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Congenital Acatalasemia: A Study of Neutrophil Functions after Provocation with Hydrogen Peroxide

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ABSTRACT. Five Swiss subjects with hereditary acatalasemia (4 homo-, 1 heterozygous) were studied by a series of neutrophil function tests. H_2O_2 was added to a polymorphonuclear neutrophil leukocyte-suspension to produce a metabolic stress; neutrophil functions related to membrane deformation were subsequently found to be depressed, *i.e.*, chemotaxis, membrane potential, and chemiluminescence. This mechanism might be one pathogenetic factor in the formation of mucosal ulcers in acatalasemic individuals. (*Pediatr Res* 19: 1187–1190, 1985)

Abbreviations

AC, acatalasemia
PMA, phorbol myristate acetate
PMN, polymorphonuclear neutrophil leukocyte

AC, characterized by very low catalase activity in blood and tissues, is inherited as an autosomal recessive trait (1, 2). It was discovered in 1946 by Takahara in Japan (3, 4) in a 10-yr-old girl with progressive mucosal gangrene. In subsequent years, the condition has been observed in many countries and in different ethnic groups (5–12).

The first cases described by Takahara (2, 4) exhibited recurring painful stomatitis, sometimes progressing to deep gangrenous ulcers and even to necrotizing lesions penetrating into maxillar or mandibular bones. In contrast, all the acatalasemic individuals observed in Switzerland by Aebi and Wyss (1) were in good health, and none had stomatologic problems.

Phagocytic function is closely connected with H_2O_2 production (13, 14) which is one of the crucial factors for intracellular killing of ingested microorganisms. On the other hand, however, H_2O_2 is a toxic substance with adverse effects to the body's own cells and tissues (15, 16). Catalase, a potent scavenger for hydrogen peroxide, protects against its damaging effect, as does the glutathion redox system.

Patients with AC offer a unique possibility to study these two effects of H_2O_2 in the human body. We therefore tested neutrophil functions with and without H_2O_2 stress of persons with AC of the Swiss type.

MATERIALS AND METHODS

Probands. The four individuals homozygous for AC have been described in 1962 by Aebi and Wyss (1). They belong to two nonrelated families. The heterozygous proband G.M. is a member of family G. At the time of blood donations all were in good health. Healthy individuals (age 21 to 50 yr) were used as controls. The number of tests and controls is given for each experiment.

Isolation of PMN. Blood was drawn in preservative-free heparin vessels and was processed within 1 to 3 h. PMN were separated after addition of dextran on a Ficoll-Hypaque gradient (17).

Determination of catalase activity. The PMN suspension (1×10^7 PMN/ml) was sonicated after addition of 0.2% (w/v) Triton x 100. The rate of oxygen release was recorded with an oxigraph (Gilson 5/6) (18). After a stable base-line had been established, 25 μl of this homogenate were added to 1.6 ml Hanks' balanced salt solution, containing 0.02 M H_2O_2. The difference in the rates of oxigen released before and after addition of H_2O_2 was taken as catalase activity.

PMN function tests. Chemotaxis was measured by the "under-agarose" method using zymosan-activated serum as a stimulant (19). The distance of migration toward zymosan-activated serum ("stimulated" migration) was compared with that on the Hanks' balanced salt solution side ("spontaneous" migration). Chemotactic activity was calculated as the difference between stimulated and spontaneous migration. One ml of the PMN suspensions was incubated in saline, the other 1 ml-fraction was simultaneously exposed to H_2O_2 generated by adding 70 mU glucose oxidase (Sigma, Munich) to 5.5 mM glucose (20). After incubation for 15 min at 37° C in a shaking water bath, the reaction was stopped with 10 ml ice cold saline, the cells were washed twice with HBSS, resuspended, and immediately filled into the prepared agarose plate.

Particle ingestion was measured by counting the yeast particles phagocytosed by 100 PMN (21).

Membrane potential for cell activation was estimated by measuring the change in membrane potential after exposure to PMA (22). Membrane depolarization was continuously followed at 470 nm (excitation) and 510 nm (emission). The results were given in relative units, taking the steady state value as 100 U (22).

Oxygen consumption was measured by an oxygen electrode and recorded continuously with an oxigraph before and after stimulation by opsonized zymosan (15 mg/ml) or PMA (1 μg/ml) (23).

O_2^- and H_2O_2 generation for these assays a Carry 219 split beam spectro-photometer was used. O_2^--dependent reduction of cytochrome c was recorded at 550 nm (24). Formation of H_2O_2 was measured at 403 nm with horseradish peroxidase as compound II (25). (Reagents purchased from Boehringer, Mannheim, Germany.)

Chemiluminescence was measured at 37° C in a luminometer (LKB 1251, Turku, Finland) after stimulation with PMA or opsonized zymosan, according to Allen *et al.* (26). Results were expressed in mV/min/10^6 PMN.

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Cytochrome b reduction by PMA was measured by scanning difference-spectra of hemoglobin-free PMNs using a Carry 219 split-beam spectrophotometer. Absorbance at 427 and 558 nm was measured and cytochrome b activity was calculated (27).

Membrane fluidity estimation (28) for PMN ruptured by mild sonication on ice were ultracentrifuged and the isolated membrane fractions (80–100 μg protein) were suspended in 3 ml HBSS and labeled with 2 μM 1.6-diphenyl-1,3,5-hexatriene (2 mM stock solution in tetrahydrofuran) for 20 min at 37° C. Steady state fluorescence anisotropy was measured under constant stirring in an amino SPF-500 spectrofluorimeter. The sample was excited by vertically polarized light at 357 nm and fluorescence elicited was analyzed at 430 nm by using optical filters (cutoff 420 nm) and by turning the monochromator into vertically and horizontally polarized positions I_V and I_H. The steady state anisotropy, r^s is obtained as $r^s = (I_V - I_H)/(I_V + 2I_H)$.

I_V and I_H were corrected for intrinsic fluorescence, light scattering, and instrumental factors prior to each determination of r^s.

PMA (stock solution 2 mg/ml in dimethylsulfoxide) was added to membranes (final concentration 21 μg/ml), and the first determination of r^s was performed within 8 s.

Table 1. *Catalase activity in PMN lysates (μmol H_2O_2 degraded/min/10^7 PMN lysate)*

Subject	
Homozygote, G.A.	4.3*
Homozygote, G.P.	5.3
Homozygote, G.L.	6.3
Homozygote, A.B.	4.9
Heterozygote, G.M.	154.9
Normal controls (n = 8)	367.2 ± 48.4†
Range	211.1–451.2

* Each figure = mean of four determinations.
† Mean ± SD.

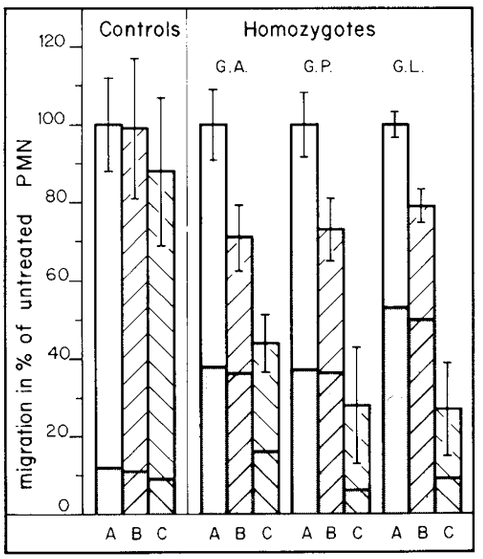

Fig. 1. Migration of PMN: effect of H_2O_2. *Whole column*, stimulated migration toward zymosan activated serum (*ZAS*). *Lower part of column*, spontaneous migration; *upper part of column*, net chemotactic migration. Since the migration distances exhibit considerable variation from one experiment to the other (674 ± 65 to 913 ± 54 μm in the normal controls), each proband is compared with his control, this latter being set to 100%. *A*, untreated PMN: *B*, preincubated PMN without H_2O_2 stress; *C*, preincubated PMN with H_2O_2 stress.

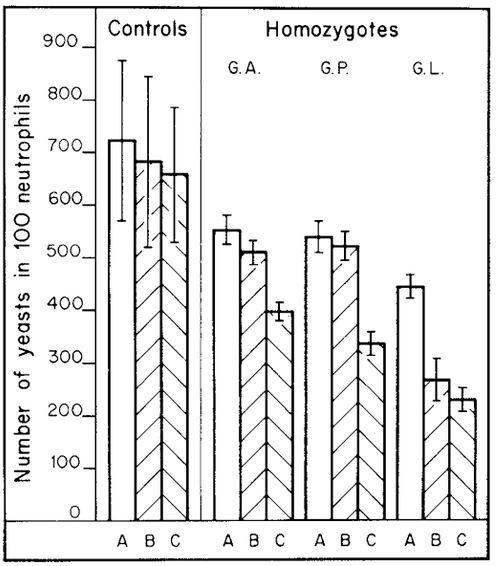

Fig. 2. Phagocytosis measured as yeast particles ingested by 100 PMNs. Effect of H_2O_2; controls; n = 5. *A*, untreated PMN; *B*, preincubated PMN without H_2O_2 stress; *C*, preincubated PMN with H_2O_2 stress.

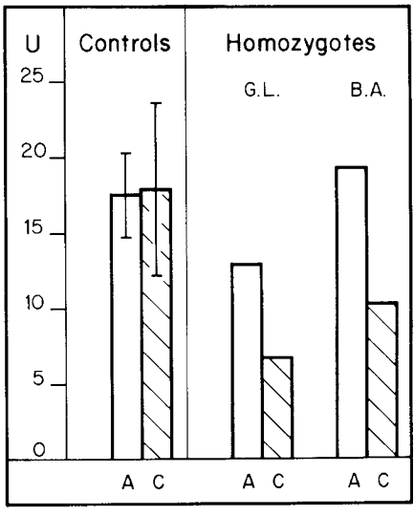

Fig. 3. Membrane potential of PMN after PMA stimulation in relative units, steady state value = 100 U. Effect of H_2O_2; controls, n = 6. *A*, untreated PMN; *C*, PMN incubated with H_2O_2

RESULTS

Catalase activity (Table 1). Under the experimental conditions used, catalase activity of four homozygous and of one heterozygous AC probands was 1–2% and 42%, respectively, compared to the normal controls calculated as 100%.

Functional tests. Chemotaxis measured with fresh PMN of three homozygous AC was indentical with that of normal PMN (Fig. 1). After preincubation for 15 min at 37° C the chemotactic activity of control cells was barely changed, but AC cells migrated distinctly slower after simple incubation in saline and considerably less well after exposure to H_2O_2 stress.

The phagocytosis assay (Fig. 2) gave similar results. While normal PMN showed only insignificant decrease after H_2O_2 stress, the cells of three homozygous AC carriers exhibited 28% decrease after incubation only, and a reduction of 49% after H_2O_2 stress.

Again, no change of membrane potential in normal PMN after 15 min exposure to H_2O_2 could be observed, but significant decreases with PMN of two AC homozygotes (Fig. 3).

Uptake of O_2 by the PMNs of one AC subject showed a high value in the absence of H_2O_2, but markedly less after H_2O_2 stress (Table 2*A*).

O_2^- production (Table 2*B*) in three homozygous AC carriers was normal in the absence of H_2O_2 but significantly reduced after H_2O_2 stress.

H_2O_2 production (Table 2*C*) was normal in the three homozygous AC carriers.

Chemiluminescence (Fig. 4) during phagocytosis was normal in all cells tested. However, after 15 min preincubation with the H_2O_2-generating system, normal PMN still produced 45% of the original chemiluminescence, whereas the homozygous acatalasemic cells were severely damaged, showing only 2.5–12.9% of their original capacity.

No difference in cytochrome b activity could be observed between normal PMN ($0.5–1 \times 10^{-17}$ mol cytochrome b/PMN) and AC-PMN exposed to H_2O_2 stress (0.66×10^{-17} mol cytochrome b/PMN).

Table 2. *Oxygen activation by PMNs*

Stimulus	Proband	No pretreatment	H_2O_2 pretreatment
A) O_2 consumption (nmol O_2/5 × 10^6 PMN/h at 37° C			
Opsonized	A.B. (heterozyg.)	1800	763
Zymosan	Control (n = 1)	1464	1452
B) O_2^- production (nmol O_2^- produced/min/10^6)			
PMA	G.A. (homozyg.)	8.6	4.9
	G.P. (homozyg.)	8.1	4.6
	G.L. (homozyg.)	5.5	3.5
	Control (n = 5)	6.3 ± 1.4	7.0 ± 1.6
C) H_2O_2 production (nmol H_2O_2 produced/min/10^6 PMN)			
PMA	G.A. (homozyg.)	0.85	
	G.P. (homozyg.)	0.57	
	G.L. (homozyg.)	0.36	
	Control	0.90 ± 0.29	

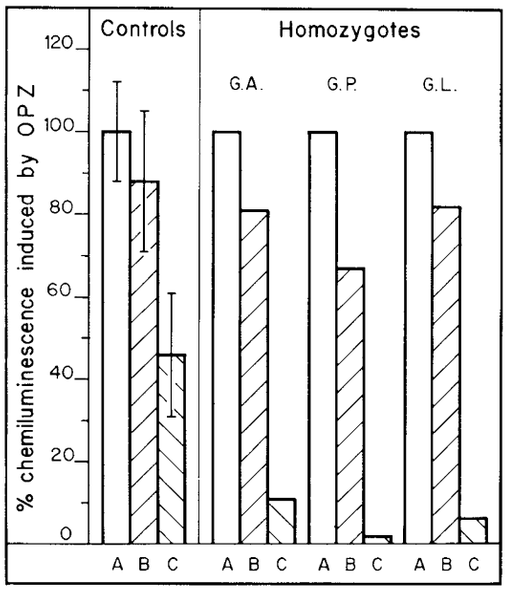

Fig. 4. Chemiluminescence of PMN after stimulation by opsonized zymosan. Effect of H_2O_2; controls, n = 5. *A*, untreated PMN; *B*, preincubated PMN without H_2O_2 stress. *C*, preincubated PMN with H_2O_2 stress.

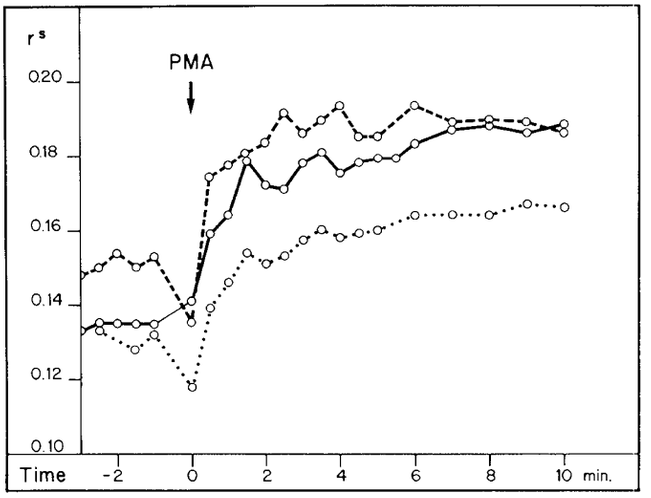

Fig. 5. Membrane fluidity of PMN after PMA stimulation. r^s, anisotropy; effect of H_2O_2. O——O, control PMN, untreated; O- - - - -O, AC, homozygous, untreated; O . . . . . O, AC, homozygous, incubated with H_2O_2.

Membrane fluidity (Fig. 5) was slightly increased in resting PMNs of AC individuals. After PMA stimulation, it slightly increased in normal controls and in AC cells after simple incubation in saline, but barely rose after preincubation with H_2O_2.

DISCUSSION

PMN provide an important protection to the body against invading microorganisms. Their functions are manyfold: migration toward the infectious focus (chemotaxis), ingestion, and intracellular killing of microorganisms. The last step is mediated by a sequence of biochemical reactions leading to the activation of oxigen. One crucial product is H_2O_2 which kills bacteria by perforating their cell walls.

As a side effect H_2O_2 may damage the body's own tissues. Effective protection against this noxious effect is provided by four mechanisms: 1) Degradation of H_2O_2 by catalase to H_2O and O_2; 2) degradation of H_2O_2 through the action of glutathione peroxidase; 3) deviation of H_2O_2 into the myeloperoxidase-halide system for bacterial killing; and 4) release of H_2O_2 outside the PMN by diffusion (29).

Herein we are exclusively concerned with the first factor, since in AC only the breakdown of H_2O_2 is impaired and the other criteria are independent of catalase activity.

The results in our patients can be compared with data obtained in experimentally produced AC. Voetman and Roos (30) added NaN_3 to normal PMN *in vitro* and observed no damage to the phagocytic functions by endogenous H_2O_2 (31). They concluded that during phagocytosis the glutathione redox system provides sufficient protection for the PMN, and that catalase becomes essential only to cope with additional exogenous H_2O_2.

Our own experiments were designed to reach approximately equivalent extracellular H_2O_2 concentrations as are observed during phagocytosis of normal PMN by endogenous production (20). This oxidative stress, although certainly not excessive for the PMN, was of longer duration than the naturally occurring H_2O_2 load during the respiratory burst. The following pathologic results were found: 1) Chemotaxis was markedly depressed; 2) membrane potential was moderately decreased; 3) O^-_2 generation was reduced by 40%, whereas cytochrome b reduction was normal; and 4) chemiluminescence was markedly reduced when particulate stimulants (opsonized zymosan or yeast) were used, but normal after stimulation with PMA in solution.

This effect of PMA is mediated by the binding of its fatty acid chain to hydrophobic structures within the cell membrane. By

this mechanism the PMNs are directly activated and the normal pathway over C3 or Fc-receptors is bypassed (32). This fact explains our observation that chemiluminescence induced by PMA is equal in congenital AC as in control neutrophils, but diminished, if particulate stimuli are used. In additional experiments (data not presented) we could confirm these alterations of chemiluminescence in normal PMNs artificially made acatalasemic by NaN_3 incubation, and we could protect them against this damage by vitamin E which is a well-known antioxidant (33).

Roos *et al.* (29) studied the ultrastructure of granulocyte membranes before and after ingestion of zymosan particles. They found no difference in the electron micrographs taken from artificially acatalasemic and from control neutrophils. This correlates well with our data on membrane fluidity before and after incubation of the cell with H_2O_2. There was no difference between AC and control PMN, and treatment with H_2O_2 had no measurable influence. We therefore conclude that PMN membranes of AC patients are not damaged by intrinsic H_2O_2.

The study of these complex interrelations is of crucial importance to understand Takahara's original findings: the patients he treated during the years 1946 to 1950 presented with deep necrotizing ulcerations, sometimes even necessitating partial resection of the mandible. In recent years, however, he never encountered such severe signs. He therefore considered concomitant exogenous factors—the development of antibiotics and their frequent application may have changed the mucosal flora or the early defense against bacterial growth (2) and improvement of the grossly deficient nutritional and hygienic conditions in Japan during the postwar years may have increased the critical level of oxidative damage.

The formation of mucosal ulcers is explained by the following vicious circle. H_2O_2 produced by bacteria in the absence of catalase accumulates locally and oxidizes Hb to methemoglobin. Since this is an insufficient O_2 carrier, the infection finally leads to progressive necrosis of the surrounding tissues (2). The well-known tendency for oral ulcer formation in the lazy leukocyte syndrome (34) and in chronic granulomatous disease (35) confirm the validity of this hypothesis.

For well-nourished not vitamin E-depleted carriers of AC who observe a satisfactory oral hygiene, apparently the other mechanisms mentioned above to dispose of H_2O_2 arising from their own respiratory burst activity and bacterial metabolism offer satisfactory protection in order to keep them asymptomatic.

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