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Altered Oxidative Metabolism, Motility, and Adherence in Phagocytic Cells from Cystinotic Children

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ABSTRACT. The present study investigates whether the metabolic abnormalities in cystinotic cells could affect nonspecific immune responses. Lymphocytes showed normal antibody-dependent cellular cytotoxicity and natural killer activity. However, cystinotic polymorphonuclear and mononuclear phagocytes exhibited altered oxidative responses as monitored by a luminol dependent chemiluminescence (CL) assay. Both isolated polymorphonuclear and mononuclear phagocytes in the absence of stimuli showed significantly increased CL production which was not found when cells were tested directly within whole blood. CL responses to a panel of stimuli differed markedly according to the type of cells and agents tested. Indeed, isolated polymorphonuclear demonstrated increased CL responses to soluble but not particulate agents, whereas isolated mononuclear phagocytes and overall cell CL responses in whole blood were found to be within the normal range regardless of the type of stimulus used. We also studied some membrane related properties of phagocytic cells. Fc and C_3b receptors were normally expressed as tested by erythrocyte-antibody and erythrocyte-antibody-complement rosette-forming cells. Nevertheless, cystinotic polymorphonuclear and mononuclear phagocytes presented decreased random and directed migrations in an under-agarose chemotaxis assay. Finally, cystinotic granulocytes showed an impaired adhesiveness in a nylon fiber assay. (*Pediatr Res* 19:1318–1321, 1985)

Abbreviations

ADCC, antibody-dependent cellular cytotoxicity
NK, natural killer
CL, chemiluminescence
FMLP, N-formyl-L-methionyl-L-leucyl-L-phenylalanine
PMA, phorbol-12-myristate-13-acetate
ZAS, zymosan-activated serum
PMN, polymorphonuclear
MN, mononuclear
EA, erythrocyte-antibody
EAC, erythrocyte-antibody-complement

Cystinosis is a recessively inherited metabolic disorder characterized biochemically by a high intracellular content of free cystine within lysosomes (1). The clinical expression of infantile cystinosis consists mainly in a Fanconi's syndrome and a progressive glomerular damage leading to chronic renal failure in the first decade of life (2). Cysteamine treatment, now undergoing clinical trials, induces a decrease of the intracellular cystine content, *in vitro* and *in vivo* (3). The disease does not recur in the graft after renal transplantation (4).

The metabolic defect in cystinosis has been the subject of recent studies. Some of them have shown a decreased efflux of cystine from isolated cystinotic lysosomes (5, 6). This suggests a defect in a specific lysosomal membrane carrier for cystine, probably ATP-dependent (6). An accelerated glutathione turnover and an increased transpeptidation have also been postulated as playing important roles in the intracellular cystine accumulation in cystinotic cells (7, 8).

Renal graft survival is better in cystinotic patients than in other transplanted children (2, 9), suggesting an altered immunological response. We investigated whether some nonspecific immune responses, such as ADCC and NK activity were modified in cystinotic patients. Since glutathione is the most important intracellular redox regulator, acts as an acceptor of oxygen radicals to prevent autotoxicity, and is also implicated in microtubule assembly (10), we studied the oxidative metabolism and other membrane-dependent properties of phagocytic cells, such as locomotion and adherence.

MATERIALS AND METHODS

Patients. We studied a group of 17 patients with infantile cystinosis, aged 2 to 10 yr (mean 5.3 yr). The glomerular filtration rate as assessed by creatinine clearance was >30 ml/min/1.73 m^2. Renal transplanted patients were not included in the study because of their immunosuppresive therapy. Since all patients, except two, were under cysteamine treatment, care was taken to obtain the blood samples at least 6 h after the last dose. Patients were free of infection at the time of the tests.

Controls. For CL assay (whole blood), 10 normal children (3 to 14 yr old) were tested. We did not find any differences between adult and children controls in this test. The CL assay on isolated cells was performed for six normal adult controls. The results were in the same range as those found in a previously studied larger normal population (39 individuals), tested under the same conditions of cell isolation. For this reason, this large pool of normal individuals was used as control population. Chemotaxis and random migration were tested for eight normal adult blood donors, four normal children (7, 8, 10, and 13 yr old) and three children on hemodialysis. For the other tests (adherence, Fc and C_3b receptors, ADCC and NK activity) each experiment included a healthy blood donor as control.

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Informed consent was obtained from the parents of cystinotic children to draw 10–15 ml of blood and from the parents of control children to draw 1–5 ml. Adult controls were volunteer healthy blood donors.

Reagents. Phenol red free Hanks' balanced salt solution, Ficoll-triosil, and minimum essential medium Eagle, were obtained from Eurobio (Paris, France), medium RPMI 1640 from Seromed (Berlin, Germany). Zymosan "A," FMLP, phorbol-12-myristate-13-acetate (PMA) and luminol (5-amino-2,3-dihydro-1,4-phtalazinedione) were purchased from Sigma Chemical Co. (St. Louis, MO), Bacto latex particles (0.81 μm) from Difco Laboratories (Detroit, MI), dimethyl sulfoxide (DMSO) from Prolabo (Paris, France), fetal calf serum from Gibco Europe Limited (Paisley, Scotland), dextran T-500 from Pharmacia Fine Chemicals (Uppsala, Sweden). Serum-treated zymosan and ZAS were prepared as follows: zymosan was suspended in saline (20 mg/ml; *i.e.* approximately 10^9 particles/ml), boiled for 60 min, washed twice, and resuspended at the same concentration in fresh human AB serum, incubated for 30 min at 37° C, washed once, resuspended in Hanks' balanced salt solution at 20 mg/ml and stored at −30° C. The zymosan-activated human AB serum was collected, filtered, and stored in 1 ml aliquots at −30°C.

Cells. Mononuclear cells were obtained by Ficoll gradient centrifugation (11) and PMN cells were isolated after a subsequent 5% dextran sedimentation and erythrocyte lysis with an ammonium chloride solution (12). In the chemotaxis assay, PMN cells were tested in the presence of contaminating erythrocytes because the ammonium chloride treatment caused decreased rates of migration.

Chemotaxis assay. The agarose method described by Nelson *et al.* (13) was used. Briefly, 10 μl of chemoattractant, 10 μl of cell suspension (2.5×10^5 PMN or 10^7 MN cells) and 10 μl of control medium were added to each well of a three-well series. After a 2 h incubation for PMN and 18 h for MN at 37° C/5% CO_2, migration was evaluated in an inverted microscope (Leitz-Diavert) with a graduated reticle (each division corresponds to a 0.235 mm distance and is arbitrarily described as 1 unit). The distance of directed migration (A) as well as random migration (B) were measured and a chemotactic index (A/B) and a chemotactic differential (A-B) were calculated for quadruplicate tests.

Chemoattractants. We used ZAS (20 mg/ml), prepared as described above, and FMLP. FMLP diluted 10^{-3} M in dimethyl sulfoxide was stored at −30° C and used at 10^{-7} M in Hanks' balanced salt solution.

PMN cell adherence assay. This test was performed as previously described (14). One ml of heparinized whole blood was drawn and filtered through a nylon fiber column packed in a Pasteur pipette. The column length was 15 mm and the fiber weight 75 mg. Total leukocyte and PMN counts were performed, in duplicate, on both the original and the effluent samples in order to calculate the percentage of PMN cell adherence.

CL assay. CL production was monitored in 100 μl of 10^{-1} diluted whole blood or PMN cell (3×10^6/ml) and MN cell (10^7/ml) suspensions, and was measured by a technique previously described (12, 15). Results are expressed as the maximal light intensity for isolated cells and as maximal light intensity corrected for 10^3 PMN + MN phagocytes in whole blood. Means were calculated from a single value obtained for each patient.

EA and EAC rosettes. Ox red blood cells were sensitized with rabbit IgG anti-ox red blood cell for EA rosettes and with rabbit IgM anti-ox RBC and complement for EAC. Fifty μl of mononuclear cells diluted 10^7/ml and 50 μl of fetal calf serum previously absorbed with ox red cells were mixed with 100 μl of 2% solution of sensitized ox red cells. After 1 h of incubation at room temperature for EA and at 4° C for EAC, the percentages of rosettes were evaluated after counting at least 400 cells.

NK activity. Different concentrations of MN cells were mixed with ^{51}Cr labeled K-562 target cells in round bottomed tubes at effector/target cell ratios of 6:1, 12:1, 25:1, and 50:1. After a 4-h incubation at 37° C/5% CO_2, the tubes were centrifuged at 150 $\times$ *g* for 5 min and the supernatant was removed for evaluation of the ^{51}Cr release.

ADCC. One hundred μl of a MN cell suspension were mixed with 100 μl (10^5) of ^{51}Cr labeled Chang cells and 100 μl of rabbit anti-Chang serum at 1/10,000 final dilution in round bottomed tubes at the following effector/target cell ratios: 5:1, 10:1, 30:1. After an overnight incubation at 37° C/5% CO_2, tubes were centrifuged at 150 $\times$ *g* and a 150-μl sample was taken from the supernatant in order to determine the ^{51}Cr release. Results for NK and ADCC were expressed as means ± SD of triplicate experiments according to the following equation: X = experimental ^{51}Cr release − spontaneous ^{51}Cr release/maximal ^{51}Cr release − spontaneous ^{51}Cr release × 100.

Results are expressed in means and SD. Significance of the results has been evaluated by the Wilcoxon's "W" test (16).

RESULTS

Random and directed migrations. Mean random migration for PMN cells (1.39 ± 0.56) from 10 cystinotic patients was lower than random migration for PMN cells (2.15 ± 0.65) from eight controls ($p < 0.01$). Directed migration toward the chemotactic factor ZAS was also decreased for cystinotic PMN cells (4.31 ± 1.20 compared to 6.06 ± 1.49) ($p < 0.03$). Random and directed migrations were also decreased in cystinotic MN cells. The mean value of random migration for nine patients was 1.90 ± 0.81 compared to 3 ± 0.96 for eight controls ($p < 0.04$). The values for directed migration were 4.64 ± 0.99 for cystinotic MN cells compared to 6.38 ± 0.96 for normal MN cells ($p < 0.01$).

Similar results were obtained in the chemotaxis assay using FMLP as the chemoattractant for PMN cells. Random migration was 1.57 ± 0.51 for cystinotic PMN cells (nine patients) compared to 2.11 ± 0.36 for six normal controls. Directed migration toward FMLP was 4.59 ± 0.93 for cystinotic PMN cells (three patients) compared to 6.10 ± 0.46 for three controls.

PMN random and directed migrations toward ZAS were also tested for four normal children and three pediatric patients on hemodialysis. Normal children showed mean random and directed migrations slightly lower than those of normal adult controls, but not significantly different. Hemodialyzed patients presented random and directed migrations lower than those of normal children. However, values obtained for cystinotic patients were lower than those for normal and hemodialyzed children (Table 1).

Chemotactic indexes (A/B) for cystinotic PMN an MN cells were not different from controls (3.10 ± 0.85 compared to 2.82 ± 0.84 for PMN cells and 2.89 ± 1.65 compared to 2.23 ± 0.42 for MN cells). The chemotactic differential (A-B) was significantly lower for cystinotic PMN and MN cells as compared to controls (2.92 ± 0.88 *versus* 3.90 ± 0.82 for PMN cells and 2.74 ± 0.81 *versus* 3.53 ± 0.56 for MN cells) ($p < 0.05$ in both cases). Similar results were obtained with FMLP-stimulated PMN cells (2.86 ± 0.69 and 2.92 ± 0.58 for cystinotic children versus 2.76 ± 0.50 and 3.86 ± 0.71 for controls for A/B and A-B, respectively).

PMN cell adherence. The PMN cell ability to adhere to nylon fiber was tested for 12 cystinotic patients and nine controls. Mean adherence values for cystinotic children were 38.38 ± 14.09 compared to 52.36 ± 9.87 for controls ($p < 0.02$).

Oxidative metabolism. Unstimulated isolated PMN and MN phagocytes (medium alone) exhibited increased CL compared to controls ($p < 0.001$ and < 0.01, respectively). Cystinotic PMN cells also showed increased CL responses after stimulation by soluble agents (PMA, FMLP) ($p < 0.001$), but not with particulate stimuli. In contrast, MN phagocytes presented normal responses regardless of the type of stimulus tested (Table 2).

The CL responses of total phagocytic cells within whole blood diluted 1/10 were not significantly different from those of controls, with or without stimulation (Table 2).

Table 1. *Comparison of PMN cell random and directed migrations (ZAS) for adult controls, normal children, hemodialysis patients, and cystinotic children tested in this study**

	Adult controls ($n = 8$)	Normal children ($n = 4$)	Hemodialysis patients ($n = 3$)	Cystinotic children ($n = 10$)
PMN directed migration (ZAS)	6.06 ± 1.49	5.95 ± 0.12	5.88 ± 0.64	4.31 ± 1.20
PMN random migration	2.15 ± 0.65	2.08 ± 0.32	1.83 ± 0.50	1.39 ± 0.56

* Values are expressed in arbitrary units.

Table 2. *CL production by cystinotic PMN and MN phagocytes (isolated and in whole blood)*

	Isolated PMN cells		Isolated MN cells		Whole blood 1/10	
	Cystinotics ($n = 11$)	Controls ($n = 39$)	Cystinotics ($n = 11$)	Controls ($n = 35$)	Cystinotic ($n = 17$)	Controls ($n = 44$)
Hanks' balanced salt solution	80040* ±48764	18410 ±13518	2018† ±1708	938 ±840	0.90 ±0.70	1.23 ±0.66
FMLP	211814* ±123312	95908 ±54343	7121 ±5479	9364 ±8678	11.24 ±6.00	13.72 ±11.64
PMA	291571* ±131812	167635 ±84070	41836 ±26227	33960 ±25155	30.19 ±16.60	47.40 ±21.88
Latex	174099 ±39925	145881 ±51440	10888 ±9419	11328 ±9879	27.99 ±17.72	34.93 ±24.54
Zymosan	466863 ±95661	413361 ±198441	104106 ±64465	114098 ±52694	167.09 ±81.80	171.22 ±82.91

Values are expressed as indicated in "Materials and methods" (*$p < 0.00$; † $p < 0.01$).

Fc and C_3b receptors. The proportions of MN cells bearing Fc and C_3b receptors (EA and EAC rosettes) in 12 cystinotic patients were not different from those of controls. EA for cystinotic patients were 15.87% ± 2.77 compared to 18.92% ± 4.58 for 10 controls. EAC for cystinotic patients were 16.39% ± 3.60 compared to 18.84% ± 4.99 for controls.

ADCC and *NK activity.* ADCC was tested for nine cystinotic patients and five normal controls. NK activity was tested in only two cystinotic patients and two controls. The results for ADCC and NK activity were within normal limits (Table 3).

Table 3. *ADCC and NK activity in cystinotic children and controls**

	Effector/target ratio	(n)	Cystinotic patients	(n)	Controls
ADCC	5:1	(9)	35.11 ± 13.59	(5)	28.55 ± 20.84
	10:1	(9)	40.97 ± 15.21	(5)	36.20 ± 20.32
	25:1	(9)	62.04 ± 9.09	(5)	56.93 ± 28.55
NK	6:1	(2)	28.25 ± 3.46	(2)	44.40 ± 9.92
	12:1	(2)	37.05 ± 0.21	(2)	52.80 ± 8.35
	25:1	(2)	44.20 ± 2.84	(2)	60.00 ± 6.23
	50:1	(2)	50.40 ± 4.53	(2)	64.00 ± 1.27

* Results are expressed as indicated in "Materials and methods."

DISCUSSION

The metabolic defect in cystinosis is expressed in leukocytes as a high intracellular free-cystine content. This abnormality has been found in polymorphonuclear leukocytes and monocytes rather than in lymphocytes (17). We studied whether this metabolic defect could modify some nonspecific immunological responses in these patients.

Lymphocyte ADCC and NK activity in cystinotic children were found to be within the normal ranges. However, our results show that cystinotic PMN and MN phagocytes present an altered oxidative metabolism as compared to controls. An increased CL production by isolated PMN and MN phagocytes was observed in the absence of stimuli. Cystinotic PMN leukocytes also exhibited an increased CL generation with soluble stimuli, PMA, and FMLP. The CL production by PMN and MN phagocytes in whole blood without stimuli was found to be normal suggesting that an increased production of free oxygen radicals could be counteracted by an excess of circulating scavengers.

Random and directed migrations were decreased in cystinotic PMN leukocytes and monocytes. Similar results were found with the two chemoattractants used: FMLP, a synthetic tripeptide, and ZAS, which acts via the C_{5a} factor of complement. Chemotactic indexes for cystinotics were normal suggesting an impaired motility rather than a chemotactic defect alone. This impaired locomotion was not correlated with the degree of renal function. Furthermore, random and directed migrations for hemodialyzed patients were higher than those of cystinotic patients. In addition, PMN leukocytes from cystinotic children presented diminished adhesiveness to nylon fiber. Both migratory capacity and adherence of phagocytic cells are essential events in the induction of the inflammatory response.

The mechanism of these alterations is not clear. First, an increased cystine reduction rate (18) and an increased level of γ-glutamyl transpeptidase (7) have been described in cystinotic cells. It could alter the ratio between reduced and oxidized glutathione. The NADPH oxidase system, which is necessary for the regeneration of glutathione (19), may thus be stimulated. On the other hand, since increased oxygen consumption and free radical metabolite generation, including superoxide, have been described during thiol oxydation (20), the increased intracellular cystine content could also be responsible for these alterations. The intracellular cystine content for the 14 cystinotic children included in this study was increased, despite cysteamine treatment, from five to 25 times the normal value (x = 2.10 ± 1.15 (SD); range 1.04 to 5.23 nmol ½ cystine/mg protein). However,

intracellular cystine content was not closely correlated with CL production (data not shown). It is unlikely that cysteamine, which could be present intracellularly during the test, plays a role. *In vitro* preincubation of control PMN cells with cysteamine at 1 mM as previously described (3), did not change the CL results (Pintos Morell G, unpublished observations).

Regardless of the origin, the increased production of oxygen radicals could induce autooxidative membrane damage (21). This damage could be responsible for the impaired motility and adherence, but does not affect the expression of Fc and C_3b receptors as shown by EA and EAC rosettes.

In conclusion, cystinotic PMN and MN phagocytes show an altered oxidative metabolism and impaired migratory and adherence capacities. These alterations could be related to an increased intracellular cystine content and/or to the abnormalities of the glutathione cycle. In order to determine accurately the nature of this altered oxidative burst, a complete study of the oxidative metabolism including oxygen consumption, superoxide, and hydrogen peroxide production as well as hexose monophosphate shunt, should be performed. The *in vitro* response of cystinotic cells to antioxidant agents could also be helpful.

The clinical implications of the *in vitro* impaired capacities of adherence and migration in cystinotic phagocytic cells are not known. However, it shoud be stressed that adherence and migration of PMN leukocytes and monocytes are essential events for leukocyte–endothelial interactions and that these interactions have been implicated in allograft rejection (22).

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