox shows less variability. In this report, the patients with CGD, two siblings, show a homozygotous dinucleotide GT deletion (△GT) at the beginning of exon 2.

We also reported a child with recurrent infections who initially received the diagnosis of G6PD deficiency. Molecular studies showed that the G6PD deficiency was due a 202 G→A mutation, the A^- variant common in African ethnic groups. The proband also exhibited severely impaired respiratory burst activity, as observed in X-linked CGD. Sequence analysis of genomic DNA showed a 264 G→A substitution at the 3' splice

junction of gp91-*phox* exon 3. The cDNA sequence showed a deletion of gp91-*phox* exon 3, giving rise to an unstable or nonfunctional mutant gp91-*phox* and to the phenotype of X-linked CGD. We propose that clinicians in face of a patient with G6PD deficiency under a severe infection episode consider the possibility of temporary or permanent impairment of the phagocytes microbicidal activity, and the eventual association of G6PD deficiency and chronic granulomatous disease.

Keywords: chronic granulomatous disease, primary immunodeficiencies, *CYBB*, mutations, neutrophils,phagocytes.

INTRODUÇÃO

CONCEITO E CLASSIFICAÇÃO DA DOENÇA GRANULOMATOSA CRÔNICA

Os reativos intermediários do oxigênio tiveram sua relevância clínica reconhecida, ao demonstrar-se que fagócitos de pacientes com a imunodeficiência primária denominada doença granulomatosa crônica (DGC), apresentam atividade microbicida defeituosa, resultado uma baixa produção de superóxido, secundária às mutações que afetam os componentes do sistema NADPH oxidase (HOLMES et al., 1966; HOLMES et al., 1967).

A DGC infantil foi descrita como uma entidade clínica em 1957, a qual acometia crianças do sexo masculino com pneumonia, linfadenite e abscessos localizados em diferentes áreas (BERENDES et al., 1957; LANDING & SHIRKEY, 1957; BRIDGES et al., 1959). Caracteriza-se clinicamente como uma imunodeficiência grave e rara (incidência estimada de 1/250.000 nascidos vivos por ano), de manifestação precoce, na qual, os quadros infecciosos por bactérias como *Staphylococcus aureus*, bacilos gram-negativos e fungos como *Aspergillus* e *Candida*, ocorrem predominantemente em locais considerados barreiras naturais do organismo (SEGAL et al., 1983; TAUBER et al., 1983). Desta maneira, o paciente apresenta infecções graves e recidivantes na pele, vias respiratórias, trato gastrointestinal e respectivos linfonodos que drenam essas áreas. Outros orgãos são: fígado, ossos, sistema nervoso central e pâncreas (FORREST et al., 1988; JOHNSTON, JR., 2001; WINKELSTEIN et al., 2000; SEGAL et al., 2000).

O defeito molecular da DGC reside na ausência ou baixa expressão de um dos componentes do sistema NADPH oxidase. Assim, na forma ligada ao sexo, é afetada a cadeia pesada do citocromo b_{558} , no caso, o componente gp91-*phox* (56% dos casos) (DINAUER et al., 1987). Nas formas autossômicas recessivas é afetado um dos componentes citosólicos do sistema NADPH oxidase, respectivamente a p47-*phox* ou p67-*phox* (respectivamente 33% e 5% dos casos) (CLARK et al., 1989); ou ainda a cadeia leve do citocromo b_{558} , o componente p22-*phox* (6% dos casos) (DINAUER et al., 1990; PARKOS et al., 1988). Até o momento não se documentou pacientes com DGC secundária com defeitos nos componentes p40-*phox*, rap1A, rac1, ou GDI. Descreveu-se recentemente um paciente com uma imunodeficiência secundária tendo o componente rac2 defeituoso (JOHNSTON JR,. 2001). Com base nestes achados, a classificação atual da DGC baseia-se

em defeitos moleculares específicos (CURNUTTE, 1988; CURNUTTE et al., 1994; WINKELSTEIN et al.,2000; SEGAL et al., 2000). O mecanismo de herança é definido pela abreviação "A" para autossômico ou "X" ligado ao sexo; o componente defeituoso da oxidase é representado pelo peso molecular da proteína afetada, "91," "22," "47," ou "67"; e o nível de expressão da proteína daquele componente é indicado pelo superescrito "0" para ausente, "+" para presente, e "-" para reduzido. O fenótipo $X91^0$ é o mais frequente, secundário há defeitos no gene *CYBB* no cromosso X, que codifica a proteína gp91-*phox* e resulta na ausência de citocromo b_{558} e atividade NADPH oxidase nula. O fenótipo $X91^-$ é menos frequente, e se refere à forma variante da DGC, laboratorialmente caracterizada por neutrófilos com baixa atividade NADPH oxidase, proporcional ao nível de citocromo b_{558} expresso (LEW et al., 1981; NEWBURGER et al., 1986; ROOS et al., 1996b; RAE et al., 1998). No fenótipo $X91^+$, o citocromo b_{558} encontra-se em níveis normais, entretanto sua atividade está diminuída ou ausente. A maioria das formas autossômica recessivas de DGC não guarda expressão residual do componente afetado (fenótipos $A22^0$, $A47^0$, e $A67^0$), entretanto formas variantes autossômicas ocasionais de DGC já foram descritas (SHURIN et al., 1983).

Dentre os defeitos gênicos de pacientes com DGC, os mais comuns são: deleções, inserções e substituições. Sendo que, a maior parte destes pacientes possui mutações exclusivas de suas famílias. A diversidade destas mutações e os múltiplos genes afetados constituem uma explicação para a heterogeneidade clínica e genética da DGC (CURNUTTE, 1993; ROOS et al., 1996a). Neste sentido, o estudo das células dos pacientes com DGC, além de ilustrar a relevância clínica dos reativos intermediários do oxigênio, possibilitou a identificação dos diversos componentes do sistema NADPH oxidase, bem como seus mecanismos de ativação (DINAUER et al., 2000).

MUTAÇÕES NA DGC LIGADA AO X

O gene *CYBB*, o qual codifica a grande subunidade glicosilada do citocromo b_{558}, denominada gp91-*phox*, contém 13 éxons e ocupa aproximadamente 30 kb da região Xp21.1 do cromossomo X (BAEHNER et al., 1986). Diversos defeitos moleculares que levam a DGC ligada ao sexo, foram identificados nas regiões codificadora, íntrons e

raramente nas regiões 5' regulatórias deste gene. (ROOS et al., 1996b; RAE et al., 1998; FREY et al., 1988; DE SAINT-BASILE et al., 1988; DINAUER et al., 1989; BOLSCHER et al.,1991; SCHAPIRO et al., 1991; de BOER et al. ,1992; RABBANI et al., 1993; ARIGA et al., 1994a; ARIGA et al., 1994b; LEUSEN et al., 1994; NEWBURGER et al. ,1994a; ARIGA et al., 1995; AZUMA et al., 1995; BU-GHANIM et al., 1995; CROSS et al. ,1995; ARIGA et al., 1998; NEWBURGER et al. ,1994b; SUZUKI et al., 1998; HEYWORTH et al., 2001; SEGAL et al., 2000).

Roos e colaboradores identificaram incialmente uma grande coleção de mutações que levam ao fenótipo de DGC, estas mutações foram disponibilizadas em uma base de dados computadorizada, (ROOS et al., 1996a), a qual, pode ser acessada pela internet no endereço http://www.helsinki.fi/science/signal/databases/x-cgdbase.html. Posteriormente mais de 300 mutações que levam ao fenótipo de DGC ligada ao X, identificadas por um grupo de pesquisadores também disponíveis no site: http://www.uta.fi/imtbioinfo/CYBBbase (HEYWORTH et al., 2001).

Os tipos de mutações que causam DGC ligada ao sexo incluem grandes deleções multigênicas, deleções e inserções menores, substituições do tipo "missense" e "nonsense", bem como defeitos de "splicing". Os principais estudos (ROOS et al., 1996b; RAE et al.,1998; WINKELSTEIN et al., 2000; SEGAL et al., 2000; HEUNG-BUM et al., 2004), mostram que as mutações se distribuem com frequência similar entre os éxons e nas bordas dos genes. Famílias não relacionadas nestes estudos serviram como base para os cálculos das frequências relativas de diferentes tipos de mutações. A heterogeneidade das mutações e a falta de um genótipo predominante mostram que a incidência mundial de DGC é consequência de muitos eventos mutacionais.

Rae e colaboradores (1998) identificaram mutações no gene *CYBB* que levaram ao fenótipo de DGC ligada ao sexo em 131 famílias consecutivas e independentes. O rastreamento por meio de SSCP ("single strand confirmation polymorphism analysis") identificou mutações em 124 famílias. O sequenciamento completo dos éxons e das regiões próximas às extremidades dos introns revelou outras 7 mutações. Neste estudo foi possível identificar 103 diferentes mutações específicas, sendo que nenhuma mutação isolada se repetiu em mais que 7 famílias independentes. Os tipos de mutações foram grandes e

pequenas deleções (11%), "frameshifts" (24%), mutações "nonsense" (23%), mutações "missence" (23%), mutações na região do "splicing" (17%) e mutações nas regiões reguladoras (2%). A distribuição das mutações ao longo do gene *CYBB* mostrou-se bastante heterogênea e não foi identificado nenhum locus preferencial para sua ocorrência.

Na América Latina, Patino e colaboradores (PATINO et al.,1999a) estudaram sete famílias não relacionadas na Colômbia e no Brasil. Neste estudo, seis mães eram portadoras de um alelo *CYBB* mutante, sendo que um dos casos tratava-se de uma mutação "de novo". Este grupo identificou uma substituição A por G no penúltimo nucleotídeo do íntron 12, quatro novas mutações "nonsense" (R91X, W106X, R157X, R290X), além de outras duas mutações "missense" (E225V, C244Y).

Mutações próximas aos sítios de "splicing" levam à DGC, interferindo com o processamento do RNA mensageiro, sendo documentadas em 39 de 251 casos nos estudos principais (ROOS et al., 1996a; RAE et al., 1998; WINKELSTEIN et al., 2000; SEGAL et al., 2000). A maior parte das mutações ocorreu nos sítios de "splicing", e resultaram no fenótipo $X91^0$ devido a deleção de um ou mais éxons, como na maioria das mutações "splicing" que levam a DGC ligada ao sexo, anteriormente documentadas (de BOER et al., 1992). Entretanto, numa minoria de casos, tais mutações levam ao fenótipo $X91^-$, devido a manutenção parcial do "splicing" normal (RAE et al., 1998). Uma destas famílias, assunto de pesquisa de nosso grupo, mostrou-se especialmente responsiva ao tratamento com interferon-gama (IFN-γ)1, com restauração quase completa da atividade oxidase *in vitro* e *in vivo*, pelo menos em parte pelo aumento dos níveis de transcritos normais (EZEKOWITZ et al., 1987; EZEKOWITZ et al,. 1988; CONDINO-NETO et al., 1997; CONDINO-NETO & NEWBURGER, 2000).

MUTAÇÕES NA DGC AUTOSSÔMICA

O número de mutações identificadas em pacientes com DGC autossômica é menor que na DGC ligada ao sexo, devido a uma menor incidência de DGC autossômica. Nove famílias com deficiência de p22-*phox*, cerca de 40 famílias com deficiência de p47-*phox* e 11 famílias com deficiência de p67-*phox* tiveram suas mutações identificadas. Os resultados indicam que as bases genético-moleculares das deficiências de p22-*phox* e

p67-*phox* são tão heterogêneas quanto às observadas nas deficiências de gp91-*phox*, ligadas ao X, enquanto os casos de deficiência de p47-*phox* são mais homogêneos (ROOS et al. 1996a; RAE et al. 1998; CROOS et al, 2000).

Em 9 famílias com mutações na p22-*phox*, 10 diferentes mutações foram descobertas em 18 alelos, incluindo deleções, inserções e substituições próximas aos sítios de "splicing", e mutações missense(CROSS et al., 1996; ROOS et al. ,1996b). Em 7 famílias os pacientes eram homozigotos para as mutações encontradas, enquanto em 2 famílias os pacientes eram heterozigotos compostos. Somente em 2 famílias não relacionadas, foram encontrados pacientes com a mesma mutação. Somente 4 polimormismos da p22-*phox* foram identificados. Portanto, pequenas alterações na composição desta proteína parecem resultar em instabilidade intrínsica ou instabilidade secundária pela baixa interação com a gp91-*phox*, ao compor o citocromo b_{558} (DINAUER et al., 2000).

As mutações que levam a DGC autossômica por defeitos na p47-phox têm sido um enigma para os pesquisados de DGC. Em 35 pacientes não relacionados com deficiência da p47-*phox* , foi identificada uma deleção de 2 nucleotídeos na repetição GTGT, correspondente às 4 primeiras bases do segundo éxon do gene *NCF1* (CHANOCK et al., 1991; CASIMIR et al., 1991; IWATA et al., 1994; VOLPP & LIN, 1993). Em 31 destes casos a deleção GT foi homozigota e em 1 dos outros 4 pacientes, outra mutação de 1 nucleotídeo foi identificada além da delecão GT. Surpreendentemente, no entanto, a amplificação por PCR do cDNA ou gDNA de indivíduos normais, também revelou a presença simultânea da sequência GTGT e do produto com a deleção GT. Isto sugeriu a existência de um pseudogene com a deleção GT, além do gene *NCF1*, pelo que a DGC autossômica por defeito da p47-*phox* se deve a recombinação entre o gene *NCF1* e o pseudogene (GORLACH et al., 1997). Recentemente, foram também descritos casos de DGC secundários com defeitos no p47-phox, por mutações não relacionadas ao pseudogene (NOACK et al., 2001; HEYWORTH et al., 2003).

Dos 11 pacientes descritos com DGC autossômica por deficiência de p67-*phox*, 11 diferentes mutações foram identificadas em 22 alelos afetados. Estas incluem mutações "missense", "nonsense", substituições nos sítios de "splicing", uma inserção de

dinucleotídeo e uma variedade de deleções, cujos tamanhos variam de alguns nucleotídeos de até 11-13 kb (AOSHIMA et al., 1996; PATINO et al., 1999b; LEUSEN et al., 1996; NUNOI et al., 1995; TANUGI-CHOLLEY et al., 1995; de BOER et al., 1994). Em alguns casos de DGC autossômica por deficiência de p67-*phox*, o nível de mRNA para p67-*phox* foi normal, mas a proteína p67-phox mostrou-se indetectável. Entretanto, em um dos pacientes com 1 deleção de 3 nucleotídeos (1718-1720), cerca de 50% da proteína estava presente. Neste paciente, a mutação prediz uma deleção na "moldura" (LYs-58) e resulta na expressão de p67-*phox* não funcional que não se transloca para a membrana plasmática (LEUSEN et al., 1996).

CORRELAÇÃO GENÓTIPO-FENÓTIPO

No geral os casos de DGC autossômica p22-*phox*^{0} e p67-*phox*^{0} são tão graves quanto os casos de DGC ligada ao X gp91-*phox*^{0}. Por outro lado, diversas comparações clínicas entre DGC ligada ao X e DGC autossômica secundária com defeitos no p47-*phox* sugerem que esta última tem evolução mais benigna (The International Chronic Granulomatous Disease Cooperative Study Group, 1991; MARGOLIS et al., 1990; WEENING et al., 1985), o que pode ser atribuída à atividade NADPH oxidase residual (CROSS & CURNUTTE, 1995; CROSS et al., 1994; BEMILLER et al., 1995; WINKELSTEIN et al., 2000). Espera-se que pacientes com o fenótipo X91^{-}, com atividade NADPH oxidase residual de 3-30% tenham evolução mais benigna do que aqueles com fenótipos X91^{0} e X91^{+} (ROOS et al.,1992).

O prognóstico de pacientes com DGC melhorou significativamente desde que a doença foi descoberta na década de cinquenta, quando era denominada "granulomatose fatal da infância". Os pilares do tratamento da DGC são: 1) prevensão das infecções por meio de imunizações e remoção das fontes de patógenos, 2) uso profilático de trimetoprin-sulfametoxazol ou outro antimicrobiano com penetração intracelular, 3) uso profilático de IFN-γ, 4) uso precoce e agressivo de antibióticos parenterais, 5) drenagem cirúrgica ou ressecção de focos infecciosos persistentes. Dos cinco itens, o mais importante é a intervenção precoce nas infecções, antes que elas resultem o comprometido do sistema imunológico do paciente com DGC (ROOS & CURNUTTE, 1999; SEGAL et al., 2000).

O IFN-γ humano recombinante está indicado na dose de 50 $\mu g/m^2$ de superfície corporal, por via subcutânea, três vezes por semana. Ele reduz o risco relativo de infecções graves em 70%. Recentemente foi demonstrado que o IFN-γ incrementa a fidelidade do "splicing" e a estabilidade dos transcritos do gene *CYBB*, corrigindo parcialmente a expressão do componente gp91-*phox*, atenuando o fenótipo de DGC ligada ao sexo em uma família especialmente responsível ao IFN-γ (RAE et al., 1998; CONDINO-NETO & NEWBURGER, 2000).

Dentre as perspectivas de cura, no que pese a dificuldade imposta pela heterogeneidade das mutações que levam ao fenótipo de DGC, o programa de terapia gênica evoluiu esperançosamente e espera-se que seja aplicada clinicamente na próxima década (ROOS & CURNUTTE, 1999; DINAUER et al., 2000; SEGAL et al., 2000; JOHNSTON Jr., 2001). Alternativamente, o mini-transplante de medula também apresenta resultados promissores (HORWITZ et al., 2001; NAGLER et al., 1999; AMROLIA et al., 2001), sendo esta, uma possível estratégia de correção fenotípica parcial em casos selecionados.

Apesar da intensa investigação clínica, bioquímica e molecular, existem ainda muitas lacunas no conhecimento dos defeitos genético-moleculares e sua correlação com os diferentes fenótipos da Doença Granulomatosa Crônica (DGC) (DINAUER et al. ,2000).

Identificar o componente alterado e a mutação subjacente que leva às variadas manifestações clínicas é muito importante, pois cada paciente com DGC e sua família têm potencialmente um defeito molecular específico. Isto é essencial para o aconselhamento genético adequado, viabilizando a terapêutica precoce e prevenção de seqüelas.

Os resultados advindos deste trabalho contribuirão para o avanço do conhecimento sobre a estrutura, função e regulação do sistema NADPH oxidase fagocítico humano; o desenvolvimento e diferenciação da série mielóide humana; os mecanismos do sistema imune inato; e, sobretudo para a construção de estratégias que permitam a correção definitiva dos defeitos genético-moleculares relacionados a esta e outras doenças.

OBJETIVOS

OBJETIVO GERAL

Identificar as alterações genético-moleculares de pacientes com DGC.

OBJETIVOS ESPECÍFICOS

Capitulo 1: *"O uso da transcripção reversa seguida de PCR no diagnóstico de doença granulomatosa crônica ligada ao X".*

Identificar o componente defeituoso nos leucócitos de pacientes com DGC, por meio de ensaio RT-PCR.

Capitulo 2: *" Genética molecular da doença granulomatosa crônica ligada ao X em pacientes latino-americanos".*

Identificar o componente defeituoso do sistema NADPH oxidase nos leucócitos de pacientes com DGC, por meio de "Western Blot" e ensaio RT-PCR.

Localizar a mutação no gene gp91-*phox* , mediante análise de polimorfismo conformacional de cadeia simples (SSCP).

Identificar a mutação específica no gene gp91-*phox*, por meio de sequenciamento enzimático do DNA amplificado.

Capitulo 3: *" Associação de deficiência de glucose-6-fosfato desidrogenase e doença granulomatosa crônica ligada ao X num paciente com anemia e infecções recorrentes".*

Correlacionar as alterações genético-moleculares com o fenótipo de um paciente estudado com associação de DGC e deficiência de G6PD.

Capitulo 4: **Doença granulomatosa crônica autossômica: relato de caso e análise genético-molecular de uma família brasileira.**

Localizar a mutação no gene p47-*phox* , mediante análise de polimorfismo conformacional de cadeia simples (SSCP) em dois irmãos.

Identificar a mutação específica no gene p47-*phox*, por meio de sequenciamento enzimático do DNA amplificado.

Correlacionar as alterações genético-moleculares com o fenótipo dos pacientes estudados.

CAPÍTULO 1

The use of reverse transcription-PCR in the diagnosis of X-linked chronic granulomatous disease

Agudelo-Flórez P.[1], *López J.A.*[1], *Redher J.*[1], *Carneiro-Sampaio, M.M.S.*[2], *Costa-Carvalho B.T.*[3], *Grumach A.S.*[4], *Condino-Neto A.*[1]

1) Centro de Investigação em Pediatria e Departamentos de Pediatria e Farmacologia, Faculdade de Ciências Médicas, Universidade Estadual de Campinas.

2) Departamento de Imunologia, Instituto de Ciências Biomédicas, Universidade de São Paulo.

3) Disciplina de Alergia, Imunologia e Reumatologia, Departamento de Pediatria, Escola Paulista de Medicina, Universidade Federal de São Paulo.

4) Laboratório de Investigação Médica 56, Departamento de Dermatologia, Faculdade de Medicina, Universidade de São Paulo.

Grants: Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP 01/14365-3 e 02/05880-4), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq 470413/03-4), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), and The United States National Institutes of Health Fogarty International Center (1RO3TW00883-01).

Author for Correspondence:

ANTONIO CONDINO-NETO, MD, PHD.

Tel: (19) 3788-8988. Fax (19) 3289-8638

E-mail: condino@lexxa.com.br ou condino@unicamp.br

Centro de Investigação em Pediatria. Faculdade de Ciências Médicas, UNICAMP, Caixa Postal 6111, Campinas, SP 13081-970, BRASIL.

Keywords: superoxide, phagocytes, primary immunodeficiency, respiratory burst, neutrophils, human.

Running title: RT-PCR and X-linked chronic granulomatous disease.

ABSTRACT

Chronic Granulomatous Disease (CGD) is an inherited disorder of the innate immune system characterized by a defective oxidative burst of phagocytes and subsequent impairment of their microbicidal activity. Mutations in one of the NADPH oxidase components affect gene expression or function of this system, leading to the phenotype of CGD. Defects in gp91-*phox* lead to X-linked CGD, responsible for approximately 70% of CGD cases. Investigation of the very heterogeneous genotype of CGD patients includes mutation analysis, northern blot or western blot assays according to the particular case. The aim of this work was to use reverse transcription (RT)-PCR for the analysis of molecular defects responsible for X-linked CGD in eight Brazilian patients and to evaluate its potential for broader application for molecular screening in CGD. Total RNA was prepared from EBV-transformed B-lymphocytes and reverse transcribed using random hexamers. The resultant cDNA was PCR-amplified by specific and overlapping pairs of primers designed to amplify three regions of the gp91-*phox* gene: exons 1-5, 3-9, and 7-13. This strategy detected defective gp91-*phox* expression in seven patients. The RT-PCR results matched clinical history, biochemical data (NBT or superoxide release assay) and available mutation analysis in four cases. In three additional cases, RT-PCR results matched clinical history and biochemical data. In another case, RT-PCR was normal despite a compatible clinical history of CGD and defective respiratory burst. We conclude that RT-PCR analysis – a simple, economical and rapid method – was appropriate for screening molecular defects in 7 of 8 X-linked CGD patients.

INTRODUCTION

Chronic granulomatous disease (CGD) is a primary immunodeficiency, originally described in 1957 as a clinical entity affecting male infants and named, at the time, fatal granulomatous disease of childhood. The main characteristics of CGD are recurrent and severe infections involving the natural barriers of the organism such as the respiratory tract and lymph nodes, and eventually inner structures such as the liver, spleen, bones, and brain (1-3). The estimated incidence of this rare disease is 1/250.000 live births per year. The infections are generally caused by catalase positive bacteria such as *Staphylococcus aureus*, gram-negative bacilli, and fungi species such as *Aspergillus, Candida* and *Nocardia* (4,5).

The NADPH-oxidase system generates superoxide and other reactive oxygen intermediates, crucial for phagocytes' microbicidal activity. The biochemical defect in CGD is an impairment of NADPH-oxidase activity and subsequent inability to destroy microorganisms (6). The main components of the NADPH oxidase system are gp91-, p22-, p47-, p67-, and p40-*phox*. Molecular defects causing CGD are generally due to absence, low expression or malfunctioning of one of the NADPH oxidase components. The X-linked form of this disease is caused by defects in gp91-*phox*, the heavy chain of the cytochrome b_{588}, and accounts for approximately 70% of all cases (7,8). The autosomal recessive forms are caused by defects in one of the cytosolic components of the NADPH oxidase (p47-*phox* or p67-*phox*, respectively 20% and 5% of cases), or the cytochrome b_{588} light chain component (p22-*phox*, 5% of cases) (9,10). CGD is a very heterogeneous condition: over 300 mutations have been registered in an internationally-maintained X-CGD database (8). The mutations have been distributed largely within the 13 exons or at exon/intron boundaries of the gp91-*phox* (*CYBB*) gene and almost 200 of these mutations are unique.

The diagnosis of CGD is generally based on the presenting clinical characteristics of CGD plus defective NADPH oxidase activity as demonstrated by abnormal nitroblue tetrazolium (NBT), dihydrorhodamine (DHR) 123, or superoxide release assays. (11,12). In the stimulated NBT test, normal individuals display nearly 100% positive cells, while in CGD patients, fewer than 5% of the cells are positive (13). In addition, cells from patients with variant CGD are positive, but show only very low activity

(6). The NBT test also detects the carriers of X-linked CGD (mothers and sisters). A definitive molecular CGD diagnosis is established in patients with abnormal NBT test or respiratory burst activity who have one of the following characteristics: a mutation in gp91, p22, p47, or p67-*phox*; absent mRNA for one of these genes detected by Northern blot analysis; and/or absent protein for one of these oxidase components by western blot. A genetic, but not molecular, diagnosis can be established by demonstration of maternal cousins, uncles, or nephews with an abnormal NBT test or respiratory burst (14). Thus, the establishment of definitive diagnosis of this rare and very heterogeneous disease requires complex and expensive methodologies such as a combination of northern blot or western blot, and single strand conformation polymorphism analysis (SSCP) followed by DNA sequencing of several family members, all performed in high complexity research laboratories.

The reverse transcription–PCR (RT-PCR) method involves the amplification of cDNA by PCR. This technique can be easily standardized in less sophisticated laboratories. It provides information about gene expression and preliminary data about the structure or size of the mRNA of the defective component. RT-PCR has seldom been used in CGD research, restricted to pathophysiology studies (15-29). To date, the potential use of this useful tool for establishing the definitive diagnosis of X-linked CGD, the most frequent form, has not been investigated extensively. The aim of this work was to analyze the potential use of RT-PCR for screening molecular defects responsible for X-linked CGD, a rare and possibly misdiagnosed immunodeficiency, in eight Brazilian patients.

METHODS

Patients

The study included 8 non-related male Brazilian patients with probable X-linked CGD (2 Blacks, 6 Caucasians; age 2-8 years; height 88-108 cm; weight 11-19 kg). The patients presented clinical histories of recurrent severe infections such as pneumonia, lymphadenitis, liver abscess, pyodermitis, and adverse reactions to BCG immunization. The patients were referred to our laboratory for biochemical and molecular diagnostic evaluation. Written informed consent was obtained from the participants prior to the study. The Medical School Ethics Committee approved the protocol in accordance to the Helsinki Convention and Brazil Ministry of Health, Resolution 196/96.

Biochemical diagnosis of CGD

The biochemical diagnosis of CGD was established according to the Pan American Group for Immunodeficiencies criteria. An impairment of the NADPH oxidase activity was demonstrated by the nitroblue tetrazolium (NBT) slide test and/or the superoxide anion release assay by peripheral blood neutrophils and mononuclear leukocytes (14,30,31). Neutrophils and mononuclear leukocytes were obtained by centrifugation of blood samples over a Ficoll-Hypaque density gradient (32).

The NBT slide test was based on the reduction of NBT to formazan by activated leukocytes (31). The assay was performed as previously published (30). More than 95% of 200 normal neutrophils stimulated with PMA (30nM) should be able to reduce NBT. Absent reaction or <5% positive cells was considered diagnostic of CGD (14).

Quantitative superoxide release by neutrophils and mononuclear leukocytes was assessed by a modified superoxide dismutase-inhibitable cytochrome *c* reduction assay as previously published (33-35). The amount of superoxide released was calculated using an extinction coefficient for cytochrome *c* of $0.21 nM^{-1}cm^{-1}$. The results were expressed as nmol of superoxide released by 10^6 cells per hour. Patients with CGD showed less than 10% of control values.

Screening molecular defects responsible for X-linked CGD

B-lymphocytes from X-linked CGD patients were transformed *in vitro* with EBV (15,16) in order to provide an abundant source of nucleic acids for molecular studies. The EBV-transformed B cell lines reproduce the biochemical and molecular defects of CGD patients (15,16,36), and avoid repeated blood collections. Briefly, peripheral blood leukocytes from X-linked CGD patients were cultured with supernatants from B95-8, an EBV-producer cell line (15,16,36,37), in RPMI 1640 medium supplemented with heat inactivated fetal bovine serum (10%), 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml of streptomycin, at 37°C, in a humid atmosphere with 5% CO_2. Cellular viability was monitored and the cultures were maintained during the study period.

RNA samples from EBV-transformed B-cell lines were prepared by the guanidine HCl method, followed by ethanol precipitation and quantification by standard methods (38,39). The cDNA samples were obtained by reverse transcription of 2 μg of total RNA with SuperScript II RT (GIBCO BRL) and random hexamers (15). The quality of the mRNA samples was checked by PCR amplification of β-actin, a constitutive gene control (bp 920-943 and bp 1494-1471) (Gen Bank accession No. NM001101).

gp91-phox gene expression was assessed by RT-PCR. Specific and overlapping pairs of primers (Gen Bank accession No. NM_000397, Table I) were used to amplify (30 cycles) three gp91-phox exonic regions: 1-5, 3-9, and 7-13. This strategy allowed us to screen all gp91-phox exons. PCR products were analyzed by agarose (2%) gel electrophoresis and stained with ethidium bromide.

gp91-phox relative gene expression was analyzed by a Image Master Software (Pharmacia-Biotech). The densitometry result for target samples was divided by the densitometry result of β-actin, a constitutive gene control, normalizing the level that was considered for analysis. The results of RT-PCR assays were compared to patients' clinical history, biochemical assays (NBT and/or superoxide release assay), and available mutation analysis data (http://www.sbi.org.br/Sbi2003/index.htm).

RESULTS

We studied 8 male patients with clinical histories of recurrent severe infections, referred to our laboratory for biochemical and molecular diagnosis of CGD. The results from the NBT slide tests and the superoxide release assays are shown in Table II. Six patients presented less than 5% positive leukocytes in the NBT slide test. Six patients showed impaired superoxide release by granulocytes and/or mononuclear leukocytes (less than 10% compared to healthy controls). Four patients had abnormal results on both tests. All patients presented at least one abnormal test and received the diagnosis of probable X-linked CGD. The mother and the sister of patient TBP presented a NBT slide test compatible with the carrier state of X-linked CGD. During the study period one patient (GM) died from pneumonia.

We next investigated the definitive diagnosis of X-linked CGD by RT-PCR analysis of gp91-*phox* gene expression. The results of the RT-PCR amplification with three sets of overlapping primers are presented in Table II. The quality of mRNA sample was checked by PCR amplification of β-actin, a constitutive gene control. Expected normal products were obtained in this case (Figures 1, 2 and 3).

The amplification of exons 1-5 detected two patients (GM and RB) with reduced gp91-*phox* gene expression (Figure 1, lanes 1 and 2, respectively). One patient (JEM, lane 8) showed absent gp91-*phox* gene expression. Two patients (GG and RS, Figure 1, lanes 3 and 7, respectively) presented a smaller PCR product (between 300 and 400 bp), suggesting a splicing defect or small deletion. Three patients presented normal gp91-*phox* expression (TPB, MF and TP; Figure 1, lanes 4, 5, and 6, respectively).

The results of RT-PCR amplification of gp91-*phox* exons 3-9 are presented in Figure 2. Two patients (RB and MF) presented reduced gp91-*phox* gene expression (Figure 2, lanes 2 and 5, respectively). Three patients (GG, RS, and JEM; lanes 3, 7, and 8, respectively) presented absent gp91-*phox* gene expression. Three patients presented normal gp91-*phox* expression (GM, TPB and TP; Figure 2, lanes 1, 4 and 6, respectively).

RT-PCR amplification of gp91-*phox* exons 7-13 is presented in Figure 3. One patient (RB) presented reduced gp91-*phox* gene expression (Figure 3, lane 2). Four patients (GM, GG, TP and JEM, lanes 1, 3, 6, and 8, respectively) presented absent gp91-*phox* gene

expression. Three patients (TPB, MF and RS; Figure 3, lanes 4, 5 and 7, respectively) presented normal gp91-*phox* expression.

In four cases, it was possible to match RT-PCR data with available mutation analysis, presented elsewhere by Patiño et al. (30) or by our group (http://www.sbi.org.br/Sbi2003/index.htm): JEM presents a $C_{469} \rightarrow T$ transition in exon 5, predicting a nonsense mutation (R157X). MF presents a nonsense substitution in exon 3, R (Arginine) 73– Stop. RS showed a 264 G→A substitution at the 3' splice junction of gp91-*phox* exon 3. The cDNA sequence showed a deletion of gp91-*phox* exon 3, giving rise to an unstable or nonfunctional mutant gp91-*phox*. GG presented a defective splicing of gp91-*phox* exon 3 (the underlying mutation was not determined yet). The other patients remain under current investigation.

Taken together, this strategy made possible the detection of defective gp91-*phox* expression in seven of eight patients. The RT-PCR results matched clinical history, biochemical data (NBT or superoxide release assay) and available mutation analysis in four cases. In three additional cases, RT-PCR results matched clinical history and biochemical data. In another case, RT-PCR was normal despite a compatible clinical history of CGD, a defective respiratory burst characterized by NBT test and superoxide release assay and NBT tests of his mother and sister compatible with X-linked CGD carrier state.

DISCUSSION

This paper reports on the biochemical and gene expression studies of 8 unrelated Brazilian male patients with a clinical history of CGD, who were referred to our laboratory for detailed investigation. CGD is a rare inherited disorder in which phagocytic cells are unable to generate superoxide anion and other reactive oxygen intermediates. We have initially established the diagnosis of probable X-linked CGD by means of biochemical tools such as the NBT slide tests and/or superoxide anion release assays. Our results have shown that all patients presented an impaired NADPH oxidase function and in turn, probable X-linked CGD.

Both methods contribute to the probable diagnosis of CGD in different ways. The NBT slide test provides information about the number of cells that reduce NBT to formazan inside the cytoplasm and the intensity of this reduction. Thus, patients with variant forms of X-linked CGD show abnormal NBT slide tests, in which most of the cells weakly reduce NBT to formazan. The NBT slide test also diagnoses female carriers of X-linked CGD. The superoxide release assay measures the reduction of cytochrome *c* by superoxide produced by activated leukocytes present in the reaction, making possible the diagnosis of variant forms of X-linked CGD. Both tests can be easily standardized in low complexity laboratories and do not require expensive equipment. The DHR test is a very sensitive method for the biochemical diagnosis of CGD, however it requires a flow cytometer, a very expensive instrument.

We investigated gp91-*phox* gene expression in EBV-transformed B lymphocytes from CGD patients by RT-PCR analysis. Specific and overlapping pairs of primers were used to amplify three regions of the gp91-*phox* gene by RT-PCR: exons 1-5, 3-9, and 7-13. This strategy made possible the detection of defective gp91-*phox* expression in seven patients. The RT-PCR results matched clinical history, biochemical data (NBT or superoxide release assay) and available mutation analysis in four cases. In three additional cases, RT-PCR results matched clinical history and biochemical data.

gp91-*phox* gene expression was reduced in all exonic regions of patient RB. This reduction can be the result of mutations leading to low RNA stability or altered transcriptional activity. Patients GM and TP showed absent expression of exons 7-13,

suggesting that the decreased expression of these more 3'-end exons can be the result of RNA instability or a splicing defect – e.g. partially correct splicing to produce a signal, but partially abnormal splicing to eliminate a primer binding site, a subject to be investigated by future genomic DNA mutation analyses.

The patient GG presented a diffuse, low abundance, smaller-sized product of exons 1-5 (Figure 1), and absent expression of other exonic regions. His mutation analysis has shown a defect at exon 3 splice site. Similarly, the mutation analysis of patient RS has also revealed a defect at exon 3 splice site. However, in this case, a smaller abundance PCR product could be detected.

The patient MF presented reduced expression of exonic regions 3-9 and 7-13, which can be the result of defective transcriptional activity in exon 3. JEM presents a nonsense mutation in exon 5 that resulted in loss of gp91-*phox* expression, as evidenced by RT-PCR analysis, a possible consequence of nonsense-mediated mRNA instability.

In one case, TPB, gp91-*phox* gene expression was normal despite a family history compatible with CGD and a defective respiratory burst activity. In this case, the diagnosis of probable X-linked CGD was based on abnormal NBT tests in his mother and his sister, compatible with a carrier state. We hypothesize that a point mutation, such as a single base substitution that does not change PCR fragment length or abundance,should be investigated in this particular case.

RT-PCR is a powerful tool to assess gene expression. This characteristic may partially explain the variability of gp91-phox gene expression among the patients included in this study, and the importance to combine overlapping pair of primers to screen the full length of the message. RT-PCR has been used in isolated case studies as part of the initial diagnosis of the different forms of CGD (17-22). RT-PCR analysis was also useful in the prenatal diagnosis of CGD, resulting from p47-*phox* deficiency (23). However it has been more commonly used for the study of gene regulation of the NADPH oxidase components in a variety of cells (15,16,24-29). To date, the potential use of this useful tool for establishing the definitive diagnosis of X-linked CGD, the most frequent form, has not been investigated extensively. Further studies should be performed to compare the sensitivity

and specificity of this test compared to other complex assays such as western or northern blots.

Overall, we have demonstrated that RT-PCR, a simple and low cost methodology, established the definitive diagnosis of X-linked CGD in 7 out of 8 cases, without the need to use complex and expensive methodologies such as northern blot, slot blot, SSCP analysis, or genomic DNA sequencing. Thus, RT-PCR may be a suitable tool for diagnosing CGD in laboratories from developing countries. It is very important to determine the definitive molecular genetic defect in order to provide the appropriate genetic counseling and prognosis to kindreds with CGD. In addition, molecular genetic studies of the human NADPH oxidase system will advance the knowledge about this crucial and ancient defense mechanism.

ACKNOWLEDGEMENTS:

The authors thank the patients and their families for participating and help, and the nurse Silvana Severino for technical assistance. The authors also thank Prof. Peter Newburger from University of Massachusetts Medical School for the critical review of this manuscript.

REFERENCES

1. Berendes H, Bridges RA, Good RA. (1957). A fatal granulomatosus of childhood: the clinical study of a new syndrome. *Minnesota Medicine,* 40:309-312.

2. Landing BH, Shirkey HS. (1957). A syndrome of recurrent infection and infiltration of the viscera by pigmented lipid histiocytes. *Pediatrics,*20:431-442.

3. Patterson EL, Milstrey R, Stokstad ELR. (1975). Effect of selenium in preventing exudative diathesis in chicks. *Proceedings of Society for Experimental Biology and Medicine*, 95:617-620.

4. Tauber AI, Borregaard N, Simons ER, Wright J. (1983). Chronic granulomatous disease A syndrome of phagocyte oxidase deficiencies. *Medicine*,62:286-309.

5. Forrest CB, Forehand JR, Axtell RA, Roberts RL, Johnston RB, Jr. (1988). Clinical features and current management of chronic granulomatous disease. *Hematololgy and Oncology Clinics of North America,* 2:253-266.

6. Curnutte JT (1993). Chronic granulomatous disease: the solving of a clinical riddle at the molecular level. *Clinical Immunology and Immunopathology, 67:*S2-15.

7. Dinauer MC, Orkin SH, Brown R, Jesaitis AJ, Parkos CA. (1987). The glycoprotein encoded by the X-linked chronic granulomatous disease locus is a component of the neutrophil cytochrome b complex. *Nature,*327:717-720.

8. Heyworth PG, Curnutte JT, Rae J, Noack D, Roos D, van Koppen E, Cross AR (2001). Hematologically important mutations: X-linked chronic granulomatous disease (second update). *Blood Cells Molecules and Diseases, 27:*16-26.

9. Clark RA, Malech HL, Gallin JI, Nunoi H, Volpp BD, Pearson DW, Nauseef WM, Curnutte JT. (1989). Genetic variants of chronic granulomatous disease: Prevalence of deficiencies of two cytosolic components of the NADPH oxidase system. *New England Journal of Medicine*, 321:647-652.

10. Cross AR, Noack D, Rae J, Curnutte JT, Heyworth PG (2000). Hematologically important mutations: the autosomal recessive forms of chronic granulomatous disease (first update). *Blood Cells Molecules and Diseases, 26:*561-565.

11. Smith RM, Curnutte JT (1991). Molecular basis of chronic granulomatous disease. *Blood, 77:*673-686.

12. Vowells SJ, Fleisher TA, Sekhsaria S,Alling DW, Maguire TE, Malech HL(1996). Genotype-dependent variability in flow cytometric evaluation of reduced nocotinamide adenine dinucleotide phosphate oxidase function in patients with chronic granulomatous disease. *Journal Pediatrics,* 128:104-107.

13. Roos D, de Boer M, Kuribayashi F, Meischl C, Weening RS, Segal AW, Ahlin A, Nemet K, Hossle JP, Bernatowska-Matuszkiewicz E, Middleton-Price H (1996). Mutations in the X-linked and autosomal recessive forms of chronic granulomatous disease. *Blood, 87:*1663-1681.

14. Conley ME, Notarangelo LD, Etzioni A. (1999). Diagnostic criteria for primary immunodeficiencies. *Clinical Immunology*, 93:190-197.

15. Condino-Neto A, Newburger PE. (1998). NADPH oxidase activity and cytochrome b558 content of human Epstein-Barr virus transformed B lymphocytes correlate with expression of genes encoding components of the the oxidase system. *Archives of Biochemistry and Biophysics,* 360:158-164.

16. Condino-Neto A, Newburger PE. (2000). Interferon-gamma improves splicing efficiency of CYBB gene transcripts in an interferon-responsive variant of X-linked chronic granulomatous disease due to a splice site consensus region mutation. *Blood,* 95:3548-3554.

17. Aoshima M, Nunoi H, Shimazu M, Shimizu S, Tatsuzawa O, Kenney RT, Kanegasaki S (1996). Two-exon skipping due to a point mutation in p67-phox--deficient chronic granulomatous disease. *Blood, 88:*1841-1845.

18. Azuma H (1994). DNA analysis of cytochrome b positive chronic granulomatous disease (a case report)). *Rinsho Byori, 42:*242-248.

19. Bonizzato A, Russo MP, Donini M, Dusi S (1997). Identification of a double mutation (D160V-K161E) in the p67phox gene of a chronic granulomatous disease patient. *Biochemical and Biophysical Research Communications, 231:*861-863.

20. Borgato L, Bonizzato A, Lunardi C, Dusi S, Andrioli G, Scarperi A, Corrocher R (2001). A 1.1-kb duplication in the p67-phox gene causes chronic granulomatous disease. *Human Genetics, 108:*504-510.

21. Noack D, Heyworth PG, Newburger PE, Cross AR (2001). An unusual intronic mutation in the CYBB gene giving rise to chronic granulomatous disease. *Biochimica et Biophysica Acta, 1537:*125-131.

22. Roesler J, Curnutte JT, Rae J, Barrett D, Patino P, Chanock SJ, Goerlach A (2000). Recombination events between the p47-phox gene and its highly homologous pseudogenes are the main cause of autosomal recessive chronic granulomatous disease. *Blood, 95:*2150-2156.

23. De Boer M, Singh V, Dekker J, Di Rocco M, Goldblatt D, Roos D (2002). Prenatal diagnosis in two families with autosomal, p47(phox)-deficient chronic granulomatous disease due to a novel point mutation in NCF1. *Prenatal Diagnosis, 22:*235-240.

24. Baehner RL, Millar-Groff S, Bringas P (1999). Developmental expression of NADPH phagocytic oxidase components in mouse embryos. *Pediatrics Research, 46:*152-157.

25. Banerjee R, Anguita J, Roos D, Fikrig E (2000). Cutting edge: infection by the agent of human granulocytic ehrlichiosis prevents the respiratory burst by down-regulating gp91phox. *Journal Immunology, 164:*3946-3949.

26. Bayraktutan U, Blayney L, Shah AM (2000). Molecular characterization and localization of the NAD(P)H oxidase components gp91-phox and p22-phox in endothelial cells. *Arteriosclerosis Thrombosis and Vascular Biology, 20:*1903-1911.

27. Cobbs S, Malech HL, Leto TL, Freeman SM, Blaese RM, Gallin JI, Lomax KJ (1992). Retroviral expression of recombinant p47phox protein by Epstein-Barr virus-transformed B lymphocytes from a patient with autosomal chronic granulomatous disease. *Blood 79:*1829-1835.

28. Gorlach A, Brandes RP, Nguyen K, Amidi M, Dehghani F, Busse R (2000). A gp91phox containing NADPH oxidase selectively expressed in endothelial cells is a major source of oxygen radical generation in the arterial wall. *Circulation Research, 87:*26-32.

29. Rouzaut A, Lopez-Moratalla N, de Miguel C (2000). Differential gene expression in the activation and maturation of human monocytes. *Archives of Biochemistry and Biophysics, 374:*153-160.

30. Patino PJ, Perez JE, Lopez JA, Condino-Neto A, Grumach AS, Botero JH, Curnutte JT, Garcia de Olarte D (1999). Molecular analysis of chronic granulomatous disease caused by defects in gp91-phox. *Human Mutation, 13:*29-37.

31. Ochs HD, Igo RP. (1973). The NBT slide test: a simple screening method for detecting chronic granulomatous disease and female carriers. *Journal Pediatrics,* 83:77-82.

32. Boyum A. (1968). Isolation of mononuclear cells and granulocytes from human blood. *Scandinavian Journal of Clinical and Laboratory Investigation*, 21(Suppl.97):1-77.

33. Condino-Neto A, Muscara MN, Grumach AS, Carneiro-Sampaio MMS, de Nucci G. (1993). Neutrophils and mononuclear cells from patients with chronic granulomatous disease release nitric oxide. *British Journal of Clinical Pharmacology,* 35:485-490.

34. McCord J, Fridovich I. (1969). Superoxide dismutase: An enzymatic function for erythrocuprein (hemocuprein). *Journal of Biological Chemistry,* 244:6044-6055.

35. Condino-Neto A, Muscara MN, Grumach AS, Bellinati-Pires R, Brandao AC, Carneiro-Sampaio MMS, de Nucci G. (1996). The effect of recombinant human interferon-gamma therapy on neutrophil and mononuclear cell nitric oxide release from patients with chronic granulomatous disease. *Journal Interferon and Cytokine Research*, 16:357-364.

36. Volkman DJ, Buescher ES, Gallin JI, Fauci AS. (1984). B cell lines as models for inherited phagocytic diseases: superoxide generation in chronic granulomatous disease and granules in Chediak-Higashi syndrome. *Journal Immunology,* 133:3006-3009.

37. Nilsson K, Klein G, Henle W, Henle G. (1971). The establishment of lymphoblastoid cell lines from adult and fetal human lymphoid tissue and its dependence on EBV. *International Journal of Cancer*, 8:443-450.

38. Ginsburg D, Handin RI, Bonthron DT, Donlon TA, Bruns GAP, Latt SA, Orkin SH. (1985). Human von Willebrand factor: Isolation of complementary DNA clones and chromosomal location. *Science*, 228:1401-1406.

39. Subrahmanyam YVBK, Baskaran N, Newburger PE, Weissman SM (1999). A modified method for the display of 3'-end restriciton fragments of cDNAs: Molecular profiling of gene expression in neutrophils. *Methods in Enzymology*, 303:272-297.

LEGENDS TO TABLES

Table I-Oligonucleotide primers used for gp91-*phox* RT-PCR analysis.

Table II-Stimulated respiratory burst activity (PMA-30nM) of granulocytes (GRA) and mononuclear leukocytes (MON) as assessed by the NBT test or superoxide release assay, and gp91-*phox* gene expression in EBV-transformed B lymphocytes from CGD patients as assessed by RT-PCR analysis. Granulocytes and mononuclear leukocytes from healthy controls released superoxide respectively in the range of 6-14 and 5-7 nmol $O_2^-/10^6$cells/60min.

Table I-Gp91-*phox* specific primers

Region	Sequence of gp91-phox primers	
	Forward	Reverse
Exons 1-5	5'- GCT CTA GAG CAT GAG GGG CTC TCC ATT TTT GTC A-3'	5'- CGG GAT CCC GAG TTC AGA GAG TGC TAC TGA ATA A – 3'
Exons 3-9	5' - GCC TGC CTG AAT TTC AAC – 3'	5' – TCA TCT GTA GCT CGA TG –3'
Exons 7-13	5'- GGA ATG CCC AAT CCC TCA G – 3'	5'- GGG CCA GAC TCA GAG TTG G – 3'

Table II-Biochemical and RT-PCR data from X-linked CGD patients.

Patient	Respiratory Burst Activity		gp91-*phox* Exons 1-5	gp91-*phox* Exons 3-9	gp91-*phox* Exons 7-13
	NBT	Superoxide	Relative Expression		
1.GM		MON 1.0	Reduced	Reduced	Absent
2.RB	<5%		Reduced	Reduced	Reduced
3.GG	<5%		Possible splicing defect	Absent	Absent
4.TPB	<5%	MON 0.27 GRA 0.22	Normal expression	Normal expression	Normal expression
5.MF	<5%	GRA 0.22	Normal expression	Reduced	Reduced
6.TP	<5%	MON 0.25 GRA 0.46	Normal expression	Reduced	Absent
7.RS		MON –0.03 GRA 0.58	Possible splicing defect	Absent	Reduced
8.JEM	<5%	MON 0.0 GRA 0.3	Absent	Absent	Absent

LEGENDS TO FIGURES

Figure 1-RT-PCR analysis of gp91-*phox* gene expression (exons 1-5) in EBV-transformed B cells lines from X-linked CGD patients: Representative agarose gel electrophoresis (2%) of PCR products stained with Ethidium bromide. Size standards (100 bp ladder); Lane 1,patient GM; Lane 2,patient RB; Lane 3, patient GG; Lanes 4, patient TPB; Lane 5,patient MF; Lane 6,patient TP; Lane 7,patient RS; lane 8,patient JEM; and Lane 9, healthy control. β-actin was used as an internal control for de RT-PCR in all samples. The lower panel shows the mean band densitometry of gp 91-*phox*/β actin relative expression (n=3).

Figure 2-RT-PCR analysis of gp91-*phox* gene expression (exons 3-9) in EBV-transformed B cells lines from X-linked CGD patients: Representative agarose gel electrophoresis (2%) of PCR products stained with Ethidium bromide Size standards (100 bp ladder); Lane 1, patient GM; Lane 2, patient RB; Lane 3, patient GG; Lanes 4, patient TPB; Lane 5, patient MF; Lane 6, patient TP; Lane 7, patient RS; lane 8, patient JEM; and Lane 9, healthy control. β-actin was used as an internal control for de RT-PCR in all samples. The lower panel shows the mean band densitometry of gp 91-*phox*/β actin relative expression (n=3).

Figure 3-RT-PCR analysis of gp91-*phox* gene expression (exons 7-13) in EBV-transformed B cells lines from X-linked CGD patients: Representative agarose gel electrophoresis (2%) of PCR products stained with Ethidium bromide. Size standards (100 bp ladder); Lane 1, patient GM; Lane 2, patient RB; Lane 3, patient GG; Lanes 4, patient TPB; Lane 5, patient MF; Lane 6, patient TP; Lane 7, patient RS; lane 8, patient JEM; and Lane 9, healthy control. β-actin was used as an internal control for de RT-PCR in all samples. The lower panel shows the mean band densitometry of gp 91-*phox*/β actin relative expression (n=3).

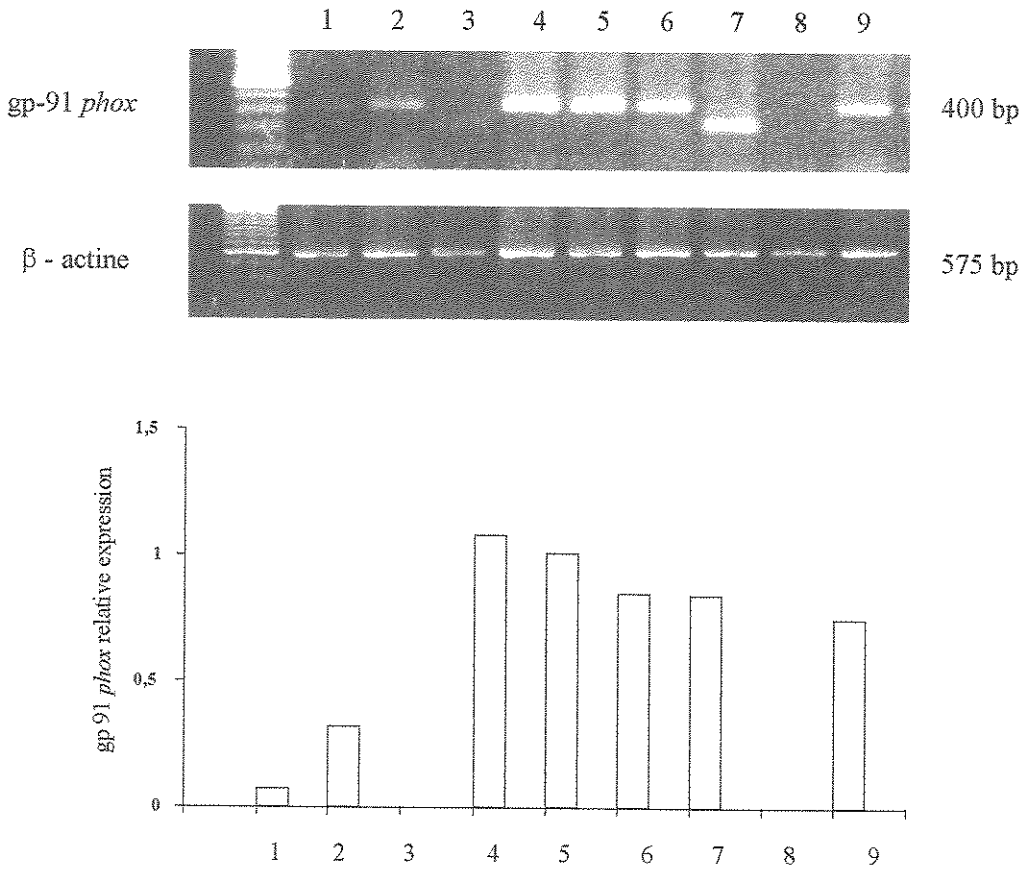
1 2 3 4 5 6 7 8 9
gp-91 *phox*
400 bp
β - actine
575 bp
gp 91 *phox* relative expression
1,5
1
0,5
0
1 2 3 4 5 6 7 8 9

FIGURE 1-

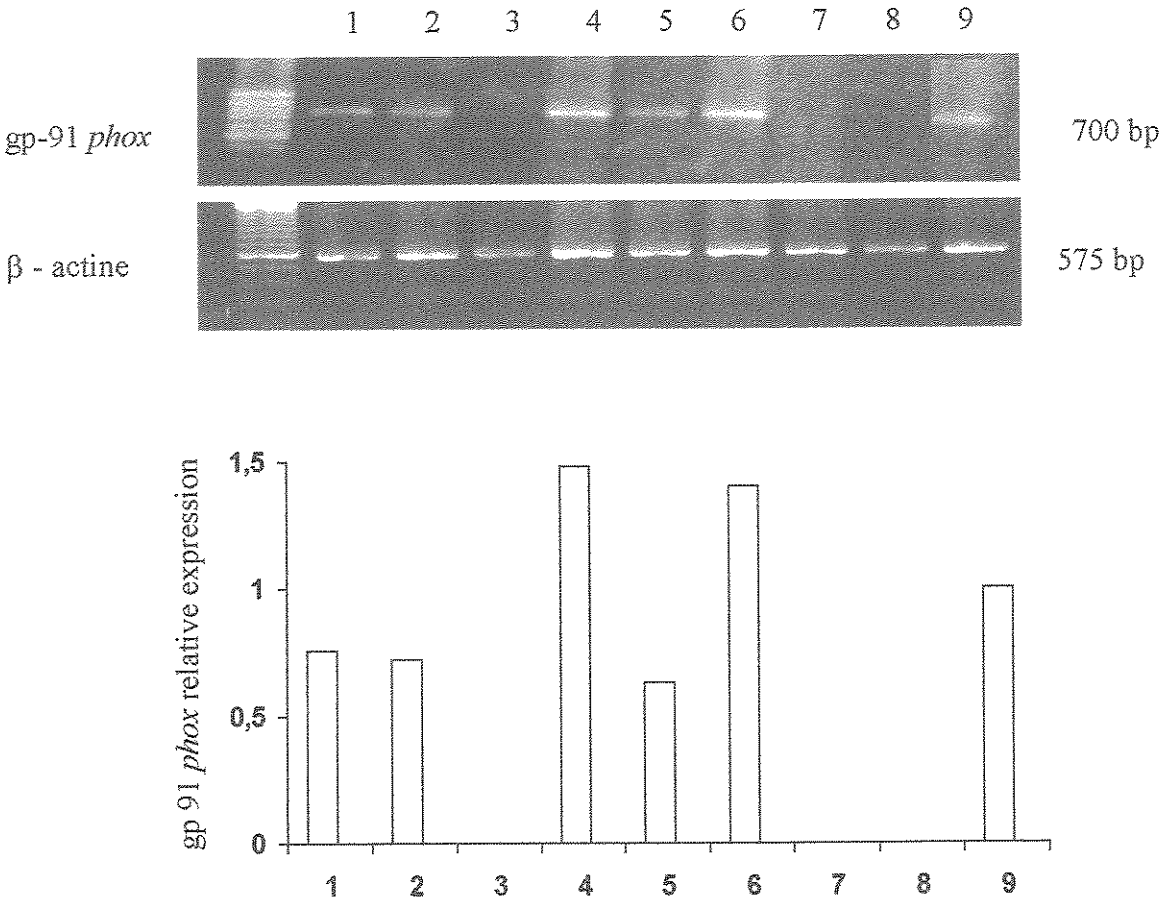
1 2 3 4 5 6 7 8 9
gp-91 *phox*
700 bp
β - actine
575 bp
gp 91 *phox* relative expression
1,5
1
0,5
0
1 2 3 4 5 6 7 8 9

FIGURE 2-

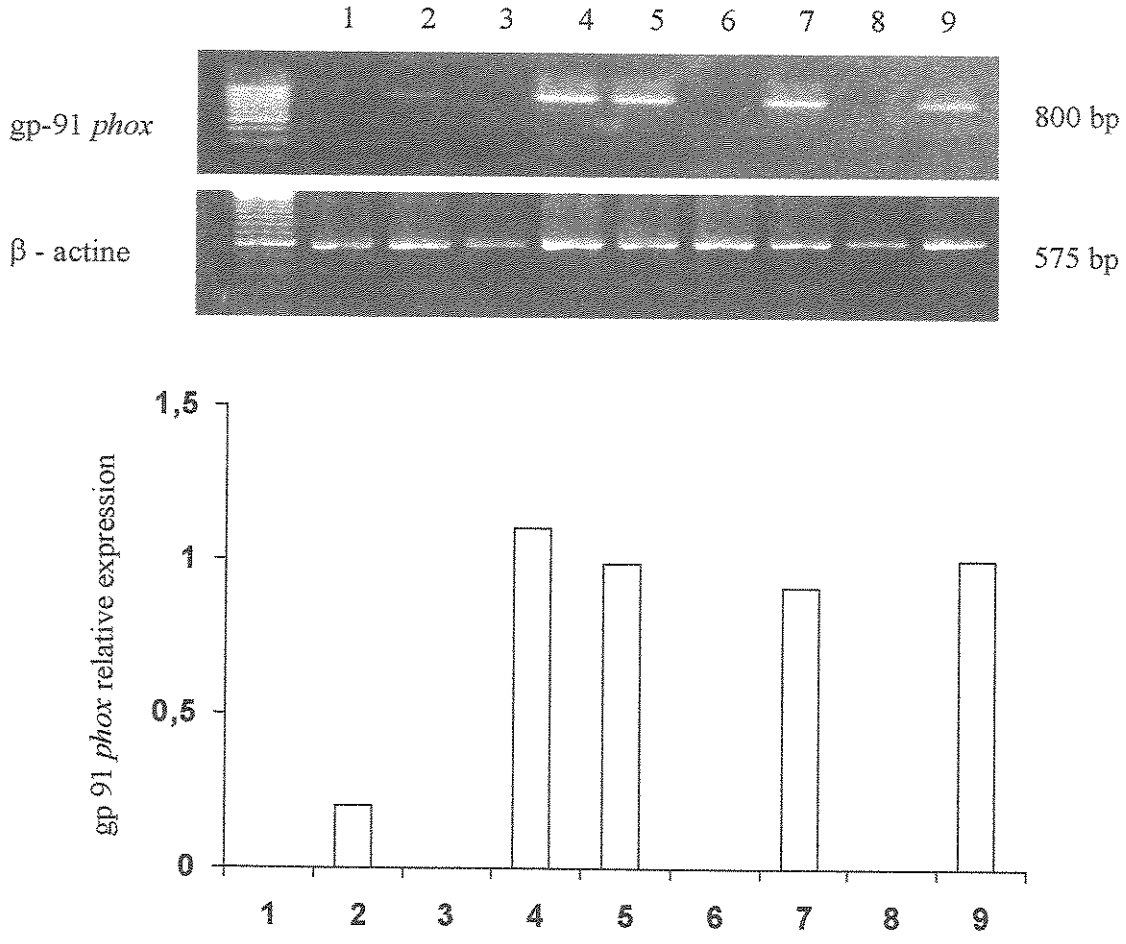
1 2 3 4 5 6 7 8 9
gp-91 *phox*
800 bp
β - actine
575 bp
gp 91 *phox* relative expression
1,5
1
0,5
0
1 2 3 4 5 6 7 8 9

FIGURE 3-

CAPÍTULO 2

The molecular genetics of X-linked Chronic Granulomatous Disease in Latin American patients

Piedad Agudelo-Flórez[1], Juan Alvaro López[1], Carolina Cardoso Prando-Andrade[1], Beatriz Tavares Costa-Carvalho[2], Monica Cornejo de Luigi[3], Persio Roxo Jr[4], Magda Maria Salles Carneiro-Sampaio[5], Peter E. Newburger[6], and Antonio Condino-Neto[1,5]*

1 - Center for Investigation in Pediatrics and Department of Pediatrics, State University of Campinas Medical School, São Paulo, Brazil.

2 - Division of Allergy, Immunology and Rheumatology, Department of Pediatrics, Federal University of São Paulo Medical School, São Paulo, Brazil.

3 – Department of Pediatrics, University of Valparaiso Medical School, Valparaiso, Chile.

4 – Department of Pediatrics, Ribeirão Preto Medical School, University of São Paulo, Brazil.

5 – Department of Immunology, Institute of Biomedical Sciences, University of São Paulo, Brazil.

6 – Department of Pediatrics, University of Massachusetts Medical School, Worcester, MA, USA.

Grants: Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP 01/14365-3 and 02/05880-4), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq 470413/03-4), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), and United States National Institutes of Health Fogarty International Center (R03TW00883).

Author for Correspondence:

Antonio Condino-Neto, MD, PhD

Department of Immunology

Institute of Biomedical Sciences

University of São Paulo

1730 Lineu Prestes Avenue, São Paulo, SP.

Brazil. CEP 05508-900.

Tel: (55) (19) 3289-3103. Fax (55) (19) 3289-8638

E-mail: condino@lexxa.com.br

Running title: *CYBB* gene mutations in Latin American patients.

ABSTRACT

Chronic granulomatous disease (CGD) is a rare primary immunodeficiency characterized by early onset recurrent severe infections. The molecular defects causing CGD are generally due to the absence, low expression or malfunctioning of one of the NADPH oxidase components. The X-linked form of the disease is caused by defects in the *CYBB* gene encoding the gp91-*phox* component (OMIM 306400). The aim of this work was to investigate the molecular genetic defects in 7 unrelated patients with X-linked CGD, from Chile and Brazil. We found an insertion c.1267_1268insA in exon 10 leading to a frameshift mutation. This mutation is a novel report. We detected two single base-pair substitutions that lead to nonsense mutations. The first was a c.95 G>A substitution in the exon 2 which predicts a stop codon W28X and the second was a c.229 C>T substitution in the exon 3 which predicts a stop codon R73X. We also identified different splice site mutations in four cases. Two patients presented a c.264 G> A substitution at the end of exon 3. The remaining two patients presented either a c.1326 + 1 G>A substitution in intron 10 or a c.1164 – 2 A>G substitution in intron 9. This last mutation is also novel. The gp91-*phox* mutations identified in these patients show a high degree of molecular heterogeneity as reported in other ethnic groups and the importance to investigate molecular genetic defects in different populations.

Keywords: chronic granulomatous disease, primary immunodeficiencies, *CYBB*, mutations, neutrophils, phagocytes.

INTRODUCTION

Chronic granulomatous disease (CGD), a rare primary immunodeficiency, was first characterized in 1957 as a clinical entity affecting male infants and originally termed "fatal granulomatous of childhood". CGD is characterized by severe recurrent infections affecting mainly the natural barriers such as the respiratory tract and lymph nodes, and eventually inner structures, such as liver, spleen, bones and brain [Berendes et al.,. 1957; Landing and Shirkey, 1957]. The estimate incidence of this disease is 1/250000 live births per year. The infections are generally caused by catalase positive bacteria, such as *Staphylococcus aureus* and gram-negative bacilli; or fungal species such as *Aspergillus and Candida* [Segal et al., 1983; Segal et al., 2000].

The molecular defects causing CGD are generally due to the absence, low expression or malfunctioning of one component of the phagocyte NADPH oxidase responsible for the generation of microbicidal reactive oxygen species [Winkelstein et al., 2000]. The X-linked form of the disease (OMIM 306400) is caused by defects in the heavy chain of the cytochrome b_{588} component (gp91-*phox*) and accounts for 56% of the cases [Dinauer et al., 1987]. The autosomal recessive forms are caused by defects in one of the cytosolic components of the NADPH oxidase (p47-*phox* and p67-*phox*, respectively 33% and 5% of the cases) [Clark et al., 1989]; or the cytochrome b_{588} light chain component (p22-*phox* , 6% of the cases) [Dinauer et al., 1990; Parkos et al., 1988]. To date, no CGD patients have been reported with defects in p40-*phox*, rap1A, rac1, or GDI components. A related immunodeficiency has recently been reported to result from a defect in the gene for Rac2 [Heyworth et al., 2003].

Genetic defects of patients with CGD described to date include 410 reported mutations in the four affected genes [Heyworth et al., 2003]. The diversity of these mutations and the multiple affected genes give an explanation for the clinical and genetic heterogeneity of CGD [Curnutte , 1993;Roos et al., 1996a].

Molecular defects leading to X-linked CGD have been identified in the coding region, introns, and (rarely) in the 5' flanking regulatory region of the *CYBB* gene [Segal et al., 2000; Roos et al.,. 1996b; Rae et al.,. 1998].Over 300 *CYBB* mutations resulting in

X-linked CGD have been registered in an international database (http://www.uta.fi/imtbioinfo/CYBBbase) [Heyworth et al., 2001].

The mutations causing X-linked CGD include large multigenic deletions, smaller deletions and insertions, missense or nonsense substitutions, and splicing defects. The mutations are distributed evenly among the exons and gene boundaries [Segal et al., 2000;Roos et al.,. 1996b; Rae et al.,. 1998] and most patients have mutations unique to their kindred. The heterogeneity of the mutations and the absence of a predominant genotype show that the worldwide incidence of CGD is a consequence of many independent mutational events.

In the only previous study from Latin America, Patino *et al* [Patino et al., 1999]. identified X-linked CGD mutations in seven independent families in Colombia and Brazil. They found a splice site mutation IV12-2A→G, four nonsense mutations (R91X, W106X, R157X, R290X), and two missense mutations (E225V, C244Y). This study found that six mothers had a mutant *CYBB* allele, and one of the cases represented a *de novo* mutation. The aim the present study was to further investigate the molecular genetic basis of defects leading to the phenotype of X-linked CGD in Latin American patients from Brazil and Chile.

METHODS

Patients

Patients with a clinical history of recurrent severe infections such as pneumonia, lymphadenitis, liver abscess, pyodermitis, and adverse reactions to BCG immunization were evaluated at the Center for Investigation in Pediatrics at State University of Campinas Medical School. A detailed clinical and family history and physical examination were performed. Patients and their families received explanations about the research plan and written informed consent was obtained. The Medical School Ethics Committee approved the research plan and the experimental procedures according to the Ministry of Health of Brazil (Resolution 196/96). Alternatively, a physician certified by the Latin-American Group of Primary Immunodeficiencies, according to the Pan-American Group of Primary Immunodeficiencies protocols [Conley et al., 1999] evaluated patients at distant locations or in other Latin-American countries for inclusion in this research protocol.

NBT tests and superoxide release assays

The biochemical diagnosis of CGD was established according to the Pan American Group for Immunodeficiency criteria, including impairment of NADPH oxidase activity demonstrated by the nitroblue tetrazolium (NBT) slide test and the superoxide anion release assay in peripheral blood neutrophils and monocytes [Conley et al., 1999; Ochs and Igo, 1973]. For this study, the cells were isolated by centrifugation of blood over a Ficoll-Hypaque density gradient [Boyum, 1968].

The NBT slide test is based on the reduction of NBT to formazan by activated leucocytes [Ochs and Igo, 1973]. The assay was performed as previously published [Patino et al., 1999]. In concurrent normal controls, more than 95% of 200 neutrophils stimulated with PMA (30nM) must be able to reduce NBT. Absence of reaction or <5% positive cells is diagnostic of CGD [Conley et al., 1999] "variant" CGD patients may show >95% positive cells, but with very little formazan production. The histochemical NBT test also detects female carriers for X-linked CGD, in whom random X inactivation generally leads to NBT reduction by >5% but <95% of phagocytes.

Quantitative superoxide release by neutrophils and monocytes was measured by the superoxide dismutase inhibitable reduction of cytochrome c assay as previously described [Condino-Neto et al., 1993; Condino-Neto et al., 1996]. Results are expressed as nmol of superoxide released by 10^6 cells per hour. Leukocytes from patients with CGD show less than 10% of control values.

Detection of gp91-phox and p22- phox by western blot assay.

Western blotting was performed as previously published [Patino et al., 1999] with some modifications. The gp91-*phox* and p22-*phox* components were detected by analysis of proteins solubilized in a 1% Triton X-100 from neutrophils or Epstein-Barr virus (EBV)-transformed B-lymphocytes (30 μg of proteins in each sample). Protein detection was performed using goat anti-human gp91-*phox* and p22-*phox* (Santa Cruz Biotechnology). Rabbit anti-goat IgG AP (Santa Cruz Biotechnology) was used as the secondary antibody. Band densitometry was performed by computer image analysis using an ImageMaster VDS System (Pharmacia). Absence of a protein component determined the CGD type.

Genotype determinations

DNA and RNA samples were extracted from neutrophils, monocytes and EBV-transformed B-lymphocytes from CGD confirmed patients and healthy controls by DNAzol® (Life Technologies, Gibco) and guanidine HCl method followed by ethanol precipitation [Ginsburg et al., 1985; Subrahmanyam et al., 1999].

EBV-transformed B cell lines reproduce the biochemical and molecular defects of CGD patients [Condino-Neto and Newburger, 1998] avoid repeated blood collections from patients, and provide an abundant source of nucleic acids for molecular studies. To prepare such cell lines, mononuclear cells were cultured with supernatants from B95-8, an EBV-producer cell line [Condino-Neto and Newburger, 1998] in RPMI 1640 medium supplemented with heat inactivated fetal bovine serum (10%), 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml of streptomycin, at 37°C, in a humid atmosphere with 5%

CO_2. Cellular viability was monitored and the cultures were maintained during the studies. Aliquots of EBV-transformed B cells were frozen in liquid nitrogen for archiving and future studies.

In order to identify the mutation responsible for the CGD phenotype, we investigated the underlying mutation by single strand conformation polymorphism (SSCP) analysis of PCR-amplified genomic DNA (gDNA) and by sequencing of the affected gene region, both according to previously published procedures [Patino et al., 1999]. The gDNA was amplified by nested PCR. Appropriate primers were designed for amplification of all 13 exons of gp91-*phox* [Patino et al., 1999].

The SSCP assay is based on changes in the electrophoretic mobility of denatured PCR products and is sensitive even to single nucleotide mutations. The polyacrylamide gels were stained with silver and the electrophoretic mobility of the PCR-amplified products from CGD patients and healthy controls were compared.

Samples of PCR-amplified gDNA or RT-PCR amplified cDNA were purified using the "Concert Rapid PCR Purification System" (Life Technologies, Gibco) and sequenced using the "DNA sequencing Kit, Big Dye Terminator Cycle Sequencing Ready Reaction for ABI 377 PE/Applied Biosystems", as previously published [Condino-Neto and Newburger , 2000].

The sequences obtained from CGD patients and healthy controls were compared to GenBank data (Accession No NM_000397) and submitted to BLAST analysis. Description of sequence changes was made in accordance with the nomenclature of the Human Genome Variation Society (www.hgvs.org/mutnomen/). The nucleotides were numbered according to the cDNA sequence used by Heyworth et al [Heyworth et al., 2001] where the start of translation is +1 and the A of ATG start codon is 13.

RESULTS

We studied 7 male unrelated patients from Chile and Brazil referred to our laboratory for biochemical and molecular diagnosis of CGD. The subjects included 2 Blacks and 5 Caucasians; age 2-8 years; height 88-108 cm; weight 11-19 kg. The patients presented clinical histories of recurrent severe infections such as pneumonia, lymphadenitis, liver abscess, pyodermitis, and adverse reactions to BCG immunization. The results from the NBT slide tests in all patients showed less than 5% positive leukocytes; five patients also demonstrated impaired superoxide release by granulocytes and/or mononuclear leukocytes (less than 10% compared to healthy controls). The mother of patient IC was found to have an NBT slide test compatible with the carrier state of X-linked CGD.

Western blot analysis of granulocytes from all seven CGD patients showed the absence of both protein components of flavocytochrome b_{558}, gp91-*phox* and p22- *phox*, indicating that all had the X-linked form of CGD with the $X91^0$ phenotype.

Sequence analysis of the *CYBB* gene (encoding gp91-*phox*) in the patient JY revealed an insertion c.1267_1268insA in exon 10 leading to a frameshift mutation (Figure 1). This mutation is novel [Heyworth et al., 2001]. We found two single base-pair substitutions that lead to nonsense mutations: in patient PT, a c.95 G>A substitution in exon 2 which predicts a stop codon W28X (Figure 2) and in patient MF a c.229 C>T substitution in exon 3 which predicts a stop codon R73X (figure 3). Both mutations were confirmed with the reverse primer. These mutations have been previously reported in the X-CGD mutations database [Heyworth et al., 2001].

Four cases were associated with different splice site mutations. Two patients (RS and GG), showed an exon 3 deletion in gp91-*phox* cDNA; in genomic DNA, each showed a substitution c.264 G> A at the 5' end of exon 3 (Figures 4, 5). Another two splice site mutations were identified, both resulting in exon 10 deletion. The first patient (IC) had a c.1326 + 1 G>A substitution in intron 10, with the mutation also detectable in his heterozygous mother (Figure 6). The second patient (Vin) demonstrated a c.1164 – 2 A>G substitution in intron 9 (Figure 7). This mutation is also novel. The other three splicing

defects described in this study were previously reported in the X-CGD mutations database [Heyworth et al., 2001].All mutations here reported are summarized in Table I.

DISCUSSION

CGD is a rare inherited disorder in which phagocytic cells are unable to generate superoxide anion and other reactive oxygen intermediates. This paper reports on the biochemical and gene expression studies of seven unrelated Brazilian and Chilean male patients with a clinical history of CGD, who were referred to our laboratory for detailed investigation. We initially established the diagnosis of CGD by means of biochemical tools such as the NBT slide tests and/or superoxide anion release assays. Our results show that all patients presented an impaired NADPH oxidase function and in turn, probable CGD.

The analysis of protein expression showed absence of cytochrome b_{558} in all patients. Because absence of either cytochrome b component destabilizes the other, patients with defects in either the gp91-*phox* or p22-*phox* gene generally show no expression of the other protein. If the specific form of the disease cannot be determined by family studies (i.e. for X-linked or autosomal inheritance patterns) or carrier detection, both genes need to be further investigated by western blot assay, as performed in this study.

X-linked CGD may result from mutations in any part of the *CYBB* gene that encodes gp91-*phox*. The seven mutations identified in this study include several different mechanisms that interfere with gp91-*phox* expression. An insertion c.1267_1268insA in exon 10 leads to a frameshift mutation and eventual downstream termination. This mutation is a novel report. Overall, mutations of this type constitute 24% of *CYBB* gene defects resulting in X-linked CGD [Rae et al., 1998]. We found two single base-pair substitutions that leave to nonsense mutations. The frequency of nonsense mutations is around 23% for X-linked CGD [Rae et al., 1998].

In four cases, splice site mutations were identified. The same mutation was found at the 5' end of exon 3 in two cases. This mutation is itself silent as a coding change, but it disrupts the donor splice site of intron 3, changing the CpG sequence to CpA. Most single nucleotide substitution mutations involve CpG sequences, which can be considered mutational hot spots. Ten similar mutations have been documented to date [Heyworth et al., 2001]. In addition, we identified two other splice site mutations which lead to exon 10 deletions.

Mutations near splice sites leading to defects in RNA processing have been observed in 39 out of 251 cases in the largest studies [Segal et al., 2000 ;Winkelstein et al., 2000; Roos et al., 1996a; Rae et al.,. 1998]. Most resulted in the $X91^0$ phenotype due to the deletion of one or more exons [de Boer et al., 1992]. However, in few cases, such mutations lead to $X91^-$ phenotype, due to the partial maintenance of the normal "splicing" [Rae et al.,. 1998]. One of these families had been shown to be extremely responsive to the treatment with IFN-γ, which almost completely restored oxidase activity *in vitro* and *in vivo* [Ezekowitz et al., 1987; Ezekowitz et al., 1988].

Our previous studies showed this response to derive at least in part from increases in the level of normally spliced transcripts [Condino-Neto and Newburger , 2000]. For this reason, we have investigated the splice site mutations reported here, for evidence of correctly and incorrectly spliced products. RT-PCR analysis has shown only a smaller PCR product (between 300 and 400 bp) between exons 1-5 in gp91-*phox* transcripts from the patients RS and GG, as presented in a previous paper [Agudelo-Florez et al., 2004a].

The mutations in gp91-*phox* demonstrated in these Latin American patients showed a high degree of molecular heterogeneity, as reported in other ethnic groups. All of the specific mutations predict structural defects that alter the expression and function of the gene product; two of them are novel. Most mutations are distributed throughout the 13 exons or at exon/intron boundaries. In this study the most common location was the splice site. The absence of a large portion of the mRNA might generate an unstable transcript, which would be degraded after its synthesis. Patient RS also presents an association of glucose-6-phosphate deficiency in addition to X-linked CGD, as previously reported [Agudelo-Florez et al., 2004b]. These cases validate the current concept that the definitive diagnosis of CGD requires the demonstration of the defective component of the NADPH oxidase system and the detection of the underlying mutation responsible for the CGD phenotype.

ACKNOWLEDGEMENT

The authors thank Jussara Rehder from Department of Pediatrics and Dulcineia Albuquerque from Division of Hematology, Department of Medicine, State University of Campinas Medical School for technical assistance.

REFERENCES

1. Berendes H, Bridges RA, Good RA. 1957. A fatal granulomatosus of childhood: the clinical study of a new syndrome. Minn Med 40:309-312.

2. Landing BH, Shirkey HS. 1957. A syndrome of recurrent infection and infiltration of the viscera by pigmented lipid histiocytes. Pediatrics 20:431-442.

3. Segal AW, Cross AR, Garcia RC, Borregaard N, Valerius NH, Soothill JF, Jones OTG. 1983. Absence of cytochrome b_{-245} in chronic granulomatous disease. A multicenter European evaluation of its incidence and relevance. N Engl J Med 308:245-251.

4. Segal HH, Leto TL, Gallin JI, Malech HL, Holland SM. 2000. Genetic, biochemical, and clinical features of chronic granulomatous disease. Medicine 79:170-200.

5. Winkelstein JA, Marino MC, Johnston RB, Jr., Boyle J, Curnutte JT, Gallin JI, Malech HL, Holland SM, Ochs HD, Quie PG, Buckley RH, Foster CB, Chanock SJ, Dickler H. 2000. Chronic granulomatous disease. Report on a national registry of 368 patients. Medicine 79:155-169.

6. Dinauer MC, Orkin SH, Brown R, Jesaitis AJ, Parkos CA. 1987. The glycoprotein encoded by the X-linked chronic granulomatous disease locus is a component of the neutrophil cytochrome b complex. Nature 327:717-720.

7. Clark RA, Malech HL, Gallin JI, Nunoi H, Volpp BD, Pearson DW, Nauseef WM, Curnutte JT. 1989. Genetic variants of chronic granulomatous disease: Prevalence of deficiencies of two cytosolic components of the NADPH oxidase system. N Engl J Med 321:647-652.

8. Dinauer MC, Pierce EA, Bruns GAP, Curnutte JT, Orkin SH. 1990. Human neutrophil cytochrome *b* light chain (p22-*phox*). Gene structure, chromosomal location, and mutations in cytochrome-negative autosomal recessive chronic granulomatous disease. J Clin Invest 86:1729-1737.

9. Parkos CA, Dinauer MC, Walker LE, Allen RA, Jesaitis AJ, Orkin SH. 1988.The primary structure and unique expression of the 22 kilodalton light chain of human neutrophil cytochrome b. Proc Natl Acad Sci USA 85:3319-3323.

10. Heyworth PG, Cross AR, Curnutte JT. 2003. Chronic Granulomatous Disease. Curr Opin Immunol 15:578-584.

11. Curnutte JT. 1993. Chronic granulomatous disease: The solving of a clinical riddle at the molecular level. Clin Immunol Immunopathol 67:S2-S15.

12. Roos D, Curnutte JT, Hossle JP, Lau YL, Ariga T, Nunoi H, Dinauer MC, Gahr M, Segal AW, Newburger PE, Giacca M, Keep NH, van Zwieten R. 1996. X-CGDbase: a database of X-CGD-causing mutations. Immunol Today 17:517521

13. Roos D, de Boer M, Kuribayashi F, Meischl C, Weening RS, Segal AW, Åhlin A, Nemet K, Hossle JP, Bernatowska-Matuszkiewicz E, Middleton-Price H. 1996. Mutations in the X-linked and autosomal recessive forms of chronic granulomatous disease. Blood 87:1663-1681.

14. Rae J, Newburger PE, Dinauer MC, Noack D, Hopkins PJ, Kuruto R, Curnutte JT. 1998. X-linked chronic granulomatous disease: Mutations in the CYBB gene encoding the gp91-*phox* component of the respiratory burst oxidase. Am J Hum Genet 62:1320-1331.

15. Heyworth PG, Curnutte JT, Rae J, Noack D, Roos D, van Koppen E, Cross AR. 2001. Hematologically important mutations: X-linked chronic granulomatous disease. Blood Cells Mol Dis 26:561-565.

16. Patino PJ, Perez JE, Lopez JA, Condino-Neto A, Grumach AS, Botero JH, Curnutte JT, Garcia de Olarte D.1999. Molecular analysis of chronic granulomatous disease caused by defects in gp91-*phox*. Human Mutation 13:29-37

17. Conley ME, Notarangelo LD, Etzioni A. 1999. Diagnostic criteria for primary immunodeficiencies. Clinical Immunology 93:190-197.

18. Ochs HD, Igo RP. 1973. The NBT slide test: a simple screening method for detecting chronic granulomatous disease and female carriers. J Pediatr 83:77-82.

19. Boyum A.1968. Isolation of mononuclear cells and granulocytes from human blood. Scand J Clin Lab Invest 21(Suppl.97):1-77.

20. Condino-Neto A, Muscara MN, Grumach AS, Carneiro-Sampaio MMS, de Nucci G. 1993. Neutrophils and mononuclear cells from patients with chronic granulomatous disease release nitric oxide. Br J Clin Pharmacol 35:485-490.

21. Condino-Neto A, Muscara MN, Grumach AS, Bellinati-Pires R, Brandao AC, Carneiro-Sampaio MMS, de Nucci G. 1996. The effect of recombinant human interferon-gamma therapy on neutrophil and mononuclear cell nitric oxide release from patients with chronic granulomatous disease. J Interferon Cytokine Res 16:357-364.

22. Subrahmanyam YVBK, Baskaran N, Newburger PE, Weissman SM. 1999. A modified method for the display of 3'-end restriciton fragments of cDNAs: Molecular profiling of gene expression in neutrophils. Meth Enzymol 303:272-297.

23. Condino-Neto A, Newburger PE. 1998. NADPH oxidase activity and cytochrome b558 content of human Epstein-Barr virus transformed B lymphocytes correlate with expression of genes encoding components of the the oxidase system. Arch Biochem Biophys 360:158-164.

24. Condino-Neto A, Newburger PE. 2000. Interferon-gamma improves splicing efficiency of CYBB gene transcripts in an interferon-responsive variant of X-linked chronic granulomatous disease due to a splice site consensus region mutation. Blood 95:3548-3554.

25. de Boer M, Bolscher BGJM, Dinauer MC, Orkin SH, Smith CIE, Åhlin A, Weening RS, Roos D. 1992. Splice site mutations are a common cause of X-linked chronic granulomatous disease. Blood 80:1553-1558.

26. Ezekowitz RAB, Orkin SH, Newburger PE. 1987. Recombinant interferon gamma augments phagocyte superoxide production and X-chronic granulomatous disease gene expression in X-linked variant chronic granulomatous disease. J Clin Invest 80:1009-1016.

27. Ezekowitz RAB, Dinauer MC, Jaffe HS, Orkin SH, Newburger PE. 1988. Partial correction of the phagocyte defect in patients with X-linked chronic granulomatous disease by subcutaneous interferon gamma. N Engl J Med 319:146-151.

28. Agudelo-Florez P, Lopez JA, Redher J, Carneiro-Sampaio MM, Costa-Carvalho BT, Grumach AS, Condino-Neto A. 2004. The use of reverse transcription-PCR in the diagnosis of X-linked chronic granulomatous disease. Braz J Med Biol Res 37:625-634

29. Agudelo-Florez P, Costa-Carvalho BT, Lopez JA, Redher J, Newburger PE, Olalla-Saad ST, Condino-Neto A. 2004. Association of glucose-6-phosphate dehydrogenase deficiency and X-linked chronic granulomatous disease in a child with anemia and recurrent infections. Am J of Hematology 75:151-156.

LEGENDS TO FIGURES

Figure 1-*CYBB* genotype of patient JY. **A.** SSCP analysis of exon 2 amplified from gDNA. Arrow shows the altered migration pattern. **B.** DNA sequence of exon 10 shows an insertion c.1267_1268 insA in the patient (lower panel) compared to a normal control (upper panel). **C.** DNA sequence downstream from the insertion demonstrates the formation of a termination site (indicated by *) in place of amino acid 430.

Figure 2-*CYBB* genotype of patient PT. **A.** SSCP analysis of exon 2 amplified from gDNA. Arrow shows the altered migration pattern. **B.** DNA sequence shows a hemizygous G > A mutation leading to the nonsense substitution W 28X.

Figure 3-*CYBB* genotype of patient MF. DNA sequence analysis in patient MF shows a C > T mutation in exon 3 leading to the nonsense substitution R 73X.

Figure 4-*CYBB* genotype of patient RS. **A.** SSCP analysis of exon 3 amplified from gDNA. Arrow shows the altered migration pattern. **B.** cDNA sequence shows a deletion of exon 3.

Figure 5-*CYBB* genotype of patient GG. **A.** SSCP analysis of exon 3 amplified from gDNA. Arrow shows the altered migration pattern. **B.** DNA sequence shows a hemizygous G >A transition in the splice site.

Figure 6-*CYBB* sequence analysis of patient IC. Left panel: The arrow in SSCP shows the altered migration pattern.. Right panel: DNA sequence shows a hemizygous G>A transition in the splice site of intron 10 in the patient (bottom tracing), while his mother (middle tracing) is heterozygous for this mutation. The top tracing is a normal control.

Figure 7-*CYBB* genotype of patient Vin. **A.** SSCP analysis of exon 10 and flanking intronic sequence amplified from gDNA. Arrow shows the altered migration pattern. **B.** DNA sequence shows a hemizygous A>G transition in the splice site of intron 9.

Table I-Mutations in *CYBB* gene identified in Latin American CGD patients.

Patient	Mutation	Position	Nucleotide Change	Product	Reference
JY	Frameshift	Exon 10	c.1267_1268insA	Termination SiteExon 10	New report
PT	Nonsense	Exon 2	c.95 G>A	Termination Site W28X	Heyworth et al., 2001.
MF	Nonsense	Exon 3	c.229 C>T	Termination Site R73X	Heyworth et al., 2001.
RS	Splicing	Exon 3	c.264 G>A	Splicing ProductExon 3 deletion	Heyworth et al., 2001.
GG	Splicing	Exon 3	c.264 G>A	Splicing ProductExon 3 deletion	Heyworth et al., 2001.
IC	Splicing	Intron 10	c.1326 + 1 G>A	Splicing ProductExon 10 deletion	Heyworth et al., 2001.
Vin	Splicing	Intron 9	c.1164 – 2 A>G	Splicing ProductExon 10 deletion	New report

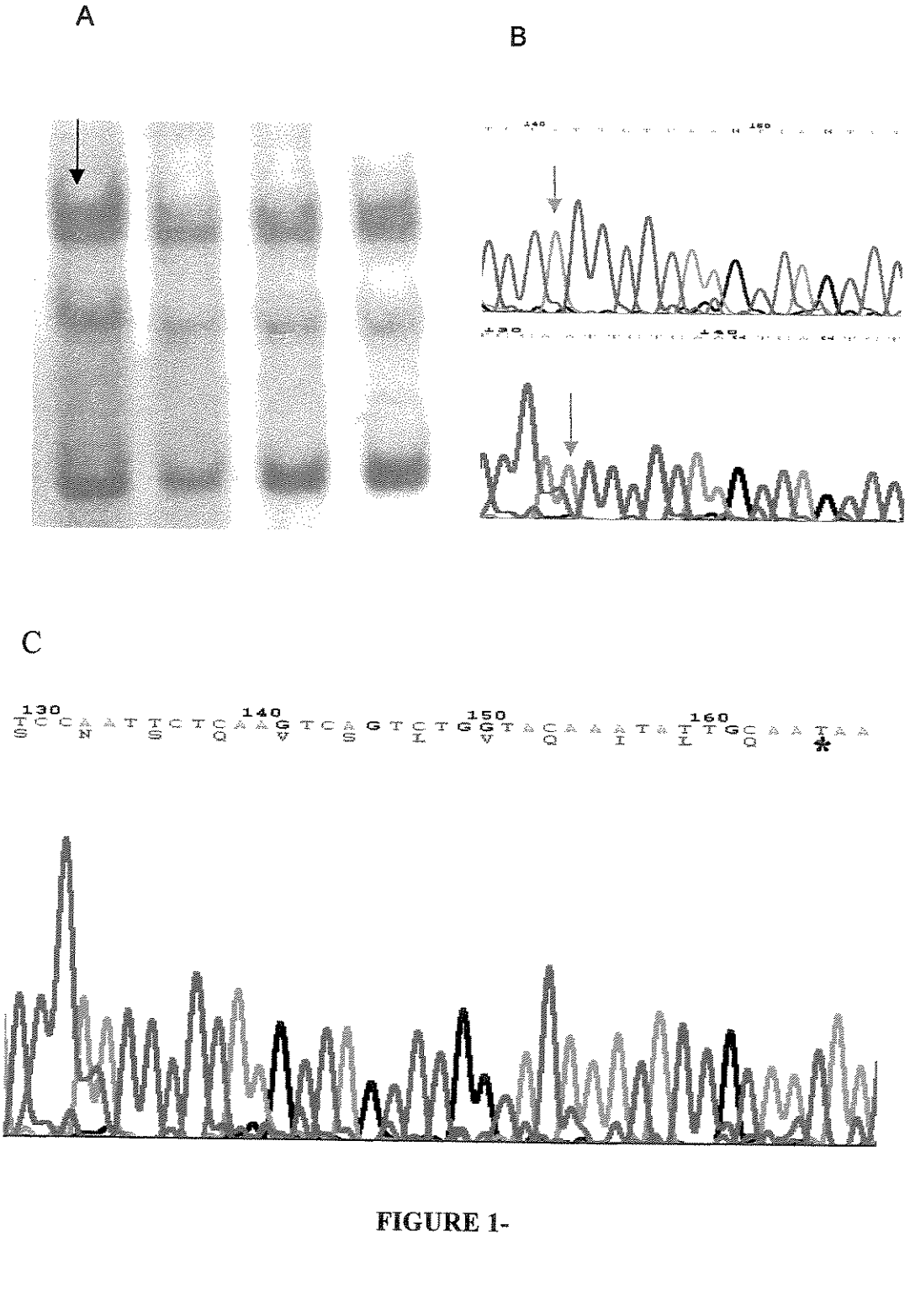

FIGURE 1-

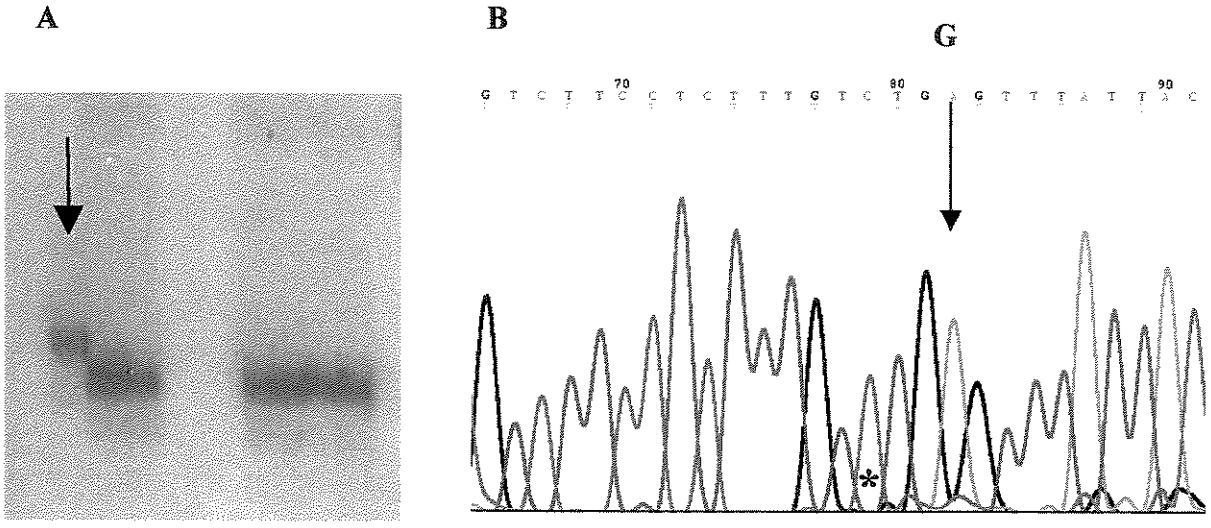

FIGURE 2-

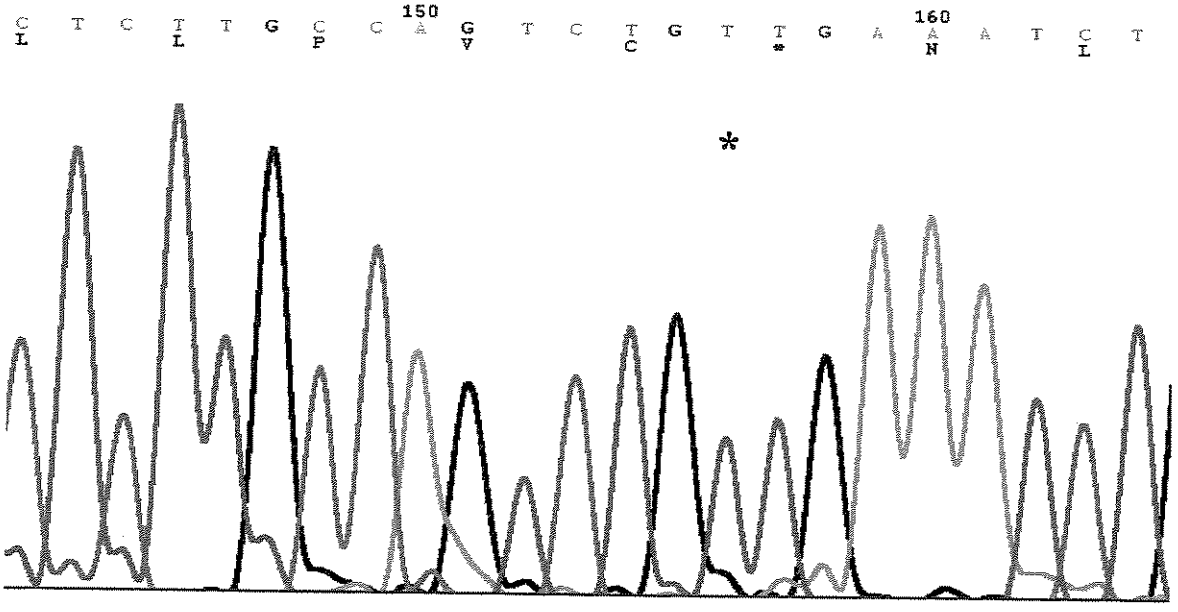

FIGURE 3-

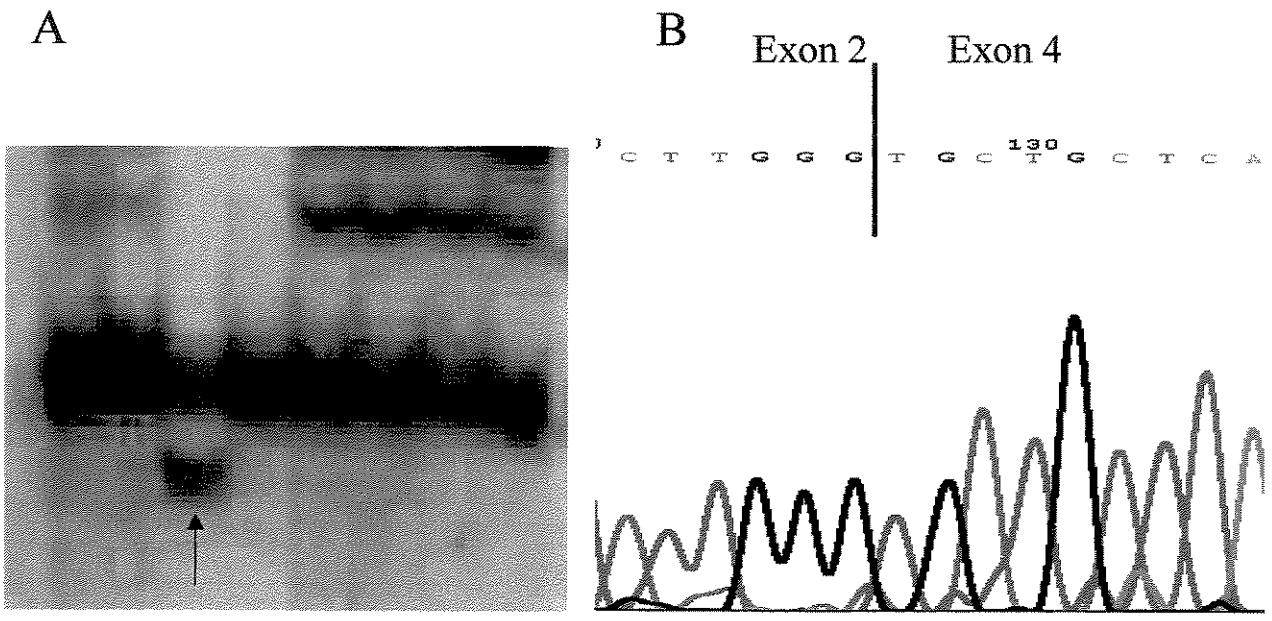

FIGURE 4-

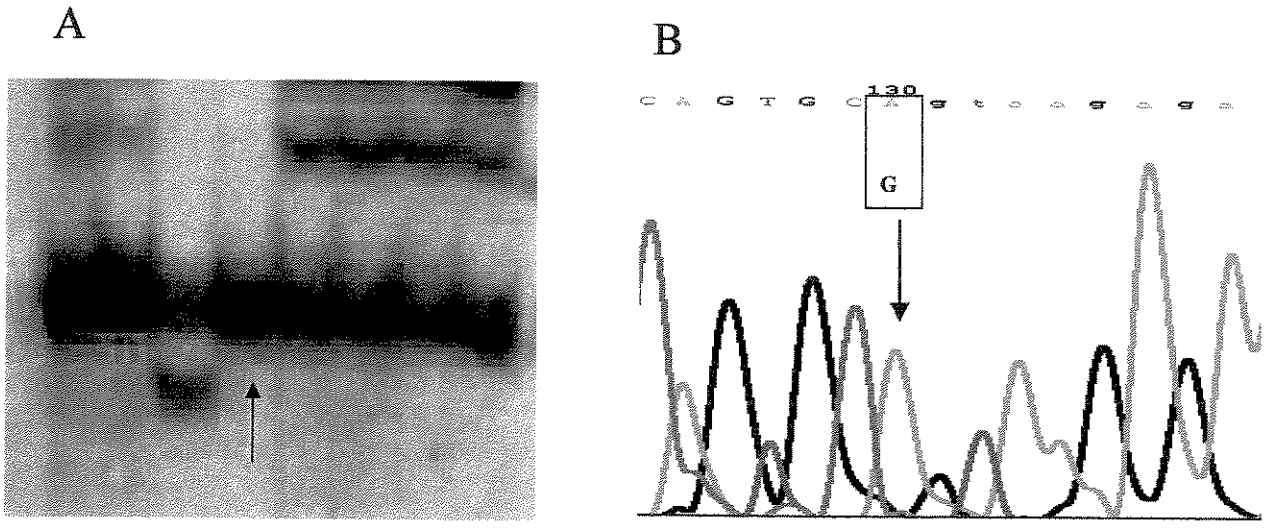

FIGURE 5-

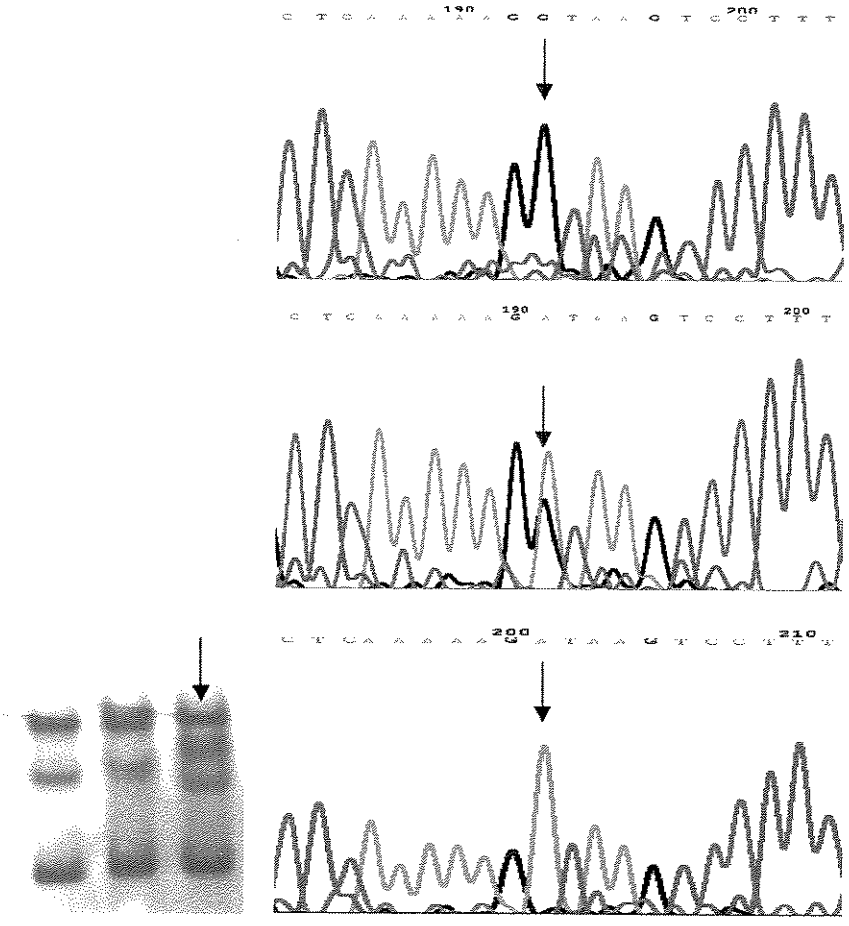

FIGURE 6

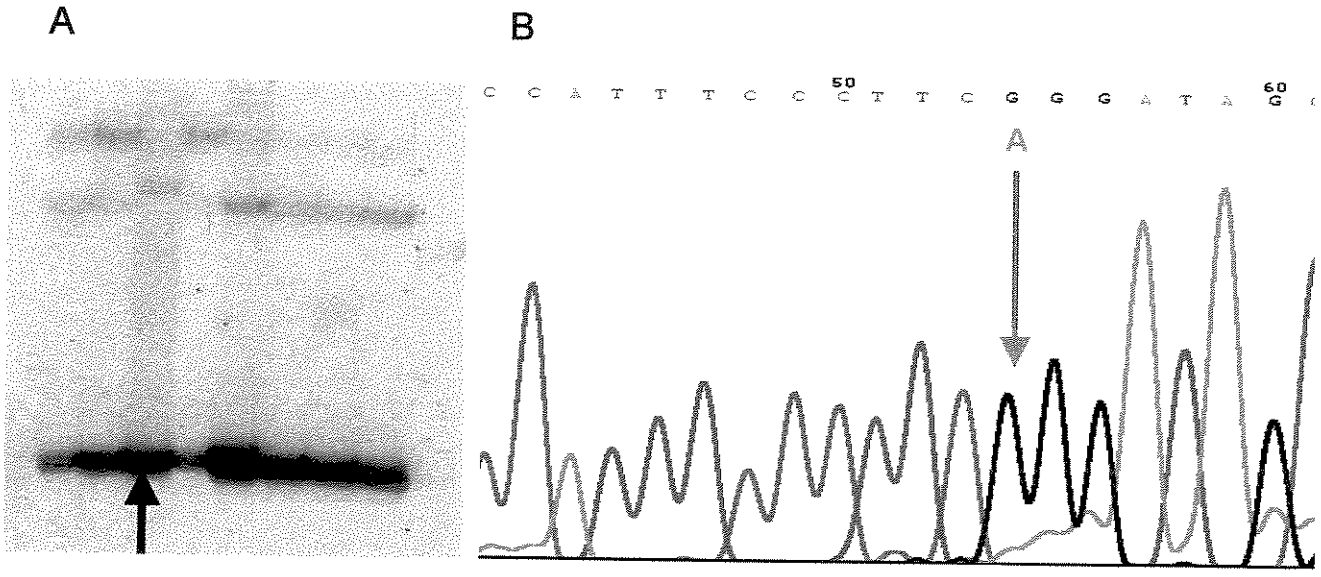

FIGURE 7-

CAPÍTULO 3

Association of glucose-6-phosphate dehydrogenase deficiency and X-linked chronic granulomatous disease in a child with anemia and recurrent infections

Piedad Agudelo-Flórez[1], Beatriz T. Costa-Carvalho[2], Juan Alvaro López[1], Jussara Redher[1], Peter E. Newburger[3], Sara Teresinha Olalla-Saad[4], and Antonio Condino-Neto[1]*.

[1]Center for Investigation in Pediatrics and Department of Pediatrics, State University of Campinas Medical School.

[2]Division of Allergy, Immunology and Rheumatology, Department of Pediatrics, Federal University of São Paulo.

[3]Department of Pediatrics, University of Massachusetts Medical School

[4]Division of Hematology, Department of Medicine, State University of Campinas Medical School.

Piedad Agudelo-Flórez and Beatriz Costa-Carvalho have contributed equally to this paper.

Running title: Association of G6PD deficiency and CGD.

Keywords: chronic granulomatous disease, G6PD deficiency, recurrent infections, anemia, and phagocytes.

***Author for Correspondence:**

Antonio Condino-Neto, MD

Tel: (55) (19) 3289-3103. Fax (55) (19) 3289-8638

E-mail: condino@lexxa.com.br

Center for Investigation in Pediatrics. State University of Campinas Medical School, UNICAMP, PO Box 6111, Campinas, SP 13081-970, BRAZIL.

Grants: Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP 01/14365-3), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq 302685/02-02), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), and The United States National Institutes of Health Fogarty International Center (1RO3TW00883-01).

ABSTRACT

Patients with severe G6PD deficiency in leukocytes may present with an impairment of the NADPH oxidase activity and a history of recurrent infections, mimicking the phenotype of chronic granulomatous disease. We here report a child with recurrent infections who initially received the diagnosis of G6PD deficiency. His erythrocyte G6PD activity was reduced: 1.8 U/ g Hb (normal: 12.1 ± 2.1 U/ g Hb). Further studies revealed that G6PD activity in neutrophils, mononuclear leukocytes, and Epstein-Barr virus-transformed B-lymphocytes from the proband was similar to healthy controls. Molecular studies showed that the G6PD deficiency was due a 202 G→A mutation, the A^- variant common in African ethnic groups. The proband also exhibited severely impaired respiratory burst activity, as observed in X-linked CGD. Sequence analysis of genomic DNA showed a 264 G→A substitution at the 3' splice junction of gp91-*phox* exon 3. The cDNA sequence showed a deletion of gp91-*phox* exon 3, giving rise to an unstable or nonfunctional mutant gp91-*phox* and to the phenotype of X-linked CGD. We propose that clinicians in face of a patient with G6PD deficiency under a severe infection episode consider the possibility of temporary or permanent impairment of the phagocytes microbicidal activity, and the eventual association of G6PD deficiency and chronic granulomatous disease.

INTRODUCTION

The phagocytic cells constitute one of the major effector mechanisms against invasive microorganisms. Phagocyte abnormalities are generally manifested by recurrent and severe infections, usually starting in early infancy. When phagocytes are activated, they increase oxygen consumption and reduce molecular oxygen to superoxide, a reaction known as the "respiratory burst", catalyzed by an enzymatic complex called the NADPH oxidase (1).

The NADPH is the electron donor used to reduce the molecular oxygen to superoxide. It is generated from glucose, via the hexose's monophosphate shunt, which also increases substantially its activity when the phagocytic cells are activated. In the first step of this pathway, glucose-6-phosphate (G6P) is converted into 6-phosphoglucolactone, catalyzed by glucose-6-phosphate dehydrogenase, and accompanied by the reduction of nicotinamide adenine dinucleotide phosphate (NADP) into NAPDH (2).

Chronic Granulomatous Disease (CGD) is a primary immunodeficiency, characterized by recurrent infections affecting mainly the natural barriers of the organism such as the respiratory tract, lymph nodes, and eventually inner structures, such as the liver, spleen, bones and brain (3). The biochemical defect in CGD is an impairment of the NADPH oxidase activity and the subsequent inability to destroy microorganisms (1). The main components of the NADPH oxidase system are gp91-*phox*, p22-*phox*, p47-*phox*, and p67-*phox*. The molecular defects causing CGD are generally due to the absence, low expression or malfunctioning of one of these NADPH oxidase components. The X-linked form of the disease is caused by defects in gp91-*phox*, the heavy chain of the cytochrome b_{588}, and accounts for approximately 60% of the cases. The *CYBB* gene encodes gp91-*phox* and is located to Xp21.1. The autosomal recessive forms are caused by defects in one of the cytosolic components of the NADPH oxidase (p47-*phox* or p67-*phox*, respectively 30% and 5% of the cases), or even the cytochrome b_{588} light chain component (p22-*phox*, 5% of the cases) (4- 6).

G6PD deficiency is a common genetic disorder in humans. Clinically, it varies from mild hemolytic anemia to chronic nonspherocytic hemolytic anemia, associated with attacks of severe anemia induced by infections and drugs. The G6PD gene is located to

Xq28. Thus, hemizygous men most frequently express the clinical symptoms (2,7). Patients with severe G6PD deficiency in leukocytes may indeed present an impairment of the NADPH oxidase activity and subsequent recurrent infections, mimicking the phenotype of chronic granulomatous disease (8, 9).

We here report on child with anemia and recurrent infections, who initially received the diagnosis of G6PD deficiency. Further studies confirmed an impairment of the NADPH oxidase system. Mutation analysis revealed a splicing defect on the exon 3 of *CYBB* gene, confirming a unusual association of X-linked chronic granulomatous disease and the African variant of G6PD deficiency.

PATIENTS AND METHODS

Case report and human subjects

The proband is a male, born in 1994 to Brazilian parents with African and Caucasian ethnic background, who presented with anemia and recurrent severe infections since he was six months old. By age five years, he had already had twelve episodes of pneumonia (two with pleural effusion), two of diarrhea (enteropathogenic *E. coli, and Salmonella*), three of otitis, four of sinusitis, one of stomatitis, two of skin abscess, and one of sepsis. He was hospitalized on eight occasions. He received the recommended immunizations, including BCG, without any side effects. His parents were not consanguineous. A three-year-old brother died from sepsis secondary to pneumonia and abscesses in the liver and lungs. His father and his sister have sickle cell trait. His physical examination findings at age five years included: weight 17 kg, height 107 cm, pallor, digital clubbing, and moderate hepatosplenomegaly.

Blood cell counts revealed anemia (hemoglobin range: 8.1 – 10.9 g/dL), neutrophilia and eosinophilia. The reticulocyte count was normal (1.3%), as well as serum unconjugated bilirubin (0.3 mg/dl). Serum immunoglobulin levels (IgA, IgG, and IgM) were increased. CD3, CD4, and CD8 cell counts were normal. Lymphocyte proliferation in response to phytohemaglutinin, pokeweed mitogen and concanavalin A was normal. The complement CH50 activity was normal. The HIV test was negative. A thoracic CT scan showed emphysema and extensive areas of pulmonary destruction. Erythrocyte G6PD activity was reduced: 1.8 U/ g Hb compared to the normal range: 12.1 ± 2.1 U/ g Hb.

Considering the recurrent infections, the hypothesis of a phagocyte abnormality was established and the patient was referred to our laboratory for further investigation. Experiments compared assays in blood samples and cell lines obtained from the proband, one patient with X-linked chronic granulomatous disease (10), one patient with G6PD deficiency (African variant) (11), and healthy controls. Written informed consent was obtained prior to the study. The Medical School Ethics Committee approved the protocol in accordance to the Helsinki Convention and the Brazilian National Health Council.

Purification and culture of blood cells

Erythrocytes were obtained by centrifugation and aspiration of plasma and buffy coat, followed by washing 3 times with saline (12). Neutrophils and mononuclear leukocytes were obtained by centrifugation of heparinized blood over a Ficoll-Hypaque density gradient (13).

B-lymphocytes were transformed *in vitro* with Epstein-Barr virus (EBV) in order to provide an abundant source of leukocytes for biochemical and molecular studies. EBV-transformed B cell lines reproduce the biochemical and molecular defects of CGD patients (14) and avoid repeated blood collections. Briefly, mononuclear leukocytes were cultured with supernatants from B95-8, an EBV-producer cell line, in RPMI 1640 medium supplemented with heat inactivated fetal bovine serum (10%), 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml of streptomycin, at 37°C, in a humid atmosphere with 5% CO_2. (15). Cellular viability was monitored and the cultures were maintained during the studies.

G6PD studies

G6PD activity was assayed as the rate of reduction of NADP to NADPH, when erythrocytes or leukocytes lysates were incubated with glucose-6-phosphate, as originally described by Beutler (16), modified by Saad. (11). Comparative studies included erythrocytes, neutrophils, mononuclear leukocytes, and EBV-transformed B-lymphocytes.

Genomic DNA was obtained from peripheral blood leucocytes using DNAzol Reagent (Life technologies, Gibco BRL). The 202 G→A mutation was investigated by allele specific oligomer hybridization, as described elsewhere (11) or digestion of the exon 4 of the G6PD gene with the restriction endonuclease *Nla*III. The Mediterranean variant was investigated by digestion of exon 6 with the restriction enzyme *Mbo*II.

NADPH oxidase studies

The biochemical diagnosis of CGD was established according to the Pan American Group for Immunodeficiencies criteria (17). NADPH oxidase activity of neutrophils and mononuclear leukocytes was measured by superoxide dismutase

(SOD)-inhibitable reduction of cytochrome *c*, as described McCord and Fridovich (18) modified by Condino-Neto (15). Oxidase activity in cells from patients with CGD generally show up to 5% of control values (17).

The definitive diagnosis of CGD requires the demonstration of the underlying mutation or the absence of mRNA for one of the NADPH oxidase components (17). RNA samples were prepared from leukocytes by the guanidine HCl method, followed by ethanol precipitation (19). Reverse transcription followed by PCR (RT-PCR) analysis was performed on total RNA prepared from EBV-transformed B-lymphocytes. The cDNA samples were obtained by reverse transcription of total RNA with SuperScript II RT (GIBCO BRL) and random hexamers (15). The cDNA samples were amplified by PCR (30 cycles) with primers specific for the *CYBB* gene encoding gp91-phox (Gen Bank NM_000397), including exons 1-5, 32F-443R. For p47-*phox* (Gen Bank NM_000265) samples were amplified by PCR with specific primers, 48F-451R. PCR products were analyzed by 2% agarose gel electrophoresis and stained with ethidium bromide.

Single strand conformation polymorphism analysis (SSCP) was used to detect the affected gene region. Fragments of genomic DNA (gDNA) were amplified by PCR using specific primers, according to previously published procedures (10). The polyacrylamide gel was stained with silver and the electrophoretic mobility of the PCR-amplified products from the proband was compared to healthy controls.

Samples from PCR-amplified gDNA or RT-PCR-amplified cDNA were purified using the "CONCERT™ Rapid PCR Purification System" (Life Technologies, Gibco) and sequenced by the DNA sequencing kit, Big Dye Terminator Cycle Sequencing Ready Reaction for ABI 377 PE/Applied Biosystems, as previously published (15). The sequences obtained from the proband and healthy controls were compared to GenBank data by BLAST analysis (http://www.ncbi.nlm.nih.gov/BLAST/).

RESULTS

G6PD studies

G6PD activity was assayed in erythrocytes, neutrophils, mononuclear leukocytes and EBV-transformed B-lymphocytes from the proband, a patient with X-linked CGD, a patient with the African (A^-) variant of G6PD deficiency, and healthy controls. As presented in Table I, the proband and the known G6PD-deficient patient showed very low erythrocyte G6PD activity. However, the G6PD activity of the proband's neutrophils, mononuclear leukocytes, and EBV-transformed B-lymphocytes leukocytes was similar to the healthy controls.

Molecular studies of the G6PD gene included the digestion of PCR-amplified gDNA with the restriction endonuclease *Nla*II. As shown in Figure 1, exon 4 of the G6PD gene was digested and the 202 G→A mutation was detected in the proband gDNA, confirming his African variant genotype.

NADPH oxidase studies

A series of studies compared superoxide release by neutrophils and mononuclear leukocytes from the proband, a patient with known X-linked CGD, a patient with the African variant of G6PD deficiency, and healthy controls. The results in Table II show severely impaired respiratory burst activity in the proband and the X-linked CGD patient, but normal respiratory burst activity in the patient with G6PD deficiency (African variant) and the healthy controls.

Because the proband showed defective respiratory burst activity, which was not present in the patient with the African variant G6PD deficiency, a mutational screen for CGD was performed. RT-PCR analysis included the amplification of p47-*phox* and gp91-*phox* transcripts, since these genes are affected in 90% of the mutations leading to the phenotype of CGD. Figure 2, shows that p47-*phox* was expressed normally in both the proband and healthy controls. On the other hand, as shown in Figure 3, gp91-*phox* expression was impaired in the proband, as in the X-linked CGD patient, compared to healthy controls, indicating the diagnosis of X-linked CGD in the proband. Moreover,

amplified fragment from his gp91-phox transcript was 300-400 bp smaller than that of healthy controls, suggesting the presence of a splicing defect.

SSCP analysis of gDNA (Figure 4) revealed a faster electrophoretic mobility of gp91-phox exon 3 in the proband sample. Figures 5 and 6 show sequence analysis of gDNA and cDNA respectively of this region in gp91-*phox*. Sequencing of exon 3 in the gDNA showed a 264 G→A substitution at the splice junction. Further sequencing of the cDNA showed a deletion of exon 3, giving rise to unstable or nonfunctional mutant gp91-phox protein and hence to the phenotype of CGD.

DISCUSSION

We have studied a male patient with a clinical history of chronic anemia and the diagnosis of erythrocyte G6PD deficiency. In addition, this patient also had recurrent severe infections, with exacerbation of the anemia during infections.

The bactericidal activity of phagocytes depends primarily on the activation of the NADPH oxidase system and the subsequent release of oxygen free radicals within the phagolysosome. G6PD is the first enzyme in the hexose monophosphate shunt pathway, in which glucose-6-phosphate (G6P) is converted to 6-phosphogluconate and simultaneously NADP+ is reduced to NADPH, the substrate for the NADPH oxidase responsible for the respiratory burst.

Assays of phagocyte oxidase activity in the proband demonstrated impaired respiratory burst activity. Thus we needed to determine if the impairment of the his respiratory burst activity was due to severe G6PD deficiency in his leukocytes or to a concurrent genetic disorder, chronic granulomatous disease.

African variants and other mild forms of G6PD deficiency do not produce only intermittent, not chronic, hemolysis in response to oxidative stress (e.g. infection, anti-malarial drugs). Only the more severe forms of G6PD deficiency, the class I variants, produce chronic hemolysis (11,20). The digestion of the proband's PCR-amplified gDNA with the restriction endonuclease *Nla*II detected the 202 G→A mutation in the exon 4 of the G6PD gene, and confirmed the African variant, the most frequent G6PD deficiency in the population of southeastern Brazil (11,21). Thus, the chronic anemia of the proband could be explained in part by the association the recurrent severe infections and the African variant phenotype of G6PD deficiency.

However, considering that the African variant of G6PD deficiency could not solely explain the clinical history of recurrent severe infections of the proband, a series of comparative biochemical assays with phagocytes from the proband, healthy controls, a patient with known X-linked CGD, and another patient with previously characterized African variant of G6PD deficiency were performed. The results showed impaired respiratory burst activity in the proband, similar to the patient with X-linked CGD, but not

observed in the phagocytes of the patient with the African variant phenotype of G6PD deficiency.

The definitive diagnosis of CGD requires the demonstration of the defective component of the NADPH oxidase system and the detection of the underlying mutation responsible for the CGD phenotype (17). Further molecular screening by RT-PCR demonstrated a smaller fragment between exons 1-5 in gp91-*phox* transcripts from the proband. SSCP analysis of his gDNA revealed faster electrophoretic mobility of gp91-*phox* exon 3. Sequence analysis of gDNA revealed a 264 G→A substitution at exon 3 splice junction. Sequencing of the cDNA showed a deletion of exon 3, giving rise to unstable or nonfunctional mutant gp91-phox and hence to the CGD phenotype, as previously reported by (4,6).

Several reports have described severe G6PD deficiency leading to an impairment of the respiratory burst activity (9,8,22-27). However, this is the first report of concurrent G6PD deficiency and CGD genotypes. The African variant of G6PD deficiency is considered mild and unlikely to affect respiratory burst activity. However, one has to consider that in patients with G6PD deficiency, localized hypoxemia and oxidative stress at sites of infection could exacerbate both conditions to produce hemolysis and further impairment of microbial killing. Future studies of infections or stress conditions *in vitro* or *in vivo* in G6PD deficient individuals may determine whether this condition can interfere in defense mechanisms dependent on respiratory burst activity or can worsen the CGD phenotype. However, our studies support the CGD phenotype as the major cause of recurrent infections in this patient, with chronic anemia secondary to the infections.

We suggest that clinicians caring for a G6PD-deficient patient with a severe infection consider the possibility of temporary or local impairment of phagocyte microbicidal activity related to lack of substrate for the NADPH oxidase. This defect can be the result of an unusual association of CGD, as in the present case, or due to the aggravation of milder G6PD phenotypes under stressful conditions.

LEGENDS TO FIGURES

Figure 1-G6PD mutational screen performed with digestion of gDNA with the restriction enzyme *Nla* III. Healthy controls show a 320 bp fragment (Lanes 2, 4, 5 and 6). The proband gDNA produces a 213 bp fragment (Lane 3) confirming a G202A/A376G mutation, typical of the African phenotype. Lane 1: 100 bp ladder size markers.

Figure 2-RT-PCR analysis of p47 *phox* gene expression. Agarose gel electrophoresis (2%) stained with Ethidium bromide of PCR products. Lane 1: molecular weight marker (100 bp); Lane 2: healthy control; Lane 3: proband with normal expression; Lane 4: negative control.

Figure 3-RT-PCR analysis of gp 91 *phox* gene expression: Agarose gel electrophoresis (2%) stained with Ethidium bromide of PCR products. Lane 1: molecular weight marker (100 bp); Lane 2: X-linked CGD no expression compared to the healthy control; Lane 3: another X-linked CGD patient with reduced expression; Lane 4: proband with a lower size product; Lane 5: healthy control.

Figure 4-SSCP analysis of genomic DNA amplified by PCR. Arrow shows abnormal electrophoretic mobility of gp91-*phox* exon 3 from the proband compared to healthy controls in all other lanes.

Figure 5-Genomic DNA sequence of the proband showing a substitution (guanine-adenine) at the 3' splice junction of gp91-*phox* exon 3.

Figure 6-cDNA sequence of the proband gp91-*phox* transcript showing deletion of exon 3.

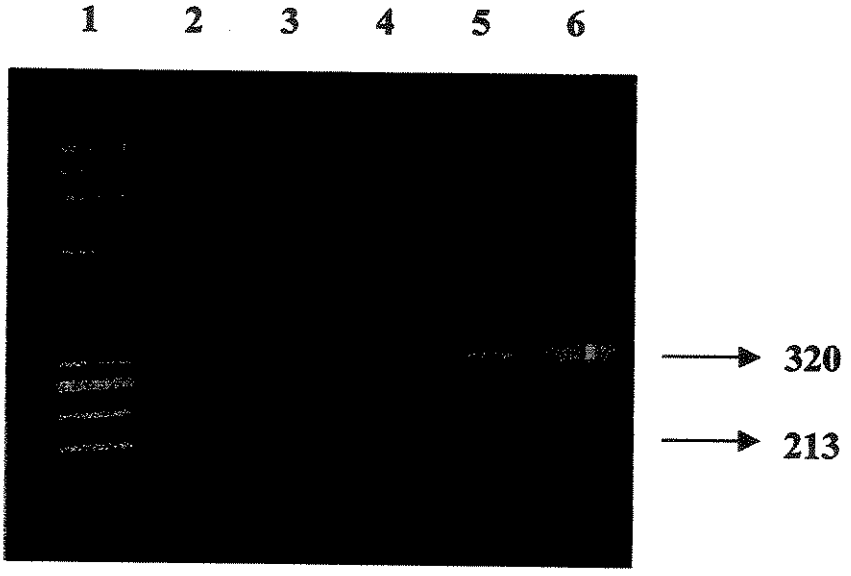

FIGURE 1-

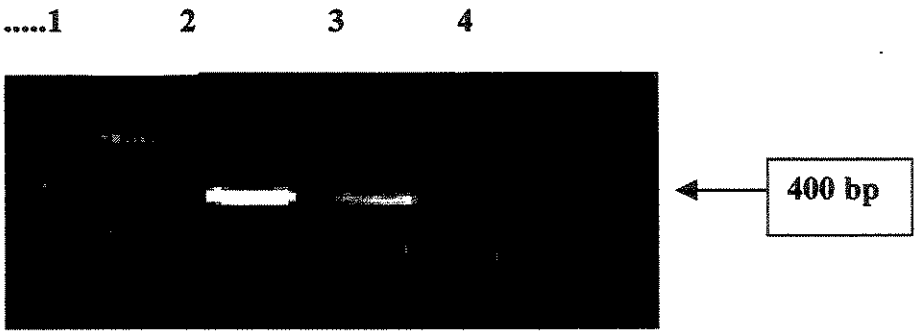

FIGURE 2-

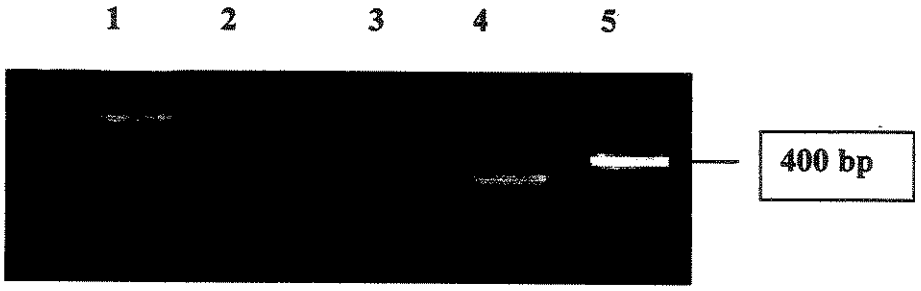

FIGURE 3-

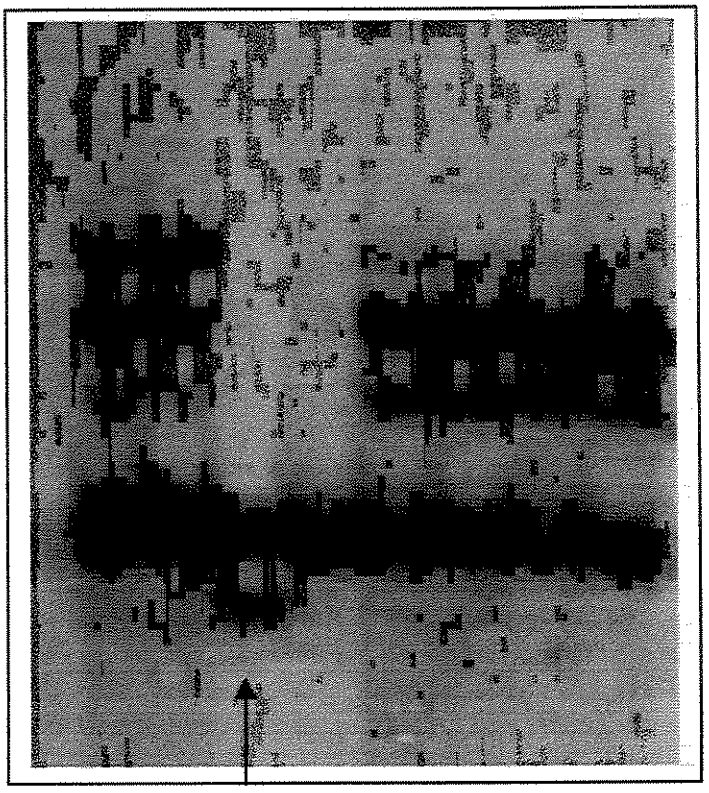

FIGURE 4-

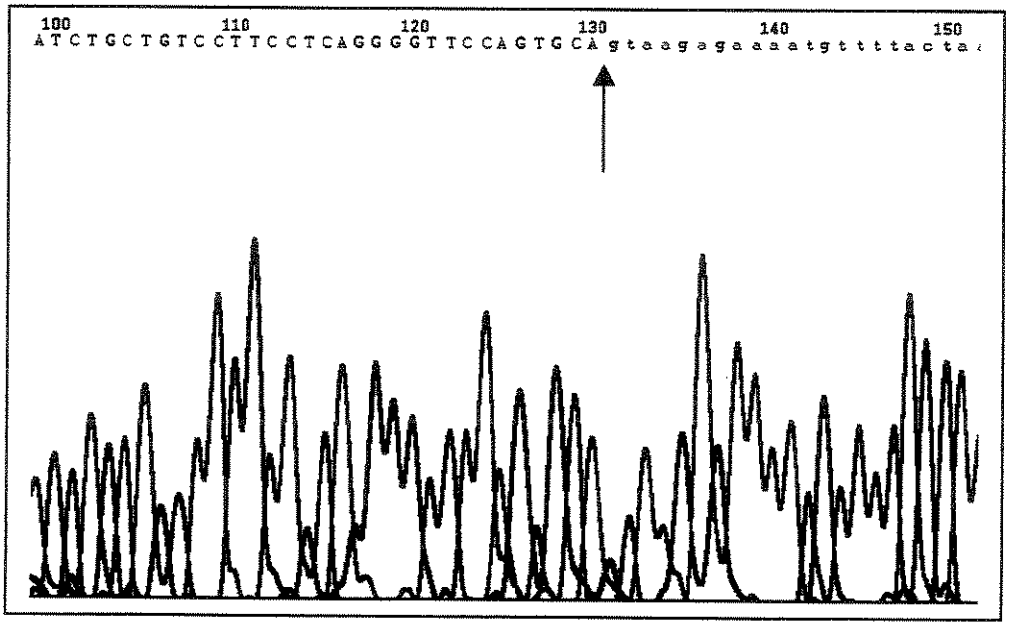
100
110
120
130
140
150
ATCTGCTGTCCTTCCTCAGGGGTTCCAGTGCAgtaagagaaaatgttttacta

FIGURE 5-

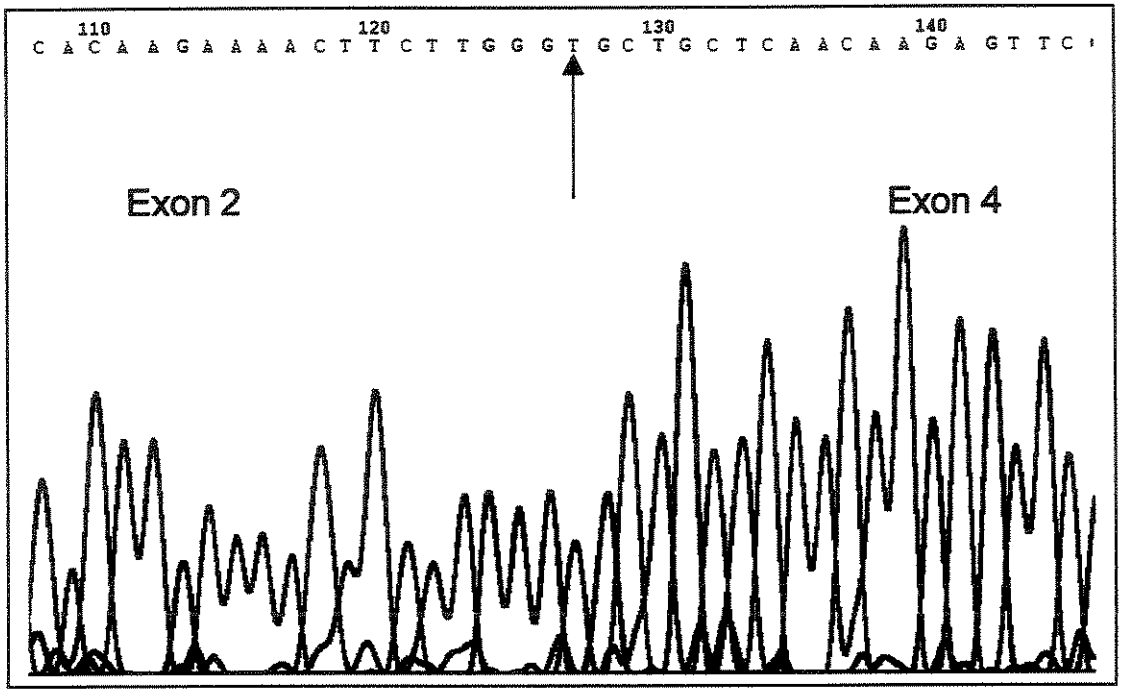
110
120
130
140
C A C A A G A A A A C T T C T T G G G T G C T G C T C A A C A A G A G T T C
Exon 2
Exon 4

FIGURE 6-

Table I-G6PD activity in red blood cells (U/g Hb), neutrophils, mononuclear leukocytes, and EBV-transformed B-lymphocytes (U/mg protein) from the proband, a X-linked CGD patient (X-CGD), a patient with the African variant of G6PD deficiency (G6PD-AV), and healthy controls (HC).

	Proband	X-CGD	G6PD-AV	HC
Eythrocytes	1.8	12.8	0.9	14.1
Neutrophils	0.4	1.0	1.8	3.1
Mononuclear leukocytes	0.8	4.2	0.5	0.5
B-lymphocytes	2.0	2.0	0.6	2.8

Values are given as mean of 3 determinations.

Table II-Superoxide release (nmol/10^6 cells/h) by neutrophils and mononuclear leukocytes from the proband, a X-linked CGD patient (X-CGD), a patient with the African variant of G6PD deficiency (G6PD-AV), and healthy controls (HC).

	Proband	X-CGD	G6PD-AV	HC
Neutrophils	0.3	0.3	19.6	9.4
Mononuclear leukocytes	0.0	0.0	4.6	5.5

Values are given as mean of 3 determinations.

REFERENCES

1. Curnutte JT, Orkin S.H, Dinauer M.C. Genetic disorders of phagocyte function. In: Stamatoyannopoulos G, Nienttuis, A.,Majerus, P.,Varmus, H., editor. The molecular basis of blood diseases. 2d edition ed. Philadelphia: W.B. Saunders Company; 1994. p. 493-522.

2. Beutler E. G6PD deficiency. Blood 1994;84:3613-3636.

3. Johnston RB, Jr. Clinical aspects of chronic granulomatous disease. Curr Opin Hematol 2001;8:17-22.

4. Roos D, de Boer M, Kuribayashi F, Meischl C, Weening RS, Segal AW, et al. Mutations in the X-linked and autosomal recessive forms of chronic granulomatous disease. Blood 1996;87:1663-81.

5. Cross AR, Noack D, Rae J, Curnutte JT, Heyworth PG. Hematologically important mutations: the autosomal recessive forms of chronic granulomatous disease (first update). Blood Cells Mol Dis 2000;26:561-5.

6. Heyworth PG, Curnutte JT, Rae J, Noack D, Roos D, van Koppen E, et al. Hematologically important mutations: X-linked chronic granulomatous disease (second update). Blood Cells Mol Dis 2001;27:16-26.

7. Beutler E, Vulliamy TJ. Hematologically Important Mutations: Glucose-6-phosphate Dehydrogenase. Blood Cells Mol Dis 2002;28:93-103.

8. van Bruggen R, Bautista JM, Petropoulou T, de Boer M, van Zwieten R, Gomez-Gallego F, et al. Deletion of leucine 61 in glucose-6-phosphate dehydrogenase leads to chronic nonspherocytic anemia, granulocyte dysfunction, and increased susceptibility to infections. Blood 2002;100:1026-30.

9. Roos D, van Zwieten R, Wijnen JT, Gomez-Gallego F, de Boer M, Stevens D, et al. Molecular basis and enzymatic properties of glucose 6-phosphate dehydrogenase volendam, leading to chronic nonspherocytic anemia, granulocyte dysfunction, and increased susceptibility to infections. Blood 1999;94:2955-62.

10. Patino PJ, Perez JE, Lopez JA, Condino-Neto A, Grumach AS, Botero JH, et al. Molecular analysis of chronic granulomatous disease caused by defects in gp91-phox. Hum Mutat 1999;13:29-37.

11. Saad ST, Salles TS, Carvalho MH, Costa FF. Molecular characterization of glucose-6-phosphate dehydrogenase deficiency in Brazil. Hum Hered 1997;47:17-21.

12. Loos JA RD, Weening RS, Houwerzijl J. Familial deficiency of glutathione reductase in human blood cells. Blood 1976;48:53.

13. Boyun A. Isolation of mononuclear cells and granulocytes from human blood. Scandinavian Journal Clinical Laboratory Investigation 1968;21(suppl 97):1-77.

14. Condino-Neto A, Newburger PE. NADPH oxidase activity and cytochrome b558 content of human Epstein-Barr-virus-transformed B lymphocytes correlate with expression of genes encoding components of the oxidase system. Arch Biochem Biophys 1998;360:158-64.

15. Condino-Neto A, Newburger PE. Interferon-gamma improves splicing efficiency of CYBB gene transcripts in an interferon-responsive variant of chronic granulomatous disease due to a splice site consensus region mutation. Blood 2000;95:3548-54.

16. Beutler E. Glucose-6-phosphate dehydrogenase deficiency. In: J.B. Stambury JBW, D.S.Fredrikson, J.L. Goldstein & M.S. Brown, editor. The Metabolic Basis of Inherited Disease. New York: McGraw-Hill; 1983. p. 1629-1653.

17. Conley ME, Notarangelo LD, Etzioni A. Diagnostic criteria for primary immunodeficiencies. Representing PAGID (Pan-American Group for Immunodeficiency) and ESID (European Society for Immunodeficiencies). Clin Immunol 1999;93:190-7.

18. McCord JM, Fridovich I. Superoxide dismutase. An enzymic function for erythrocuprein (hemocuprein). J Biol Chem 1969;244:6049-55.

19. Maniatis T, Fritsch EF and Sambrook J. Molecular cloning. A laboratory manual. 2nd ed. New York, N Y: Cold Spring Harbor Laboratory Press; 1990.

20. Beutler E. G6PD: population genetics and clinical manifestations. Blood Rev 1996;10:45-52.

21.Compri MB, Saad ST, Ramalho AS. [Genetico-epidemiological and molecular investigation of G-6-PD deficiency in a Brazilian community]. Cad Saude Publica 2000;16:335-42.

22. Baehner RL, Johnston RB, Jr., Nathan DG. Comparative study of the metabolic and bactericidal characteristics of severely glucose-6-phosphate dehydrogenase-deficient polymorphonuclear leukocytes and leukocytes from children with chronic granulomatous disease. J Reticuloendothel Soc 1972;12:150-69.

23. Gray GR, Stamatoyannopoulos G, Naiman SC, Kliman MR, Klebanoff SJ, Austin T, et al. Neutrophil dysfunction, chronic granulomatous disease, and non-spherocytic haemolytic anaemia caused by complete deficiency of glucose-6-phosphate dehydrogenase. Lancet 1973;2:530-4.

24. Corberand J, De Larrard B, Vergnes H, Carriere JP. Chronic granulomatous disease with leukocytic glucose-6-phosphate dehydrogenase deficiency in a 28-month-old girl. Am J Clin Pathol 1978;70:296-300.

25. Bellanti JA, Cantz BE, Schlegel RJ. Accelerated decay of glucose 6-phosphate dehydrogenase activity in chronic granulomatous disease. Pediatr Res 1970;4:405-11.

26. Rutenberg WD, Yang MC, Doberstyn EB, Bellanti JA. Multiple leukocyte abnormalities in chronic granulomatous disease: a familial study. Pediatr Res 1977;11:158-63.

27.Erickson RP, Stites DP, Fudenberg HH, Epstein CJ. Altered levels of glucose-6-phosphate dehydrogenase stabilizing factors in X-linked chronic granulomatous disease. J Lab Clin Med 1972;80:644-53.

CAPÍTULO 4

Doença Granulomatosa Crônica Autossômica: Relato de Caso e Análise Genético-Molecular de uma Família Brasileira.

Autosomal Chronic Granulomatous Disease: Case Report and Mutation Analysis of Two Brazilian Siblings.

Carolina Prando-Andrade[1], Piedad Agudelo-Florez[2], Juan A. Lopez[3], Maria Aparecida de Souza Paiva[4], Beatriz T. Costa-Carvalho[5], Antonio Condino-Neto[6].

1- Mestre em Pediatria, aluna de Doutorado em Farmacologia da Faculdade de Ciência Médicas (FCM) da Unicamp.

2- Aluna de Doutorado em Pediatria da FCM Unicamp.

3- Mestre em Imunologia, aluno de Doutorado em Farmacologia da FCM Unicamp.

4- Chefe do Setor de Pneumologia Pediátrica do Hospital dos Servidores do Estado do Rio de Janeiro.

5- Doutora em Pediatria, Professora Adjunta da Disciplina de Alergia, Imunologia Clínica e Reumatologia do Depto. de Pediatria da UNIFESP-EPM.

6- Livre-Docente, Professor Associado do Departamento de Pediatria da FCM Unicamp.

***Apoio Financeiro**: Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP 01/14365-3 e 02/05880-4), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq 302685/02, 470413/03) e The United States National Institutes of Health Fogarty International Center (1RO3TW00883-01).

Autor para correspondência:

Antonio Condino-Neto

TEL: (19) 3788 89 88. Fax (19) 3289-8638

E-mail: condino@lexxa.com.br

Centro de Investigação em Pediatria, Faculdade de Ciências Médicas, Universidade Estadual de Campinas, CP 6111, Campinas SP 13081-970. Brasil.

RESUMO

Objetivo: Relatar dois casos de irmãos com Doença Granulomatosa Crônica. A Doença Granulomatosa Crônica é uma imunodeficiência primária caracterizada por atividade microbicida deficiente. Mutações no gene que codifica a proteína p47-phox (*NCF-1)* estão presentes em 30% dos casos de DGC. Esta forma da doença é de herança autossômica recessiva e resulta em fenótipo de evolução mais benigna e início tardio em relação à forma ligada ao X, que corresponde a 56% dos casos.

Descrição: Caso 1- paciente feminina, iniciou infecções de repetição aos 10 anos, com impetigo, seguida de pneumonia grave seis meses após. A gravidade da infecção pulmonar associada a abscesso hepático e sua refratariedade ao tratamento demandaram investigação laboratorial para imunodeficiência, com teste do *nitroblue tetrazolium* e dosagem de ânion superóxido compatíveis com Doença Granulomatosa Crônica. A avaliação dos familiares confirmou o mesmo diagnóstico em seu irmão (**Caso 2**), que também iniciou infecções de repetição com impetigo aos 10 anos e pneumonia seis meses após, porém tratada com sucesso ambulatorialmente. A análise de polimorfismo conformacional de cadeia simples revelou alteração da mobilidade eletroforética do exon 2 do gene *NCF-1*. Identificou-se uma deleção dos nucleotídeos GT no exon 2 por sequenciamento do DNA.

Comentários: Este estudo mostra a importância da avaliação de familiares, mesmo quando não apresentam história clínica típica de Doença Granulomatosa Crônica. A identificação da mutação e sua correlação com o fenótipo dos pacientes é importante para estabelecer o prognóstico e o aconselhamento genético.

Palavras-chave: doença granulomatosa crônica, imunodeficiência, NADPH oxidase, p47-phox.

ABSTRACT

Objective: To report two siblings with Chronic Granulomatous Disease. Chronic Granulomatous Disease is a primary immunodeficiency due to a defect in phagocytes respiratory burst, resulting in abnormal microbicidal activity. Mutations in p47-phox gene (*NCF-1*) are present in about 30% of Chronic Granulomatous Disease patients and they have a better prognosis and later onset of recurrent infections comparing with X linked Chronic Granulomatous Disease, present in about 56% of patients.

Description: Case 1- a girl who presented skin infections since age 10 years, followed by severe pneumonia and liver abscess, which prompted laboratory investigation for immunodeficiency. The nitroblue tetrazolium test and superoxide release were consistent with the diagnosis of Chronic Granulomatous Disease. Laboratory tests of the parents and siblings revealed the diagnosis of the disease in her brother (**Case** 2), who also started presenting skin infections at age 10, however followed by non severe pneumonia. Single-strand conformational polymorphism analysis detected abnormal electrophoretic mobility of exon 2 of *NCF-1* gene. Sequence DNA analysis revealed a dinucleotide GT deletion in exon 2.

Comments: It is important to evaluate the relatives of a Chronic Granulomatous Disease patient, even if they do not present a typical clinical course of the disease. Defining the mutation and its correlation with phenotype is important to provide appropriate genetic counseling and clinical prognosis.

Key words: chronic granulomatous disease, immunodeficiency, NADPH oxidase, p47-phox.

INTRODUÇÃO

A Doença Granulomatosa Crônica (DGC) é uma síndrome caracterizada por alterações genéticas que determinam um defeito na produção dos reativos intermediários do oxigênio, interferindo na capacidade dos leucócitos destruírem microrganismos fagocitados [1].

A incidência da DGC é de aproximadamente 1:250000 nascidos vivos [2].

Uma alteração genética na gp91-phox, p22-phox, p47-phox ou p67-phox, proteínas que fazem parte do sistema NADPH oxidase, pode determinar falha da expressão e/ou função deste sistema transportador de elétrons, levando ao fenótipo da DGC [3].

A forma mais comum de DGC é causada por um defeito na gp91-phox, que tem padrão hereditário ligado ao cromossoma X (X-DGC, 56%), seguida pelas formas autossômicas recessivas por alteração na p47-phox (A47-DGC, 30%), p22-phox (A22-DGC, 5 a 7%) e p67-phox (A67-DGC, 5 a 7%)[2].

Clinicamente, a DGC caracteriza-se por infecções de repetição por microrganismos catalase-positivos, principalmente *Staphylococcus aureus*, bactérias gram-negativas e *Aspergillus*, além de microrganismos intracelulares, como *Pneumocystis carinii* e *Mycobacterium spp*, que acometem com freqüência pele, pulmões, fígado, baço, linfonodos e ossos [2]. Pode ocorrer formação de granulomas inflamatórios em trato gastrointestinal e vias urinárias, causando sintomas obstrutivos em esôfago, estômago ou duodeno [4], além de manifestações que assemelham-se à doença inflamatória intestinal [5].

Os pacientes com herança ligada ao X apresentam manifestações mais precoces, no 1° ano de vida, e o diagnóstico é feito, em geral, antes dos 2 anos [2,6]. Além disso, as infecções costumam ser mais graves e o número de hospitalizações maior em relação aos pacientes com herança autossômica recessiva [2,6].

De acordo com o Grupo Pan-Americano de Estudo em Imunodeficiências Primárias (PAGID), o diagnóstico de DGC é estabelecido para o indivíduo do sexo masculino ou feminino com teste do NBT ou explosão respiratória de neutrófilos alterados (menor que 5% dos controles) e que apresentem: mutação na gp91-*phox*, p22-*phox*, p47-*phox* ou p67-*phox* ou ausência de mRNA observado por análise de "Northern blot"

para um dos genes citados ou mãe, primos, tios ou sobrinhos com alteração no teste de NBT ou explosão respiratória [7].

O teste do NBT em lâmina é um método bastante útil para o diagnóstico da DGC, e também permite detectar as portadoras do defeito (mãe, irmãs) nos casos de X-DGC [8].

Este estudo apresenta a análise clínica, bioquímica e molecular de dois irmãos com DGC autossômica recessiva por defeito na p47-*phox*.

Descrição dos casos

Caso 1- Adr.O.M., sexo feminino.

Recebeu imunização básica completa, sem reações adversas e apresentava desenvolvimento pôndero-estatural e neuropsicomotor adequados.

Em junho de 1992, com 10 anos de idade, apresentou pneumonia tratada por dois períodos de 15 dias com ampicilina, seguidos de 45 dias de esquema tríplice para tuberculose, sem melhora. Recebeu então cefalotina e cloranfenicol, evoluindo com discreta melhora clínica e radiológica. A biópsia pulmonar realizada nesta fase do tratamento evidenciou reação inflamatória inespecífica com infiltrado linfo-histiocitário e presença de granulócitos de células gigantes multinucleadas e circundados por fibrose; com cultura deste material negativa para fungos e bactérias. A cultura de lavado broncoalveolar foi positiva para *Candida sp* e *Streptococcus pneumoniae*.

A paciente evoluiu com pneumopatia crônica necessitando novas internações em fevereiro, para tratamento de pneumonia e abscesso hepático, e em abril de 1993. Nesta época foi estabelecido o diagnóstico de DGC por redução do NBT em granulócitos estimulados com PMA (1μg/ml) <5% em relação ao controle sadio e à mãe da paciente. Do mesmo modo, a produção de ânion superóxido em resposta ao estímulo com PMA (30nM) na presença de citocromo C (80μM) foi muito baixa em relação ao controle sadio e à mãe (Tabela 1).

Recebeu alta da última internação com ciprofloxacina e interferon gama humano recombinante, apresentando controle moderado da pneumopatia.

No ano seguinte interrompeu o uso de interferon gama e desde então mantém uso irregular de sulfametoxazol e trimetoprim. Evoluiu com melhora importante da pneumopatia, com função pulmonar normal. No período de nove anos de acompanhamento apresentou bartolinite (1999) e uma pneumonia (2003), ambas tratadas ambulatorialmente com sucesso.

Caso 2- Adm.O.M., sexo masculino.

Assim como sua irmã recebeu imunização básica completa, sem reações adversas e sempre apresentou desenvolvimento pôndero-estatural e neuropsicomotor adequados.

Apresentou três episódios de impetigo acometendo região axilar bilateral e membros inferiores no ano de 2000. Em janeiro de 2001 apresentou pneumonia tratada por 10 dias com sulfametoxazol e trimetoprim.

Entre os meses de agosto e dezembro de 2001 apresentou mais dois episódios de impetigo, com resposta adequada a tratamento antibiótico.

Para determinar qual o componente afetado do sistema NADPH oxidase, foi realizado inicialmente análise de polimorfismo conformacional de cadeia simples (SSCP). Sabendo-se que a grande maioria dos pacientes com deficiência nesta proteína apresentam uma deleção homozigota dos nucleotídeos GT no início do exon 2 do gene *NCF-1*, realizou-se SSCP para este exon, observando-se um padrão de migração distinto nos dois pacientes, em comparação com o controle (Figura 1).

A seguir foi realizado o sequenciamento desta região específica do DNA utilizando-se o kit comercial "DNA sequencing Kit, Big Dye Terminator Cycle Sequencing Ready Reaction for ABI 377 PE/Applied Biosystems", conforme previamente publicado, confirmando que ambos os pacientes apresentam a deleção GT (ΔGT) no início do exon 2 (Figura 2).

Comentários

As alterações na atividade da NADPH oxidase, características da DGC, resultam em deficiência importante que envolve a etapa inicial da defesa às infecções. Como há amplo contato entre agentes do meio externo e a pele e as mucosas do trato respiratório e gastrointestinal, barreiras naturais do organismo, estes locais, juntamente com seus tecidos adjacentes, constituem os alvos primários de invasão microbiana e sítios freqüentes de processos infecciosos em pacientes com DGC [2,9]. Ambos os pacientes deste estudo apresentaram início tardio de quadros infecciosos, e apenas Adr.O.M. apresentou

infecção grave. Além das infecções de pele e pulmões e abscesso hepático eles não apresentaram outros quadros infecciosos freqüentes na DGC, como abscessos de partes moles, adenite supurada e osteomielite.

As infecções pulmonares constituem a forma mais comum de infecção invasiva na DGC [2], e o envolvimento pulmonar está associado a aumento significativo da morbidade e da mortalidade, uma vez que pneumonias de repetição podem evoluir para bronquiectasia e fibrose pulmonar.

Embora a topografia dos processos infecciosos sejam sugestivas de imunodeficiência de fagócitos, a evolução favorável contrasta com as descrições de pacientes com DGC [10]. Recentemente, a casuística de 368 pacientes apresentada por Winkelstein et al. (2000) demonstrou maior precocidade em relação ao início dos sintomas e maior taxa de mortalidade nos pacientes com X-DGC ($p<0,02$) [2]. Além disso, foi observada maior prevalência de manifestações inflamatórias causando obstrução gástrica e urinária e de determinado tipos de infecção (abscesso perirretal, adenite supurativa e septicemia) em pacientes com X-DGC [2].

Segundo alguns autores, a evolução mais favorável observada na A47-DGC poderia ser atribuída a uma produção residual de superóxido e peróxido de hidrogênio por neutrófilos deficientes de p47-*phox* [11]. Entretanto, observamos níveis extremamente baixos nos pacientes deste estudo, semelhantes aos níveis de outros pacientes brasileiros com X-DGC [12].

Outra característica importante da A47-DGC é o fato de se encontrar a mesma mutação no gene *NCF-1* entre as diferentes famílias, ao contrário da X-DGC, onde existem 300 mutações descritas, 200 das quais exclusivas para cada família [13]. De 82 pacientes com alteração na p47-*phox* estudados até o ano de 2000, 74 apresentavam a mesma mutação, uma deleção homozigota do nucleotídeo GT, correspondente às 4 primeiras bases do segundo exon do gene *NCF-1*. Os demais pacientes eram heterozigotos para a deleção GT (n=6) ou apresentavam outra mutação associada à deleção GT (n=2)[14]. A elevada frequência da deleção GT no início do exon 2 do gene *NCF-1* em pacientes não aparentados com alteração na p47*phox*, é explicada pela presença de um pseudogene altamente homólogo, o qual por meio de fenômenos de recombinação meiótica faz com que

o gene normal seja eliminado ou convertido à sequência anormal [15]. Duas novas mutações no gene *NCF-1* foram recentemente identificadas: uma mutação homozigota G579A, com a formação de um "stop codon", sendo os pais deste paciente heterozigotos para tal mutação e no segundo caso, mutação heterozigota G579A [16].

Estudos que mostrem aspectos clínicos e moleculares correlacionados na DGC são importantes para o estabelecimento de prognóstico, aconselhamento genético adequado e eventual terapêutica no futuro.

A pesquisa de DGC em familiares de pacientes já diagnosticados deve ser considerada, mesmo quando os familiares não apresentem sinais e sintomas clínicos característicos. Talvez as formas autossômicas recessivas de DGC possam ocorrer com maior freqüência do que o observado até o momento, se pesquisadas com mais freqüência e profundidade.

REFERÊNCIAS BIBLIOGRÁFICAS

1. Curnutte JT. Chronic granulomatous disease: the solving of a clinical riddle at the molecular level. Clin Immunol Immunopathol 1993;67(3 Pt 2):S2-15.

2. Winkelstein JA, Marino MC, Johnston RB, Jr., Boyle J, Curnutte J, Gallin JI, et al. Chronic granulomatous disease. Report on a national registry of 368 patients. Medicine (Baltimore) 2000;79(3):155-69.

3. Leusen JH, Verhoeven AJ, Roos D. Interactions between the components of the human NADPH oxidase: intrigues in the phox family. J Lab Clin Med 1996;128(5):461-76.

4. Renner WR, Johnson JF, Lichtenstein JE, Kirks DR. Esophageal inflammation and stricture: complication of chronic granulomatous disease of childhood. Radiology 1991;178(1):189-91.

5. Al-Binali AM, Scott B, Al-Garni A, Montgomery M, Robertson M. Granulomatous pulmonary disease in a child: an unusual presentation of Crohn's disease. Pediatr Pulmonol 2003;36(1):76-80.

6. Liese J, Kloos S, Jendrossek V, Petropoulou T, Wintergerst U, Notheis G, et al. Long-term follow-up and outcome of 39 patients with chronic granulomatous disease. J Pediatr 2000;137(5):687-93.

7. Conley ME. Diagnostic guidelines--An International Consensus document. Clin Immunol 1999;93(3):189.

8. Ochs H, Igo, R.P. The NBT slide test: a simple screnningmethod for detecting chronic granulomatous disease and female carriers. J Pediatr 1973;83:77-82.

9. Segal BH, Leto TL, Gallin JI, Malech HL, Holland SM. Genetic, biochemical, and clinical features of chronic granulomatous disease. Medicine (Baltimore) 2000;79(3):170-200.

10. Bridges RA, Berendes, H., Good, R. A. A fatal granulomatosis disease of childhood. The clinical, pathological and laboratory features of a new syndrome. Am J Dis Child 1959;97:387-408.

11. Cross AR, Yarchover JL, Curnutte JT. The superoxide-generating system of human neutrophils possesses a novel diaphorase activity. Evidence for distinct regulation of electron flow within NADPH oxidase by p67-phox and p47-phox. J Biol Chem 1994;269(34):21448-54.

12. Andrade CCP. Aspectos clínicos de pacientes sob suspeita de imunodeficiência fagocitária. [dissertação de mestrado]. Campinas: Universidade Estadual de Campinas; 2003.

13. Heyworth PG, Curnutte JT, Rae J, Noack D, Roos D, van Koppen E, et al. Hematologically important mutations: X-linked chronic granulomatous disease (second update). Blood Cells Mol Dis 2001;27(1):16-26.

14. Roesler J, Curnutte JT, Rae J, Barrett D, Patino P, Chanock SJ, et al. Recombination events between the p47-phox gene and its highly homologous pseudogenes are the main cause of autosomal recessive chronic granulomatous disease. Blood 2000;95(6):2150-6.

15. Vazquez N, Lehrnbecher T, Chen R, Christensen BL, Gallin JI, Malech H, et al. Mutational analysis of patients with p47-phox-deficient chronic granulomatous disease: The significance of recombination events between the p47-phox gene (NCF1) and its highly homologous pseudogenes. Exp Hematol 2001;29(2):234-43.

16. de Boer M, Singh V, Dekker J, Di Rocco M, Goldblatt D, Roos D. Prenatal diagnosis in two families with autosomal, p47(phox)-deficient chronic granulomatous disease due to a novel point mutation in NCF1. Prenat Diagn 2002;22(3):235-40.

Legendas das Figuras

Figura 1-Polimorfismo Conformacional de Cadeia Simples de pacientes com DGC: Perfil migratório do exon 2 do gene *NCF-1*. Note-se o perfil migratório alterado nos pacientes (Adr, Ade), com relação aos indivíduos normais (N).

Figura 2-Sequenciamento do DNA genômico de pacientes com DGC e controle: Análise da seqüência do exon 2 do gene *NCF-1*. **A:** Paciente Adr. Observa-se uma deleção GT (ΔGT) no início do exon 2. **B:** Paciente Ade. Observa-se a mesma mutação ΔGT no início do exon 2. **C:** Controle, note-se a dupla seqüência do gene *NCF-1* pela presença do pseudogene.

Tabela 1-Dosagem de ânion superóxido dos pacientes com DGC.

Paciente	PMN esp*	PMN PMA†	MONO esp‡	MONO PMA§
Adr.O.M.	1,0	1,03	-1,33	0,37
Ade.O.M.	-0,01	1,94	0,37	0,37
Controle	5,12	13,04	1,75	12,94

*liberação espontânea de ânion superóxido por polimorfonucleares

†liberação de ânion superóxido por polimorfonucleares estimulados com forbol miristato acetato (PMA)

‡liberação espontânea de ânion superóxido por mononucleares

§liberação de ânion superóxido por mononucleares estimulados com PMA

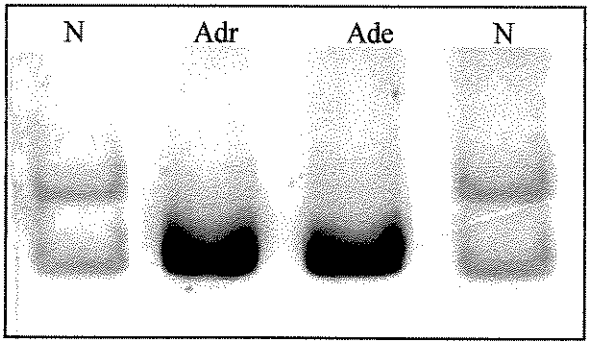

FIGURE 1-

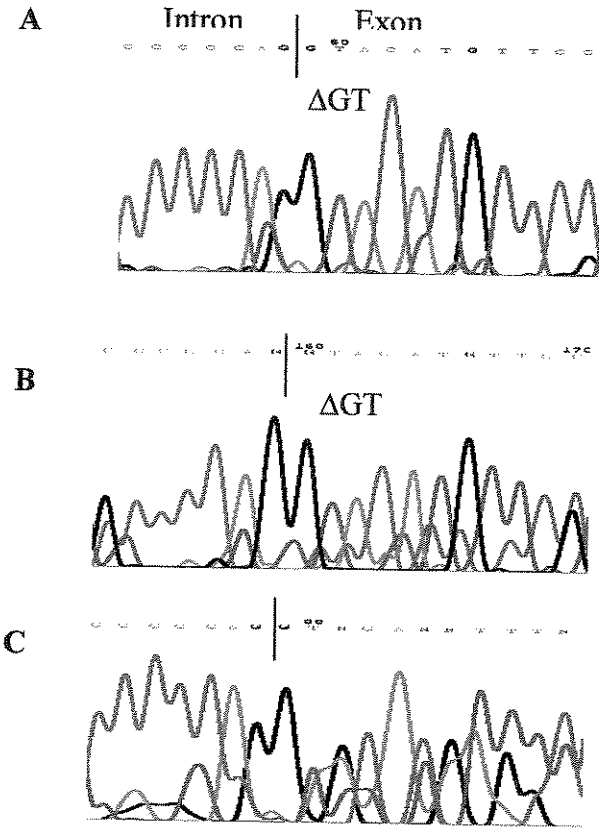

FIGURE 2-

DISCUSSÃO GERAL

A DGC é causada por defeitos moleculares que levam à ausência, baixa expressão ou mau funcionamento de um dos componentes do sistema NADPH oxidase. A maior parte dos pacientes com DGC possuem mutações exclusivas de suas famílias. A diversidade destas mutações e os múltiplos genes afetados constituem uma explicação para a heterogeneidade clínica e genética da DGC (CURNUTTE et al.,1993; ROOS et al., 1996a). Desta maneira, o estudo das células dos pacientes com DGC, além de ilustrar a relevância clínica dos reativos intermediários do oxigênio, possibilita a identificação dos diversos componentes da NADPH oxidase, bem como seus mecanismos de ativação (DINAUER et al.,2000).

O gene *CYBB* que codifica a proteína gp91-*phox* contém 13 éxons e ocupa aproximadamente 30 kb da região Xp21.1 do cromossomo X, compreende 570 aminoácidos (aa) distribuídos em quatro domínios. O primeiro é um domínio N-terminal (aa 1-277), o segundo é o domínio de união ao FAD (aa 278-397) com o domínio de união ao NADPH (aa 398-483 e 504-570) e por último um "loop over" no domínio de união ao NADPH (484-503) (ROOS et al.,1996b).

A DGC ligada ao X resulta de mutações em qualquer destes domínios de gp91-*phox*. Os defeitos neste gene resultam na ausência de citocromo b_{558}, com são grandes deleções multigênicas, deleções , inserções menores, substituições do tipo "missense" e "nonsense", bem como defeitos de "splicing". Mais de 300 mutações que levam ao fenótipo de DGC ligada ao X têm sido identificadas até a data (HEYWORTH et al.,2001).

Na América Latina, Patino e colaboradores (PATINO et al.,1999a) estudaram 7 famílias não relacionadas na Colômbia e no Brasil. Neste estudo, 6 mães eram portadoras de um alelo *CYBB* mutante, sendo que um dos casos deveu-se a mutação "de novo". Identificou-se uma substituição A por G no penúltimo nucleotídeo do íntron 12, quatro novas mutações "nonsense" (R91X, W106X, R157X, R290X), além de outras duas mutações "missense" (E225V, C244Y).

Com o presente estudo, determinamos no gene *CYBB* 7 mutações (AGUDELO-FLOREZ et al.,2004 submetido). No paciente JY, encontramos uma inserção c. 1267_1268 ins A, afetando o domínio de união ao NADPH, resultando numa perda de interação da proteína gp91-*phox* com os outros componentes citosólicos do sistema

NADPH oxidase. Esta mutação aqui descrita é inédita na literatura (HEYWORTH et al, 2001). Descrevemos também duas substituições "nonsense" W28X no paciente PT e R73X no paciente MF, que resultam num erro de leitura da janela ("frameshift"), originando "stop codons" prematuros. Estas mutações afetam o domínio N-terminal que afetam a união da gp91-*phox* à membrana e a interação com a proteína p22-*phox*.

Em quatro casos, diferentes erros de "splicing" foram encontrados. Duas mutações (pacientes RS e GG) deste tipo levaram a deleção do éxon 3 da proteína gp91-*phox* , quando uma substituição G>A foi evidenciada no final do éxon 3. As outras duas mutações encontradas, levaram à deleção do éxon 10, a primeira (paciente IC) foi uma substituição de uma G>A no íntron 10 e a outra (paciente Vin) foi uma substituição de uma A>G íntron 9. Neste último caso, mais um inédito erro de "splicing" foi aqui documentado (HEYWORTH et al.,2001).

Mutações próximas aos sítios de "splicing" são uma causa freqüente de DGC ligada ao sexo, estas mutações interferem com o processamento do RNA mensageiro, sendo documentadas em 39 de 251 casos em estudos anteriores (WINKELSTEIN et al.,2000; SEGAL et al.,2000; ROOS et al., 1996a; RAE et al.,1998). A maioria ocorreu nos sítios de "splicing", e resultaram no fenótipo $X91^0$ devido a deleção de um ou mais ´rxons, como na maioria das mutações "splicing" que levam a DGC ligada ao sexo, anteriormente documentadas (de BOER et al.,1992). Entretanto, numa minoria de casos, tais mutações levam ao fenótipo $X91^-$, devido à manutenção parcial do "splicing" normal (RAE et al., 1998). O estudo do fenótipo destes pacientes pela análise Western Blot evidenciou a ausência de expressão da proteína gp91-*phox*. Quando as mutações levam a completa ausência ou diminuição dos níveis de tradução da proteína, o gene sofreu deleção parcial ou total, podendo haver a possibilidade de síntezar-se um produto protéico (ou mRNA) aberrante ou não estável. As mutações aqui relatadas produzem significativos disturbios bioquímicos demonstrados pela ausência da atividade do "burst" oxidativo, acompanhada pela instabilidade do mRNA de gp 91- *phox*, de sua proteína ou de ambos, resultando em níveis indetectavéis desta proteína por Western Blot e um quadro clinico grave, como relatado na dissertação de Mestrado, que apresentou o curso clinico de nossos pacientes (PRANDO-ANDRADE, 2003).

As mutações no gene gp91-*phox* determinadas nestes pacientes Latino-Americanos mostraram um alto grau de heterogeneidade molecular, como é relatados em outros grupos étnicos. Todas estas mutações específicas predizem defeitos estruturais que alteram a expressão e função do produto gênico. A maioria das mutações é distribuída ao longo dos 13 éxons ou nas bordas éxon/íntron. Neste estudo, a mutação mais comum foi a do sitio de "splicing". A ausência de uma grande porção de mRNA pode gerar um transcrito instável, o qual pode ser degradado depois de ser sintetizado. O Paciente RS também apresentou uma associação com a deficiência de G6PD além de DGC ligado ao X, como foi descrito por AGUDELO-FLOREZ et al.(2004b). Este caso valida nosso conceito de que o diagnóstico definitivo de DGC necessita a demonstração do componente defeituoso do sistema NADPH oxidase e a detecção da mutação responsável pelo fenótipo de DGC.

O número de mutações identificadas em pacientes com DGC autossômica é menor que na DGC ligada ao sexo. Publicou-se a caracterização molecular de dois irmãos (PRANDO- ANDRADE et al.,2004 *in press*), com DGC causada por deficiência de p47-*phox* (gene *NCF-1*). O estudo demonstrou uma deleção homozigota de 2 nucleotídeos na repetição GTGT, correspondente às 4 primeiras bases do segundo éxon do gene. A alta freqüência da deleção GT no início do exon 2 em pacientes não relacionados, é explicada pela presença de um pseudogene altamente homólogo que sob eventos de recombinação, elimina o gene normal ou converte este numa seqüência anormal.

Os resultados indicam que as bases genético-moleculares da deficiência de gp91-*phox*, ligada ao X são heterogêneas, enquanto as observadas na deficiência de p47-*phox* são mais homogêneas. A correlação genótipo-fenótipo determina que a clínica da DGC ligada ao X é grave, enquanto a DGC autossômica secundária aos defeitos na p47-*phox*, tem evolução mais benigna (WEENING et al., 1985; MARGOLIS et al.,1990), o que pode ser atribuída a uma atividade NADPH oxidase residual (WINKELSTEIN et al.,2000).

Paralelamente, determinou-se também, a expressão do componente gp91-*phox* do sistema NADPH oxidase, por RT-PCR, nos leucócitos de 8 pacientes com DGC (AGUDELO-FLOREZ et al.,2004a). Oligonucleotídeos específicos foram desenhados para amplificar três regiões exônicas do gene gp91-*phox* por RT-PCR: 1-5, 3-9, e 7-13.

Com esta estratégia foi possível detectar a expressão defeituosa de gp91-*phox* em sete pacientes. A expressão da proteína gp91-*phox* esteve reduzida no paciente RB em todas as regiões exônicas. Esta redução sugestiona uma mutação que leva a uma baixa estabilidade do RNA ou uma atividade transcricional alterada.

Os pacientes GM e TP apresentaram expressão ausente na região exônica 7-13, o que pode ser o resultado de mutações "nonsense", e deve ser confirmado em uma futura análise mutacional. O paciente GG apresentou um produto de tamanho menor e pouco abundante nos exons 1-5 e uma expressão ausente nas outras regiões exônicas. A análise da mutação deste paciente mostrou um defeito no sítio do "splicing" do exon 3. De maneira similar, a análise da mutação do paciente RS, revelou um defeito no sítio de "splicing" também no exon 3, embora neste caso um produto de PCR abundante se evidenciou.

O paciente MF apresentou uma expressão reduzida nas regiões 3-9 e 7-13, resultado da mutação "nonsense" no éxon 3. JEM apresenta uma mutação "nonsense" no exon 5 (PATINO et al, 1999a) que leva a uma perda de expressão de gp91-*phox*, como foi evidenciado pela análise RT-PCR.

No caso, TPB, a expressão do gene gp91-*phox* foi normal embora este paciente tivesse um histórico compatível com DGC e um defeito na atividade do "burst" oxidativo. Neste caso o diagnóstico provável de DGC ligada ao X, baseou-se nos testes de NBT, revelando o perfil de portadora em sua mãe e irmã. Este achado sugere existir um defeito pós-transcricional neste caso.

Estes estudos mostram que o RT-PCR é uma ferramenta de grande potencial para determinar alterações na expressão gênica. Esta característica pode explicar em parte a variabilidade na expressão gênica do gene gp91-*phox*. Entre os pacientes incluídos neste estudo a importância de combinar pares de primers para fazer um "screening" de todo o mRNA. Além disso, através da metologia de RT-PCR, pode-se estudar a expressão do mRNA, permitindo assumir o diagnóstico definitivo de DGC segundo os critérios atuais (CONLEY et al.,1999), sendo uma alternativa ao método "northern blot", de alta complexidade e elevado custo.

Diante disso, outras metodologias mais sensíveis estão agora disponíveis para aprofundar e continuar este estudo, entre elas, Cromatografia Liquida de Alta Pressão em condições denaturantes (DHPLC) que já foi ussada para o diagnóstico prenatal da DGC

ligada ao X (CHIEN, S. et al., 2003) e PCR em Tempo Real que ajudou a determinar os genótipos polimorficos de p22-*phox* e sua relação com a expressão do sistema NADPH oxidase em pacientes hipertensos (WYCHE, K.E. et al, 2004). A disponibilização destas metodologias e sua utilização para estudar pacientes com DGC e diferentes aspectos relacionados com o sistema NADPH oxidase, ampliarão o espetro de detecção mutacional que teve este trabalho, e elucidarão outros aspectos do complexo genótipo-fenótipo da DGC.

CONCLUSÃO GERAL

Os dados obtidos com o presente trabalho permitem concluir que:

O teste RT-PCR é uma ferramenta com bom potencial para determinar a expressão gênica e pode explicar em parte a variabilidade na expressão gênica do gene gp91-*phox* entre os pacientes incluídos neste estudo.

O teste RT-PCR, por estudar a expressão do mRNA, permite assumir o diagnóstico definitivo de DGC segundo os critérios atuais, sendo uma alternativa ao método "northern blot", de alta complexidade e elevado custo.

As mutações no gene gp91-*phox* determinadas nestes pacientes Latino-Americanos mostraram um alto grau de heterogeneidade molecular, como é relatado em outros grupos étnicos. Todas estas mutações específicas predizem defeitos estruturais que alteram a expressão e função do produto gênico.

Propomos aos clínicos que frente a um paciente com deficiência de G6PD com episódios de infecções graves recorrentes, considerem a possibilidade de um defeito fagocítico e uma eventual associação com DGC.

As bases genético-moleculares da deficiência de gp91-*phox*, ligada ao X são heterogêneas, enquanto as observadas na deficiência de p47-*phox* são mais homogêneas.

O diagnóstico definitivo de DGC requer a demonstração do componente defeituoso do sistema NADPH oxidase e a detecção da mutação responsável pelo fenótipo de DGC.

REFERÊNCIAS BIBLIOGRÁFICAS

AGUDELO-FLOREZ, P; LOPEZ, J.A; REDHER, J. et al.. The use of reverse transcription-PCR in the diagnosis of X-linked chronic granulomatous disease. Braz. J. Med. Biol. Res, 37:625-634,2004a.

AGUDELO-FLOREZ, P; COSTA-CARVALHO, BT; LOPEZ, JA. et al. Association of glucose-6-phosphate dehydrogenase deficiency and X-linked chronic granulomatous disease in a child with anemia and recurrent infections. **Am. J. of Hematology,** 75:151-156, 2004b.

AGUDELO-FLÓREZ, P; LÓPEZ, JA; PRANDO-ANDRADE, C. et al. The molecular genetics of X-linked Chronic Granulomatous Disease in Latin American patients. 2004, Human Mutation. Submetido.

AMROLIA, P.; GASPAR, H.B.; HASSAN, A., et al. Nonmyeloablative stem cell transplantation for congenital immunodeficiencies. **Blood,** 96:1239-1246, 2001.

AOSHIMA, M.; NUNOI, H.; SHIMAZU, M., et al. Two-exon skipping due to a point mutation in p67-phox-deficient chronic granulomatous disease. **Blood,** 88:1841-1845, 1996.

ARIGA, T.; SAKIYAMA, Y.; FURUTA, H.; MATSUMOTO, S. Molecular genetic studies of two families with X-linked chronic granulomatous disease: Mutation analysis and definitive determination of carrier status in patients' sisters. **Eur.J.Haematol,** 52:99-102, 1994a.

ARIGA, T.; SAKIYAMA, Y.; MATSUMOTO, S. Two novel point mutations in the cytochrome b 558 heavy chain gene, detected in two Japanese patients with X-linked chronic granulomatous disease. **Hum.Genet,** 94:4411994b.

ARIGA, T.; SAKIYAMA, Y; MATSUMOTO, S. A 15-base pair (bp) palindromic insertion associated with a 3-bp deletion in exon 10 of the gp91-phox gene, detected in two patients with X-linked chronic granulomatous disease. **Hum.Genet,**. 96:6-8, 1995.

ARIGA, T.; FURUTA, H.; CHO, K.;SABIYAMA, Y. Genetic analysis of 13 families with X-linked chronic granulomatous disease reveals a low proportion of sporadic patients and a high proportion of sporadic carriers. **Pediatr.Res,**. 44:85-92, 1998.

AZUMA, H., OOMI, H., SASAKI, K., et al. A new mutation in exon 12 of the gp91-phox gene leading to cytochrome b-positive X-linked chronic granulomatous disease. **Blood,** 85:3274-3277, 1995.

BAEHNER, R.L., KUNKEL, L.M., MONACO, A.P., et al. DNA linkage analysis of X chromosome-linked chronic granulomatous disease. **Proc.Natl.Acad.Sci.USA**, 83:3398-3401, 1986.

BEMILLER, L.S.; ROBERTS, D.H.; STARKO, K.M.;CURNUTTE, J.T. Safety and effectiveness of long-term interferon gamma therapy in patients with chronic granulomatous disease. **Blood Cells Mol.Dis,**. 21:239-247, 1995.

BERENDES, H.; BRIDGES, R.A; GOOD, R.A. A fatal granulomatosus of childhood: the clinical study of a new syndrome. **Minn.Med,**. 40:309-312, 1957.

BOLSCHER, B.G.J.M.; DE BOER, M.; DE KLEIN, A.; WEENING, R.S.; ROOS, D. Point mutations in the □-subunit of cytochrome b_{558} leading to X-linked chronic granulomatous disease. **Blood,** 77:2482-2487, 1991.

BRIDGES, R.A.; BERENDES, H.; GOOD, R.A. A fatal granulomatous disease of childhood. The clinical, pathological, and laboratory features of a new syndrome. **Am.J.Dis.Child,** 97:387-408, 1959.

BU-GHANIM, H.N.; SEGAL, A.W.; KEEP, N.H.; CASIMIR, C.M. Molecular analysis in three cases of X91$^-$ variant chronic granulomatous disease. **Blood,** 86:3575-3582, 1995.

CASIMIR, C.M.; BU-GHANIM, H.N.; RODAWAY, A.R.F.; BENTLEY, D.L.; ROWE, P.; SEGAL, A.W. Autosomal recessive chronic granulomatous disease caused by deletion at a dinucleotide repeat. **Proc.Natl.Acad.Sci.USA,** 88:2753-2757, 1991.

CHANOCK, S.J.; BARRETT, D.M.; CURNUTTE, J.T.; ORKIN, S.H. Gene structure of the cytosolic component phox-47 and mutations in autosomal recessive chronic granulomatous disease. **Blood,** 78:165a, 1991 (Abstract)

CHIEN, S.H; LEE, C.N; HUNG, C; TSAO, P.SU, Y.; HSIEH, F. Rapid prenatal diagnosis of X-linked chronic granulomatous disease using a denaturing high-performance liquid chromatography (DHPLC) system. **Prenat. Diagn,** 23:1092-1096, 2003.

CLARK, R.A.; MALECH, H.L.; GALLIN, J.I.; et al. Genetic variants of chronic granulomatous disease: Prevalence of deficiencies of two cytosolic components of the NADPH oxidase system. **N.Engl.J.Med,** 321:647-652, 1989.

CONDINO-NETO, A.; RAE, J.; PADDEN, C.; WHITNEY, C.; CURNUTTE, J.T; NEWBURGER, P.E. An intronic mutation in CYBB gene leading to RNA instability and variant X-linked chronic granulomatous disease. **Blood** , 90:599, 1997 (Abstract).

CONDINO-NETO, A.; NEWBURGER, P.E. Interferon-gamma improves splicing efficiency of CYBB gene transcripts in an interferon-responsive variant of X-linked chronic granulomatous disease due to a splice site consensus region mutation. **Blood,** 95:3548-3554, 2000.

CONLEY, M.E.; NOTARANGELO, L.D; ETZIONI, A. Diagnostic criteria for primary immunodeficiencies. **Clinical Immunology** , 93:190-197, 1999.

CROSS, A.R.; YARCHOVER, J.L.; CURNUTTE, J.T. The superoxide-generating system of human neutrophils possesses a novel diaphorase activity. Evidence for distinct regulation of electron flow within NADPH oxidase by p67-*phox* and p47-*phox*. **J.Biol.Chem,** 269:21448-21454, 1994.

CROSS, A.R.; RAE, J. ; CURNUTTE, J.T. Cytochrome b_{-245} of the neutrophil superoxide-generating system contains two nonidentical hemes. Potentiometric studies of a mutant form of $gp91^{phox}$. **J.Biol.Chem,** 270:17075-17077, 1995.

CROSS, A.R ; CURNUTTE, J.T. The cytosolic factors p47-phox and p67-phox have distinct roles in the regulation of electron flow in NADPH oxidase. **J.Biol.Chem,** 270:6543-6548, 1995.

CROSS, A.R.; CURNUTTE, J.T.; RAE, J.; HEYWORTH, P.G. Hematologically important mutations: X-linked chronic granulomatous disease. **Blood Cells Mol.Dis,** 22:90-95, 1996.

CROOS, A.R.;NOACK, D.;RAE, J.CURNUTTE, J.T.; HEYWORTH, P.G. Hematologically important mutations:The autosomal Recessive forms of Chronic Granulomatous Disease (First Update). **Blood Cells Mol.Dis,** 26:561-565, 2000.

CURNUTTE, J.T. The classification of chronic granulomatous disease. **Hematol./Oncol.Clin.N.Am,** 2:241-252, 1988.

CURNUTTE, J.T. Chronic granulomatous disease: The solving of a clinical riddle at the molecular level. **Clin.Immunol.Immunopathol,** 67:S2-S15, 1993.

CURNUTTE, J.T.; ORKIN, S.H.; DINAUER, M.C. Genetic disorders of phagocyte function. In: *The Molecular Basis of Blood Diseases*, edited by Stamatoyannopoulos, G., Neinhuis, A.W., Majerus, P.W. and Varmus, H. Philadelphia: W.B. Saunders, 1994, p. 493-540.

de BOER, M., BOLSCHER, B.G.J.M., DINAUER, M.C., et al. Splice site mutations are a common cause of X-linked chronic granulomatous disease. **Blood,** 80:1553-1558, 1992.

de BOER, M.; HILARIUS-STOKMAN, P.M.; HOSSLE, J.-P., et al. Autosomal recessive chronic granulomatous disease with absence of the 67-kD cytosolic NADPH oxidase component: Identification of mutation and detection of carriers. **Blood**, 83:531-536, 1994.

DE SAINT-BASILE, G.; BOHLER, M.C.; FISCHER, A., et al. Xp21 DNA microdeletion in a patient with chronic granulomatous disease, retinitis pigmentosa, and McLeod phenotype. **Hum.Genet,** 80:85-89, 1988.

DINAUER, M.C.; ORKIN, S.H.; BROWN, R.; JESAITIS, A.J.;PARKOS, C.A. The glycoprotein encoded by the X-linked chronic granulomatous disease locus is a component of the neutrophil cytochrome b complex. **Nature,** 327:717-720, 1987.

DINAUER, M.C.; CURNUTTE, J.T.; ROSEN, H. ;ORKIN, S.H. A missense mutation in the neutrophil cytochrome b heavy chain leading to X-linked chronic granulomatous disease. **J.Clin.Invest,**. 84:2012-2016, 1989.

DINAUER, M.C.; PIERCE, E.A.; BRUNS, G.A.P.; CURNUTTE, J.T.;ORKIN, S.H. Human neutrophil cytochrome *b* light chain (p22-*phox*). Gene structure, chromosomal location, and mutations in cytochrome-negative autosomal recessive chronic granulomatous disease. **J.Clin.Invest,**. 86:1729-1737, 1990.

DINAUER, M.C.; LEKSTROM-HIMES, J.A.; DALE, D.C. Inherited neutrophil disorders: molecular basis and new therapies. **Hematology** *(Am.Soc.Hematol.Educ.Program)* 303-318, 2000.

EZEKOWITZ, R.A.B.; ORKIN, S.H.; NEWBURGER, P.E. Recombinant interferon gamma augments phagocyte superoxide production and X-chronic granulomatous disease gene expression in X-linked variant chronic granulomatous disease. **J.Clin.Invest,**. 80:1009-1016, 1987.

EZEKOWITZ, R.A.B.; DINAUER, M.C.; JAFFE, H.S.; ORKIN, S.H.; NEWBURGER, P.E. Partial correction of the phagocyte defect in patients with X-linked chronic granulomatous disease by subcutaneous interferon gamma. **N.Engl.J.Med**, 319:146-151, 1988.

FORREST, C.B.; FOREHAND, J.R.; AXTELL, R.A.; ROBERTS, R.L. ;JOHNSTON, R.B., JR. Clinical features and current management of chronic granulomatous disease. **Hematol./Oncol.Clin.N.Am,** 2:253-266, 1988.

FREY, D.; MÄCHLER, M.; SEGER, R.A.; SCHMID, W. ;ORKIN, S.H. Gene deletion in a patient with chronic granulomatous disease and McCleod syndrome: Fine mapping of the Xk gene locus. **Blood,** 71:252-255, 1988.

GORLACH, A.; LEE, P.; ROESLER, J., et al. A p47-phox pseudogene carries the most common mutation causing p47-phox deficient chronic granulomatous disease. **J.Clin.Invest,** 100:1907-1918, 1997.

HEUNG-BUM, O.; JOON, P.;WOOCHANG, L.; SOO J.Y.; JIN, H.Y.; SUN-YOUNG O. Molecular Analysis of X-linked Chronic Granulomatous Disease in Five Unrelated Korean Patients. **J Korean Med Sci,** 19:218-22, 2004.

HEYWORTH, P.G., CURNUTTE, J.T., RAE, J., et al. Hematologically important mutations: X-linked chronic granulomatous disease. **Blood Cells Mol.Dis,**. 26:561-565, 2001.

HEYWORTH, P.G; CROSS, A.R.; CURNUTTE, J.T. Chronic Granulomatous Disease. **Curr Opin Immunol,** 15:578-584, 2003

HOLMES, B.; QUIE, P.G.; WINDHORST, D.B.;GOOD, R.A. Fatal granulomatous disease of childhood. An inborn abnormality of phagocytic function. **Lancet** i:1225-1228, 1966.

HOLMES, B.; PAGE, A.R.; GOOD, R.A. Studies of the metabolic activity of leukocytes from patients with a genetic abnormality of phagocyte function. **J.Clin.Invest,**. 46:1422-1432, 1967.

HORWITZ, M.E., BARRETT, A.J., BROWN, M.R., et al. Treatment of chronic granulomatous disease with nonmyeloablative conditioning and a T-cell-depleted hematopoietic allograft. **N.Engl.J.Med**, 344:881-888, 2001.

IWATA, M.; NUNOI, H.; YAMAZAKI, H., et al. Homologous dinucleotide (GT or TG) deletion in Japanese patients with chronic granulomatous disease with p47-phox deficiency. **Biochem.Biophys.Res.Commun,**. 199:1372-1377, 1994.

JOHNSTON, R.B., Jr. Clinical aspects of chronic granulomaotus disease. **Curr.Opin.Hematol** 8:17-22, 2001.

LANDING, B.H. ;SHIRKEY, H.S. A syndrome of recurrent infection and infiltration of the viscera by pigmented lipid histiocytes. **Pediatrics,** 20:431-442, 1957.

LEUSEN, J.H.W., DE BOER, M., BOLSCHER, B.G.J.M., et al. A point mutation in gp91-*phox* of cytochrome b_{558} of the human NADPH oxidase leading to defective translocation of the cytosolic proteins p47-*phox* and p67-*phox*. **J.Clin.Invest,**. 93:2120-2126, 1994.

LEUSEN, J.H.W.; VERHOEVEN, A.J. ; ROOS, D. Interactions between the components of the human NADPH oxidase: Intrigues in the *phox* family. **J.Lab.Clin.Med,** 128:461-476, 1996.

LEW, P.D.; SOUTHWICK, F.S.; STOSSEL, T.P.; WHITIN, J.C.; SIMONS, E.R.; COHEN, H.J. A variant of chronic granulomatous disease: Deficient oxidative metabolism due to a low-affinity NADPH oxidase. **N.Engl.J.Med,** 305:1329-1333, 1981.

MARGOLIS, D.M.; MELNICK, D.A.; ALLING, D.W.; GALLIN, J.I. Trimethoprim-sulfamethoxazole prophylaxis in the management of chronic granulomaotus disease. **J.Infect.Dis,** 162:723-726, 1990.

NAGLER, A.; ACKERSTEIN, A.; KAPELUSHNIK, J.; OR, R.; NAPARSTEK, E.; SLAVIN, S. Donor lymphocyte infusion post-non-myeloablative allogeneic peripheral blood stem cell transplantation for chronic granulomaotus disease. **Bone Marrow Transplant**, 24:339-342, 1999.

NEWBURGER, P.E., LUSCINSKAS, F.W., RYAN, T., et al. Variant chronic granulomatous disease: Modulation of the neutrophil defect by severe infection. **Blood**,68:914-919, 1986.

NEWBURGER, P.E.; MALAWISTA, S.E.; DINAUER, M.C., et al. Chronic granulomatous disease and glutathione peroxidase deficiency, revisited. **Blood**, 84:3861-3869, 1994a.

NEWBURGER, P.E.; SKALNIK, D.G.; HOPKINS, P.J.; EKLUND, E.A.;CURNUTTE, J.T. Mutations in the promoter region of the gene for gp91-phox in X-linked chronic granulomatous disease with decreased expression of cytochrome b_{558}. **J.Clin.Invest,**. 94:1205-1211, 1994b.

NOACK, D.; RAE, J.; CROSS, A.R., et al. Autosomal recessive chronic granulomatous disease caused by defects in NCF-1, the gene encoding the phagocyte p47-phox: mutations not arising in the NCF-1 pseudogenes. **Blood,** 97:305-311, 2001.

NUNOI, H.; IWATA, M.; TATSUZAWA, S., et al. AG dinucleotide insertion in a patient with chronic granulomatous disease lacking cytosolic 67-kD protein. **Blood,** 86:329-333, 1995.

PARKOS, C.A.; DINAUER, M.C.; WALKER, L.E.; ALLEN, R.A.; JESAITIS, A.J.; ORKIN, S.H. The primary structure and unique expression of the 22 kilodalton light chain of human neutrophil cytochrome b. **Proc.Natl.Acad.Sci,**. *USA* 85:3319-3323, 1988.

PATINO, P.J., PEREZ, J.E., LOPEZ, J.A., et al. Molecular analysis of chronic granulomatous disease caused by defects in gp91-phox. **Human Mutation,** 13:29-37, 1999a.

PATINO, P.J., RAE, J., NOACK, D., et al. Molecular characterization of autosomal recessive chronic granulomatous disease caused by a defect of the nicotinamide adenine dinucleotide phosphate (Reduced form) oxidase component p67-phox. **Blood,** 94:2505-2514, 1999b.

PRANDO-ANDRADE, C., AGUDELO-FLOREZ, P., LOPEZ, J.A., et al. Doença Granulomatosa Crônica Autossômica: Relato de Caso e Análise Genético-Molecular de uma Família Brasileira, 2004 Journal de Pediatria. *In press.*

PRANDO-ANDRADE, C. Aspectos clinicos de pacientes sob suspeita de imunodeficiencia fagocitaria. Universidade Estadual de Campinas,Campinas, SP [s.n], 2003.

RABBANI, H., DE BOER, M., ÅHLIN, A., et al. A 40-base-pair duplication in the gp91-*phox* gene leading to X-linked chronic granulomatous disease. **Eur.J.Haematol,** 51:218-222, 1993.

RAE, J., NEWBURGER, P.E., DINAUER, M.C., et al. X-linked chronic granulomatous disease: Mutations in the CYBB gene encoding the gp91-phox component of the respiratory burst oxidase. **Am.J.Hum.Genet,**. 62:1320-1331, 1998.

ROOS, D., DE BOER, M., BORREGAARD, N., et al. Chronic granulomatous disease with partial deficiency of cytochrome b_{558} and incomplete respiratory burst: Variants of the X-linked, cytochrome b_{558}-negative form of the disease. **J.Leukocyte Biol,**. 51:164-171, 1992.

ROOS, D.; CURNUTTE, J.T.; HOSSLE. J.P.; LAU, Y.L.; ARIGA, T.; NUNOI, H, et al R. X-CGDbase: a database of X-CGD-causing mutations. **Immunol Today,**17:517521, 1996a.

ROOS, D., DE BOER, M., KURIBAYASHI, F., et al. Mutations in the X-linked and autosomal recessive forms of chronic granulomatous disease. **Blood,** 87:1663-1681, 1996b.

ROOS, D. ; CURNUTTE, J.T. Chronic Granulomatous Disease. In: *Primary Immunodeficiency Diseases: A Molecular and Genetic Approach*, edited by Ochs, H.D., Smith, C.I.E. and Puck, J.M. New York: Oxford University Press, 1999, p. 353-374.

SCHAPIRO, B.L.; NEWBURGER, P.E.; KLEMPNER, M.S.; DINAUER, M.C. Chronic granulomatous disease presenting in a 69-year-old man. **N.Engl.J.Med,** 325:1786-1790, 1991.

SEGAL, A.W., CROSS, A.R., GARCIA, R.C., et al. Absence of cytochrome b_{245} in chronic granulomatous disease. A multicenter European evaluation of its incidence and relevance. **N.Engl.J.Med,** 308:245-251, 1983.

SEGAL, H.H.; LETO, T.L.; GALLIN, J.I.; MALECH, H.L.;HOLLAND, S.M. Genetic, biochemical, and clinical features of chronic granulomatous disease. **Medicine,** 79:170-200, 2000.

SHURIN, S.B.; COHEN, H.J.; WHITIN, J.C.; NEWBURGER, P.E. Impaired granulocyte superoxide production and prolongation of the respiratory burst due to a low-affinity NADPH-dependent oxidase. **Blood,** 62:564-571, 1983.

SUZUKI, S.; KUMATORI, A.; HAAGEN, I.A., et al. PU.1 as an essential activator for the expression of gp91-phox gene in human peripheral neutrophils, monocytes, and B lymphocytes. **Proc.Natl.Acad.Sci.US,** 95:6085-6090, 1998.

TANUGI-CHOLLEY, L.C.; ISSARTEL, J.-P.; LUNARDI, J.; FREYCON, F.; MOREL, F. VIGNAIS; P.V. A mutation located at the 5' splice junction sequence of intron 3 in the $p67^{phox}$ gene causes the lack of $p67^{phox}$ mRNA in a patient with chronic granulomatous disease. **Blood**, 85:242-249, 1995.

TAUBER, A.I.; BORREGAARD, N.; SIMONS, E.R.; WRIGHT, J. Chronic granulomatous disease : A syndrome of phagocyte oxidase deficiencies. **Medicine**, 62:286-309, 1983.

The International Chronic Granulomatous Disease Cooperative Study Group. A controlled trial of interferon gamma to prevent infection in chronic granulomatous disease. **N.Engl.J.Med,** 324:509-5160, 1991.

VOLPP, B.D. ;LIN, Y. In vitro molecular reconstitution of the respiratory burst in B lymphoblasts from p47-*phox*-deficient chronic granulomatous disease. **J.Clin.Invest,**, 91:201-207, 1993.

WEENING, R.S.; ADRIAANSZ, L.H.; WEEMAES, C.M.R.; LUTTER, R. ;ROOS, D. Clinical differences in chronic granulomatous disease in patients with cytochrome b-negative or cytochrome b-positive neutrophils. **J.Pediatr,** 107:102-104, 1985.

WINKELSTEIN, J.A., MARINO, M.C., JOHNSTON, R.B., JR., et al. Chronic granulomatous disease. Report on a national registry of 368 patients. **Medicine,** 79:155-169, 2000.

WYCHE, K.E.; WANG, S.S.; GRIENDLING, K.K.; DIKALOV, S.I.; AUSTIN, H.; RAO, S. et al. C242T CYBA polymorphism of the NADPH oxidase is associated with reduced respiratory burst in human neutrophils. **Hypertension.** 43:1246-1251, 2004

ANEXOS

Brazilian Journal of Medical and Biological Research (2004) 37: 625-634
ISSN 0100-879X

The use of reverse transcription-PCR for the diagnosis of X-linked chronic granulomatous disease

P. Agudelo-Flórez[1], J.A. López[1], J. Redher[1], M.M.S. Carneiro-Sampaio[2], B.T. Costa-Carvalho[3], A.S. Grumach[4] and A. Condino-Neto[1]

[1]Centro de Investigação em Pediatria e Departamentos de Pediatria e Farmacologia, Faculdade de Ciências Médicas, Universidade Estadual de Campinas, Campinas, SP, Brasil
[2]Departamento de Imunologia, Instituto de Ciências Biomédicas, Universidade de São Paulo, São Paulo, SP, Brasil
[3]Disciplina de Alergia, Imunologia e Reumatologia, Departamento de Pediatria, Escola Paulista de Medicina, Universidade Federal de São Paulo, São Paulo, SP, Brasil
[4]Laboratório de Investigação Médica 56, Departamento de Dermatologia, Faculdade de Medicina, Universidade de São Paulo, São Paulo, SP, Brasil

Abstract

Chronic granulomatous disease (CGD) is an inherited disorder of the innate immune system characterized by a defective oxidative burst of phagocytes and subsequent impairment of their microbicidal activity. Mutations in one of the NADPH-oxidase components affect gene expression or function of this system, leading to the phenotype of CGD. Defects in gp91-*phox* lead to X-linked CGD, responsible for approximately 70% of CGD cases. Investigation of the highly heterogeneous genotype of CGD patients includes mutation analysis, Northern blot or Western blot assays according to the particular case. The aim of the present study was to use reverse transcription (RT)-PCR for the analysis of molecular defects responsible for X-linked CGD in eight Brazilian patients and to assess its potential for broader application to molecular screening in CGD. Total RNA was prepared from Epstein B virus-transformed B-lymphocytes and reverse transcribed using random hexamers. The resulting cDNA was PCR-amplified by specific and overlapping pairs of primers designed to amplify three regions of the gp91-*phox* gene: exons 1-5, 3-9, and 7-13. This strategy detected defective gp91-*phox* expression in seven patients. The RT-PCR results matched clinical history, biochemical data (nitroblue tetrazolium or superoxide release assay) and available mutation analysis in four cases. In three additional cases, RT-PCR results matched clinical history and biochemical data. In another case, RT-PCR was normal despite a clinical history compatible with CGD and defective respiratory burst. We conclude that this new application of RT-PCR analysis - a simple, economical and rapid method - was appropriate for screening molecular defects in 7 of 8 X-linked CGD patients.

Correspondence
A. Condino-Neto
Centro de Investigação em Pediatria
FCM, UNICAMP
Caixa Postal 6111
13081-970 Campinas, SP
Brasil
Fax: +55-19-3289-8638
E-mail: condino@lexxa.com.br or condino@unicamp.br

Research supported by FAPESP (Nos. 01/14365-3, 01/14365-3 and 02/05880-4), CNPq (No. 470413/03-4), CAPES, and The United States National Institutes of Health Fogarty International Center (No. 1RO3TW00883-01).

Received February 5, 2003
Accepted February 10, 2004

Key words
- Superoxide
- Phagocytes
- Primary immunodeficiency
- Respiratory burst
- Neutrophils
- Human

Introduction

Chronic granulomatous disease (CGD) is a primary immunodeficiency originally described in 1957 as a clinical entity affecting male infants and named, at the time, fatal granulomatous disease of childhood. The main characteristics of CGD are recurrent and severe infections involving the natural barriers of the organism such as the respiratory tract and lymph nodes, and eventually inner structures such as the liver, spleen, bones, and brain (1-3). The estimated incidence of this rare disease is 1/250,000 live births per year. The infections are generally caused by catalase-negative bacteria such as *Staphylococcus aureus*, Gram-negative bacilli, and fungal species such as *Aspergillus*, *Candida* and *Nocardia* (4,5).

The NADPH-oxidase system generates superoxide and other reactive oxygen intermediates, crucial for the microbicidal activity of phagocytes. The biochemical defect in CGD is an impairment of NADPH-oxidase activity and subsequent inability to destroy microorganisms (6). The main components of the NADPH-oxidase system are gp91-, p22-, p47-, p67-, and p40-*phox*. Molecular defects causing CGD are generally due to absence, low expression or malfunctioning of one of the NADPH-oxidase components. The X-linked form of this disease is caused by defects in gp91-*phox*, the heavy chain of cytochrome b_{588}, and accounts for approximately 70% of all cases (7,8). The autosomal recessive forms are caused by defects in one of the cytosolic components of NADPH oxidase (p47- or p67-*phox*, respectively in 20 and 5% of cases), or the cytochrome b_{588} light chain component (p22-*phox*, 5% of cases) (9,10). CGD is a highly heterogeneous condition: over 300 mutations have been registered in an internationally maintained X-CGD database (8). The mutations have been distributed largely within the 13 exons or at the exon/intron boundaries of the gp91-*phox* (*CYBB*) gene and almost 200 of these mutations are unique.

The diagnosis of CGD is generally based on the clinical characteristics of the disease plus defective NADPH-oxidase activity as demonstrated by abnormal nitroblue tetrazolium (NBT), dihydrorhodamine 123, or superoxide release assays (11,12). In the stimulated NBT test, normal individuals display nearly 100% positive cells, while in CGD patients fewer than 5% of the cells are positive (13). In addition, cells from patients with variant CGD are positive, but show only very low activity (6). The NBT test also detects the carriers of X-linked CGD (mothers and sisters). A definitive molecular CGD diagnosis is established in patients with an abnormal NBT test or respiratory burst activity who have one of the following characteristics: a mutation in gp91-, p22-, p47-, or p67-*phox*; absent mRNA for one of these genes detected by Northern blot analysis; and/or absent protein for one of these oxidase components by Western blot. A genetic, but not molecular, diagnosis can be established by demonstration of maternal cousins, uncles, or nephews with an abnormal NBT test or respiratory burst (14). Thus, the establishment of a definitive diagnosis of this rare and highly heterogeneous disease requires complex and expensive methodologies such as a combination of Northern blot or Western blot, and single-strand conformation polymorphism analysis (SSCP) followed by DNA sequencing of several family members, all performed in high complexity research laboratories.

The reverse transcription-PCR (RT-PCR) method involves the amplification of cDNA by PCR. This technique can be easily standardized in less sophisticated laboratories. It provides information about gene expression and preliminary data about the structure or size of the mRNA of the defective component. RT-PCR has seldom been used in CGD research, being limited to pathophysiology studies (15-29). To date, the potential use of this useful tool for establishing the definitive

diagnosis of X-linked CGD, the most frequent form, has not been extensively investigated. The aim of the present study was to evaluate the use of RT-PCR for screening molecular defects responsible for X-linked CGD, a rare and possibly misdiagnosed immunodeficiency, in eight Brazilian patients.

Patients and Methods

Patients

The study included 8 unrelated male Brazilian patients with probable X-linked CGD (2 Blacks and 6 Caucasians; age 2-8 years; height 88-108 cm; weight 11-19 kg). The patients presented clinical histories of recurrent severe infections such as pneumonia, lymphadenitis, liver abscess, pyodermitis, and adverse reactions to BCG immunization. They were referred to our laboratory for biochemical and molecular diagnostic evaluation. Written informed consent was obtained from the participants prior to the study. The Medical School Ethics Committee approved the protocol in accordance to the Helsinki Convention and Brazil Ministry of Health, Resolution 196/96.

Biochemical diagnosis of CGD

The biochemical diagnosis of CGD was established according to the Pan American Group for Immunodeficiencies criteria. An impairment of NADPH-oxidase activity was demonstrated by the NBT slide test and/or the superoxide anion release assay by peripheral blood neutrophils and mononuclear leukocytes (14,30,31). Neutrophils and mononuclear leukocytes were obtained by centrifugation of blood samples over a Ficoll-Hypaque density gradient (32).

The NBT slide test was based on the reduction of NBT to formazan by activated leukocytes (31). The assay was performed as previously described (30). More than 95% of 200 normal neutrophils stimulated with 30 nM phorbol 12-myristate 13-acetate (PMA) should be able to reduce NBT. Absent reaction or <5% positive cells was considered to indicate a diagnosis of CGD (14).

Quantitative superoxide release by neutrophils and mononuclear leukocytes was assessed by a modified superoxide dismutase-inhibitable cytochrome c reduction assay (33-35). The amount of superoxide released was calculated using an extinction coefficient of 0.21 nM/cm for cytochrome c. The results are reported as nmol superoxide released by 10^6 cells per hour. Patients with CGD showed less than 10% of control values.

Screening molecular defects responsible for X-linked CGD

B-lymphocytes from X-linked CGD patients were transformed *in vitro* with Epstein B virus (EBV) (15,16) in order to provide an abundant source of nucleic acids for molecular studies. The EBV-transformed B cell lines reproduce the biochemical and molecular defects of CGD patients (15,16,36), and eliminate the need for repeated blood collections. Briefly, peripheral blood leukocytes from X-linked CGD patients were cultured with supernatants from B95-8, an EBV-producer cell line (15,16,36,37), in RPMI 1640 medium supplemented with heat-inactivated fetal bovine serum (10%), 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml of streptomycin, at 37°C, in a humid atmosphere with 5% CO_2. Cell viability was monitored and the cultures were maintained throughout the study period.

RNA samples from EBV-transformed B-cell lines were prepared by the guanidine HCl method, followed by ethanol precipitation and quantification by standard methods (38,39). The cDNA samples were obtained by reverse transcription of 2 µg of total RNA with SuperScript II RT (GIBCO BRL) and random hexamers (15). The quality of the mRNA samples was checked by PCR amplification of ß-actin, a constitutive gene con-

trol (bp 920-943 and bp 1494-1471) (Gen Bank accession No. NM001101).

gp91-*phox* gene expression was assessed by RT-PCR. Specific and overlapping pairs of primers (Gen Bank accession No. NM_000397, Table 1) were used to amplify (30 cycles) three gp91-*phox* exonic regions: 1-5, 3-9, and 7-13. This strategy permitted us to screen all gp91-*phox* exons. PCR products were analyzed by 2% agarose gel electrophoresis and stained with ethidium bromide.

Relative gp91-*phox* gene expression was analyzed with the Image Master Software (Pharmacia-Biotech). The densitometry result for target samples was divided by the densitometry result of ß-actin, a constitutive gene control, normalizing the level considered for analysis. The results of RT-PCR assays were compared to patient clinical his-

Table 1. Oligonucleotide primers used for gp91-*phox* RT-PCR analysis.

Region	Sequence of gp91-*phox* primers	
	Forward	Reverse
Exons 1-5	5'-GCT CTA GAG CAT GAG GGG CTC TCC ATT TTT GTC A-3'	5'-CGG GAT CCC GAG TTC AGA GAG TGC TAC TGA ATA A-3'
Exons 3-9	5'-GCC TGC CTG AAT TTC AAC-3'	5'-TCA TCT GTA GCT CGA TG-3'
Exons 7-13	5'-GGA ATG CCC AAT CCC TCA G-3'	5'-GGG CCA GAC TCA GAG TTG G-3'

Table 2. Stimulated respiratory burst activity of granulocytes (GRA) and mononuclear leukocytes (MON) as assessed by the nitroblue tetrazolium (NBT) test or superoxide release assay, and gp91-*phox* gene expression in Epstein B virus-transformed B lymphocytes from patients with chronic granulomatous disease as assessed by RT-PCR analysis.

Patient	Respiratory burst activity		Relative gp91-*phox* expression		
	NBT	Superoxide	Exons 1-5	Exons 3-9	Exons 7-13
1. G.M.		MON 1.0	Reduced	Reduced	Absent
2. R.B.	<5%		Reduced	Reduced	Reduced
3. G.G.	<5%		Possible splicing defect	Absent	Absent
4. T.P.B.	<5%	MON 0.27 GRA 0.22	Normal expression	Normal expression	Normal expression
5. M.F.	<5%	GRA 0.22	Normal expression	Reduced	Reduced
6. T.P.	<5%	MON 0.25 GRA 0.46	Normal expression	Reduced	Absent
7. R.S.		MON -0.03 GRA 0.58	Possible splicing defect	Absent	Reduced
8. J.E.M.	<5%	MON 0.0 GRA 0.3	Absent	Absent	Absent

NBT is reported as percent of positive cells. Normal individuals display nearly 100% positive cells. GRA and MON from healthy controls releeased superoxide in the range of 6-14 and 5-7 nmol O_2^- 10^6 cells^{-1} 60 min^{-1}, respectively.

tory, biochemical assays (NBT and/or superoxide release assay), and available mutation analysis data (http://www.sbi.org.br/Sbi2003/index.htm).

Results

We studied 8 male patients with clinical histories of recurrent severe infections, referred to our laboratory for biochemical and molecular diagnosis of CGD. The results of the NBT slide tests and the superoxide release assays are shown in Table 2. Six of the patients presented less than 5% positive leukocytes in the NBT slide test. Six patients showed impaired superoxide release by granulocytes and/or mononuclear leukocytes (less than 10% compared to healthy controls). Four patients had abnormal results in both tests. All patients presented at least one abnormal test and received the diagnosis of probable X-linked CGD. The mother and the sister of patient T.B.P. presented an NBT slide test compatible with the carrier status of X-linked CGD. During the study period one patient (G.M.) died from pneumonia.

We next investigated the definitive diagnosis of X-linked CGD by RT-PCR analysis of gp91-*phox* gene expression. The results of RT-PCR amplification with three sets of overlapping primers are presented in Table 2. The quality of the mRNA sample was checked by PCR amplification of ß-actin, a constitutive gene control. The expected normal products were obtained in this case (Figures 1, 2 and 3).

The amplification of exons 1-5 detected two patients (G.M. and R.B.) with reduced gp91-*phox* gene expression (Figure 1, lanes 1 and 2, respectively). One patient (J.E.M., lane 8) showed absent gp91-*phox* gene expression. Two patients (G.G. and R.S.; Figure 1, lanes 3 and 7, respectively) presented a smaller PCR product (between 300 and 400 bp), suggesting a splicing defect or small deletion. Three patients presented normal gp91-*phox* expression (T.P.B., M.F. and T.P.; Figure 1, lanes 4, 5, and 6, respectively).

The results of RT-PCR amplification of gp91-*phox* exons 3-9 are presented in Figure

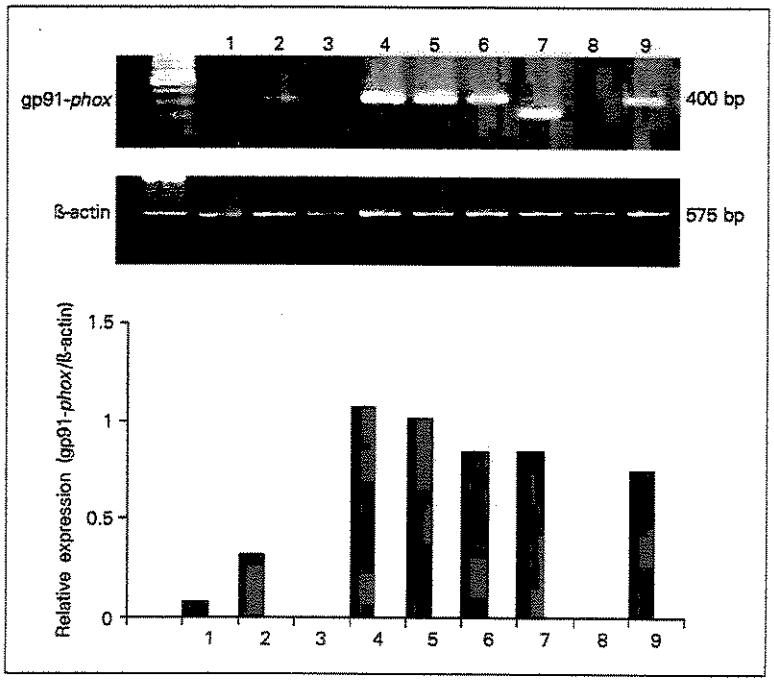

Figure 1. RT-PCR analysis of gp91-*phox* gene expression (exons 1-5) in Epstein B virus-transformed B cells from patients with X-linked chronic granulomatous disease. Representative 2% agarose gel electrophoresis of PCR products stained with ethidium bromide. Size standards (100-bp ladder); *Lane 1*, patient G.M.; *lane 2*, patient R.B.; *lane 3*, patient G.G.; *lane 4*, patient T.P.B.; *lane 5*, patient M.F.; *lane 6*, patient T.P.; *lane 7*, patient R.S.; *lane 8*, patient J.E.M., and *lane 9*, healthy control. ß-actin was used as an internal control for RT-PCR in all samples. The lower panel shows the mean band densitometry of gp91-*phox*/ß-actin relative expression (N = 3).

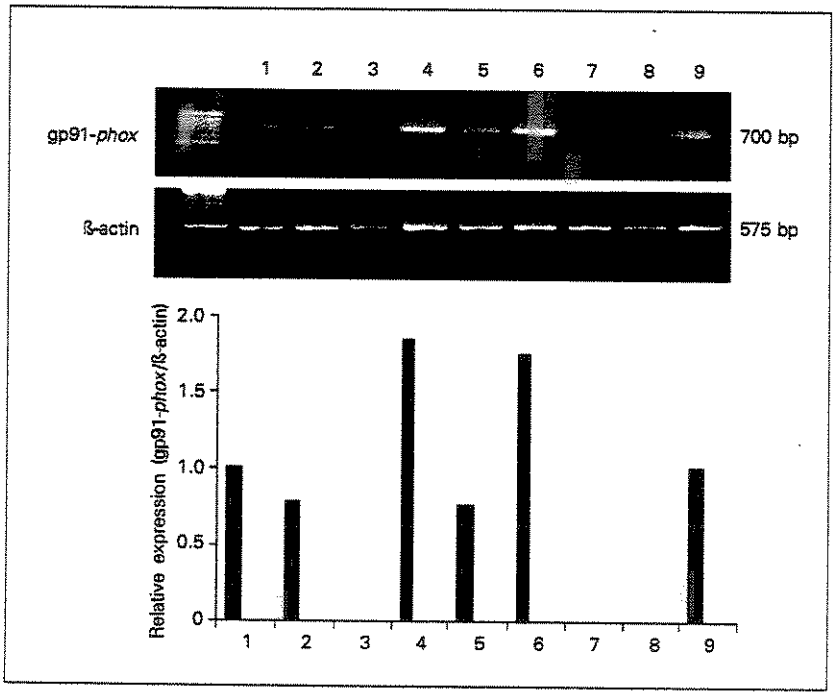

Figure 2. RT-PCR analysis of gp91-*phox* gene expression (exons 3-9) in Epstein B virus-transformed B cell lines from patients with X-linked chronic granulomatous disease. Representative 2% agarose gel electrophoresis of PCR products stained with ethidium bromide. Size standards (100-bp ladder); *Lane 1*, patient G.M.; *lane 2*, patient R.B.; *lane 3*, patient G.G.; *lane 4*, patient T.P.B.; *lane 5*, patient M.F.; *lane 6*, patient T.P.; *lane 7*, patient R.S.; *lane 8*, patient J.E.M., and *lane 9*, healthy control. ß-actin was used as an internal control for RT-PCR in all samples. The lower panel shows the mean band densitometry of gp91-*phox*/ß-actin relative expression (N = 3).

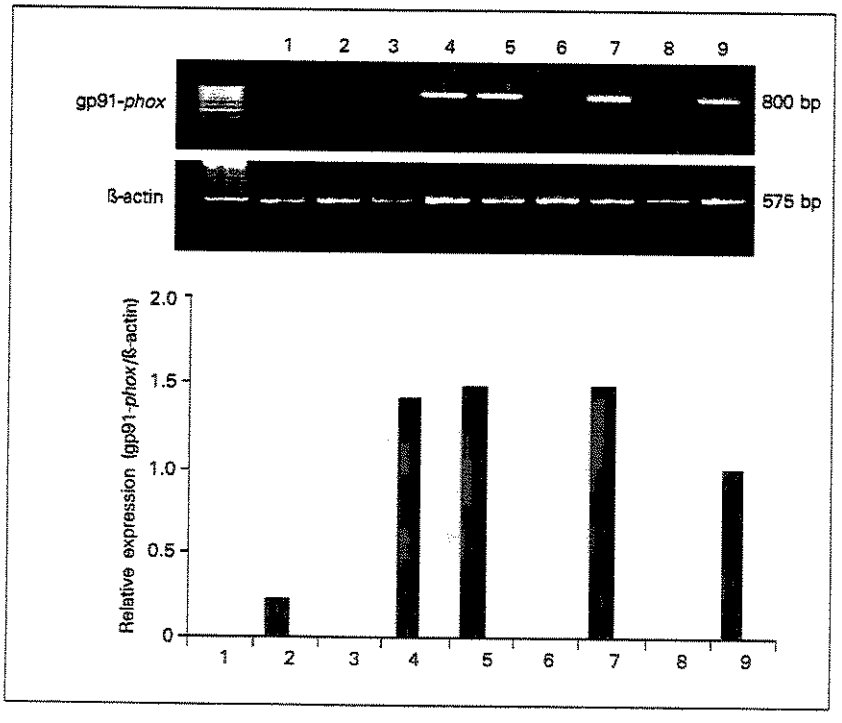

Figure 3. RT-PCR analysis of gp91-*phox* gene expression (exons 7-13) in Epstein B virus-transformed B cell lines from X-linked CGD patients. Representative 2% agarose gel electrophoresis of PCR products stained with ethidium bromide. Size standards (100-bp ladder); *Lane 1*, patient G.M.; *lane 2*, patient R.B.; *lane 3*, patient G.G.; *lane 4*, patient T.P.B.; *lane 5*, patient M.F.; *lane 6*, patient T.P.; *lane 7*, patient R.S.; *lane 8*, patient J.E.M., and *lane 9*, healthy control. ß-actin was used as an internal control for RT-PCR in all samples. The lower panel shows the mean band densitometry of gp91-*phox*/ß-actin relative expression (N = 3).

2. Two patients (R.B. and M.F.) presented reduced gp91-*phox* gene expression (Figure 2, lanes 2 and 5, respectively). Three patients (G.G., R.S., and J.E.M.; lanes 3, 7, and 8, respectively) presented absent gp91-*phox* gene expression. Three patients presented normal gp91-*phox* expression (G.M., T.P.B. and T.P.; Figure 2, lanes 1, 4 and 6, respectively).

RT-PCR amplification of gp91-*phox* exons 7-13 is presented in Figure 3. One patient (R.B.) presented reduced gp91-*phox* gene expression (Figure 3, lane 2). Four patients (G.M., G.G., T.P. and J.E.M., lanes 1, 3, 6, and 8, respectively) presented absent gp91-*phox* gene expression. Three patients (T.P.B., M.F. and R.S.; Figure 3, lanes 4, 5 and 7, respectively) presented normal gp91-*phox* expression.

In four cases, it was possible to match RT-PCR data with available mutation analysis, presented elsewhere by Patiño et al. (30) or by our group (http: //www.sbi.org.br/Sbi2003/index.htm): J.E.M. presented a $C_{469} \rightarrow T$ transition in exon 5, predicting a nonsense mutation (R157X). M.F. presented a nonsense substitution in exon 3, R (arginine) 73-Stop. R.S. showed a 264 G→A substitution at the 3' splice junction of gp91-*phox* exon 3. The cDNA sequence showed a deletion of gp91-*phox* exon 3, giving rise to an unstable or nonfunctional mutant gp91-*phox*. G.G. presented a defective splicing of gp91-*phox* exon 3 (the underlying mutation has not yet been determined). The other patients continue to be under investigation.

As a whole, this strategy permitted the detection of defective gp91-*phox* expression in seven of eight patients. The RT-PCR results matched clinical history, biochemical data (NBT or superoxide release assay) and available mutation analysis in four cases. In three additional cases, RT-PCR results matched clinical history and biochemical data. In another case, RT-PCR was normal despite a clinical history compatible with CGD, a defective respiratory burst characterized by the NBT test and superoxide release assay and NBT tests of his mother and sister compatible with X-linked CGD carrier status.

Discussion

This paper reports on the biochemical and gene expression studies of 8 unrelated Brazilian male patients with a clinical history of CGD, who were referred to our laboratory for detailed investigation. CGD is a rare inherited disorder in which phagocytic cells are unable to generate superoxide anion and other reactive oxygen intermediates. We initially established the diagnosis of probable X-linked CGD by means of biochemical methods such as the NBT slide tests and/or superoxide anion release assays. Our results showed that all patients presented impaired NADPH-oxidase function and, in turn, probable X-linked CGD.

Both methods contribute to the probable diagnosis of CGD in different ways. The NBT slide test provides information about the number of cells that reduce NBT to formazan inside the cytoplasm and the intensity of this reduction. Thus, patients with variant forms of X-linked CGD show abnormal NBT slide tests, in which most of the cells weakly reduce NBT to formazan. The NBT slide test also detects female carriers of X-linked CGD. The superoxide release assay measures the reduction of cytochrome c by superoxide produced by activated leukocytes present in the reaction, permitting the diagnosis of variant forms of X-linked CGD. Both tests can be easily standardized in low complexity laboratories and neither requires expensive equipment. The dihydrorhodamine 123 test is a highly sensitive method for the biochemical diagnosis of CGD; however, it requires a flow cytometer, a very expensive instrument.

We evaluated gp91-*phox* gene expression in EBV-transformed B lymphocytes from CGD patients by RT-PCR analysis.

Specific and overlapping pairs of primers were used to amplify three regions of the gp91-*phox* gene by RT-PCR: exons 1-5, 3-9, and 7-13. This strategy permitted the detection of defective gp91-*phox* expression in 7 of 8 patients. The RT-PCR results matched clinical history, biochemical data (NBT or superoxide release assay) and available mutation analysis in four cases. In three additional cases, RT-PCR results matched clinical history and biochemical data.

gp91-*phox* gene expression was reduced in all exonic regions of patient R.B. This reduction can be the result of mutations leading to low RNA stability or altered transcriptional activity. Patients G.M. and T.P. showed absent expression of exons 7-13, suggesting that the decreased expression of these additional 3'-end exons can be the result of RNA instability or a splicing defect, e.g., partially correct splicing to produce a signal, but partially abnormal splicing to eliminate a primer binding site, a subject to be investigated by future genomic DNA mutation analyses.

Patient G.G. presented a diffuse, low abundance, smaller-sized product of exons 1-5 (Figure 1), and absent expression of other exonic regions. His mutation analysis showed a defect at the exon 3 splice site. Similarly, the mutation analysis of patient R.S. also revealed a defect at the exon 3 splice site. However, in this case, a less abundant PCR product could be detected.

Patient M.F. presented reduced expression of exonic regions 3-9 and 7-13, which can be the result of defective transcriptional activity in exon 3. J.E.M. presented a nonsense mutation in exon 5 that resulted in loss of gp91-*phox* expression, as evidenced by RT-PCR analysis, a possible consequence of nonsense-mediated mRNA instability.

In one case, patient T.P.B., gp91-*phox* gene expression was normal despite a family history compatible with CGD and a defective respiratory burst activity. In this case, the diagnosis of probable X-linked CGD was based on abnormal NBT tests in his mother and his sister, compatible with carrier status. We hypothesize that a point mutation, such as a single base substitution that does not change PCR fragment length or abundance, should be investigated in this particular case.

RT-PCR is a powerful tool to assess gene expression. This characteristic may partially explain the variability of gp91-*phox* gene expression among the patients included in this study, and the importance to combine overlapping pair of primers to screen the full length of the message. RT-PCR has been used in isolated case studies as part of the initial diagnosis of the different forms of CGD (17-22). RT-PCR analysis was also useful in the prenatal diagnosis of CGD, resulting from p47-*phox* deficiency (23). However, it has been more commonly used for the study of gene regulation of the NADPH-oxidase components in a variety of cells (15,16,24-29). To date, the potential use of this useful tool for establishing the definitive diagnosis of X-linked CGD, the most frequent form, has not been investigated extensively. Further studies should be performed to compare the sensitivity and specificity of this test compared to other complex assays such as Western or Northern blots.

Overall, we have demonstrated that RT-PCR, a simple and low cost methodology, established the definitive diagnosis of X-linked CGD in 7 of 8 cases, without the need to use complex and expensive methodologies such as Northern blot, slot blot, SSCP analysis, or genomic DNA sequencing. Thus, RT-PCR may be a suitable tool for diagnosing CGD in laboratories in developing countries. It is very important to determine the definitive molecular genetic defect in order to provide the appropriate genetic counseling and prognosis to kindreds with CGD. In addition, molecular genetic studies of the human NADPH-oxidase system will advance the knowledge about this crucial and ancient defense mechanism.

Acknowledgments

The authors thank the patients and their families for participating and for their help, and the nurse Silvana Severino for technical assistance. The authors also thank Prof. Peter Newburger, University of Massachusetts Medical School, for a critical review of this manuscript.

References

1. Berendes H, Bridges RA & Good RA (1957). A fatal granulomatosus of childhood: the clinical study of a new syndrome. *Minnesota Medicine*, 40: 309-312.
2. Landing BH & Shirkey HS (1957). A syndrome of recurrent infection and infiltration of the viscera by pigmented lipid histiocytes. *Pediatrics*, 20: 431-442.
3. Patterson EL, Milstrey R & Stokstad ELR (1975). Effect of selenium in preventing exudative diathesis in chicks. *Proceedings of the Society for Experimental Biology and Medicine*, 95: 617-620.
4. Tauber AI, Borregaard N, Simons ER & Wright J (1983). Chronic granulomatous disease A syndrome of phagocyte oxidase deficiencies. *Medicine*, 62: 286-309.
5. Forrest CB, Forehand JR, Axtell RA, Roberts RL & Johnston Jr RB (1988). Clinical features and current management of chronic granulomatous disease. *Hematology/Oncology Clinics of North America*, 2: 253-266.
6. Curnutte JT (1993). Chronic granulomatous disease: the solving of a clinical riddle at the molecular level. *Clinical Immunology and Immunopathology*, 67: S2-S15.
7. Dinauer MC, Orkin SH, Brown R, Jesaitis AJ & Parkos CA (1987). The glycoprotein encoded by the X-linked chronic granulomatous disease locus is a component of the neutrophil cytochrome b complex. *Nature*, 327: 717-720.
8. Heyworth PG, Curnutte JT, Rae J, Noack D, Roos D, van Koppen E & Cross AR (2001). Hematologically important mutations: X-linked chronic granulomatous disease (second update). *Blood Cells, Molecules and Diseases*, 27: 16-26.
9. Clark RA, Malech HL, Gallin JI, Nunoi H, Volpp BD, Pearson DW, Nauseef WM & Curnutte JT (1989). Genetic variants of chronic granulomatous disease: Prevalence of deficiencies of two cytosolic components of the NADPH oxidase system. *New England Journal of Medicine*, 321: 647-652.
10. Cross AR, Noack D, Rae J, Curnutte JT & Heyworth PG (2000). Hematologically important mutations: the autosomal recessive forms of chronic granulomatous disease (first update). *Blood Cells, Molecules and Diseases*, 26: 561-565.
11. Smith RM & Curnutte JT (1991). Molecular basis of chronic granulomatous disease. *Blood*, 77: 673-686.
12. Vowells SJ, Fleisher TA, Sekhsaria S, Alling DW, Maguire TE & Malech HL (1996). Genotype-dependent variability in flow cytometric evaluation of reduced nicotinamide adenine dinucleotide phosphate oxidase function in patients with chronic granulomatous disease. *Journal of Pediatrics*, 128: 104-107.
13. Roos D, de Boer M, Kuribayashi F et al. (1996). Mutations in the X-linked and autosomal recessive forms of chronic granulomatous disease. *Blood*, 87: 1663-1681.
14. Conley ME, Notarangelo LD & Etzioni A (1999). Diagnostic criteria for primary immunodeficiencies. *Clinical Immunology*, 93: 190-197.
15. Condino-Neto A & Newburger PE (1998). NADPH oxidase activity and cytochrome b558 content of human Epstein-Barr virus transformed B lymphocytes correlate with expression of genes encoding components of the oxidase system. *Archives of Biochemistry and Biophysics*, 360: 158-164.
16. Condino-Neto A & Newburger PE (2000). Interferon-gamma improves splicing efficiency of CYBB gene transcripts in an interferon-responsive variant of X-linked chronic granulomatous disease due to a splice site consensus region mutation. *Blood*, 95: 3548-3554.
17. Aoshima M, Nunoi H, Shimazu M, Shimizu S, Tatsuzawa O, Kenney RT & Kanegasaki S (1996). Two-exon skipping due to a point mutation in p67-phox-deficient chronic granulomatous disease. *Blood*, 88: 1841-1845.
18. Azuma H (1994). DNA analysis of cytochrome b positive chronic granulomatous disease (a case report). *Rinsho Byori*, 42: 242-248.
19. Bonizzato A, Russo MP, Donini M & Dusi S (1997). Identification of a double mutation (D160V-K161E) in the p67phox gene of a chronic granulomatous disease patient. *Biochemical and Biophysical Research Communications*, 231: 861-863.
20. Borgato L, Bonizzato A, Lunardi C, Dusi S, Andrioli G, Scarperi A & Corrocher R (2001). A 1.1-kb duplication in the p67-phox gene causes chronic granulomatous disease. *Human Genetics*, 108: 504-510.
21. Noack D, Heyworth PG, Newburger PE & Cross AR (2001). An unusual intronic mutation in the CYBB gene giving rise to chronic granulomatous disease. *Biochimica et Biophysica Acta*, 1537: 125-131.
22. Roesler J, Curnutte JT, Rae J, Barrett D, Patino P, Chanock SJ & Goerlach A (2000). Recombination events between the p47-phox gene and its highly homologous pseudogenes are the main cause of autosomal recessive chronic granulomatous disease. *Blood*, 95: 2150-2156.
23. De Boer M, Singh V, Dekker J, Di Rocco M, Goldblatt D & Roos D (2002). Prenatal diagnosis in two families with autosomal, p47(phox)-deficient chronic granulomatous disease due to a novel point mutation in NCF1. *Prenatal Diagnosis*, 22: 235-240.
24. Baehner RL, Millar-Groff S & Bringas P (1999). Developmental expression of NADPH phagocytic oxidase components in mouse embryos. *Pediatric Research*, 46: 152-157.
25. Banerjee R, Anguita J, Roos D & Fikrig E (2000). Cutting edge: infection by the agent of human granulocytic ehrlichiosis prevents the respiratory burst by down-regulating gp91phox. *Journal of Immunology*, 164: 3946-3949.
26. Bayraktutan U, Blayney L & Shah AM (2000). Molecular characterization and localization of the NAD(P)H oxidase components gp91-phox and p22-phox in endothelial cells. *Arteriosclerosis, Thrombosis and Vascular Biology*, 20: 1903-1911.
27. Cobbs S, Malech HL, Leto TL, Freeman SM, Blaese RM, Gallin JI & Lomax KJ (1992). Retroviral expression of recombinant p47phox protein by Epstein-Barr virus-transformed B lymphocytes from a

patient with autosomal chronic granulomatous disease. *Blood*, 79: 1829-1835.

28. Gorlach A, Brandes RP, Nguyen K, Amidi M, Dehghani F & Busse R (2000). A gp91phox containing NADPH oxidase selectively expressed in endothelial cells is a major source of oxygen radical generation in the arterial wall. *Circulation Research*, 87: 26-32.
29. Rouzaut A, Lopez-Moratalla N & de Miguel C (2000). Differential gene expression in the activation and maturation of human monocytes. *Archives of Biochemistry and Biophysics*, 374: 153-160.
30. Patiño PJ, Perez JE, Lopez JA, Condino-Neto A, Grumach AS, Botero JH, Cumutte JT & Garcia de Olarte D (1999). Molecular analysis of chronic granulomatous disease caused by defects in gp91-phox. *Human Mutation*, 13: 29-37.
31. Ochs HD & Igo RP (1973). The NBT slide test: a simple screening method for detecting chronic granulomatous disease and female carriers. *Journal of Pediatrics*, 83: 77-82.
32. Boyum A (1968). Isolation of mononuclear cells and granulocytes from human blood. *Scandinavian Journal of Clinical and Laboratory Investigation*, 21 (Suppl 97): 1-77.
33. Condino-Neto A, Muscara MN, Grumach AS, Carneiro-Sampaio MMS & de Nucci G (1993). Neutrophils and mononuclear cells from patients with chronic granulomatous disease release nitric oxide. *British Journal of Clinical Pharmacology*, 35: 485-490.
34. McCord J & Fridovich I (1969). Superoxide dismutase: An enzymatic function for erythrocuprein (hemocuprein). *Journal of Biological Chemistry*, 244: 6044-6055.
35. Condino-Neto A, Muscara MN, Grumach AS, Bellinati-Pires R, Brandao AC, Carneiro-Sampaio MMS & de Nucci G (1996). The effect of recombinant human interferon-gamma therapy on neutrophil and mononuclear cell nitric oxide release from patients with chronic granulomatous disease. *Journal of Interferon and Cytokine Research*, 16: 357-364.
36. Volkman DJ, Buescher ES, Gallin JI & Fauci AS (1984). B cell lines as models for inherited phagocytic diseases: superoxide generation in chronic granulomatous disease and granules in Chediak-Higashi syndrome. *Journal of Immunology*, 133: 3006-3009.
37. Nilsson K, Klein G, Henle W & Henle G (1971). The establishment of lymphoblastoid cell lines from adult and fetal human lymphoid tissue and its dependence on EBV. *International Journal of Cancer*, 8: 443-450.
38. Ginsburg D, Handin RI, Bonthron DT, Donlon TA, Bruns GAP, Latt SA & Orkin SH (1985). Human von Willebrand factor: Isolation of complementary DNA clones and chromosomal location. *Science*, 228: 1401-1406.
39. Subrahmanyam YVBK, Baskaran N, Newburger PE & Weissman SM (1999). A modified method for the display of 3'-end restriciton fragments of cDNAs: Molecular profiling of gene expression in neutrophils. *Methods in Enzymology*, 303: 272-297.

American Journal of Hematology 75:151–156 (2004)

Association of Glucose-6-phosphate Dehydrogenase Deficiency and X-Linked Chronic Granulomatous Disease in a Child With Anemia and Recurrent Infections

Piedad Agudelo-Flórez,[1] Beatriz T. Costa-Carvalho,[2] Juan Alvaro López,[1] Jussara Redher,[1] Peter E. Newburger,[3] Sara Teresinha Olalla-Saad,[4] and Antonio Condino-Neto[1]*

[1] Center for Investigation in Pediatrics and Department of Pediatrics, State University of Campinas Medical School, Campinas, Brazil
[2] Division of Allergy, Immunology and Rheumatology, Department of Pediatrics, Federal University of São Paulo, São Paulo, Brazil
[3] Department of Pediatrics, University of Massachusetts Medical School, Worcester, Massachusetts
[4] Division of Hematology, Department of Medicine, State University of Campinas Medical School, Campinas, Brazil

Patients with severe leukocyte G6PD deficiency may present with impairment of NADPH oxidase activity and a history of recurrent infections, mimicking the phenotype of chronic granulomatous disease. We report herein a child with recurrent infections who initially received the diagnosis of G6PD deficiency. His erythrocyte G6PD activity was reduced: 1.8 U/g Hb (normal: 12.1 ± 2.1 U/g Hb). Further studies revealed that G6PD activity in neutrophils, mononuclear leukocytes, and Epstein-Barr virus-transformed B-lymphocytes from the proband was similar to healthy controls. Molecular studies showed that the G6PD deficiency was due a 202 G→A mutation, the A⁻ variant common in African ethnic groups. The proband also exhibited severely impaired respiratory burst activity, as observed in X-linked CGD. Sequence analysis of genomic DNA showed a 264 G→A substitution at the 3′ splice junction of gp91-*phox* exon 3. The cDNA sequence showed a deletion of gp91-*phox* exon 3, giving rise to an unstable or nonfunctional mutant gp91-*phox* and to the phenotype of X-linked CGD. We propose that clinicians treating a patient with G6PD deficiency during a severe infection episode consider the possibility of temporary or permanent impairment of the phagocytes' microbicidal activity and the eventual association of G6PD deficiency and chronic granulomatous disease. Am. J. Hematol. 75:151–156, 2004. © 2004 Wiley-Liss, Inc.

Key words: chronic granulomatous disease; G6PD deficiency; recurrent infections; anemia; phagocytes

INTRODUCTION

The phagocytic cells constitute one of the major effector mechanisms against invasive microorganisms. Phagocyte abnormalities are generally manifested by recurrent and severe infections, usually starting in early infancy. When phagocytes are activated, they increase oxygen consumption and reduce molecular oxygen to superoxide, a reaction known as the "respiratory burst", catalyzed by an enzymatic complex called the NADPH oxidase [1].

NADPH is the electron donor used to reduce the molecular oxygen to superoxide. It is generated from glucose, via hexose's monophosphate shunt, which also substantially increases its activity when the phagocytic cells are activated. In the first step of this pathway,

Piedad Agudelo-Flórez and Beatriz Costa-Carvalho have contributed equally to this paper.

Contract grant sponsor: Fundação de Amparo à Pesquisa do Estado de São Paulo; Contract grant number: FAPESP 01/14365-3; Contract grant sponsor: Conselho Nacional de Desenvolvimento Científico e Tecnológico; Contract grant number: CNPq 302685/02-02; Contract grant sponsor: Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES); Contract grant sponsor: The United States National Institutes of Health Fogarty International Center; Contract grant number: 1RO3TW00883-01.

*Correspondence to: Antonio Condino-Neto, M.D., Center for Investigation in Pediatrics. State University of Campinas Medical School, UNICAMP, P.O. Box 6111, Campinas, SP 13081-970, Brazil. E-mail: condino@lexxa.com.br

Received for publication 8 April 2003; Accepted 24 September 2003

Published online in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/ajh.10477

glucose-6-phosphate (G6P) is converted into 6-phosphoglucolactone, catalyzed by glucose-6-phosphate dehydrogenase, and accompanied by the reduction of nicotinamide adenine dinucleotide phosphate (NADP) into NAPDH[2].

Chronic granulomatous disease (CGD) is a primary immunodeficiency, characterized by recurrent infections affecting mainly the natural barriers of the organism such as the respiratory tract, lymph nodes, and eventually inner structures, such as the liver, spleen, bones, and brain [3]. The biochemical defect in CGD is impairment of the NADPH oxidase activity and the subsequent inability to destroy microorganisms [1]. The main components of the NADPH oxidase system are gp91-*phox*, p22-*phox*, p47-*phox*, and p67-*phox*. The molecular defects causing CGD are generally due to the absence, low expression, or malfunction of one of these NADPH oxidase components. The X-linked form of the disease is caused by defects in gp91-*phox*, the heavy chain of the cytochrome b_{588}, and accounts for approximately 60% of the cases. The *CYBB* gene encodes gp91-*phox* and is located at Xp21.1. The autosomal recessive forms are caused by defects in one of the cytosolic components of the NADPH oxidase (p47-*phox* or p67-*phox*, respectively 30% and 5% of the cases), or even the cytochrome b_{588} light chain component(p22-*phox*, 5% of the cases) [4–6].

G6PD deficiency is a common genetic disorder in humans. Clinically, it varies from mild hemolytic anemia to chronic nonspherocytic hemolytic anemia, associated with attacks of severe anemia induced by infections and drugs. The G6PD gene is located to Xq28. Thus, hemizygous men most frequently express the clinical symptoms [2,7]. Patients with severe G6PD deficiency in leukocytes may indeed present impairment of the NADPH oxidase activity and subsequent recurrent infections, mimicking the phenotype of chronic granulomatous disease [8,9].

We report herein on a child with anemia and recurrent infections, who initially received the diagnosis of G6PD deficiency. Further studies confirmed an impairment of the NADPH oxidase system. Mutation analysis revealed a splicing defect on the exon 3 of *CYBB* gene, confirming an unusual association of X-linked chronic granulomatous disease and the African variant of G6PD deficiency.

PATIENTS AND METHODS

Case Report and Human Subjects

The proband is a male, born in 1994 to Brazilian parents with African and Caucasian ethnic background, who presented with anemia and recurrent severe infections since he was 6 months old. By age 5 years, he had already had 12 episodes of pneumonia (two with pleural effusion), two of diarrhea (enteropathogenic *Escherichia coli* and *Salmonella*), three of otitis, four of sinusitis, one of stomatitis, two of skin abscess, and one of sepsis. He was hospitalized on eight occasions. He received the recommended immunizations, including BCG, without any side effects. His parents were not consanguineous. A 3-year-old brother died from sepsis secondary to pneumonia and abscesses in the liver and lungs. His father and his sister have sickle cell trait. His physical examination findings at age 5 years included weight, 17 kg; height, 107 cm; pallor; digital clubbing; and moderate hepatosplenomegaly.

Blood cell counts revealed anemia (hemoglobin range: 8.1–10.9 g/dL), neutrophilia, and eosinophilia. The reticulocyte count was normal (1.3%) as was the serum unconjugated bilirubin (0.3 mg/dL). Serum immunoglobulin levels (IgA, IgG, and IgM) were increased. CD3, CD4, and CD8 cell counts were normal. Lymphocyte proliferation in response to phytohemaglutinin, pokeweed mitogen, and concanavalin A was normal. The complement CH50 activity was normal. The HIV test was negative. A thoracic CT scan showed emphysema and extensive areas of pulmonary destruction. Erythrocyte G6PD activity was reduced: 1.8 U/g Hb compared to the normal range of 12.1 ± 2.1 U/g Hb.

Considering the recurrent infections, the hypothesis of a phagocyte abnormality was established and the patient was referred to our laboratory for further investigation. Experiments compared assays in blood samples and cell lines obtained from the proband, one patient with X-linked chronic granulomatous disease [10], one patient with G6PD deficiency (African variant) [11], and healthy controls. Written informed consent was obtained prior to the study. The Medical School Ethics Committee approved the protocol in accordance with the Helsinki Convention and the Brazilian National Health Council.

Purification and Blood Cell Cultures

Erythrocytes were obtained by centrifugation and aspiration of plasma and buffy coat, followed by washing 3 times with saline [12]. Neutrophils and mononuclear leukocytes were obtained by centrifugation of heparinized blood over a Ficoll-Hypaque density gradient[13].

B lymphocytes were transformed in vitro with Epstein-Barr virus (EBV) in order to provide an abundant source of leukocytes for biochemical and molecular studies. EBV-transformed B-cell lines reproduce the biochemical and molecular defects of CGD patients [14] and avoid repeated blood collections. Briefly, mononuclear leukocytes were cultured with supernatants

from B95-8, an EBV-producer cell line, in RPMI 1640 medium supplemented with heat-inactivated fetal bovine serum (10%), 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL of streptomycin, at 37°C, in a humid atmosphere with 5% CO_2[15]. Cellular viability was monitored, and the cultures were maintained during the studies.

G6PD Studies

G6PD activity was assayed as the rate of reduction of NADP to NADPH, when erythrocyte or leukocyte lysates were incubated with glucose-6-phosphate, as originally described by Beutler [16] and modified by Saad [11]. Comparative studies included erythrocytes, neutrophils, mononuclear leukocytes, and EBV-transformed B lymphocytes.

Genomic DNA was obtained from peripheral blood leucocytes using DNAzol Reagent (Life Technologies, Gibco BRL, Bethesda, MD). The 202 G→A mutation was investigated by allele-specific oligomer hybridization, as described elsewhere [11], or by digestion of exon 4 of the G6PD gene with the restriction endonuclease *Nla*III. The Mediterranean variant was investigated by digestion of exon 6 with the restriction enzyme *Mbo*II.

NADPH Oxidase Studies

The biochemical diagnosis of CGD was established according to criteria of the Pan American Group for Immunodeficiencies [17]. NADPH oxidase activity of neutrophils and mononuclear leukocytes was measured by superoxide dismutase (SOD)-inhibitable reduction of cytochrome *c*, as described McCord and Fridovich [18] and modified by Condino-Neto [15]. Oxidase activity in cells from patients with CGD generally show upto 5% of control values [17].

Definitive diagnosis of CGD requires the demonstration of the underlying mutation or the absence of mRNA for one of the NADPH oxidase components [17]. RNA samples were prepared from leukocytes by the guanidine-HCl method, followed by ethanol precipitation [19]. Reverse transcription followed by PCR (RT-PCR) analysis was performed on total RNA prepared from EBV-transformed B lymphocytes. The cDNA samples were obtained by reverse transcription of total RNA with SuperScript II RT (Gibco BRL) and random hexamers [15]. The cDNA samples were amplified by PCR (30 cycles) with primers specific for the *CYBB* gene encoding gp91-*phox* (GenBank accession number 000397), including exons 1–5, 32F-443R. For p47-*phox* (GenBank accession number 000265), samples were amplified by PCR with specific primers, 48F-451R. PCR products were analyzed by 2% agarose gel electrophoresis and stained with ethidium bromide.

Single-strand conformation polymorphism analysis (SSCP) was used to detect the affected gene region. Fragments of genomic DNA (gDNA) were amplified by PCR using specific primers, according to previously published procedures [10]. The polyacrylamide gel was stained with silver, and the electrophoretic mobility of the PCR-amplified products from the proband was compared to healthy controls.

Samples from PCR-amplified gDNA or RT-PCR-amplified cDNA were purified using the CONCERT™ Rapid PCR Purification System (Life Technologies, Gibco) and sequenced by the DNA sequencing kit, Big Dye Terminator Cycle Sequencing Ready Reaction for ABI 377 PE/Applied Biosystems, as previously published [15]. The sequences obtained from the proband and healthy controls were compared to GenBank data by BLAST analysis (http://www.ncbi.nlm.nih.gov/BLAST/).

RESULTS

G6PD Studies

G6PD activity was assayed in erythrocytes, neutrophils, mononuclear leukocytes, and EBV-transformed B lymphocytes from the proband, a patient with X-linked CGD, a patient with the African (A^-) variant of G6PD deficiency, and healthy controls. As presented in Table I, the proband and the known G6PD-deficient patient showed very low erythrocyte G6PD activity. However, the G6PD activity of the proband's neutrophils, mononuclear leukocytes, and EBV-transformed B lymphocytes was similar to the healthy controls.

Molecular studies of the G6PD gene included the digestion of PCR-amplified gDNA with the restriction endonuclease *Nla*II. As shown in Fig. 1, exon 4 of the G6PD gene was digested and the 202 G→A mutation was detected in the proband gDNA, confirming his African variant genotype.

TABLE I. G6PD Activity in Red Blood Cells (U/g Hb), Neutrophils, Mononuclear Leukocytes, and EBV-Transformed B Lymphocytes (U/mg protein) From the Proband, an X-Linked CGD Patient (X-CGD), a Patient With the African Variant of G6PD Deficiency (G6PD-AV), and Healthy Controls (HC)*

	Proband	X-CGD	G6PD-AV	HC
Erythrocytes	1.8	12.8	0.9	14.1
Neutrophils	0.4	1.0	1.8	3.1
Mononuclear leukocytes	0.8	4.2	0.5	0.5
B lymphocytes	2.0	2.0	0.6	2.8

*Values are given as mean of 3 determinations.

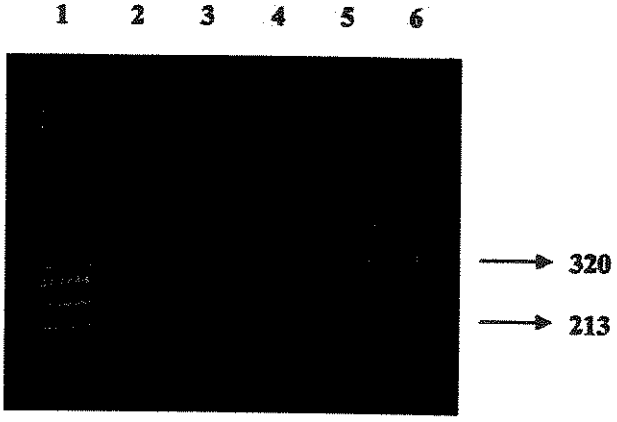

Fig. 1. G6PD mutational screen performed with digestion of gDNA with the restriction enzyme *Nla*III. Healthy controls show a 320-bp fragment (lanes 2, 4, 5, and 6). The proband gDNA produces a 213-bp fragment (lane 3) confirming a G202A/A376G mutation, typical of the African phenotype. Lane 1: 100-bp ladder size markers.

NADPH Oxidase Studies

A series of studies compared superoxide release by neutrophils and mononuclear leukocytes from the proband, a patient with known X-linked CGD, a patient with the African variant of G6PD deficiency, and healthy controls. The results in Table II show severely impaired respiratory burst activity in the proband and the X-linked CGD patient but normal respiratory burst activity in the patient with G6PD deficiency (African variant) and the healthy controls.

Because the proband showed defective respiratory burst activity, which was not present in the patient with the African variant G6PD deficiency, a mutational screen for CGD was performed. RT-PCR analysis included the amplification of p47-*phox* and gp91-*phox* transcripts, as these genes are affected in 90% of the mutations leading to the phenotype of CGD. Figure 2 shows that p47-*phox* was expressed normally in both the proband and healthy controls. On the other hand, as shown in Fig. 3, gp91-*phox* expression was impaired in the proband, as in the X-linked CGD patient, compared to healthy controls, indicating the diagnosis of X-linked CGD in the proband. Moreover, amplified

TABLE II. Superoxide Release (nmol/10^6 cells/hr) by Neutrophils and Mononuclear Leukocytes From the Proband, an X-Linked CGD Patient (X-CGD), a Patient With the African Variant of G6PD Deficiency (G6PD-AV), and Healthy Controls (HC)*

	Proband	X-CGD	G6PD-AV	HC
Neutrophils	0.3	0.3	19.6	9.4
Mononuclear leukocytes	0.0	0.0	4.6	5.5

*Values are given as mean of 3 determinations.

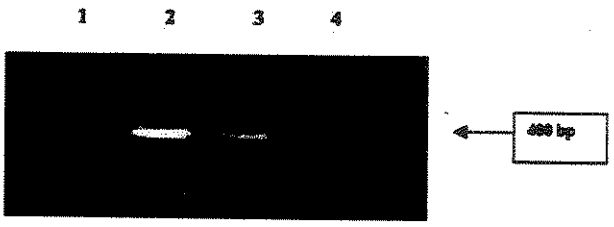

Fig. 2. RT-PCR analysis of p47-*phox* gene expression. Agarose gel electrophoresis (2%) stained with ethidium bromide of PCR products. Lane 1, molecular weight marker (100 bp); lane 2, healthy control; lane 3, proband with normal expression; lane 4, negative control.

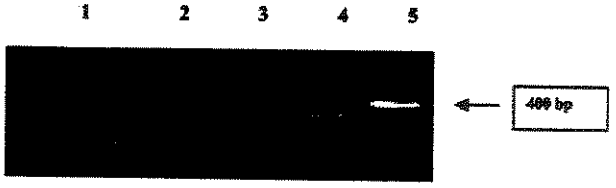

Fig. 3. RT-PCR analysis of gp91-*phox* gene expression. Agarose gel electrophoresis (2%) stained with ethidium bromide of PCR products. Lane 1, molecular weight marker (100 bp); lane 2, X-linked CGD no expression compared to the healthy control; lane 3, another X-linked CGD patient with reduced expression; lane 4, proband with a lower size product; lane 5, healthy control.

fragment from his gp91-*phox* transcript was 300–400 bp smaller than that of healthy controls, suggesting the presence of a splicing defect.

SSCP analysis of gDNA (Fig. 4) revealed a faster electrophoretic mobility of gp91-*phox* exon 3 in the proband sample. Figures 5 and 6 show sequence analysis of gDNA and cDNA, respectively, of this region in gp91-*phox*. Sequencing of exon 3 in the gDNA showed a 264 G→A substitution at the splice junction. Further sequencing of the cDNA showed a deletion of exon 3, giving rise to unstable or nonfunctional mutant gp91-*phox* protein and hence to the phenotype of CGD.

DISCUSSION

We have studied a male patient with a clinical history of chronic anemia and a diagnosis of erythrocyte G6PD deficiency. In addition, this patient also had recurrent severe infections, with exacerbation of the anemia during infections.

The bactericidal activity of phagocytes depends primarily on the activation of the NADPH oxidase system and the subsequent release of oxygen free radicals within the phagolysosome. G6PD is the first enzyme in the hexose monophosphate shunt pathway, in which glucose-6-phosphate (G6P) is converted to 6-phosphogluconate and $NADP^+$ is reduced simultaneously to

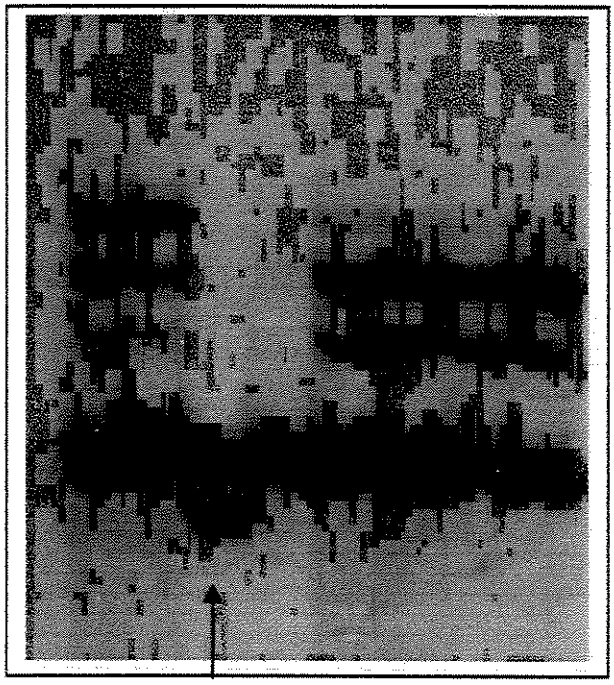

Fig. 4. SSCP analysis of genomic DNA amplified by PCR. Arrow shows abnormal electrophoretic mobility of gp91-*phox* exon 3 from the proband compared to healthy controls in all other lanes.

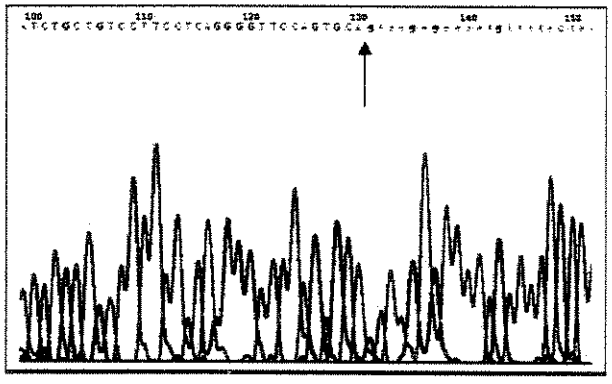

Fig. 5. Genomic DNA sequence of the proband showing a substitution (guanine→adenine) at the 3′ splice junction of gp91-*phox* exon 3. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

NADPH, the substrate for the NADPH oxidase responsible for the respiratory burst.

Assays of phagocyte oxidase activity in the proband demonstrated impaired respiratory burst activity. Thus we needed to determine if the impairment of this respiratory burst activity was due to severe G6PD deficiency in his leukocytes or to a concurrent genetic disorder, i.e., chronic granulomatous disease.

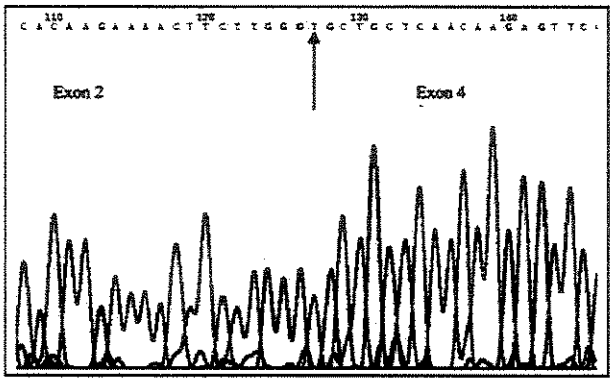

Fig. 6. cDNA sequence of the proband gp91-*phox* transcript showing deletion of exon 3. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

African variants and other mild forms of G6PD deficiency do not produce only intermittent (not chronic) hemolysis in response to oxidative stress (e.g., infection, antimalarial drugs). Only the more severe forms of G6PD deficiency, the class I variants, produce chronic hemolysis [11,20]. The digestion of the proband's PCR-amplified gDNA with the restriction endonuclease *Nla*II detected the 202 G→A mutation in the exon 4 of the G6PD gene and confirmed the African variant, the most frequent G6PD deficiency in the population of southeastern Brazil [11,21]. Thus, the chronic anemia of the proband could be explained in part by the association the recurrent severe infections and the African variant phenotype of G6PD deficiency.

However, considering that the African variant of G6PD deficiency could not solely explain the clinical history of recurrent severe infections of the proband, a series of comparative biochemical assays with phagocytes from the proband, healthy controls, a patient with known X-linked CGD, and another patient with previously characterized African variant of G6PD deficiency were performed. The results showed impaired respiratory burst activity in the proband, similar to the patient with X-linked CGD, but this was not observed in the phagocytes of the patient with the African variant phenotype of G6PD deficiency.

Definitive diagnosis of CGD requires the demonstration of the defective component of the NADPH oxidase system and the detection of the underlying mutation responsible for the CGD phenotype [17]. Further molecular screening by RT-PCR demonstrated a smaller fragment between exons 1 and 5 in gp91-*phox* transcripts from the proband. SSCP analysis of his gDNA revealed faster electrophoretic mobility of gp91-*phox* exon 3. Sequence analysis of gDNA revealed a 264 G→A substitution at exon 3

splice junction. Sequencing of the cDNA showed a deletion of exon 3, giving rise to unstable or nonfunctional mutant gp91-*phox* and hence to the CGD phenotype, as previously reported [4,6].

Several authors have described severe G6PD deficiency leading to an impairment of the respiratory burst activity [9,8,22–27]. However, this is the first report of concurrent G6PD deficiency and CGD genotypes. The African variant of G6PD deficiency is considered mild and unlikely to affect respiratory burst activity. However, one has to consider that in patients with G6PD deficiency, localized hypoxemia and oxidative stress at sites of infection could exacerbate both conditions to produce hemolysis and further impairment of microbial killing. Future studies of infections or stress conditions in vitro or in vivo in G6PD-deficient individuals may determine whether this condition can interfere in defense mechanisms dependent on respiratory burst activity or can worsen the CGD phenotype. However, our studies support the CGD phenotype as the major cause of recurrent infections in this patient, with chronic anemia secondary to the infections.

We suggest that clinicians caring for a G6PD-deficient patient with a severe infection consider the possibility of temporary or local impairment of phagocyte microbicidal activity related to lack of substrate for the NADPH oxidase. This defect can be the result of an unusual association of CGD, as in the present case, or due to the aggravation of milder G6PD phenotypes under stressful conditions.

REFERENCES

1. Curnutte JT, Orkin SH, Dinauer MC. Genetic disorders of phagocyte function. In: Stamatoyannopoulos G, Nienttuis A, Majerus P, and Varmus H, editors. The molecular basis of blood diseases. 2nd edition. Philadelphia: W.B. Saunders Company; 1994. p 493–522.
2. Beutler E. G6PD deficiency. Blood 1994;84:3613–3636.
3. Johnston RB Jr. Clinical aspects of chronic granulomatous disease. Curr Opin Hematol 2001;8:17–22.
4. Roos D, de Boer M, Kuribayashi F, et al. Mutations in the X-linked and autosomal recessive forms of chronic granulomatous disease. Blood 1996;87:1663–1681.
5. Cross AR, Noack D, Rae J, Curnutte JT, Heyworth PG. Hematologically important mutations: the autosomal recessive forms of chronic granulomatous disease (first update). Blood Cells Mol Dis 2000;26:561–565.
6. Heyworth PG, Curnutte JT, Rae J, et al. Hematologically important mutations: X-linked chronic granulomatous disease (second update). Blood Cells Mol Dis 2001;27:16–26.
7. Beutler E, Vulliamy TJ. Hematologically important mutations: glucose-6-phosphate dehydrogenase. Blood Cells Mol Dis 2002;28: 93–103.
8. van Bruggen R, Bautista JM, Petropoulou T, et al. Deletion of leucine 61 in glucose-6-phosphate dehydrogenase leads to chronic nonspherocytic anemia, granulocyte dysfunction, and increased susceptibility to infections. Blood 2002;100:1026–1030.
9. Roos D, van Zwieten R, Wijnen JT, et al. Molecular basis and enzymatic properties of glucose 6-phosphate dehydrogenase volendam, leading to chronic nonspherocytic anemia, granulocyte dysfunction, and increased susceptibility to infections. Blood 1999; 94:2955–2962.
10. Patino PJ, Perez JE, Lopez JA, et al. Molecular analysis of chronic granulomatous disease caused by defects in gp91-*phox*. Hum Mutat 1999;13:29–37.
11. Saad ST, Salles TS, Carvalho MH, Costa FF. Molecular characterization of glucose-6-phosphate dehydrogenase deficiency in Brazil. Hum Hered 1997;47:17–21.
12. Loos JA RD, Weening RS, Houwerzijl J. Familial deficiency of glutathione reductase in human blood cells. Blood 1976;48:53.
13. Boyun A. Isolation of mononuclear cells and granulocytes from human blood. Scand J Clin Lab Invest 1968;21 (Suppl 97):1–77.
14. Condino-Neto A, Newburger PE. NADPH oxidase activity and cytochrome b_{558} content of human Epstein-Barr-virus-transformed B lymphocytes correlate with expression of genes encoding components of the oxidase system. Arch Biochem Biophys 1998; 360:158–164.
15. Condino-Neto A, Newburger PE. Interferon-γ improves splicing efficiency of CYBB gene transcripts in an interferon-responsive variant of chronic granulomatous disease due to a splice site consensus region mutation. Blood 2000;95:3548–3554.
16. Beutler E. Glucose-6-phosphate dehydrogenase deficiency. In: Stambury JBW, Fredrikson DS, Goldstein JL, and Brown MS, editors. The metabolic basis of inherited disease. New York: McGraw-Hill; 1983. p 1629–1653.
17. Conley ME, Notarangelo LD, Etzioni A. Diagnostic criteria for primary immunodeficiencies. Representing PAGID (Pan-American Group for Immunodeficiency) and ESID (European Society for Immunodeficiencies). Clin Immunol 1999;93:190–197.
18. McCord JM, Fridovich I. Superoxide dismutase. An enzymic function for erythrocuprein (hemocuprein). J Biol Chem 1969;244: 6049–6055.
19. Maniatis T, Fritsch EF, Sambrook J. Molecular cloning. A laboratory manual. 2nd edition. Plainview, NY: Cold Spring Harbor Laboratory Press; 1990.
20. Beutler E. G6PD: population genetics and clinical manifestations. Blood Rev 1996;10:45–52.
21. Compri MB, Saad ST, Ramalho AS. [Genetico-epidemiological and molecular investigation of G-6-PD deficiency in a Brazilian community]. Cad Saude Publ 2000;16:335–342.
22. Baehner RL, Johnston RB Jr. Nathan DG. Comparative study of the metabolic and bactericidal characteristics of severely glucose-6-phosphate dehydrogenase-deficient polymorphonuclear leukocytes and leukocytes from children with chronic granulomatous disease. J Reticuloendothel Soc 1972;12:150–169.
23. Gray GR, Stamatoyannopoulos G, Naiman SC, et al. Neutrophil dysfunction, chronic granulomatous disease, and non-spherocytic haemolytic anaemia caused by complete deficiency of glucose-6-phosphate dehydrogenase. Lancet 1973;2:530–534.
24. Corberand J, De Larrard B, Vergnes H, Carriere JP. Chronic granulomatous disease with leukocytic glucose-6-phosphate dehydrogenase deficiency in a 28-month-old girl. Am J Clin Pathol 1978;70:296–300.
25. Bellanti JA, Cantz BE, Schlegel RJ. Accelerated decay of glucose 6-phosphate dehydrogenase activity in chronic granulomatous disease. Pediatr Res 1970;4:405–411.
26. Rutenberg WD, Yang MC, Doberstyn EB, Bellanti JA. Multiple leukocyte abnormalities in chronic granulomatous disease: a familial study. Pediatr Res 1977;11:158–163.
27. Erickson RP, Stites DP, Fudenberg HH, Epstein CJ. Altered levels of glucose-6-phosphate dehydrogenase stabilizing factors in X-linked chronic granulomatous disease. J Lab Clin Med 1972;80:644–653.