

Chronic granulomatous disease. Diagnosis by the dihydrorhodamine assay

Enfermedad granulomatosa crónica. Diagnóstico mediante el ensayo de dihidrorodamina

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What do we know about the subject matter of this study?

There is extensive information on the subject under investigation. Diagnoses of CGD through the DHR assay have been reported for two decades, as well as detection of carriers and inheritance patterns, however, in Paraguay is the first time that such information is obtained.

What does this study contribute to what is already known?

The study reinforces existing information on cases of CGD, carriers and inheritance patterns identified using the DHR assay. These data are relevant for countries in the region that are not yet working with this technique and for our country where we recently implemented it through this study.

Abstract

Chronic granulomatous disease (CGD) is characterized by an alteration of the neutrophil oxidative function. Its inheritance patterns are linked to the X chromosome (X-linked CGD) and autosomal recessive (AR CGD). The dihydrorhodamine (DHR) assay is used for the diagnosis and detection of carriers and provides information on inheritance patterns. **Objective:** To detect CGD cases in children with recurrent infections and to evaluate their female relatives through the DHR assay to identify carriers and obtain information about possible inheritance patterns. **Patients and Method:** 107 patients (< 18 years of age) with clinical suspicion of CGD such as pneumonia, lymphadenopathies, and abscesses were included, referred by physicians from public hospitals between 2014 and 2017. Six female relatives of children with CGD were also included. The DHR assay was performed on all patient samples and the results were expressed as neutrophils stimulation index (SI). **Results:** The median age of patients was 3 years and 62/107 of them were male. The average SI was 39.7 ± 13.8 and a complete shift of DHR was found in 101/107 children. In 2/107 children, no DHR shift was observed (SI = 1.0) indicating possible X-linked CGD, and a third child showed a slight DHR shift (SI = 4.8) compatible with AR CGD. 5/6 female relatives presented a bimodal pattern, showing a carrier status. **Conclusions:** Three cases of CGD and five female carriers were detected through the DHR assay, being the first time that this technique was used in Paraguay. Information on the most likely inheritance patterns, two X-linked CGD, and one AR CGD case was also obtained.

Keywords:

Chronic Granulomatous Disease; X-linked Chronic Granulomatous Disease; dihydrorhodamine-123; Paraguay

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Introduction

Chronic granulomatous disease (CGD) is a rare genetic pathology, with an approximate incidence of 1:250,000 live births. CGD is caused by a phagocyte NADPH oxidase complex defect, mainly in neutrophils, resulting in deficient production of reactive oxygen species (ROS). This mechanism is essential for the elimination of bacteria and fungi¹. NADPH oxidase complex is composed of two cellular membrane proteins, gp91phox and p22phox, coded in *CYBB* and *CYBA* genes, respectively, in addition to three other cytosolic components, p47phox, p67phox and p40phox, coded by the *NCF1*, *NCF2*, and *NCF4* genes, respectively. There are two CGD inheritance patterns, one linked to the X chromosome (X-linked CGD) and another one of autosomal recessive form (AR CGD). Mutations in the *CYBB* gene lead to the X-linked pattern of the disease and represent 70% of the cases. Autosomal recessive mutations in *NCF1* (p47phox) occur in 20% of the cases, whereas mutations in the *CYBA* (p22phox) and the *NCF2* (p67phox) genes, each represents 5% of the reported cases^{2,3}. Usually, children with X-linked CGD present more severe infections, the onset of symptoms is earlier and mortality is higher than in the AR CGD^{1,2}.

CGD is diagnosed by the absence or markedly reduced oxidase activity of the neutrophils when they are stimulated to produce ROS in the so-called “respiratory burst”. The most frequently used tests to evaluate the respiratory burst are the nitroblue tetrazolium (NBT) reduction test and the dihydrorhodamine (DHR) oxidation assay. NBT reduction has disadvantages such as subjectivity and visual reading of a reduced number of cells, whereas DHR assay is a quick test with high sensitivity for CGD diagnosis⁴. The dihydrorhodamine diffuse inside the cell through the plasmatic membrane where it reaches the mitochondria and is oxidated to rhodamine, which emits an intense fluorescence that is measured by flow cytometry⁵.

In addition, the DHR assay provides information about possible CGD inheritance patterns through the identification of carriers among the female relatives on the mother’s side of the affected patients. Female carriers show two populations of phagocytes (bimodal pattern) in flow cytometry, one does not activate and the other one does it normally, in which case the inheritance pattern could be X-linked CGD. These women are at risk of developing autoimmune conditions such as discoid lupus erythematosus, CGD type infections and/or inflammatory bowel disease⁶⁻⁸.

Reports on CGD in Paraguay are very limited^{9,10} and the National Primary Immunodeficiency (PID) Center registered only eight cases in 21 years between 1992 and 2013 (unpublished data). Those patients were diagnosed using the NBT assay and there is no

information about the CGD inheritance pattern in these children. Data currently available in this center are not enough to contact these patients. On the other hand, we presume that the clinical suspicion from physicians is deficient, which could be due to the lack of knowledge of the pathology or because of thinking that is a “very rare” disease. Also, the availability and access to diagnostic tests are limited in our country. Considering all the above, we assume that CGD could be underdiagnosed and under-registered in Paraguay. In 2013, we initiated the standardization and implementation of the DHR assay at the *Instituto de Investigaciones en Ciencias de la Salud*, the only center in the country that provides screening tests for CGD. This was the first time in Paraguay that this technique was used. The objective of this study was to detect CGD cases in a child population with recurrent infections and to evaluate their female relatives on the mother’s side using the DHR assay, in order to identify carriers and obtain information about the most likely inheritance patterns of the disease.

Patients and Method

Patients

The participants were of both sexes and younger than 18 years of age. Children with clinical features suggestive of CGD were referred by specialist physicians to the investigation center, from different public hospitals of the country, between March 2014 and July 2017. Female relatives on the mother’s side of children diagnosed with CGD during this study were also included and their carrier status was evaluated.

Selection criteria

Children with clinical features suggestive of CGD were selected with the collaboration of specialists physicians (pediatricians, infectious disease specialists, allergists and immunologists) from five public reference hospitals of the country, whom referred patients with clinical manifestations (≥ 2 times per year) such as severe and/or recurrent pneumonia, lymphadenopathies, recurrent bacterial and fungal infections on skin/mucosa, cutaneous and/or organ abscesses, prolonged fever of unknown origin, and osteomyelitis. Children with hematologic oncological diseases were excluded, as well as patients with secondary immunodeficiencies caused by drugs and/or HIV infection. Female relatives of the children diagnosed with CGD during this study were also included. Samples of their mothers, a grandmother and two aunts on the maternal side, who agreed to participate in this work, were also analyzed.

Blood samples

1 ml of blood was taken through venipuncture and

collected in a tube with EDTA as anticoagulant. In the case of hospitalized patients, the sample was preserved at room temperature and immediately transported to the investigation center. The DHR assay was carried out in a time that did not exceed four hours after the blood extraction.

Evaluation of the neutrophils oxidative function using the DHR assay

The evaluation was performed following the protocol described by Acosta et al.¹¹, including some modifications. In summary, 4 ml of lysing solution NH₄Cl 0.83g% were added to 100 µl of blood for 3 minutes, then this was centrifuged at 1200 rpm for 3 minutes and the cells were resuspended in 1 mL of PBS buffer. Two tubes were identified as S (stimulated) and NS (non-stimulated) with 340 µL of cell suspension each, 150 µL of DHR solution (Invitrogen, Oregon, USA) were added to a final concentration of 87µM and they were incubated at 37°C for 5 minutes, then, 150 µl of PMA 3.54µM solution (Phorbol 12-myristate 13-acetate, Sigma-Aldrich, USA) were added to the S tube and 150 µl of PBS to the NS tube, incubating them for 15 minutes at 37 °C. Later, samples were acquired with a FACS Calibur flow cytometer (Beckton Dickinson, San José CA, USA) and analyzed using CellQuestPro software. Results were expressed as neutrophils stimulation index (SI), ratio between the fluorescence mean intensity (FMI) of the S tube and the FMI of the NS tube.

Ethical disclosures

Oral and written consent form of the study was obtained from the parents or guardians of children. Female relatives on the mother's side of the children with CGD also signed informed consent and carriers received genetic counseling. All data from the patients were protected with absolute confidentiality. The protocol was approved by the Research Ethics Committee from the *Instituto de Investigaciones en Ciencias de la Salud*.

Statistical analysis

The data were loaded into a Microsoft Excel spreadsheet version 8.0 and analyzed with the statistical software Epi Info™ CDC version 7.2 for Windows. Categorical variables were described as absolute and relative frequencies, whereas the quantitative variables were expressed as medians and interquartile ranges (IQR), or averages and standard deviations (SD) according to the variable distribution.

Results

In this study, 107 patients were included, from which 58% (62/107) were male and their ages ranged

from 1 month to 15 years (median: 3 years). The more frequent clinical manifestations were pneumonia 45% (48/107), skin infections 18% (19/107), lymphadenopathies 13% (14/107), and abscesses 9% (10/107). The age of onset of these clinical manifestations presented a median of 2 years (IQR: 1 – 6 years). In 23% (24/107) of the patients, family history related to primary immunodeficiency and/or chronic granulomatous disease was observed, such as at least one brother or sister with recurrent infections (12%), and death of male relatives younger than two years of age (4%). Consanguinity between the parents was not observed in any child. Regarding the oxidative function of neutrophils ("respiratory burst") evaluated through the DHR assay, when considering the total population, an average SI of 39.7±13.8 was found (IQR: 32.0-50.6). However, in 3/107 children, SI values as low as 1.0 (patients #1 and #2) and 4.8 (patient #3) were observed. Table 1 shows in detail the described characteristics.

Table 1. Characterization of the study population

Characteristics	Total population n = 107	Patients with CGD		
		#1	#2	#3
Male:female	62:45	m	m	m
Age in years; median (IQR)	3 (1-7)	1	1	7
SI by DHR assay; mean±SD	39.7 ± 13.8	1.0	1.0	4.8
<i>Clinical manifestation; n (%)</i>				
Age at onset in years; median (IQR)	2 (1-6)	0.3	0.2	3
Pneumonia	48 (45)	+	+	+
Skin infection	19 (18)	+	-	-
Lymphadenopathy	14 (13)	-	+	+
Abscess (cutaneous and/or organ)	10 (9)	+	-	+
Prolonged fever of unknown origin	8 (7)	-	+	-
Osteomyelitis	4 (4)	-	-	-
Meningitis	2 (2)	-	-	-
Sepsis	2 (2)	+	-	-
<i>Family history</i>				
Brother or sister with recurrent infections	13 (12)	+	-	-
Female relatives (maternal side) with AD	6 (6)	-	+	-
Death of males younger than 2 years of age	4 (4)	-	+	-
Diagnosed case of PID in the family	1 (1)	-	-	-
Consanguinity between the parents	0 (0)	-	-	-

%; percentage; m: male; (IQR): interquartile range; SI: neutrophil stimulation index; SD: standard deviation; ≥: equal to or greater than; (+): observed characteristic; (-): unobserved characteristic; AD: autoimmune disease; PID: primary immunodeficiency. CGD: Chronic granulomatous disease

Regarding the DHR histograms obtained through flow cytometry, those corresponding to non-stimulated assays showed a peak or basal fluorescence signal (black line without filling in figure 1 histograms). By stimulating with PMA (gray filled curves in Figure 1 histograms) in the neutrophils of 101/107 patients, a complete fluorescence signal shift was observed (Figure 1A), indicating a normal respiratory burst of neutrophils in these patients. However, in 2/107 of the children, the patients #1 and #2 previously mentioned (both one-year-old boys), no shift was observed in the fluorescence histogram (figure 1B), resulting in both cases an SI of 1.0. These findings indicate an absence of neutrophil respiratory burst. In a third child (patient #3, a seven-year-old boy) a slight shift of the fluorescence signal was observed with a SI = 4.8 (figure 1C), which is interpreted as a very low respiratory burst production in the neutrophils of this patient.

The collection of new samples was requested for patients #1, #2 and #3 and the evaluation of these second samples provided the same results of SI through the DHR assay. At the same time that patient samples from this study were processed, a sample of an apparently healthy subject was also processed in order to have a normal control of neutrophil respiratory burst. All samples from healthy controls showed a complete fluorescence shift when stimulating neutrophils with PMA and their histograms were the same as shown in figure 1A.

Up to this point, we described the normal neutrophil respiratory burst observed in 101/107 patients and healthy controls, as well as lack of or very low respiratory burst production found in patients #1, #2 and #3. Contrary to these results, an unusual DHR pattern was observed in the histograms of 3/107 children. Regarding the shift and base width of the fluorescence signal, incomplete or shorter fluorescence shifts and peaks with broader base were found in these patients, resulting the following SI values 20.3 (Figure 1D), 17.9 (Figure 1E), and 14.6 (Figure 1F). These results indicate a lower neutrophil respiratory burst production compared to healthy controls. As in the previous cases, the collection of new samples of these patients and their mothers' samples was requested to corroborate the results. These samples were not collected in the course of this study.

The mothers of patients #1, #2 and #3 were evaluated with the DHR assay and, in the case of patient #2, the assay was also carried out in two aunts and in the grandmother, all of them on the mother's side. In the stimulated assays of 4/6 of the evaluated women, including the mothers of patients #1 and #2, one of the aunts and the grandmother of patient #2, two peaks of fluorescence (bimodal pattern) were observed that corresponded to a population of non-activated neu-

trophils (20-30% with SI=1.0-1.7) and another population of neutrophils (70-80% with SI=32.9-38.5) that were normally activated (Figure 1G). The other aunt of patient #2 also presented a bimodal pattern, but the population of activated neutrophils was 48% with an SI=34.7 (Figure 1H). A bimodal pattern in the DHR assay indicates carrier status and the most likely inheritance pattern in these cases is X-linked CGD.

In the mother of patient #3, no bimodal pattern was observed, the DHR histogram showed only one population of neutrophils with normal stimulation and SI=42.3 (Figure 1I). It can be inferred that the most likely inheritance pattern is AR CGD.

Discussion

The production of ROS by phagocytes in the so-called "respiratory burst" is an essential component of the innate immune response. Genetic defects in any protein subunits of NADPH oxidase complex, lead to CGD that is clinically characterized by recurrent bacterial and fungal infections and granulomatous inflammation¹².

In this study, the most frequent clinical manifestations observed in the evaluated child population were pneumonia, skin infections, lymphadenopathies, and abscesses. The literature refers to pneumonia, suppurative lymphadenitis, cutaneous abscesses and/or abscesses in different organs as predominant clinical manifestations of CGD^{2,3,13}. Therefore, clinical suspicion in the patients referred to participate in this study was consistent with what was reported as suggestive clinical features of this pathology.

In patients #1, #2 and #3, the CGD diagnosis can be established because they showed features that are consistent with the reports about this pathology^{2,3}, such as clinical manifestations, an abnormal SI in the DHR assay, family history, and early age at the onset of symptomatology. On the other hand, in the children that presented clinical characteristics suggestive of CGD but their SI was normal, the diagnosis of this pathology can be ruled out. However, in these patients, it would be interesting to evaluate other immune system components such as lymphocytes subpopulations, immunoglobulins concentrations, and complement system proteins to rule out other primary immunodeficiencies different from the one that was screened in this study, especially if the symptomatology persists.

In this experience, the oxidative function of neutrophils was measured using de DHR assay, and we observed that the average SI in children with clinical suspicion of CGD but with normal neutrophil function, presented a good concordance with what was reported for healthy children^{11,14}. These findings show

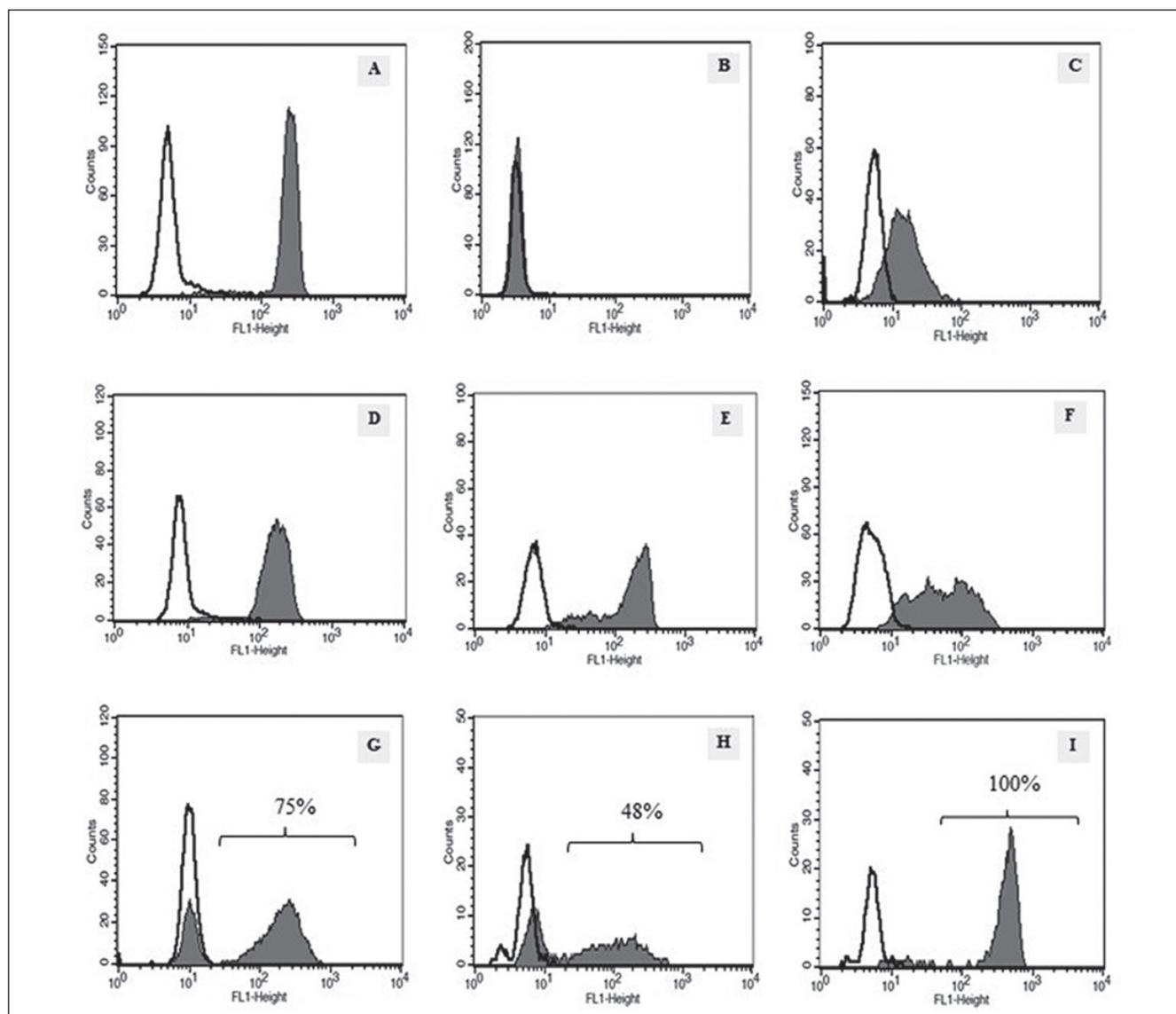


Figure 1. Fluorescence histograms obtained in the DHR assay by flow cytometry. A) Complete fluorescence shift with PMA stimulation observed in 101/107 children with recurrent infections and in healthy controls. B) Non shifted fluorescence signal indicating an absence of neutrophil respiratory burst in patients #1 and #2 with X-linked CGD (SI=1.0). C) Slight fluorescence shift showing a very low neutrophil respiratory burst production in patient #3 with CGD of probable AR inheritance pattern (SI=4.8). D), E) and F) Shorter fluorescence shift and broader base peak in 3/107 patients with an unusual DHR pattern indicating an abnormal neutrophil respiratory burst production compared with healthy controls (SI=20.3, 17.9 and 14.6, respectively). G) Two fluorescence peaks (bimodal pattern) with 75% activated neutrophils found in 4/6 female relatives of patients #1 and #2 indicating a carrier status and X-linked inheritance pattern of CGD. H) Bimodal pattern with 48% of normal activation observed in one of the aunts of the patient #2, also showing a carrier status. I) Absence of bimodal pattern with a single fluorescence peak of 100% neutrophil activation in the mother of patient #3, showing the absence of carrier status in the mother and probable AR CGD in the patient. *Counts: counted cells; FL1-Height: rhodamine fluorescence; Black line without filling: basal fluorescence signal in non stimulated assays; Gray filled curves: fluorescence signal in stimulated assays with PMA*

what other authors have previously proposed¹⁵⁻¹⁷, the efficacy of the DHR test to discriminate very accurately a CGD case from others that are not affected by this pathology, even though the clinical manifestations were compatible with those presented in CGD.

The CGD diagnosis was usually made by the NBT test. However, this test presents disadvantages such as

subjectivity, and currently the conversion evaluation from dihydrorhodamine to rhodamine through flow cytometry is more used. The DHR assay provides information about the most likely inheritance patterns of CGD because in most of the X-linked CGD cases no changes are observed in the DHR when stimulating the neutrophils, whereas, the autosomal recessive cases

(AR CGD) show a slight shift of the DHR and a broad-based histogram^{4,5}. Patients #1 and #2 of this study presented a histogram without shift in the stimulated assay and SI of 1.0, this finding indicates an X-linked CGD^{4,15,18}. In patient #3, the histogram showed a slight fluorescence shift and SI of 4.8, which is compatible with AR CGD⁴. Vowells et al.^{16,17} found in patients with CGD gp91phox y p47phox SI average values of 1.3 and 13.2 respectively. Our SI values in patients #1, #2 and #3 were very similar to those of these reports.

The detection of female carriers using the DHR assay indicates that the inheritance pattern of CGD could be linked to the X chromosome¹⁵. Regarding the evaluation of female relatives of children diagnosed with CGD, we observed that 5/6 of these women, relatives of the patients #1 and #2, showed a bimodal pattern in the DHR assay, which indicates carrier status and that the most likely inheritance pattern in this family is X-linked CGD. This finding coincides with the DHR patterns observed in these children. This inheritance pattern is the most frequent around the world^{19,20}, it is so that several studies of other countries report that approximately 2/3 of the patients present the form of CGD linked to the X chromosome²¹. However, the AR CGD is reported as the most common form of CGD in parts of the world with high consanguinity rates^{22,23}. Contrary to what was observed in the female relatives of patients #1 and #2, in the mother of patient #3, only one cell population with normal activation was found. If the mother does not show a bimodal pattern, there is more than one possibility regarding the CGD inheritance pattern in the family. A *de novo* mutation in the *CYBB* gene of the patient is a plausible explanation, that is, the mutation is present in the X chromosome but was not inherited from the mother. The other possibility, which would be the most likely, is that there is an autosomal recessive inheritance pattern in the family²⁴. In fact, in patient #3, the DHR pattern that was observed is compatible with AR CGD. On the other hand, only one female carrier showed less than 50% of activated neutrophils. It is important to know this normal activation percentage in carriers since women with less than 20% of normally activated neutrophils have higher risk of suffering severe infections²⁵.

We also decided to discuss the case of the three children in whom we found a different pattern of the DHR, specifically a smaller fluorescence shift and a peak with a broader base. In these cases, it should be considered that an atypical X-linked CGD is described, in which a DHR pattern overlaps or even present a higher SI than the one observed in p47phox-AR CGD^{4,26}. Besides, complete myeloperoxidase deficiency²⁷, as well as a blood sample that is not preserved and transported in optimal conditions²⁶, could result in histograms with incomplete fluorescence shifts. Molecular assays that

would allow the definition of atypical cases of X-linked CGD are still not available in Paraguay. Thus, in order to have a more precise interpretation of the results of these three patients, we requested the collection of new samples from the children and their mothers, but we have not achieved this up to this date.

We consider important to mention that these three children (patients #1, #2 and #3) were referred to a physician specialized in PID to initiate preventive treatment. It is important to emphasize that, once all of the patient's data have been evaluated, both the clinical characteristics and the neutrophils function results measured with the DHR assay, as well as the family history and even the presence of female carriers in the family, and that they are consistent to establish the CGD diagnosis, the following step is the immediate start of the prophylactic oral therapy with antibiotic and antifungal, currently considered the conventional management of patients affected by this pathology. In some countries, the interferon-gamma (IFN- γ) treatment is added, even though the effectiveness of this treatment is not yet clear. Nowadays, the allogeneic transplantation of hematopoietic stem cells is the only curative treatment, and the gene therapy has been tested in several centers²⁸. However, those treatments are not yet available in Paraguay, so we resort to the combined use of trimethoprim/sulfamethoxazole and itraconazole, with the purpose of reducing the frequency and severity of the infections which substantially improves the quality of life of the affected patients.

We pretend to join efforts in order to make possible the access to hematopoietic stem cells transplant in Paraguay, to offer the possibility of a healing treatment to the patients with CGD, especially the ones with X-linked inheritance pattern that show a more serious prognostic.

Finally, since the prognosis of the disease depends on the inheritance pattern, we emphasize that it is very important to evaluate the female relatives on the mother's side of children with CGD, in order to detect the carrier status and thus determine if it is an X-linked CGD or an AR CGD. Genetic counseling should be offered to female carriers and inform them about their higher risk of developing autoimmune diseases. The possibility of identifying the most likely inheritance pattern through a test that is quick, sensitive and low cost as the DHR assay, has a great value in countries like Paraguay, where the molecular techniques to confirm the CGD genotype are not yet available. Given this situation, we highlight the need to work on the implementation of the molecular technique for the genotypic study of patients with CGD in the future, because this method is the Gold Standard for diagnosis and detection of the inheritance pattern in the family. However, the objective clinical evaluation of the pa-

tient and the application of the DHR assay allow us to establish with high accuracy the phenotypic diagnosis of this disease, which is crucial to be done early for the immediate therapeutic approach of the patient.

Conclusions

In conclusion, we detected three CGD cases in a child population with recurrent infection and five female carriers were identified through the DHR assay, being the first time that this technique was used in Paraguay. Information about the most likely inheritance patterns was also obtained in this study, two cases of probable X-linked CGD and one probable case of AR CGD. According to the diagnostic records, these three cases detected in a period of three years could indicate an improvement of the CGD diagnosis in Paraguay.

Ethical Responsibilities

Human Beings and animals protection: Disclosure the authors state that the procedures were followed ac-

ording to the Declaration of Helsinki and the World Medical Association regarding human experimentation developed for the medical community.

Data confidentiality: The authors state that they have followed the protocols of their Center and Local regulations on the publication of patient data.

Rights to privacy and informed consent: The authors have obtained the informed consent of the patients and/or subjects referred to in the article. This document is in the possession of the correspondence author.

Conflicts of Interest

Authors declare no conflict of interest regarding the present study.

Financial Disclosure

Authors state that no economic support has been associated with the present study.

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