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Chronic Granulomatous Disease in Two Sisters

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Two sisters with chronic granulomatous disease (CGD) have been studied. The diagnosis was suggested by the histopathological findings from the spleen and lymph nodes of the proband and confirmed by the low values obtained in the following tests performed on polymorphonuclear leukocytes (PMN): chemiluminescence, nitroblue tetrazolium (NBT) reduction, killing of *Staphylococcus aureus*, and O_2^- production. NADPH oxidase activity was not detected in the homogenates of the patients' PMN but cytochrome *b* was normally present. In addition, PMN depolarization induced by phorbol-myristate acetate was absent, thus suggesting a defect of the activation mechanism of the respiratory enzyme. The normal depolarization induced by ouabain indicated that the membrane polarity regulated by the Na/K pump in the patients' cells was not affected. The low, but not completely absent, respiratory activity of the patients' PMN could suggest an X-linked mode of inheritance with incomplete Lyonization. From a clinical point of view, one sister had mild symptoms whereas the other was almost symptomless, thus confirming once more the heterogeneity of CGD syndrome.

KEY WORDS: Chronic granulomatous disease; female.

INTRODUCTION

Chronic granulomatous disease (CGD) is a pathological condition characterized by defective bactericidal activity of phagocytic cells, directed mainly against catalase-positive microorganisms.

From a clinical point of view, patients with this disorder suffer severe recurrent infections, mostly in the pulmonary and cutaneous areas, and they fail to respond to appropriate antibiotic therapy (1).

The phenotypic expression of the disease is very variable and ranges from mild to severe. Several investigators have attributed the biochemical defect to an absence of NADPH-oxidase or cytochrome *b* (2, 3), to a defect of the "triggering" system (4), or to the presence of "low-affinity" NADPH-oxidase (5).

CGD is a familial disease and its transmission to males clearly indicates an X-linked recessive disorder which can be indirectly verified by family studies. These usually reveal normal fathers but mothers who are asymptomatic heterozygous carriers and a male-to-female ratio of 6–7:1 (1).

The pathogenesis of the defect and its mode of inheritance in female patients have not yet been clearly identified. However, a close observation of familial cases, particularly in the females, could lead to the clarification of the genetic transmission in these cases. It has recently been proposed that the mode of inheritance in females could be either autosomal recessive or X linked, and the phenotypic expression of the disease could probably be explained by a mechanism of chromosome inactivation in accord with Lyon's hypothesis (6).

In this paper we report a study of two sisters with CGD.

MATERIALS AND METHODS

A 17-year-old girl, child of a marriage between third cousins, was hospitalized owing to an acute febrile illness. She had a history of recurrent respiratory infections since infancy.

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During hospitalization her blood cultures were negative, but *Staphylococcus epidermidis* and *Escherichia coli* were isolated from urine samples. She was also splenectomized owing to a suspected systemic disease and fragments of spleen, liver, and lymph nodes were prepared for histology.

The patient had three sisters but one had died of pneumonia at the age of 13 years. Another sister aged 9 years had had recurrent pneumonia since birth. At the age of 7 years she had a submandibulary lymphadenopathy and histology revealed the presence of granulomatous tissue, initially erroneously interpreted as tuberculous. Her eldest sister and her parents are in good health and her paternal and maternal grandparents, uncles, aunts, and their families are alive and well.

Preliminary immunological investigations demonstrated that the proband had normal levels of salivary IgA and serum IgG, IgA, IgM, and C_3, C_4 complement components. Antinuclear antibodies and rheumatoid factor were negative. Polymorphonuclear (PMN) phagocytic and chemotactic activities were also normal.

Enzymatic studies demonstrated normal levels of myeloperoxidase (MPO) and glucose-6-phosphate dehydrogenase (G-6-PD).

The diagnosis of CGD in the proband and her sister was made by the *Staphylococcus aureus* killing assay (7), quantitative (8) and cytologic (9) nitroblue tetrazolium (NBT) test, and chemiluminescence (10) test.

Reagents

Modified Krebs–Ringer phosphate (KRP) medium had 119 m*M* NaCl, 4.75 m*M* KCl, 0.50 m*M* $CaCl_2$, 1.19 m*M* $MgSO_4 \cdot 7H_2O$, 16.6 m*M* sodium phosphate buffer, pH 7.4, 5.56 m*M* glucose.

Zymosan (zymosan A, Sigma Chemical Co., St. Louis, MO) was suspended in 0.9% NaCl at a concentration of 20 mg/ml and boiled for 10 min. After washing, it was resuspended in KRP at a concentration of 10 mg/ml.

Serum-treated zymosan (STZ) was prepared by incubating the zymosan suspension with a pool of fresh human serum (final concentration of serum, 15%) at 37°C for 30 min under continuous shaking. After centrifugation and washing, STZ was resuspended at a concentration of 10 mg/ml.

502 A *Staphylococcus aureus* strain was cultured for 18 hr in nutrient broth (Eugon broth, Difco) and then measured spectrophotometrically at 628 nm, with the optical density (OD) adjusted to 0.23 and the suspension diluted in nutrient broth (1:5).

Phorbol-12-myristate-13-acetate (PMA), luminol, nitroblue tetrazolium (NBT) sodium salt, cytochrome *c* type VI, superoxide dismutase (SOD), *N*-ethylmaleimide (NEM), and diethylenetriaminepentaacetic acid (DPTA) were obtained from Sigma Chemical Co., St. Louis, MO.

PMA was dissolved in dimethylsulfoxide (DMSO) at a concentration of 0.2 mg/ml and stored frozen at −70°C prior to use. A molar absorption coefficient of 24.5 M^{-1} cm^{-1} was used for cytochrome *c* at 550–468 nm. NBT was dissolved in KRP at a concentration of 0.2% and stored at 4°C prior to use. Luminol, 10^{-3} *M*, in DMSO was stored at room temperature in the dark and, prior to use, dissolved in KRP.

Latex beads (0.81μ Ø) were supplied by Serva Feinbiochemica. Dextran (dextran T 500) and Lymphoprep were obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). Dextran was dissolved in 0.9% NaCl at a concentration of 4.5%. Sterile Hank's balanced salt solution (HBSS), pH 7.4, and phosphate-buffered saline (PBS), pH 7.4, were acquired from GIBCO. The fluorescent dye 3,3′-dipropylthiocarbocyanine [di-S-C_3(5)] was a generous gift of Dr. Alan Waggoner. Pyridine was supplied by E. Merck and AG Darmstadt.

Whole Blood Tests

(a) O_2^- production was evaluated as reported by P. Bellavite *et al.* (11). Aliquots of 0.1 ml of blood were added to 10-ml tubes containing 0.4 ml of KRP and 1.5 mg of cytochrome *c*. For each sample, six tubes were prepared with the following components: tubes 1 and 2, controls; tubes 3 and 4, with 0.5 mg of opsonized zymosan; and tubes 5 and 6, with 0.5 μg of PMA. Tubes 1, 3, and 5 each contained 30 μg of SOD.

All the tubes were incubated at 37°C for 15 min with continuous shaking. Incubation was completed by the addition of 2 ml of ice-cold KRP containing 1 m*M* NEM and the tubes were centrifuged at 1500*g* for 10 min. The OD of the cell-free supernatant was determined at 550–468 nm, and the activity calculated by the following formula:

$$\text{nmol } O_2^-/0.1 \text{ ml blood} = 102 \times (OD_a - OD_b),$$

where a is a tube without SOD and b is a tube with SOD.

(b) Chemiluminescence activity was determined according to a modification of the method of P. De Sole *et al.* (12). To a 1.0-ml final volume of KRP were added the following: 5 μ*M* luminol, 0.5 mg of zymosan, and 20 μl of a normal donor serum. After 45 min at room temperature in the dark, 100 μl of diluted blood (1:200 with KRP) was added. In other tests, 0.5 μg of PMA or polystyrene beads (latex particles/PMN = 500/1) was substituted for zymosan and serum. In these experiments, no incubation period was necessary. Chemiluminescence was monitored with a Packard Tri-Carb scintillation counter, mod. 2335, on the tritium setting and in the out of coincidence mode. Specific activity was evaluated by dividing the value at the plateau (cpm_{max}) by the PMN present in the vial.

PMN Isolation

Peripheral venous blood was collected with heparin (10 U/ml). PMN isolation and purification were accomplished by dextran sedimentation (4 vol of blood + 1 vol of dextran) for 45 min. The leukocyte-rich supernatant was layered on a Lymphoprep density gradient and centrifuged at 400*g* for 20 min. PMN were collected from the pellet and erythrocytes were lysed by hypotonic shock (30 sec with 0.2–3% NaCl). After washing, PMN were suspended in KRP at useful concentrations for the various tests.

Homogenate Preparation

PMN at a concentration of 2×10^7/ml were incubated in 1.0 ml of KRP medium at 37°C. After 3 min, 2.5 μl of 0.2 mg/ml PMA in DMSO was added for stimulated activity; to a similar sample, 2.5 μl of DMSO was added for resting activity.

Both samples were incubated at 37°C for 3 min; then the cell suspension was immediately centrifuged at 8000*g* and 4°C for 30 sec. The pellet was resuspended with an ice-cold solution containing 0.25 *M* saccharose, 1 m*M* sodium bicarbonate, 1 m*M* sodium azide, and 1 m*M* phenylmethylsulfonylfluoride in a final volume of 1.0 ml. The cell suspension was then sonicated twice for 5 sec at 100 W and 0°C.

NADPH-Oxidase Test

Approximately 30 μl of homogenate was added to a solution containing 0.15 *M* Na-phosphate buffer, pH 7.0, 1 m*M* Na-azide, 0.05% Na-deoxycholate, 1 m*M* DTPA, 80 μ*M* cytochrome *c*, and 150 μ*M* NADPH in a 1.0-ml final volume at 22°C. The reference cuvette also contained 30μg of SOD. The reduction of cytochrome *c* by O_2^- production was followed at 550 nm in a Perkin-Elmer spectrophotometer, mod. 576. The oxidase activity was calculated from the reduction rate by means of the molar extinction coefficient 21.1 mM^{-1} cm^{-1} (13).

Membrane Depolarization

Membrane depolarization studies were accomplished by the fluorescent probe 3,3′-dipropylthiocarboxcyanine as described by P. J. Sims *et al.* (14) and applied by B. Seligmann and J. I. Gallin (15) and F. Rossi *et al.* (16).

To 2.0 ml of KRP were added 4×10^6 PMN, 2 μ*M* di-S-C_3(5), 500 IU of catalase, and 25 μg of SOD. Activation of the PMN was started by the addition of 0.4 μg/ml of PMA. Membrane depolarization was also checked by treating the PMN with the addition of 10^{-4} *M* ouabain.

The difference in fluorescent intensity of di-S-C_3(5) was recorded at 37°C with a spectrofluorimeter (Ciampolini, Italy); excitation and emission wavelengths were 622 and 660 nm, respectively.

Quantitative NBT Test

One-tenth milliliter of PMN suspension (2×10^7 cells/ml), 0.4 ml of NBT solution, and 0.05 ml of 0.1% latex particles (0.81 μ Ø), previously dialyzed against 0.15 *M* PBS, pH 7.4, were incubated at 37°C for 20 min in 0.4 ml of PBS. The control did not contain latex particles. After incubation, the samples were centrifuged at 400*g* for 10 min, the supernatant was removed, and the pellet was resuspended. The reduced dye was extracted with 4.0 ml of pyridine and measured spectrophotometrically at 515 nm. The difference of absorbance between sample and control was considered.

PMN Bactericidal Assay

Mixtures containing 0.1 ml of bacterial suspension in sterile HBSS, 0.4 ml of fresh normal serum, diluted 1:4 in sterile HBSS, and 0.5 ml of PMN at 10^7/ml were incubated at 37°C.

Samples of 0.01 ml were obtained at 0 min and after 60 min. These, in turn, were diluted in 2 ml of

distilled water and 0.1-ml samples mixed in nutrient agar for pour plate cultures.

Plates were incubated overnight at 37°C and the number of viable bacteria was determined by colony count. The results were calculated by the following formula:

$$\text{killing activity (\%)} = \frac{\text{No. of colonies } (t = 60')}{\text{No. of colonies } (t = 0')} \times 100.$$

NBT Slide Test

One-tenth milliliter of PMN suspension (1×10^6 cells/ml), 0.1 ml of NBT, and 5 μl of PMA solution (0.2 mg/ml) were incubated in a water bath at 37°C for 5 min.

After incubation, 1 drop of the mixture and 1 drop of Samson stain were mounted on clean glass slides and 200 cells counted for each sample and classified as NBT positive or NBT negative. Controls were unstimulated PMN incubated without PMA solution. All tests were performed in duplicate.

Cytochrome b *Spectra*

The homogenate preparation was first centrifuged at 250*g* for 10 min, the pellet discarded, and the supernatant centrifuged at 27,000*g* for 20 min in a refrigerated centrifuge.

The supernatant was discarded and the pellet (subcellular pellet) resuspended in 5 m*M* potassium phosphate buffer, pH 8.0, containing 20% glycerol, at a concentration of approximately 1 mg/ml.

The spectral absorption measurement was carried out in a Beckman DU 8 spectrophotometer. Samples were reduced by the addition of a few particles of dithionite.

Table I. NBT Tests, Chemiluminescence, and Bactericidal Activity of PMN from the Patients and Their Relatives

Subject	NBT slide test (%)[a]	Quantitative NBT test (OD)	Killing assay (%)[b]	Chemiluminescence (cpm/PMN)
Father	93	0.49	26	105
Mother	88	0.35	43	75
1st daughter[d]	6	0.06	62	0[c]
2nd daughter[e]	6	0.05	66	0[c]
3rd daughter[f]	90	0.42	24	125
Normal value[g]	≥90	>0.20	≤50	80–130

[a]% of reducing PMN.
[b]% of viable bacteria after 60 min of incubation in comparison to the basal values.
[c]No response was obtained even if different stimuli (1 μg/ml PMA; latex, 1000 particles/cell) and higher concentrations of luminol were employed.
[d]17-year-old girl, affected sister.
[e]9-year-old girl, affected sister.
[f]Healthy daughter.
[g]Obtained from 100 normals.

Table II. O_2^- Production in Whole Blood from the Patients and Their Relatives

Subject	O_2^- production (nmol/15 min/10^6 PMN) Resting	+ opsonized zymosan (1 mg/ml)	PMA (0.5 μg/ml)
Father	1.0	51.2	78.4
Mother	1.3	73.1	84.7
1st daughter[a]	0.6	2.7	1.5
2nd daughter[b]	1.5	1.5	1.0
Normal control	4.4	106.4	74.8

[a]See Table I, footnote *d*.
[b]See Table I, footnote *e*.

RESULTS

The results (means of two determinations on two different occasions) of the NBT slide test, quantitative NBT test, bactericidal assay, and chemiluminescence assay are reported in Table I. As shown in this table, the *S. aureus* killing assay was normal in the father, mother, and healthy daughter, but both sisters with the disease had defective bactericidal activity.

Similar data were found in both the NBT tests and the chemiluminescence assay. It must be stressed that a faint reduction in the NBT slide test was not observed either in the mother or in the patients.

Table II, showing O_2^- production in whole blood, demonstrates an almost absent O_2^- production in

Table III. NADPH-Dependent O_2^- Production by Homogenates of PMN from the Patients and Their Relatives

Subject	O_2^- production (nmol O_2^-/min/10^7 PMN) Resting	+ PMA
Father	2.1	17.2
Mother	2.9	14.8
1st daughter[a]	1.6	1.0
2nd daughter[b]	1.2	1.4
Normal control	1.3	17.2
Normal range	0–3.5	10.1–28.5

[a]See Table I, footnote *d*.
[b]See Table I, footnote *e*.

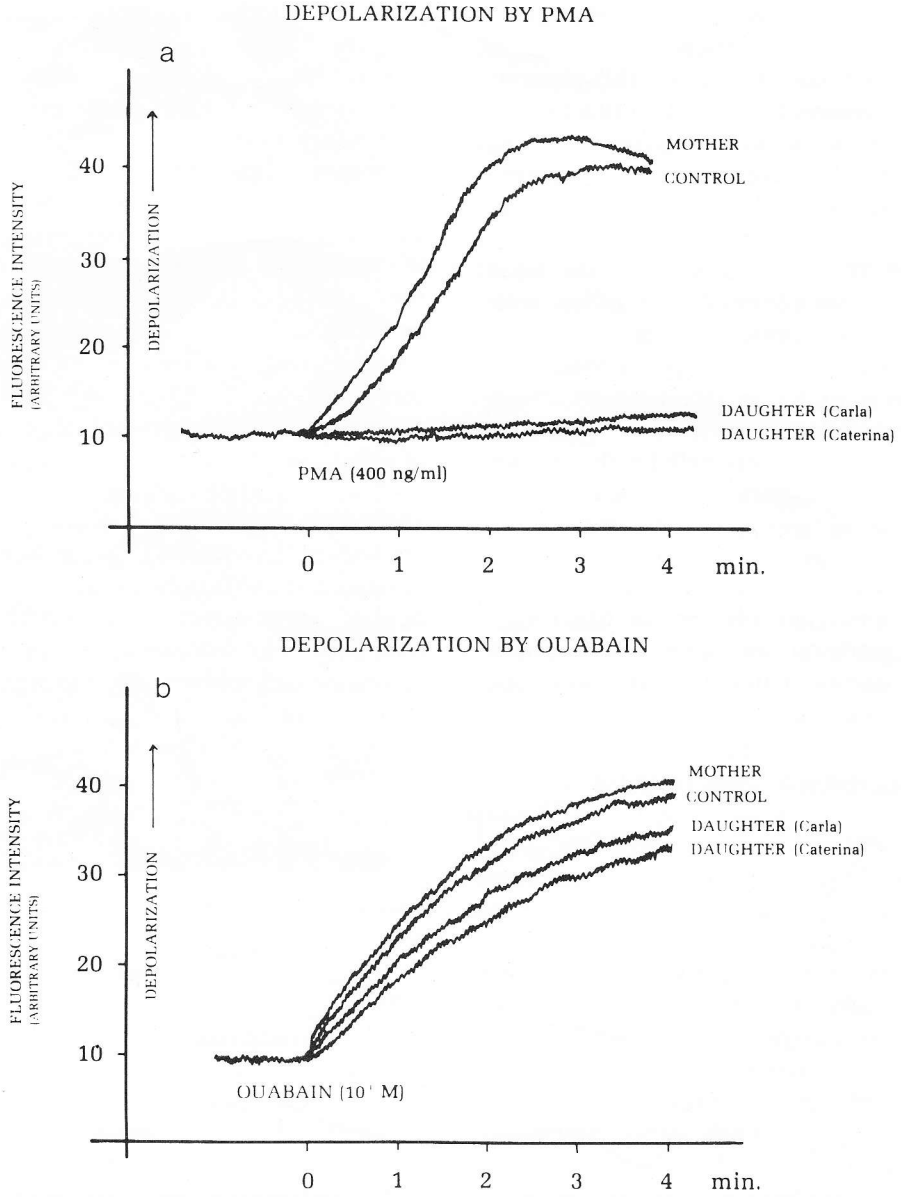


Fig. 1. Polymorphonuclear depolarization in two sisters with CGD induced by PMA (a) and ouabain (b). PMN, 4×10, were incubated for 3–5 min with the fluorescent probe 3,3′-dipropylthiocarbocyanine and the fluorescence was recorded before and after the addition of PMA or of ouabain.

the PMN of both patients with particulate (opsonized zymosan) and soluble (PMA) stimuli. At the same time, normal results were found in the parents and eldest sister, and as expected, NADPH-dependent O_2^- production (NADPH-oxidase activity) was absent in both the patients' homogenates (Table III).

Membrane depolarization was lacking in the patients' PMN when stimulated with PMA (Fig. 1a) but was normal when induced with ouabain (Fig. 1b). In addition, cytochrome *b* was present at normal levels in the PMN of the proband (Fig. 2). The other sister was not tested.

Histopathological Studies

Histopathological examination showed no evidence of a malignant lymphoma, but on the other hand, a large number of histiocytes whose cyto-

plasm was filled with a yellow-brown, granular, ceroid-like autofluorescent pigment were found in the spleen and lymph nodes (Fig. 3). This pigment typically stained greenish-blue with Giemsa and demonstrated its lipoid nature by pronounced sudanophilia. These cells were found in the spleen, mainly in the periarteriolar lymphoid sheets and in the marginal zone of the lymphoid follicles.

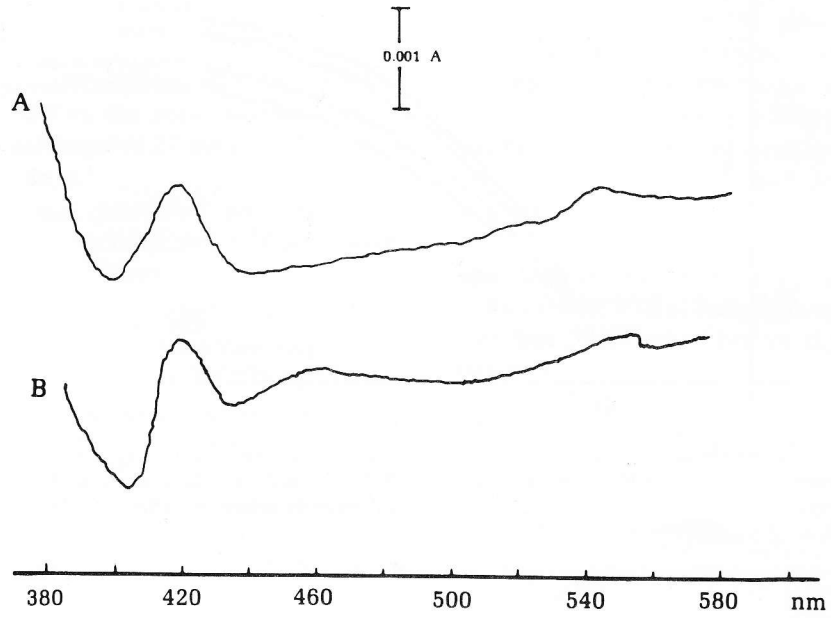
Sections of the liver showed Kupffer cells filled with ceroid and hepatocytes often swollen with small amounts of cytoplasmic pigment.

Ceroid-filled histiocytes were found in the lymph nodes mainly lying in the paracortical zone and small scattered granulomas were also separately found, composed chiefly of epithelial cells without necrosis or giant cells.

DISCUSSION

The clinical phenotype spectrum of CGD syndrome is very broad; on one hand, cases with a severe infectious symptomatology of the skin, mucosa, and internal organs can be observed, and on the other, cases with mild symptoms are found. In this study, the patients presented an apparently mild symptomatology characterized by recurrent respiratory infections. It should be stressed, concerning this, that the atypical, mild symptomatology of the proband and her sister did not indicate such a diagnosis, which was entirely suggested by the histopathological examination and later confirmed by laboratory data. Moreover, the presence of ceroid-laden histiocytes throughout the reticuloendothelium is a well-known morphological feature of the disease (18–21) and, although not pathognomonic, may be considered a quite typical finding of CGD.

On the other hand, the diagnosis of the proband's sister was based on the absence *in vitro* of PMN respiratory activity. In fact, the lack of PMN respiratory burst activation is the hallmark of CGD and depends on a defect of the NADPH-oxidase system. Due to the complexity of this system and of its activating mechanisms, the biochemical defect can



A = Carla: CGD female

B = normal control

Fig. 2. Reduced–oxidized spectra of subcellular particles obtained from homogenate of PMN from one of the CGD patients (A) and a control (B). A, 90 μg of protein/ml; B, 101 μg of protein/ml.

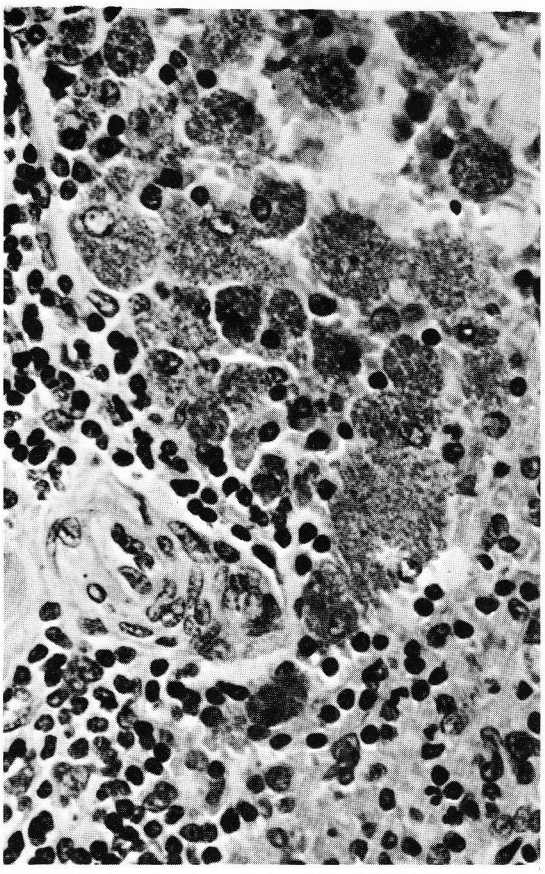


Fig. 3. A cluster of ceroid-laden histiocytes in the spleen. Hematoxylin–eosin. ×400.

be located at different levels: quantitative and/or qualitative impairment of the NADPH-oxidase (1–3), cytochrome *b* (22, 23), or receptional system (24). In the patients described here, the lack of metabolic response to PMA, to opsonized zymosan, and to latex beads rules out the possibility of a defect of the recognition mechanism of PMN similar to that reported by Weening *et al.* (24). The presence of cytochrome *b* indicates that at least a component of the oxidase system is present in our patients, but a functional impairment of cytochrome *b* cannot be excluded. The fact that NADPH-oxidase was not activated and that the PMN were not depolarized by PMA suggests a defect of the activation mechanism of the respiratory enzyme.

The syndrome has rarely been described in females and, even less, in sisters. To our knowledge, CGD syndrome has been reported only a few times in sisters (23–28), and some of these cases, as those described here, showed a mild symptomatology.

Regarding inheritance, CGD in males is clearly X-linked, while genetic transmission in females seems difficult to identify, but it has been hypothesized that it may depend either on an autosomic recessive mechanism or on an X-linked one with unfavorable Lyonization. This uncertainty in ascribing the cases to one or other mechanism of inheritance is linked to the difficulty of detecting a carrier state in the mothers of the patients. In classical X-linked CGD, the mothers show a mixed PMN population, some with normal and some with absent respiratory activity, but a few authors have reported normal NBT reduction in mothers of male patients with a clear pattern of X-linked inheritance (29).

The mother of the patients reported in this paper showed values below normal in the NBT slide test and chemiluminescence assay and which, although slight, may be suggestive of a carrier state. On the other hand, the normal functional pattern in the other tests does not agree with such an interpretation. Furthermore, the low but not absent respiratory activity of both patients' PMN, documented by the NBT slide test and O_2^- production, is suggestive of an X-linked mode of inheritance with incomplete Lyonization.

NADPH-oxidase with a low affinity for the substrate has recently been described in a case of X-linked CGD (5). This case presented mild symptomatology and a faint NBT reduction in nearly all PMN and in half of the mother's PMN. Since faint NBT reduction was not seen either in the mother or in the patients, the hypothesis of low-affinity NADPH-oxidase can be ruled out.

In conclusion, even though the biochemical defect of this disease has been known for the past 15 years, we think that new studies, both biochemical and genetical, and a greater utilization of laboratory tests for PMN respiratory activity are necessary to better define and increase our understanding of CGD syndrome.

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