H₂O₂-nonproducing *Streptococcus pyogenes* strains: survival in stationary phase and virulence in chronic granulomatous disease

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The production of hydrogen peroxide (H₂O₂) and related phenotypes were studied with *Streptococcus pyogenes* strains isolated from cases of pharyngitis or severe group A streptococcal infections. Of the 46 strains examined (34 from severe infections and 12 from pharyngitis cases), 25 strains accumulated H₂O₂ in the culture medium when grown under glucose-limited, aerobic conditions, whereas the rest of the strains did not. There was no correlation between these traits and the type of disease from which each strain had been isolated. The H₂O₂-nonproducing strains tested in this study belonged to T type 3 or T type 12. The accumulation of H₂O₂ started when the culture reached the late exponential phase. A rapid loss of cell viability accompanied H₂O₂ accumulation but was completely prevented by the addition of a catalase, indicating that the lethality was actually caused by H₂O₂. Cells of H₂O₂-nonproducing strains were resistant to killing by phagocytes from patients with chronic granulomatous disease (CGD), whereas those of H₂O₂-producing strains were subject to killing. Subcutaneous inoculation of 10⁵ c.f.u. H₂O₂-nonproducing *S. pyogenes* strains into the hind footpads of CGD mice provoked more prominent swelling of the footpad than did H₂O₂-producing strains. The mortality rate in the CGD mice infected with the H₂O₂-nonproducing strains was higher than that produced by the H₂O₂-producing strains. It is suggested that H₂O₂-nonproducing *S. pyogenes* strains are prevalent in humans and that they may be a potential threat to the health of CGD patients.

Keywords: group A streptococci, hydrogen peroxide, CGD, gp91-phox knockout mice

INTRODUCTION

Streptococcus pyogenes is the causative agent of a variety of human diseases, including pharyngitis, impetigo, necrotizing fasciitis, toxic-shock-like syndrome, and post-streptococcal diseases (acute glomerulonephritis and rheumatic fever) (Holm *et al.*, 1992; Shanley *et al.*, 1996; Talkington *et al.*, 1993). Of these, necrotizing fasciitis and toxic-shock-like syndrome, often referred to as severe group A streptococcal infections, are particularly notable in that they are extremely life-threatening. Although a number of factors have been

implicated in the virulence of this micro-organism, relatively little is known about what distinguishes this severe form from other common streptococcal diseases.

During the course of our study on pathogenicity factors of *S. pyogenes* strains in relation to the severe form of infection, we found that some strains were killed in the early stationary phase of growth under aerobic conditions, while others survived for more than 24 h after entering the stationary phase. Subsequent studies revealed that this loss of viability could be accounted for by a build-up of hydrogen peroxide (H₂O₂) in the culture medium, and established the presence of a dichotomy between positive and negative H₂O₂ accumulation in *S. pyogenes*. Streptococci possess superoxide dismutase, and many strains are known to produce H₂O₂. There have been many studies on H₂O₂

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Abbreviations: BHI, brain-heart infusion; CGD, chronic granulomatous disease.

production by oral streptococci (Garcia-Mendoza *et al.*, 1993; Holmberg & Hallander, 1973; LeBien & Bromel, 1975; Liebana *et al.*, 1993; Willcox & Drucker, 1988), and this characteristic has often been important for the classification of these species (McLeod & Gordon, 1922; Whiley *et al.*, 1990). However, there are few reports on H₂O₂ production by *S. pyogenes* (Gibson *et al.*, 2000).

It has been known that H₂O₂-nonproducing organisms are common causes of infections in patients with chronic granulomatous disease (CGD) (Segal *et al.*, 2000). CGD is an inherited disease involving phagocyte dysfunction, and is characterized by recurrent pyogenic infections that usually begin early in life (Gallin *et al.*, 1983; Roos, 1994). The NADPH oxidase complex in CGD granulocytes produces deficient levels of H₂O₂. This results in impaired intracellular killing (Roos, 1994), which is compensated for partially by the H₂O₂ production of some infecting micro-organisms. Here we report the impaired bactericidal activity of CGD granulocytes against H₂O₂-nonproducing *S. pyogenes* strains.

Recently, gene targeting has led to the development of mouse models for both X-linked (gp91-phox⁻) and autosomal recessive (p47-phox⁻) forms of CGD (Jackson *et al.*, 1995; Pollock *et al.*, 1995). To compare the virulence of H₂O₂-producing and H₂O₂-nonproducing strains for CGD *in vivo*, we infected X-linked CGD mice with *S. pyogenes* strains (via the footpads of the animals). H₂O₂-nonproducing *S. pyogenes* strains provoked prominent footpad swelling and were more lethal to CGD mice.

METHODS

Bacterial strains, culture and characterization. The *S. pyogenes* strains used in this study comprised 34 isolates from severe group A streptococcal infections and 12 from pharyngitis cases. All of the strains were epidemiologically unrelated to each other. Strains were cultured in brain-heart infusion (BHI) broth (pH 7.4, 0.2% glucose concentration; Eiken Chemical) at 37 °C with shaking under aerobic conditions. C medium [0.5% Protease Peptone no. 3 (Difco), 1.5% yeast extract (Difco), 10 mM K₂HPO₄, 0.4 mM MgSO₄ and 17 mM NaCl], was adjusted to pH 7.5 (Lyon *et al.*, 1998) and used as a low-glucose basal medium to study the effects of glucose on H₂O₂ accumulation (see below). To monitor growth at intervals, the OD₆₆₀ of bacterial cultures was measured. To count c.f.u., 10% sheep-blood agar plates were used. To test for catalase activity, cells were scraped from a culture plate and were then suspended in a drop of 3% H₂O₂ on a slide. Strains were judged catalase-positive when bubbles were observed in the suspension. Thioredoxin peroxidase activity in a crude cell extract was measured by the method described in a previous report (Jeong *et al.*, 1999). T typing was performed by the method described in a manual published by the World Health Organization (Rotta & Facklam, 1980).

H₂O₂ measurement. Samples (0.1 ml) were taken from test cultures, and the cells were removed by centrifugation. H₂O₂ was measured essentially by the method reported by Guilbault *et al.* (1966). A solution containing homovanillic acid at 18 µg ml⁻¹ and horseradish peroxidase at 2 µg ml⁻¹ was prepared in 0.1 M sodium phosphate buffer (pH 7.0), and a 1 ml portion of it was mixed with 10 µl supernatant of each sample. The fluorescence intensity of the mixture was recorded

at room temperature in a spectrophotofluorometer (model RF-500; Shimadzu) at a λ_{ex} of 315 nm and a λ_{em} of 425 nm (Guilbault *et al.*, 1966). When a plateau was reached, a known amount of authentic H₂O₂ was added as internal reference, and the resulting increment in the fluorescence intensity was recorded; this was then used to convert the final fluorescence intensities for the samples into H₂O₂ concentrations.

Effects of glucose on H₂O₂ accumulation. To test the effect of glucose on H₂O₂ accumulation, strains to be tested were cultured in C medium and glucose-supplemented C medium (the final glucose concentration was 1%, w/v) at 37 °C with shaking under aerobic conditions. At intervals, samples were taken to measure c.f.u. and the concentration of H₂O₂. The samples were serially diluted 10-fold with PBS then plated onto 10% sheep blood agar. The plates were incubated at 37 °C and the c.f.u. were counted after 18 h. The concentrations of H₂O₂ in the supernatants of the samples were measured by using the above method.

H₂O₂ sensitivity of organisms. The H₂O₂ sensitivity of organisms was measured using the method described by Cleary & Larkin (1979). Strains to be tested were cultured in BHI broth at 37 °C. At early stationary phase, the bacteria in 5 ml culture broth were washed with sterile PBS then resuspended in 5 ml PBS. H₂O₂ was added to each suspension to a final concentration of 5 µmol ml⁻¹. The suspension was incubated at 37 °C under anaerobic conditions; at 0, 2, 4, 6 and 8 h after the addition of H₂O₂, samples were taken, and catalase (final concentration, 200 U ml⁻¹) was added to each sample to eliminate the H₂O₂. After serial 10-fold dilution of the suspension with PBS, 0.1 ml was plated onto blood agar. The plates were incubated at 37 °C, and c.f.u. were counted after 18 h.

Lactate-sensitivity assays at different pH values. To study the lactate sensitivity of the *S. pyogenes* strain, cells were grown in BHI broth (pH 7.4) at 37 °C. The cells (at early stationary phase) were washed twice and resuspended in PBS with or without sodium lactate, which was adjusted to the desired pH with HCl. The suspensions were incubated at 37 °C with gentle shaking. At intervals, 200 µl aliquots of the samples were transferred to test tubes, and, after serial 10-fold dilution with PBS, 0.1 ml was plated onto blood agar. The plates were incubated at 37 °C, and c.f.u. were counted after 18 h.

Intracellular bacterial killing by granulocytes of CGD patients. Human peripheral blood was obtained from three patients with CGD (patients 1, 2 and 3) and from three healthy adults (controls). The granulocytes were separated aseptically by the dextran method (Kaplan *et al.*, 1968). Patients 1 and 2 were diagnosed as having X-CGD (gp91-phox⁻). Patient 3 was diagnosed with autosomal recessive-type CGD (p47-phox⁻). Early stationary-phase *S. pyogenes* cells were used in the assay. Cells in broth cultures were collected by centrifugation then washed twice with sterile PBS. Intracellular killing by granulocytes was studied by using the method described by Hiemstra *et al.* (1992). *In vitro* phagocytosis of serum-opsonized *S. pyogenes* was carried out for 5 min at 37 °C at a bacterium (c.f.u)/granulocyte ratio of 1:1 in Hanks' balanced salt solution containing 0.1% (w/v) gelatin (gelatin-HBSS). Non-phagocytized bacteria were removed, and phagocytosis was stopped by washing twice with cold gelatin-HBSS. Approximately 5 × 10⁶ granulocytes containing phagocytosed bacteria were suspended in 1 ml gelatin-HBSS containing 10% (v/v) fresh human serum from healthy adults of the AB blood group, and 0.25 ml portions were transferred to four sterile 1.5 ml microtubes (12310MTB1.5; Iwaki Glass). The granulocytes in the microtubes were cultured at 37 °C under

slow rotation (10 r.p.m). At 0, 30, 60 and 120 min, one sample tube each was taken and its inner wall scraped with a sterile scraper (Techno Plastic Products) to remove adhering granulocytes. The cells were well suspended and then disrupted by dilution with distilled water containing 0.01% (w/v) BSA followed by vigorous mixing. The lysate was spread on blood-agar plates to determine the numbers of viable bacteria.

Animals. Male and female X-linked CGD mice (Pollock *et al.*, 1995), which were produced by knockout of gp91-phox of C57BL/6 mice, were donated by Dr Dinuer (Riley Children's Hospital, Indianapolis, IN, USA) and Dr Kume (Division of Genetic Therapeutics, Center for Molecular Medicine, Jichi Medical School, Tochigi, Japan), and were reproduced in a specific pathogen-free animal room in our laboratory. Male, 6-week-old, X-linked CGD mice were used in the experiment. C57BL/6 mice of the same age and sex were also used as controls. They were purchased from Japan SLC.

Experimental infection, footpad swelling and mortality. One group consisted of three mice infected with one *S. pyogenes* strain; they were kept in one cage. Subcutaneous infection was performed by inoculating the suspension of *S. pyogenes* strains (10⁵ c.f.u. in a volume of 0.05 ml PBS) into the left hind footpads of mice, using a 27-gauge needle and a 1 ml syringe. As a control, 0.05 ml PBS was injected into the right hind footpad. The footpad thickness was measured by using a dial thickness gauge caliper (model G; Ozaki) at 24 and 48 h after inoculation; the difference between the thicknesses of left and right footpads was taken as the footpad swelling. The mortality of the CGD and control mice was observed until 10 d after infection. The mortality rates were determined by counting the number of dead mice in each group (consisting of 12 mice infected with four H₂O₂-producing and four H₂O₂-nonproducing strains, respectively).

Statistics. The correlation between H₂O₂ production and the type of disease was analysed using Fisher's exact test. Statistical analysis of the results of intracellular killing assay and animal experiments was performed with analysis of variance followed by a Student's *t*-test. Data monitored over time were compared by using the area under the curve.

RESULTS

Bacterial growth curves and H₂O₂ production

Bacterial growth in BHI broth and the concentration of H₂O₂ accumulated in four representative strains are shown in Fig. 1. H₂O₂ accumulated in the culture media of strains SP2 and MK5 in the late exponential and early stationary phases, whereas it was undetectable in those of strains SP1 and ME198 throughout the whole culture period. The turbidity of the cultures of the four strains was maintained or found to decrease only slowly during the stationary phase. The numbers of viable cells of strains SP2 and MK5, but not those of strains SP1 and ME198, decreased rapidly in the stationary phase. When catalase (final concentration, 200 U ml⁻¹) was added to the culture, the viability loss in strains SP2 and MK5 was prevented (data not shown). These results indicated that *S. pyogenes* strains were heterogeneous with respect to H₂O₂ production, which was responsible for the rapid loss of viability observed in the stationary phase of the culture. We therefore examined all of the 46 test strains for H₂O₂ production; the results obtained are shown,

with reference to T types, in Table 1. Of the 34 strains that were isolated from patients with severe streptococcal disease, 15 strains (44.1%) did not produce H₂O₂. Six strains out of 12 isolates (50%) from patients with pharyngitis did not produce H₂O₂. There was no correlation between H₂O₂ production and the type of disease from which each strain had been isolated (*P* = 0.75). All of the H₂O₂-nonproducing strains, which were epidemiologically unrelated to each other, belong to T types 3 or 12. Neither catalase activity nor thioredoxin peroxidase activity was detected in any strain (data not shown).

H₂O₂ sensitivity

The sensitivity to H₂O₂ of strains SP2 and MK5 (H₂O₂ producers) and strains SP1 and ME198 (H₂O₂ non-producers) was compared. All strains were killed at equal rates in the presence of H₂O₂ at a concentration of 5 µmol ml⁻¹, and a complete loss of viability was observed by 8 h (data not shown). It was also found that when H₂O₂ was added directly to stationary-phase cultures of strains SP1 and ME198 (H₂O₂ nonproducers) to a final concentration of 5 µmol ml⁻¹, a complete loss of viability was observed by 8 h, and the H₂O₂ concentration of the cultures had been maintained for this period (data not shown).

Effects of glucose on H₂O₂ production and bacterial survival

It is known that H₂O₂ production by *S. pyogenes* is depressed when the micro-organism is cultured in a glucose-rich medium (Gibson *et al.*, 2000). To test the effects of glucose in the culture medium on H₂O₂ production, two strains (SP1 and SP2) were cultured in C medium and glucose-supplemented C medium. When C medium was used, the results were the same as those with BHI broth (Fig. 1). Thus, H₂O₂ accumulated in the C medium of strain SP2, but not at all in that of strain SP1 (Fig. 2a, c). The numbers of viable cells of strain SP2, but not of strain SP1, decreased rapidly in the stationary phase because of H₂O₂ accumulation, as they did not decrease in the presence of catalase (data not shown). After cultivation in C medium for 36 h, the cultures showed final pH values in the range 6.6–6.7.

When glucose was added to C medium at a final concentration of 1% (w/v), the cultures of strain SP2, like those of strain SP1, did not accumulate H₂O₂ (Fig. 2b, d). However, despite the fact that two strains produced no detectable H₂O₂, they were killed very rapidly in the stationary phase (Fig. 2b, d). This apparent deviation from H₂O₂-dependent cell killing may be largely explained by the effect of the lactic acid derived from the glucose supplementation. Thus, the pH of the culture in glucose-enriched C medium was found to drop as low as 4.8 in the stationary phase. It was also found that 0.75% sodium lactate at pH 5.0 (a condition thought to be somewhat less severe than that actually present in the stationary phase culture) was capable of

Fig. 1. Bacterial growth in BHI broth and H₂O₂ production of *S. pyogenes* strains SP1 (a), SP2 (b), ME198 (c) and MK5 (d). SP1 and SP2 are strains isolated from patients with severe group A streptococcal infection; ME198 and MK5 are strains isolated from patients with pharyngitis. ●, Concentration of H₂O₂ in the culture medium; ○, OD₆₆₀; ■, numbers of viable bacteria.

Table 1. H₂O₂ production and T type of 46 *S. pyogenes* strains examined in this study

Source	H ₂ O ₂ production	T type								Total
		1	3	4	6	11	12	28	B3264	
Severe group A streptococcal infection	+	3	2	6	4	1	2	1		19
	—		12				3			15
Pharyngitis	+	2		1	2				1	6
	—		5				1			6
Total	+	5	2	7	6	1	2	1	1	25
	—		17				4			21

lowering the viability of SP1 cells by two orders of magnitude within a 3 h period (data not shown).

Intracellular killing by granulocytes of CGD patients

Intracellular killing of *S. pyogenes* strains was assayed by using human granulocytes from three CGD patients and three healthy control adults. *S. pyogenes* strains used in this assay were two H₂O₂-producers (strains SP2 and MK5) and two H₂O₂ nonproducers (strains SP1 and

ME198). Strains SP1 and SP2 were isolated from the patients with severe group A infection. Strains MK5 and ME198 were isolated from the patients with pharyngitis. Fig. 3 shows the mean percentage of viable intracellular bacteria in normal and CGD granulocytes over 120 min after *in vitro* phagocytosis. In granulocytes from three CGD patients, strains SP2 and MK5 (H₂O₂-producers) showed similar change over 120 min, and the killing rates did not differ significantly (all *P* > 0.05) at all three time points. Therefore, the data for these two strains

Fig. 2. Effects of glucose on H₂O₂ production. Strains SP1 (a, b) and strain SP2 (c, d) were cultured in C medium (a, c) and in glucose-supplemented C medium (b, d). ■, Numbers of viable bacteria; ●, concentration of H₂O₂ in the culture medium.

phagocytosed by the granulocytes from three CGD patients were pooled, and their mean data ('H₂O₂-producing strains phagocytosed by CGD granulocytes') are shown in Fig. 3. Likewise, as strains SP1 and ME198 (H₂O₂ nonproducers) showed similar changes (all $P > 0.05$), their data were pooled as 'H₂O₂-nonproducing strains phagocytosed by CGD granulocytes' (Fig. 3). In granulocytes from three normal control granulocytes, the difference between the two strains and the individual difference were also not significant (all $P > 0.05$). Therefore, the results fell into four groups, i.e. H₂O₂-nonproducing and H₂O₂-producing strains phagocytosed by normal and CGD granulocytes.

In CGD granulocytes, the survival levels of intracellular H₂O₂-nonproducing organisms were almost 100% after a 120 min period, whereas those of H₂O₂-producing organisms decreased by 50% at 120 min (Fig. 3). There was a significant difference between the two groups in terms of the killing by CGD granulocytes ($P = 0.0001$). These results demonstrated that the ability of *S. pyogenes* cells to produce H₂O₂ rendered them susceptible to intracellular killing by CGD granulocytes. On the other hand, normal granulocytes killed both H₂O₂-producers and H₂O₂ nonproducers at the same

rate ($P = 0.90$) (Fig. 3). The percentages of viable H₂O₂-nonproducing and H₂O₂-producing organisms in normal granulocytes were significantly lower than those in CGD granulocytes over 120 min, respectively (both $P = 0.0001$). Thus, cells of H₂O₂-nonproducing strains were totally resistant to killing by phagocytes from patients with CGD, whereas those of H₂O₂-producing strains were subject to moderate killing.

Mouse footpad infection

To clarify the virulence of H₂O₂-nonproducing strains for CGD *in vivo*, we used X-linked CGD mice and performed subcutaneous infection by inoculating *S. pyogenes* strains into the footpads of the mice. Footpad swelling has been used for the assessment of inflammation and may reflect the defect of initial killing of bacteria by neutrophils. C57BL/6 mice were used as the control. *S. pyogenes* strains used in this experimental infection were four H₂O₂-producers (strains SP2, MK5, ME1410 and ME1123) and four H₂O₂ nonproducers (strains SP1, ME198, ME206 and ME1125). Strains SP1, SP2, ME206 and ME1410 were isolated from patients with severe group A streptococcal infection; the others were isolated from patients with pharyngitis. Fig. 4

Fig. 3. Intracellular killing of *S. pyogenes* strains by human granulocytes from three CGD patients (dashed lines) and three normal control adults (solid lines). *S. pyogenes* strains used in this assay were two H_2O_2 -producers (strains SP2 and MK5) and two H_2O_2 nonproducers (strains SP1 and ME198). ○, two H_2O_2 -nonproducing strains phagocytosed by CGD granulocytes; □, two H_2O_2 -producing strains phagocytosed by CGD granulocytes; ●, two H_2O_2 -nonproducing strains phagocytosed by normal granulocytes; ■, two H_2O_2 -producing strains phagocytosed by normal granulocytes. Data are expressed as the mean ($\pm$ SD) of percentages of viable intracellular bacteria obtained from six experiments, that is, three individuals and two bacterial strains.

shows the footpad swelling of CGD and control mice at 48 h after infection with eight strains. The mean ($\pm$ SD) footpad swelling of CGD mice inoculated with H_2O_2 -nonproducing and H_2O_2 -producing strains was 2.42 ($\pm$ 0.49) mm and 0.34 ($\pm$ 0.46) mm at 24 h, and 3.16 ($\pm$ 0.43) mm and 0.58 ($\pm$ 0.65) mm at 48 h after inoculation, respectively. In CGD mice, the swelling of the footpads infected with H_2O_2 -nonproducing strains was more prominent than that of footpads infected with H_2O_2 -producing strains at both time points (both $P = 0.001$). On the other hand, in control C57BL/6 mice, the mean ($\pm$ SD) swelling of footpads infected with H_2O_2 nonproducers and H_2O_2 -producers was, respectively, 0.20 ($\pm$ 0.20) mm and 0.17 ($\pm$ 0.18) mm at 24 h, and 0.33 ($\pm$ 0.36) mm and 0.15 ($\pm$ 0.15) mm at 48 h after infection. There were no differences between the two groups at both time points ($P = 0.82$ and 0.31, respectively). The footpad swelling of CGD mice, in comparison to control mice, was significantly greater for challenges with both H_2O_2 -nonproducing ($P = 0.0001$) and H_2O_2 -producing strains ($P = 0.0174$) at 48 h after infection. Within 10 d after inoculation, some CGD mice were dead because of systemic infection. Fig. 5 shows the survival curves of the CGD and control mice after the infection of footpads. The mortality rate at day 10 in CGD mice infected with H_2O_2 -nonproducing strains was higher than that for mice infected with H_2O_2 -producing strains (92% versus 42%, respectively). No control mice died within the same period.

Fig. 4. Footpad swelling of CGD mice (hatched columns) and control C57BL/6 mice (solid black columns) at 48 h after infection with *S. pyogenes* strains. The *S. pyogenes* strains used in this experimental infection comprised four H_2O_2 -producers (strains SP2, MK5, ME1410 and ME1123) and four H_2O_2 nonproducers (strains SP1, ME198, ME206 and ME1125). Strains SP1, SP2, ME206 and ME1410 were isolated from patients with severe group A infection; the others were isolated from patients with pharyngitis. Data are expressed as the means of results from three mice ($\pm$ SD).

Fig. 5. Survival curves of CGD (dotted lines) and control (solid lines) mice after inoculation of their footpads with H_2O_2 -producing and H_2O_2 -nonproducing *S. pyogenes* strains. The mice and the *S. pyogenes* strains used in this experiment were the same as those used in the experiments presented in Fig. 4. ○, Four H_2O_2 -nonproducing strains inoculated into the footpads of 12 CGD mice; □, four H_2O_2 -producing strains inoculated into the footpads of 12 CGD mice; ●, four H_2O_2 -nonproducing strains inoculated into the footpads of 12 control C57BL/6 mice; ■, four H_2O_2 -producing strains inoculated into the footpads of 12 control C57BL/6 mice.

DISCUSSION

The ability to produce and excrete H₂O₂, manifested at low glucose concentrations, has been documented recently for a strain of *S. pyogenes* (Gibson *et al.*, 2000). In the present study, we found that clinical isolates of this bacterium could be divided into two classes characterized by the presence or absence of H₂O₂ accumulation in the culture medium. Of the 46 strains examined, 21 were H₂O₂ nonproducers. The H₂O₂ accumulation was growth-phase dependent; it started when the culture was in the late exponential phase, the maximum level being attained in the early stationary phase. The rapid viability loss in H₂O₂-producing strains during the stationary phase was due to the toxicity of endogenously produced H₂O₂ (since it was abolished by the addition of catalase to the medium). Furthermore, there was no difference between H₂O₂-producing and H₂O₂-non-producing strains in terms of sensitivity to exogenous H₂O₂.

The rapid cell death in the glucose-rich medium during the stationary phase was apparently independent of H₂O₂ production. This cell death was probably due to undissociated lactic acid (thought to be capable of crossing the plasma membrane) that was increased in the bacterial cells under weakly acid conditions (pH < 5) and caused cell damage. The toxicity of organic acids produced by fermentation is generally explained by the transmembrane flux of undissociated acids under the acidic conditions (Russell & Diez-Gonzalez, 1998; Shimokawa & Nakayama, 1999).

One potentially significant finding was that all the H₂O₂-nonproducing strains, which were epidemiologically unrelated to each other, belonged to either T type 3 or T type 12. This apparent correlation must be studied with an expanded array of clinical isolates before its meaning is considered any further; however, it must be pointed out that both T types have H₂O₂-producing members as well. There was no correlation between the ability of a strain to accumulate H₂O₂ and the severity of the infection from which it had been isolated (Table 1).

The H₂O₂-producing phenotype was shown to affect the fate of bacterial cells within phagocytes. Cells of H₂O₂-producers were moderately susceptible to intracellular killing by CGD granulocytes deficient in H₂O₂ generation by the NADPH oxidase system, whereas those of H₂O₂ nonproducers were totally resistant to it. This finding is consistent with the well-known fact that bacteria that do not produce H₂O₂ constitute the main cause of pyogenic infections in CGD patients (Segal *et al.*, 2000). Furthermore, we noted that H₂O₂-nonproducing *S. pyogenes* strains caused prominent inflammation (Fig. 4) and higher mortality (Fig. 5) in CGD mice. This suggests the possibility that the H₂O₂-nonproducing *S. pyogenes* strains would be important causative agents of severe infections in the patients with CGD. However, this relationship is not readily recognizable in clinical cases of streptococcal infections. Thus, Gallin *et al.* (1983) reported that out of 119 major

febrile episodes in 14 CGD patients, *Streptococcus* was isolated only rarely (three *Streptococcus pneumoniae* strains). Regelman *et al.* (1983) found only five *Streptococcus* strains (three *S. pneumoniae* and two *Streptococcus intermedius*) among 93 micro-organisms isolated from 15 CGD patients. It is known that *S. pneumoniae* produces H₂O₂ (Spellerberg *et al.*, 1996; Ukada *et al.*, 1959). However, the significance of its isolation from CGD patients is unclear, because it is the most common cause of pneumonia, even in healthy individuals. On the other hand, our previous study demonstrated that *S. intermedius*, an H₂O₂ nonproducer (Whiley *et al.*, 1990), was the causative agent of severe infection (brain abscess) in a CGD patient, and its pathogenicity was attributed to the lack of H₂O₂ production (Nagatomo *et al.*, 1999).

The rarity of *S. pyogenes* infection in CGD patients may be due to an early start of antimicrobial therapy and the virtual absence of drug-resistant strains. Although *S. pyogenes* infections are presently not common in CGD patients, it would be of interest to examine the H₂O₂-production phenotypes of isolates from such rare cases. In contrast to CGD granulocytes, normal granulocytes killed H₂O₂-producers and H₂O₂ nonproducers with practically equal efficiency. This may be taken to suggest that the H₂O₂ phenotype of *S. pyogenes* should be of little significance as a virulence factor for infections in healthy individuals.

Also interesting is the biochemistry of H₂O₂ production and related matters in this organism. Accumulation of H₂O₂ in the culture medium in large amounts is a unique feature common among lactic acid bacteria (streptococci, lactococci, enterococci and lactobacilli). At variance with most other organisms (in which H₂O₂ is derived largely from the superoxide anion), lactic acid bacteria are generally believed to produce H₂O₂ mainly through oxidation of substrates such as NADH by H₂O₂-producing oxidases (Thomas & Pera, 1983; Murphy & Condon, 1984; Marty-Teyssset *et al.*, 2000; Gibson *et al.*, 2000). In fact, a homology search of the *S. pyogenes* genome database has revealed the genes of putative H₂O₂-producing oxidases for NADH, α -glycerophosphate and lactate, and their involvement in H₂O₂ production has been suggested (Gibson *et al.*, 2000). In accordance with the widely held view, we are also of the opinion that at least a major part of the H₂O₂ accumulated in *S. pyogenes* cultures is produced by one or more of the above-mentioned oxidases.

The accumulation of H₂O₂ was repressed by the presence of high concentrations of glucose, and was manifested only after cell growth had slowed down in the late exponential phase. This apparent 'glucose effect' has also been well documented for lactic acid bacteria. Although precise mechanisms for these phenomena still remain unclear, their biological significance might be evident if one takes account of the fact that the above-mentioned oxidase reactions are functionally linked to the 'thioclastic' pathway of pyruvate metabolism. This ATP-coupled pathway is known to operate under

glucose-limited conditions in a variety of lactic acid bacteria, and is energetically advantageous (for a review, see Stouthamer, 1978). In fact, the *S. pyogenes* genome database shows the presence of the complete set of genes for the thioclastic pathway. Finally, the dichotomy between the presence and absence of H₂O₂ accumulation, a situation also widely seen among lactic acid bacteria, awaits explanation. Although not discussed here, a role for the H₂O₂-degrading system must be considered when such a dichotomy is addressed. Attempts to answer these unsolved questions are under way in the authors' laboratory.

In conclusion, we found that H₂O₂-nonproducing *S. pyogenes* strains resisted intracellular killing by CGD phagocytes, and we demonstrated their virulence to CGD mice *in vivo*. Our findings raise the possibility that H₂O₂-nonproducing *S. pyogenes* strains could be a potential threat to CGD patients, though such cases have not yet been documented.

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