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Assessment of oral ascorbate in three children with chronic granulomatous disease and defective neutrophil motility over a 2-year period

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SUMMARY

Two brothers and their sister with chronic granulomatous disease, elevated levels of serum IgE and defective neutrophil motility were treated with a single oral daily dose of 1 g sodium ascorbate as a supplement to prophylactic trimethoprim–sulphamethoxazole therapy for 2 years. Laboratory tests of neutrophil functions were performed prior to ascorbate therapy and repeated at 1-monthly intervals for 6 months and at 6-monthly intervals thereafter. Introduction of ascorbate to the therapeutic regimen was accompanied by slight increases in neutrophil hexose monophosphate shunt activity and staphylocidal activity and good improvement of neutrophil motility in all three children. The improved staphylocidal activity was not due to ascorbate-mediated inhibition of neutrophil or serum catalase activities or to detectable increases in superoxide and H_2O_2 production or activity of the MPO/H_2O_2/halide system. Both male children have remained free from obvious infection since ascorbate was added to their therapeutic regimen; their sister has experienced one urinary tract infection during a period when treatment with prophylactic co-trimoxazole and ascorbate was inadvertently stopped. All three children have gained weight.

INTRODUCTION

Chronic granulomatous disease (CGD) becomes manifest in early childhood as a greatly increased susceptibility to pyogenic infections due to the reduced capacity of the patient's neutrophils to destroy certain species of bacteria (Quie *et al.*, 1967). The fundamental abnormality is the inability of the polymorphonuclear leucocytes (PMN) to undergo the burst of metabolic activity which normally accompanies phagocytosis (Holmes, Page & Good, 1967). Hexose monophosphate shunt (HMS) activity (Holmes *et al.*, 1967) and generation of superoxide (Curnutte, Whitten & Baboir, 1974) and H_2O_2 (Root, 1975) are impaired in the PMN of children with CGD. PMN random and directional migratory responses *in vivo* (Kaplan, Laxdal & Quie, 1968) and *in vitro* (Ward & Schlegel, 1969; Nelson *et al.*, 1979) have been reported to be normal. Phagocytosis (Stossel, Root & Vaughan, 1972), granule enzyme content and post-phagocytic degranulation (Baehner, Karnovsky & Karnovsky, 1969) are also normal in this condition.

Ascorbic acid has been reported to increase HMS activity in normal PMN and PMN from a child with CGD *in vitro* (Cooper, McCall & De Chatelet, 1971). This agent has also been reported to enhance PMN motility and staphylocidal activity in a child with Chediak–Higashi syndrome *in*

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vitro (Boxer *et al.*, 1976). We have previously reported that sodium and calcium ascorbate improved the markedly abnormal random migration and chemotaxis *in vitro* of PMN from three children with the unusual combination of CGD, impaired PMN motility and raised serum levels of immunoglobulin E (Anderson & Dittrich, 1979). In this report we describe our findings after 2 years of the effects of ascorbate-supplemented prophylactic antimicrobial therapy on the defective motility and post-phagocytic metabolic and antimicrobial activities of PMN from these three children.

PATIENTS AND METHODS

Patients. Two brothers aged 9 years 8 months and 3 years and their sister aged 7 years, who are the offspring of a first-cousin marriage, were referred for further immunological study in April 1978. The elder brother had a history of frequent bacterial infections including juvenile acne, persistent suppurative otitis media, recurrent attacks of sinusitis which were difficult to treat and one episode of lobar pneumonia in 1974. His sister developed suppurative inguinal lymphadenitis after minor surgery to the knee in January 1973. She also has a history of recurrent upper respiratory tract infections and suffered a severe pneumonia affecting the mid-lobe from December 1975 to March 1976 which resulted in fibrosis of the mid-lobe. The youngest child presented with acute omphalitis shortly after birth. At the age of 2 weeks he developed a perianal abscess which resulted in a chronic perianal fistula. All three children are allergic to cat hair, house dust and grass, but have no known drug allergies (Table 1). CGD was diagnosed in all three children in March 1976 and treatment with prophylactic chemotherapeutic agents was commenced (trimethoprim–sulphamethoxazole). Although these agents lowered the frequency of infection both elder children failed to thrive and had subsequent episodes of pneumonia in June 1977, the boy remaining pyrexial until November 1977. His sister also had bronchiolitis and sinusitis.

Prior to testing of immune function (before and during ascorbate intake) both elder children were free from infection as determined by clinical and laboratory criteria (normal leucocyte and differential counts, normal serum immunoglobulins, negative C-reactive protein test and normal levels of the complement components C3 and C4 and total haemolytic complement). The youngest child had a chronic perianal fistula at the time of the first investigation.

Preliminary investigations showed that all three children had normal levels of salivary IgA, serum IgG, IgM and IgA and complement components C3 and C4 and total haemolytic complement. However, serum IgE levels were elevated in all three children (Table 1). Lymphocyte transformation induced by the mitogens phytohaemagglutinin and concanavalin A was normal in all three children. Serum opsonic activity for *Staphylococcus aureus* and *Candida albicans* was normal for each child as were the rates of ingestion of these micro-organisms and degranulation (post-phagocytic lysozyme and myeloperoxidase release) by PMN from the patients. However, PMN from all three children on initial investigation showed markedly abnormal random motility and motility towards the leucoattractants endotoxin-activated normal and autologous serum (EAS), and the synthetic chemotactic tripeptide F-Met-Leu-Phe (5×10^{-10} M) (Table 1). There was no evidence of cell-directed inhibitors of cell motility or inability to generate normal leucotactic activity upon endotoxin interaction with the patient's sera (Anderson & Dittrich, 1979). The semi-quantitative nitroblue tetrazolium (NBT) test was completely negative in all three children (normal value 90% NBT-positive PMN; Anderson & Dittrich, 1979). Enzymatic studies have shown normal myeloperoxidase (MPO), glucose-6-phosphate dehydrogenase, 6-phosphogluconic acid dehydrogenase, glutathione reductase and glutathione peroxidase activities in the PMN of each child. PMN post-phagocytic HMS activity, superoxide and H_2O_2 generation and MPO-mediated iodination of ingested *C. albicans* were also markedly impaired in each child (Table 2).

Ascorbate and antimicrobial prophylaxis. Each patient received a single oral daily dose of 1 g sodium ascorbate (Redoxon, Roche (Pty) Ltd, Johannesburg). This dose of ascorbate was selected on the basis of *in vitro* experiments on the effects of varying doses of ascorbate on leucocyte function (Anderson *et al.*, 1980a). The children received prophylactic trimethoprim–sulphamethoxazole (Bactrim, Roche) in a single 480-mg dose daily. This prophylactic antimicrobial agent was always stopped 1 week prior to testing of PMN functions.

Table 1. Summary of the clinical and immunological findings on the three children with CGD on initial investigation

						Allergen sensitivity by the RAST test				PMN motility (cells/HPF)		
Patient	Age (years)	Sex	Height (m)	Weight (kg)	Total serum IgE (iu/ml)	Grass*	Mite†	House-dust	Cat	Random motility	Chemotaxis to EAS	Chemotaxis to C5a
1	9·6	M	1·31 (10th)‡	26·1 (10th)‡	8,000	2	3	2	2	2 (30)§	32 (180)	19 (120)
2	3	M	0·97 (70th)	12·5 (50th)	250	1	1	1	2	0	45	15
3	7	F	1·12 (<3rd)	14·1 (<3rd)	450	1	3	0	3	0	11	13

* *Cynodon dactylon.*
† *Dermatophagoides pteronyssinus.*
‡ Height and weight percentiles.
§ Lower limit of normal value (1 s.d. below the mean value).

Neutrophil chemotaxis. This was performed as previously described (Anderson *et al*., 1979) using the leucoattractants endotoxin-activated autologous and normal serum and the synthetic chemotactic tripeptide *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (F-Met-Leu-Phe; Miles Laboratories, Indiana, USA) at a final concentration of 5×10^{-10} M. Random migration was evaluated in the absence of a gradient. Results are expressed as PMN/microscope high power field (cells/HPF). HMS activity was measured as previously described (Anderson *et al*., 1979) using PMN suspensions of > 90% purity, 10% fresh autologous serum and *Candida albicans* at a ratio of 1 PMN : 5 micro-organisms. Results are expressed as nmol [1-^{14}C]glucose metabolized.

Superoxide generation. This was assessed by reduction of ferricytochrome *c* (Cyt. *c*.) as described by Curnutte *et al*. (1974). Pure PMN (5×10^6) were mixed with pre-opsonized *C. albicans* at a ratio of 1 : 50 in the presence of 100 μmol Cyt. *c*. Post-phagocytic superoxide production was expressed as the difference (in nmol reduced Cyt. *c*.) between systems containing no superoxide dismutase (SOD) and those containing 200 u SOD (Sigma Chemical Co., St Louis, Missouri, USA). Reaction systems, which contained a final volume of 1 ml were incubated on a turntable at 37°C/15 min after which the tubes were centrifuged and the supernatants assayed spectrophotometrically at 550 nm for reduced Cyt. *c*. The amount of reduced Cyt. *c*. was calculated using an absorbance coefficient of 15·5 mM at 550 nm (Margoliash & Frohwirt, 1959).

H_2O_2 production. H_2O_2 production was measured according to the method of Root *et al*. (1975). Reaction systems contained 1×10^7 PMN, 5×10^8 opsonized *C. albicans*, and 1 mM sodium azide in a final reaction volume of 1 ml 0·15 M phosphate-buffered saline, pH 7·2, supplemented with 10 mM glucose. Tubes were incubated at 37°C/15 min on a turntable and the supernatants assayed for extracellular H_2O_2 according to the extent of reduction of scopoletin (Sigma Chemical Co.) fluorescence. Each reaction tube contained 2·5 ml distilled H_2O, 50 μl of horseradish peroxidase (Sigma Chemical Co.) at a stock concentration of 13·8 u/ml, 20 μl of 200 μM scopoletin and 50 μl of the cell-free supernatant. Standard curves were constructed in the range 0·1–10 nmol of H_2O_2. The H_2O_2-dependent reduction in fluorescence was monitored on a Perkin–Elmer model 204 Hitachi fluorescence spectrophotometer at an exciter wavelength of 390 nm and an analyser wavelength of 460 nm. Results are expressed as nmol/10^7 PMN/15 min.

MPO-mediated iodination of ingested C. albicans. This was performed as previously described (Anderson *et al*., 1979) by determination of the amount (nmol) of ^{125}I incorporated into tricarboxylic acid-precipitated protein using a PMN : *C. albicans* ratio of 1 : 10.

Staphylocidal activity. Staphylocidal activity was performed according to the viable colony counting method of Quie *et al*. (1967) following incubation periods of 30, 60 and 90 min. PMN post-phagocytic antimicrobial activity was evaluated using PMN : *S. aureus* ratios of 1 : 5 and 1 : 10 in the presence of 10% autologous serum.

Effects of ascorbate, sulphamethoxazole and trimethoprim on catalase activity in vitro. These were determined according to the spectrophotometric method of Sinha (1972) based on the H_2O_2-mediated reduction of dichromate in acetic acid. The effects of the drugs were assessed at a concentration range of 0·2–200 μg individually and in combination. Each reaction system consisted of 2·5 ml 0·05 M PBS, pH 7·2, 0·3 ml of each drug, 0·1 ml of canine liver catalase (Sigma Chemical Co.) containing 3 enzyme units and 0·1 ml of H_2O_2 to give a final concentration of 0·09 M. The systems were incubated for 10 min at 37°C (on the linear portion of the reaction curve) after which 1 ml was transferred to 2 ml of the dichromate/acetic acid solution (a mixture of 5% $K_2Cr_2O_7$ with glacial acetic acid at a ratio of 1 : 3). The mixture was heated at 80°C/10 min in a water bath and residual H_2O_2 determined spectrophotometrically at 570 nm. Where combinations of sulphamethoxazole and trimethoprim were investigated the ratio used was 5 : 1. Controls consisted of the drugs alone, the drugs with H_2O_2 only and the drugs with catalase only. This method was preferred to the direct monitoring of catalase-mediated decomposition of H_2O_2 at 240 nm since ascorbate does not absorb at this wavelength.

Effects of these agents on catalase activity in vitro. Six adult volunteers were assessed for the *in vitro* effect of these agents on catalase activity. Leucocyte and serum catalase activities were measured before and after ingestion of 4 × 480 mg co-trimoxazole tablets ingested over a 24-hr period. Catalase activity and serum co-trimoxazole levels were measured 3 hr after ingestion of the last tablet. To assess the effects of ascorbate on catalase activity *in vitro* the same volunteers received

a single intravenous infection of 1 g of sodium ascorbate (injectable Redoxon). Leucocyte and serum catalase activities and vitamin C levels were measured before and 1 hr after the injection. Catalase activities were measured in the CGD children before and 2 hr after ingestion of a single 1-g oral dose of sodium ascorbate. Leucocyte catalase activities were measured as above using sonicates of pure PMN suspensions. Serum catalase was measured by incubating 0·2 ml serum, 0·7 ml PBS and 0·1 ml of 1 M H_2O_2 for 10 min. The reaction was terminated and protein precipitated by the addition of 2 ml of 1 M tricarboxylic acid and the supernatants (1 ml) assayed for H_2O_2. Results are expressed as enzyme units calculated from a standard curve of 0–10 units of canine liver catalase.

Serum and leucocyte ascorbate levels. These were measured by the 2,4-dinitrophenyl-hydrazine method described by Attwood *et al.* (1974).

RESULTS

Neutrophil motility and serum IgE levels

Introduction of ascorbate to the therapeutic regimen correlated with a sustained improvement in PMN motility although lower limit of normal values were not attained. Results for migration for autologous EAS over a 2-year period are shown in Fig. 1. All three children showed an initial drop in serum IgE levels with subsequent fluctuations which rose to greater than initial values in the two youngest children. RAST sensitivities remained unchanged throughout (results not shown).

Post-phagocytic superoxide and H_2O_2 production and MPO-mediated iodination of *C. albicans* remained unchanged following ascorbate therapy. However, HMS activity showed a consistent slight increase in all three children (Table 2).

Catalase activity *in vitro* was inhibited by sodium ascorbate in a dose-dependent fashion (Fig. 2). However, sulphamethoxazole and trimethoprim together and individually had no inhibitory effects on catalase activity and did not affect the sodium ascorbate-mediated inhibition of catalase (results not shown).

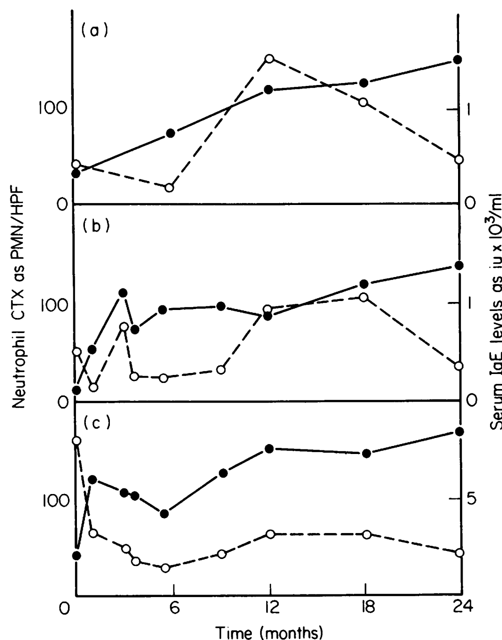

Fig. 1. Neutrophil migration to autologous EAS and serum IgE levels in three children with CGD prior to and during 2 years of ascorbate ingestion. (a) Youngest male child (not re-investigated until 6 months after commencement of ascorbate therapy); (b) results on his sister and (c) results obtained with the eldest (male) child.

Table 2. Neutrophil HMS activity, superoxide and H_2O_2 generation and myeloperoxidase-mediated iodination of *C. albicans* before and during ascorbate therapy in three children with CGD

Time (months)	HMS activity (nmol [1-^{14}C]glucose metabolized/60 min)	Superoxide generation (nmol reduced Cyt. *c*/5 × 10^6 PMN/15 min)	H_2O_2 production (nmol/10^7 PMN/15 min)	MPO-mediated protein iodination (nmol ^{125}I precipitated)
0	0·82±0·06	n.d.	0·2±0·05	n.d.
	(15·1 ±2·3)*	(44±8·2)*	(5·7±0·41)*	(0·86±0·14)*
6	2·7 ±0·52	n.d.	0·3±0·09	n.d.
12	2·3 ±0·64	n.d.	0·4±0·04	n.d.
18	2·5±0·53	n.d.	0·3±0·06	0·03±0·01
24	2·2 ±0·62	n.d.	0·4±0·06	0·04±0·01

* Mean ± standard error of 10 control subjects.
† Before ascorbate.
n.d. = Not detectable.

Serum and PMN catalase activities, however, were unaffected following intravenous injection of sodium ascorbate in normal individuals despite the achievement of ascorbate levels in serum which mediated considerable inhibition of catalase activity *in vitro* (Table 3). Ingestion of co-trimoxazole in normal volunteers gave a mean serum level of 47·7±6·2 μg/ml but had no effect on PMN and serum catalase activities (results not shown). Ingestion of 1 g ascorbate by the three children was not accompanied by significant reduction in PMN or serum catalase activities (Table 3).

Staphylocidal activity was slightly increased at a ratio of 1 PMN : 5 *S. aureus* following ingestion of 1 g sodium ascorbate in the PMN of each child but less evident at the higher ratio of 1 PMN : 10 *S. aureus* (Table 4). No significant alterations in staphylocidal activity were observed in PMN from normal volunteers following ascorbate injections (Table 4).

Clinical features

Since the start of ascorbate therapy the chronic perianal fistula has healed in the youngest child (after 3 months of ascorbate) and both male children have remained free from clinically obvious infection. Their sister has suffered one urinary tract infection of unknown origin but which responded to a 2-week course of ampicillin; this infection followed a period when the child attended a 2-week school holiday camp during which she neglected to take prophylactic chemotherapeutics and ascorbate. All three children have gained height and weight. The girl, who upon initial investigation was 60% of the minimum expected height and weight for her age is now in the third

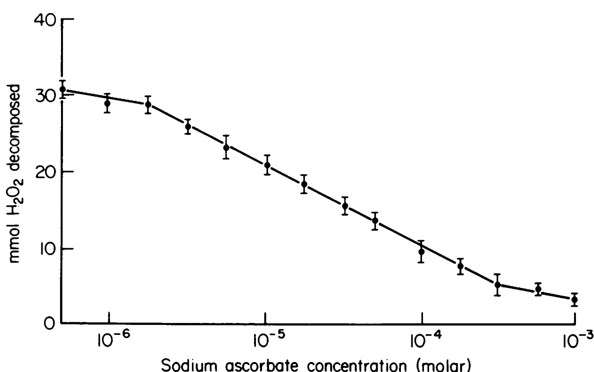

Fig. 2. The effects of ascorbate on catalase activity *in vitro*.

Table 3. Serum and neutrophil catalase activities and ascorbate levels before and after intravenous ascorbate in adult volunteers and oral ascorbate in three children with CGD

	Serum ascorbate (μg/ml)	Serum catalase (enzyme units/ml)	Leucocyte ascorbate (μg/10^8 cells)	Neutrophil catalase (enzyme units/10^6 PMN)
Adult controls				
Before ascorbate*	12·8±1·2†	8·8±1·1	2·9±0·52	9·03±0·80
After ascorbate	41·0±3·3	8·1±1·3	4·6±0·68	8·60±0·74
Children with CGD				
Before ascorbate	10·8±1·3†	9·3±1·4	3·0±0·61	10·20±1·90
After ascorbate	26·3±0·9	8·9±1·9	4·9±1·9	9·50±1·83

* Adult volunteers received 1 g injectable sodium ascorbate intravenously and the CGD children received 1 g orally.

† Results as mean and standard error of 10 normal adults and three children with CGD.

Table 4. PMN antimicrobial activity prior to and following ascorbate ingestion in three children with CGD

	PMN antimicrobial activity using:					
	A PMN:*S. aureus* ratio of 1:5			A PMN:*S. aureus* ratio of 1:10		
	30 min	60 min	90 min	30 min	60 min	90 min
Normal adults						
Before ascorbate	83 ±1·2*	93 ±1·1	95 ±0·8	77 ±1·8	85 ±1·6	90 ±0·7
After ascorbate	85 ±0·9	96 ±1·0	97 ±1·0	79 ±0·9	87 ±1·3	93 ±0·8
Children with CGD						
Before ascorbate						
Child 1	70·0±1·3	73·6±1·4	75·8±1·6	65·4±1·1	73·1±1·5	76·9±1·9
Child 2	69·6±1·2	74·0±1·8	74·4±0·9	64·2±0·9	67·0±1·2	70·2±1·3
Child 3	65·9±0·8	69·6±1·7	70·9±1·1	62·4±1·3	65·6±1·3	67·4±0·8
After ascorbate						
Child 1	79·2±2·1	82·7±1·9	84·0±0·9	71·8±1·4	75·9±2·1	79·4±1·8
Child 2	81·3±1·6	81·2±1·5	82·6±1·2	67·1±1·1	70·0±1·3	71·6±1·2
Child 3	77·4±1·7	79·0±0·9	81·2±1·2	66·0±1·2	68·6±1·2	70·0±1·0

* Results as mean and standard error for five normal adults.

percentile for height and weight; the eldest boy is on the 25th percentile for weight. Pre-investigation height and weight data are shown in Table 1. Height and weight percentile were calculated from the growth charts issues by the Children's Medical Center, Boston, USA.

DISCUSSION

The association of CGD and reduced PMN motility is unusual. The few reports on this subject suggest that PMN motility is normal in children with CGD (Ward & Schlegel 1969; Nelson *et al.*, 1979). The report by Nelson *et al.* (1979) refers to unpublished work by Cates & Quie who found no abnormality of PMN motility in a series of eight patients with CGD. Ward & Schlegel (1969) described a child with abnormal PMN antimicrobial activity for Gram-negative micro-organisms

and impaired PMN motility due to a serum inhibitor of chemotaxis; IgE levels were not performed on this child and the clinical status of the child with regard to existing infection at the time of the initial investigation was not detailed. The children studied in this investigation differ from the child described by Ward & Schlegel in that no serum inhibitors of leucotaxis were detected (Anderson & Dittrich, 1979) and their PMN have defective antimicrobial activity for *S. aureus*. It is possible that the defective PMN motility in these children is not linked to the CGD but may be secondary to existing bacterial infection (Mowat & Baum, 1971) or hyperimmunoglobulinaemia E (Hill & Quie, 1974). It is unlikely that the former is responsible since each child was assessed as being free from infection by clinical and laboratory criteria on each occasion prior to testing. The high serum IgE levels found in all three children could be implicated, although it has been our experience that the abnormalities of PMN motility found in some children with hyperimmunoglobulinaemia E are of the acquired transient type (Anderson *et al.*, 1980b). We have shown that sulphamethoxazole and trimethoprim individually and in combination *in vitro* and ingestion of co-trimoxazole by normal volunteers has no effect on neutrophil motility or HMS activity (Anderson *et al.*, 1980c). Ascorbate therapy was accompanied by increased PMN motility which was sustained throughout the 2-year period in each child. However, normal responses were not attained. The increased PMN motility was accompanied by initial decreases in total serum IgE in each child. Subsequent fluctuations to greater than pre-ascorbate levels were observed in the two youngest children. Specific IgE levels remained unaffected throughout.

Ascorbate therapy was associated with slight sustained increases in post-phagocytic HMS activity in each child which were not accompanied by detectable superoxide or H_2O_2 production and MPO-mediated iodination of ingested *C. albicans*. This suggests that the ascorbate may participate in the series of reactions proposed by De Chatelet, Cooper & McCall (1972) with generation of NADP from NADPH and subsequent stimulation of HMS activity not linked to H_2O_2 production. Ascorbate ingestion was accompanied by increased staphylocidal activity at low PMN:*S. aureus* ratios. The increased staphylocidal activity could not be linked to increased superoxide production or activity of the MPO/H_2O_2/halide system. Inhibition of catalase seemed a potential mechanism, since CGD is associated with infections due to catalase-positive micro-organisms (Kaplan *et al.*, 1968). Ascorbate caused inhibition of catalase activity *in vitro*, which has been previously reported by Orr (1967). However, no inhibition of PMN or serum catalase was found following ascorbate ingestion in the patients or after intravenous injection of ascorbate in normal volunteers. It is possible that PMN and serum catalases are refractory to ascorbate-mediated inhibition. However, we have recently found (unpublished findings) that they are inhibited by ascorbate *in vitro*. The most likely explanation for the absence of *in vivo* inhibition is the auto-oxidation of ascorbate to dehydroascorbate which may not possess catalase inhibitory activity. This possibility is currently under investigation. We have also been unable to demonstrate potentiation of co-trimoxazole by sodium ascorbate (10^{-5}–10^{-3} M) against *S. aureus in vitro* (unpublished findings). A possible explanation for increased PMN staphylocidal activity following ascorbate is the generation of antimicrobial factors which accompanies the oxidation of ascorbate (Ericsson & Lundbeck, 1955; Miller, 1969; De Chatelet *et al.*, 1972).

Increased PMN motility and staphylocidal activity was accompanied by a decrease in the frequency of infection and increased weight and growth rate in all three children. This could be due to improved PMN motility with increased numbers of phagocytic cells available to engulf potential pathogenic micro-organisms. This would be expected to result in low PMN: bacteria ratios which, as described in this report, are more efficiently destroyed by neutrophils from patients with CGD. Ascorbate in the three children with CGD has proved to be a useful supplement to prophylactic chemotherapeutics. Further evaluation of the usefulness of ascorbate is required in children with CGD and normal PMN motility.

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