


Superoxide anions produced by *Streptococcus pyogenes* group A-stimulated keratinocytes are responsible for cellular necrosis and bacterial growth inhibition

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Abstract

Gram-positive *Streptococcus pyogenes* (group A *Streptococcus* or GAS) is a major skin pathogen and interacts with keratinocytes in cutaneous tissues. GAS can cause diverse suppurative and inflammatory infections, such as cellulitis, a common acute bacterial dermo-hypodermatitis with a high morbidity. Bacterial isolation yields from the lesions are low despite the strong local inflammation observed, raising numerous questions about the pathogenesis of the infection. Using an *in vitro* model of GAS-infected keratinocytes, we show that the major ROS produced is the superoxide anion (O_2^-), and that its production is time- and dose-dependent. Using specific modulators of ROS production, we show that O_2^- is mainly synthesized by the cytoplasmic NADPH oxidase. Superoxide anion production leads to keratinocyte necrosis but incomplete inhibition of GAS growth, suggesting that GAS may be partially resistant to the oxidative burst. In conclusion, GAS-stimulated keratinocytes are able to develop an innate immune response based on the production of ROS. This local immune response limits GAS development and induces keratinocyte cell death, resulting in the skin lesions observed in patients with cellulitis.

Keywords

ROS, *Streptococcus pyogenes*, cellulitis, cell death, inflammation

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Introduction

Infections of skin and soft tissues are characterized by microbial invasion of the epidermis, dermis and subcutaneous tissues generally inducing an immune response from the host. Various bacteria are able to trigger such infections, and they include the Gram-positive bacterium *Streptococcus pyogenes* (Group A *Streptococcus* or GAS), a major pathogen that interacts with keratinocytes in the epidermis. GAS are members of the pyogenic group of streptococci associated with infections in humans, and can be classified into more than 200 types based on the sequence of the 5' variable region of the major surface M protein gene (*emm* types).¹ GAS can cause numerous types of suppurative and inflammatory infections, ranging from mild

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superficial infections (impetigo) to more invasive and potentially life-threatening diseases (cellulitis, necrotizing fasciitis and toxic shock syndromes). Cellulitis is a common acute bacterial dermo-hypodermatitis associated with high morbidity: its incidence has been estimated to be 10–100/100,000 of the population/yr; 20–50% of cases require hospitalization; and the estimated mortality is 0.5%.² However, skin and blood specimen cultures are rarely positive for the causative bacterium, with reported isolation rates between 14% and 40% for skin cultures and between 2% and 3% for blood cultures.³ This low bacteriological yield contrasts with the often substantial local inflammation, raising numerous questions about the pathogenesis of cellulitis, which remains incompletely understood.

GAS strains may enter the skin through abrasions and other types of lesion allowing the bacteria to penetrate the epidermis and to adhere to keratinocytes via a receptor-mediated process.⁴ This invasion process involves the expression by GAS of many virulence factors facilitating the internalization of the bacteria into epithelial cells by a mechanism involving matrix and plasma proteins.^{5–7} The prominent M protein is important for the attachment of GAS to keratinocytes in skin infections, mediating the recognition of CD46 and $\alpha 5\beta 1$ -integrin.^{8–11} The fibronectin-binding protein F may mediate the attachment of GAS to Langerhans cells,^{12,13} and GAS plasminogen-binding proteins have been shown to interact with $\alpha 1\beta 1$ and $\alpha 5\beta 1$ integrins.¹⁴ Once adhesion and colonization have been established, bacterial enzymes and exotoxins (streptolysin, erythrogenic toxin, DNase, streptokinase, proteinase, amylase and esterase) contribute to local inflammation and the dissemination of GAS.¹⁵

Keratinocytes are the major cell lineage in the epidermis, a multilayered stratified epithelium of the skin that is the essential first line of defense against microbial infection. It has repeatedly been shown that keratinocytes are more than a mere passive barrier to infection, and that they play an active role in the host innate immune response. Following stimulation of the PRR-related pathways by certain pathogens, keratinocytes produce various pro-inflammatory molecules that contribute to inducing the direct migration of various subpopulations of immune cells, which, in turn, amplify the local inflammatory response.¹⁶ Several surface molecules expressed by GAS are specifically recognized by cognate PRRs on the surface of host cells; the signaling adaptor MyD88 is involved in the subsequent cytokine production, although the PRRs specifically involved are not known. Indeed, GAS strains activate the p38 MAPK and NF- κ B pathways via MyD88-dependent and MyD88-independent pathways without the involvement of TLR2, TLR4 or TLR9.^{17,18} Adherent streptococcal strains, such as *S. pyogenes* JRS4, induce the transcription of the IL-1 α , IL-1 β , IL-6 and IL-8

genes and the release of prostaglandin E₂ (PGE₂).¹⁹ The cytokine response is believed to be critical for the inflammatory response, but the production of ROS may also be involved, especially during the early phase of inflammation. Indeed, in other models of skin infection involving different pathogens, keratinocytes rapidly produce ROS, contributing to the destruction of the bacteria and to the production of pro-inflammatory cytokines.^{20,21}

We report a study of the pro-inflammatory response of keratinocytes stimulated by cellulitis-related GAS strains in an *in vitro* model of infection. We investigated ROS production during the course of the immune response and the role of these molecules in both the inhibition of bacterial growth and keratinocyte cell death.

Materials and methods

Bacteria

Six *S. pyogenes* strains isolated from patients with cellulitis were obtained from the National Reference Center for Streptococci (<https://www.cnr-strep.fr>) (Table 1). They were classified on the basis of *emm* gene typing and included members of the three principal *emm* types encountered in French hospitals.²² Bacteria were grown in Todd Hewit broth (100 ml; Becton Dickinson), at 37°C, with shaking (250 rpm) and harvested by centrifugation at 7000 g for 10 min at 4°C. The exponential growth phase was identified experimentally as the point at which the OD₆₂₀ reached 0.30 ± 0.03, corresponding to 3–4 h of culture. Stationary phase bacteria were obtained after 18 h. Bacterial pellets were washed in about 30 ml cold sterile PBS [1.5 mM KH₂PO₄, 2.7 mM Na₂HPO₄·7H₂O, 0.15 M NaCl (pH 7.4)], centrifuged again and suspended in PBS or DMEM (Invitrogen, Cergy Pontoise, France). We used this suspension to prepare

Table 1. GAS strains used in this study.

Bacterial strain				
Simplified number ^a	Reference number ^b	Origin	Diagnostic	Genotype ^c
6AB	207801079	blood	cellulitis	<i>emm</i> 89
6EF	207800872	blood	cellulitis	<i>emm</i> 89
13F3	207800844	blood	cellulitis	<i>emm</i> 28
6G10	207800162	blood	cellulitis	<i>emm</i> 1
13H10	207800871	blood	cellulitis	<i>emm</i> 1
8I3	207800374	blood	cellulitis	<i>emm</i> 28

^aSimplified number to identify GAS strains used in this study. ^bReference number of GAS strains used by the French National Reference Center for Streptococci. ^cThe genotype according to Protein M typing based on sequencing of the *emm* gene.

dilutions of 10^4 – 10^9 CFU/ml used immediately to stimulate keratinocytes at 37°C, such that the multiplicities of infection (MOI) with 0.1 ml of inoculum were 0.001–100 bacteria per cell. Bacteria inactivation was achieved by heating the bacterial suspension in PBS at 75°C for 45 min and harvested by centrifugation at 7000 g for 10 min at 4°C.

Cell culture and incubation assay

Immortalized human keratinocytes (HaCaT cell line) were cultured in DMEM with 0.1–10% (FCS; Invitrogen), 20 mM L-glutamine, 1 mM sodium pyruvate and antibiotic/antimycotic solution (10 U/ml penicillin, 10 mg/ml streptomycin, 0.25 mg/ml amphotericin), and primary human keratinocytes (NHEK cell line; Lonza, Walkersville, MD, USA) cells were cultured in the serum-free keratinocyte basal medium (KBM-2; Lonza), both at 37°C under a humidified atmosphere containing 5% CO₂ as previously described.²³ Regular checks were carried out to ensure that the cell lines remained free from *Mycoplasma* infection. Cells at about 60–70% confluence were stimulated with GAS suspension, the concentration of which was adjusted to an appropriate value with DMEM supplemented with 0.1% FCS without antibiotics, for the desired period of time, at 37°C, under 5% CO₂.

Spectrofluorimetric assessment of ROS production

HaCaT cells (2×10^4 /well) were used to seed 96-well plates (Corning Costar, Brumath, France). The cells were incubated for 18 h, washed three times in PBS and incubated with 100 µl of a suspension of GAS (OD_{620 nm} = 1.0) for various times between 15 min and 24 h. The cells were washed three times, 50 µl of 5 mM dihydroethidium [DHE, to assay superoxide anions (O₂^{•−}) or 5 mM H₂-2',7'-dichlorodihydrofluorescein diacetate (DCFDA; to assay H₂O₂) or 5 mM 4,5-diaminofluorescein diacetate (DAF2-DA; to assay NO) was added per well, and fluorescence intensity was recorded hourly, for 4 h. Fluorescent probes were purchased from Molecular Probes (Eugene, OR, USA). The fluorescence excitation/emission maxima were 495/515 nm for DAF2-DA, 480/610 nm for DHE and 507/525 nm for H₂-DCFDA. At the end of the experiment, crystal violet assays were used to determine the number of adherent cells, as described below. The results of O₂^{•−}, H₂O₂ and NO assays were read by spectrofluorimetry in a Fusion spectrofluorimeter (PackardBell, Paris, France). The amount of ROS in each sample was calculated as follows: ROS rate (arbitrary units/min/10⁶ cells) = [fluorescence intensity (arbitrary units) at T4 h – fluorescence intensity (arbitrary units) at T0/240 min/number of adherent cells as determined in the crystal violet assay]. Values are expressed in arbitrary units (AU).

Origin of GAS-induced superoxide anions and modulation of their levels

HaCaT cells (2×10^4 /well) were used to seed 96-well plates and were incubated for 18 h in complete medium alone or in complete medium supplemented with the following: 2 mM diethyldithiocarbamate (or DDC or SOD inhibitor), 400 mM Cu(II)₂(3,5-diisopropylsalicylate)₄ (or CuDIPS a Cu/Zn SOD mimic), 100 mM Mn(III)tetrakis(4-benzoic acid) (or MnTBAP a MnSOD mimic), 40 mM rotenone (inhibitor of mitochondrial complex I), 40 mM antimycin (inhibitor of mitochondrial complex III), 40 mM diphenyliodonium (inhibitor of NADPH oxidase) or 40 mM allopurinol (inhibitor of xanthine oxidase). The cells were then washed three times in PBS and incubated with 100 µl of a suspension of GAS (OD_{620 nm} = 1.0) for various times between 15 min and 24 h. The cells were washed three times, 50 µl of 5 mM DHE was added to each well and fluorescence intensity was recorded hourly, for 4 h, as described above. At the end of the experiment, crystal violet assays were used to determine the number of adherent cells. The abundance of O₂^{•−} was calculated as described above.

Spectrofluorimetric analysis of cell death

Cell death was estimated spectrofluorimetrically with the fluorescent probe YO-PRO-1 (Molecular Probes) and a Fusion spectrofluorimeter (Packard Bell). HaCaT cells (2×10^4 /well) were used to seed 96-well plates and were incubated for 18 h in complete medium alone or in complete medium supplemented with the following: 2 mM diethyldithiocarbamate, 40 µM rotenone, 40 µM antimycin, 40 µM diphenyliodonium, 40 µM allopurinol, 1600 µM reduced glutathione, 3200 µM N-acetylcysteine, 400 µM CuDIPS, 100 µM MnTBAP, 800 µM D,L-buthionine-[S,R]-sulfoximine, 400 µM aminotriazol or 20 U PEG-catalase. Cells were then washed three times in PBS and 100 µl of a suspension of GAS (OD_{620 nm} = 0.5) in complete medium was added to each well and the incubation continued for 24 h. The cells were washed three times in PBS and incubated with 10 µM YO-PRO-1 for 30 min. Absorbance was read at an excitation wavelength of 480 nm and an emission wavelength of 525 nm for each sample, and cell death was evaluated by determining the fluorescence intensity (AU), as a measure of cell membrane disruption.

Flow cytometry assessment of cell death

HaCaT cells were incubated with or without GAS at an MOI of 0.01, 1.0 or 100, for 6 h at 37°C. The cells were washed twice with cold PBS, harvested by trypsin treatment, washed with PBS, incubated in 1 ml of PBS supplemented with both YOPRO-1 and propidium iodide

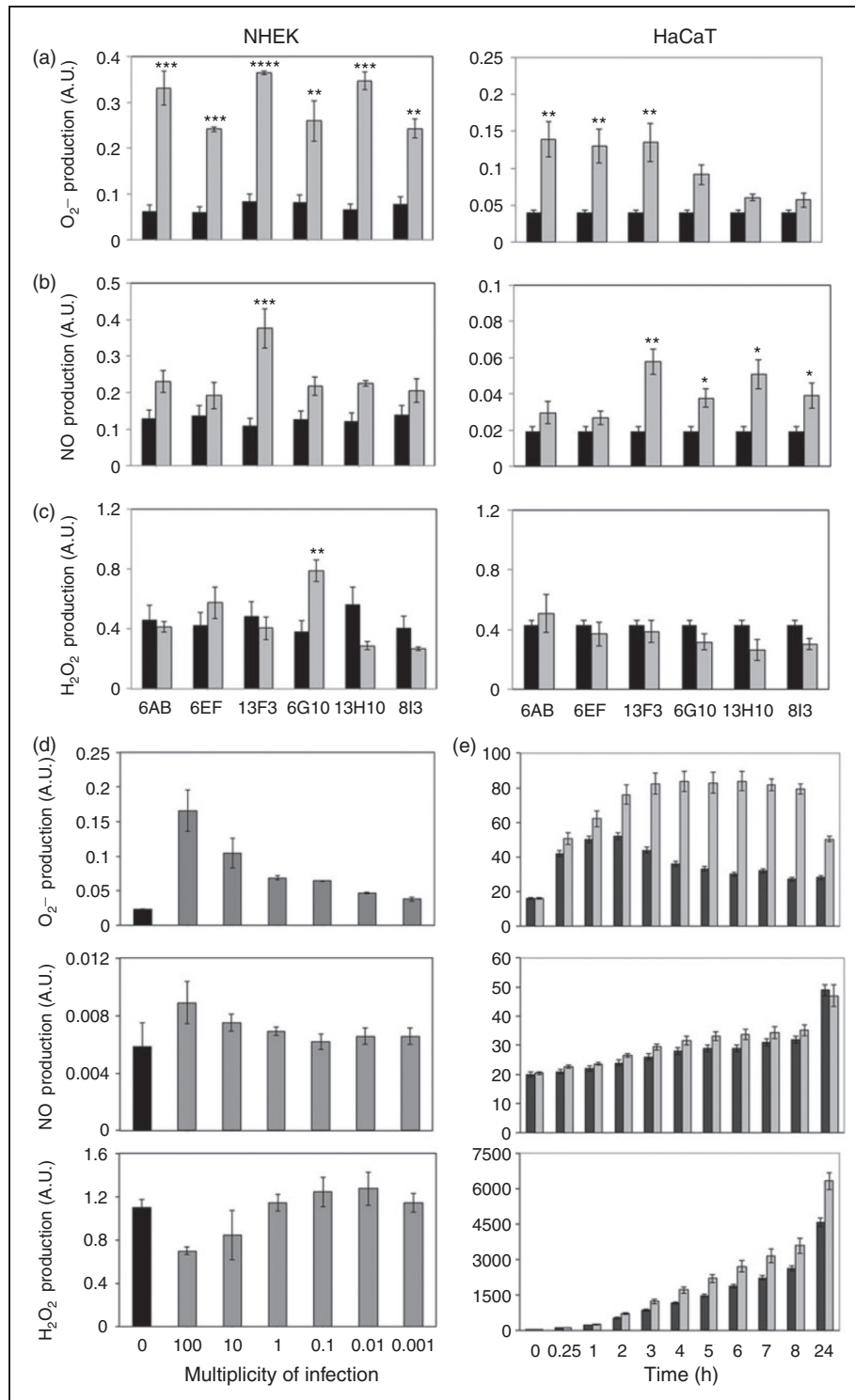


Figure 1. Evaluation of ROS production by GAS-stimulated keratinocytes. NHEK and HaCaT cells were incubated for 6 h with (gray bar) or without (black bar) cellulitis-related GAS strains (Table 1) at an MOI of 10. (a) O₂⁻, (b) NO and (c) H₂O₂ were assayed by spectrofluorimetry, as described in the 'Materials and methods'. (d) Dose dependence was determined by incubating HaCaT cells for 6 h with strain 6G10 at an MOI of 100, 10, 1, 0.1, 0.01 and 0.001. (e) HaCaT cells were incubated with strain 6G10 at an MOI of 10 and ROS production assayed at various times between 0.25 and 24 h. The values shown are means \pm SD of three independent experiments. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$.

for 15 min at 4°C in the dark, and then analyzed by flow cytometry (FACScalibur; Becton Dickinson, Mountain View, CA, USA). Control experiments involved incubating cells with PBS alone and with YOPRO-1 and propidium iodide separately.

Inhibition of GAS growth by keratinocytes

HaCaT cells (2×10^4 /well) were used to seed 96-well plates and were incubated for 18 h in complete medium. The cells were then washed three times in PBS and incubated with 50 μ l of the GAS suspension (MOI = 1) in DMEM supplemented with 10% FCS without antibiotics at 37°C. After 3 h, 150 μ l of liquid Todd Hewitt medium was added to each well and the plates were incubated for 18 h at 37°C. GAS growth was then evaluated by determining the OD₆₂₀ of the culture supernatant. A control experiment was run in parallel, with GAS in DMEM supplemented with 10% FCS with no antibiotics. To assess the effect of O₂⁻ production by the HaCaT cells on GAS growth, the HaCaT cells were pretreated for 18 h with 40 mM DPI, 2 mM DDC and 50 mM MnTBAP; the cells were then stimulated with GAS and the bacteria were cultured as described above.

Cell viability assays

Crystal violet staining was used to determine the numbers of adherent cells in 96-well plates. Briefly, after experimental incubations, the culture medium was discarded and the cells were incubated with 0.5% crystal violet solution (Sigma) for 30 min at room temperature (21°C). The cells were washed in PBS, 100% methanol was added and absorbance at 540 nm was measured spectrophotometrically.

The MTT [1-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazan] assay was used to assess cell viability in 96-well plates. The cells were incubated with 0.2% MTT in cell culture medium for 4 h at 37°C. The MTT solution was then discarded and DMSO was added to solubilize the MTT-formazan crystals formed in living cells. After thorough mixing, absorbance was measured at 540 nm.

Statistical analysis

The statistical significance of differences between experimental groups was analyzed by ANOVA. Values of $P < 0.05$ were considered significant.

Results

GAS-stimulated keratinocytes produce O₂⁻

We assessed the ability of the six GAS strains isolated from patients with cellulitis to induce the production of the major ROS involved in oxidative stress. The

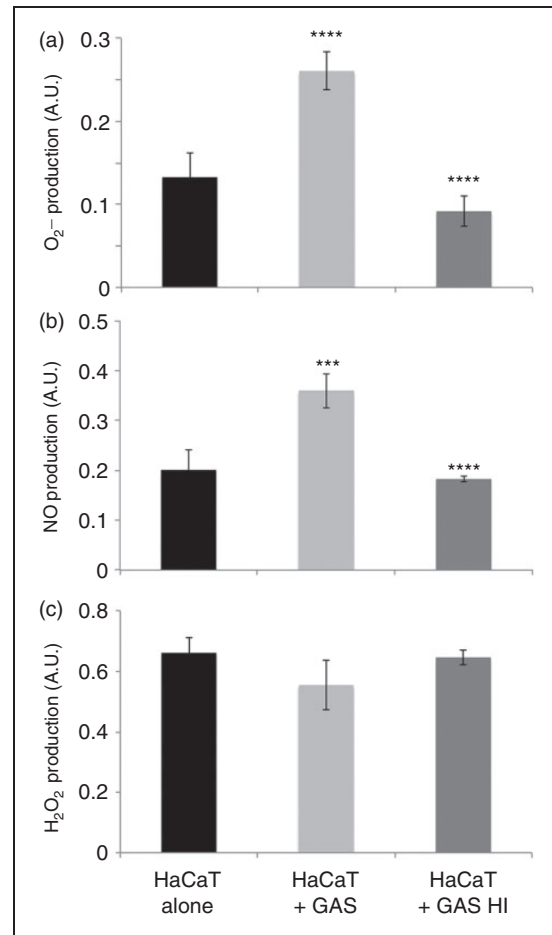


Figure 2. Evaluation of ROS production by heat-inactivated (HI) GAS-stimulated keratinocytes. HaCaT cells were incubated for 6 h with live (light gray bar) or with HI (dark gray bar) or without (black bar) 6G10 GAS strains (Table 1) at an MOI of 10. (a) O₂⁻, (b) NO and (c) H₂O₂ were assayed by spectrofluorimetry, as described in the 'Materials and methods'. The values shown are means \pm SD of three independent experiments. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$.

production of O₂⁻ and NO by keratinocytes was significantly stimulated in response to all six GAS strains tested (Figure 1a, b), but the production of H₂O₂ was not (Figure 1c). Indeed, five of the GAS strains tested did not induce the production of H₂O₂, the only exception being strain 6G10. ROS production was the same in cultured primary NHEKs and HaCaT cells which were used in subsequent experiments. The strain 6G10, displayed continuous reproducible ROS production and was used in further experiments to verify the specificity of the ROS production. The production of O₂⁻ and, to a lesser extent, NO, by HaCaT keratinocytes was stimulated by the 6G10 strain in a dose-dependent manner, whereas H₂O₂ production was not. At the highest GAS concentration, O₂⁻ and NO levels were 86% ($P < 0.001$) and 44% ($P = 0.09$) higher, respectively, than in the control (Figure 1d).

O_2^- levels increased significantly 1 h after GAS stimulation ($P < 0.05$), peaking 3 h after stimulation ($P < 0.001$). By contrast, we observed no time-dependent significant increase in NO production and H_2O_2 production increased very slowly, reaching a significant peak only after 5 h of stimulation ($P < 0.05$) (Figure 1e). However, heat-inactivated bacterial strains are not able to induce ROS production (Figure 2). Thus, stimulation with live GAS rapidly induced the production of large amounts of O_2^- by keratinocytes.

The origin of the O_2^- and the detoxification pathways in GAS-stimulated keratinocytes

Superoxide anions may be produced by the cytosolic enzymes NADPH oxidase and xanthine oxidase, or by mitochondrial complex I or III of the respiratory chain. By using specific chemical inhibitors of the mitochondrial respiratory chain or cytoplasmic enzymes potentially responsible for O_2^- production, we investigated the source of the O_2^- produced. The prior treatment of keratinocytes with rotenone and antimycin, which inhibit complexes I and III of the mitochondrial respiratory chain, respectively, had no significant effect on the production of O_2^- . By contrast, prior treatment with DPI, an NADPH oxidase inhibitor, significantly decreased O_2^- production ($P = 0.04$). Incubation with allopurinol, a xanthine oxidase inhibitor, had no effect (Figure 3). The inhibition of superoxide dismutase (SOD) with a specific inhibitor, diethyldithiocarbamate (DDC), resulted in significantly higher levels of

O_2^- ($P < 0.001$). Moreover, the use of two SOD mimics, MnTBAP and CuDIPS, resulted in significantly lower levels of O_2^- ($P < 0.0001$) (Figure 3), while the vehicles used to dissolve the ROS modulators are not (data not shown). Therefore, the O_2^- produced by NADPH oxidase in GAS-stimulated keratinocytes is presumably converted into H_2O_2 by the enzyme SOD.

O_2^- -induced cytotoxicity in GAS-stimulated keratinocytes

ROS, including O_2^- , are highly toxic to cells so we investigated GAS-stimulated keratinocyte cell death and the role of O_2^- in this phenomenon. We used YO-PRO-1 staining to evaluate the capacity of the six GAS strains to induce cell death. All but one (6EF) of the strains tested induced massive, significant ($P < 0.001$) levels of keratinocyte cell death (Figure 4a). GAS-induced death was detected within 30 min of infection, and was time- (Figure 4b) and dose-dependent (Figure 4c). We studied the nature of GAS-induced keratinocyte cell death, by a combination of YO-PRO-1 and propidium iodide staining, and analyzed keratinocytes by flow cytometry after stimulation with GAS strain 6G10. After 6 h of incubation, the cell death was mostly necrotic and cell death rates were dose-dependent (Figure 4d). We then investigated the role of O_2^- in GAS-induced cell death, by treating the keratinocytes with specific modulators of enzymes involved in the production and detoxification of O_2^- before incubation with GAS. Inhibition of O_2^- production with DPI or the

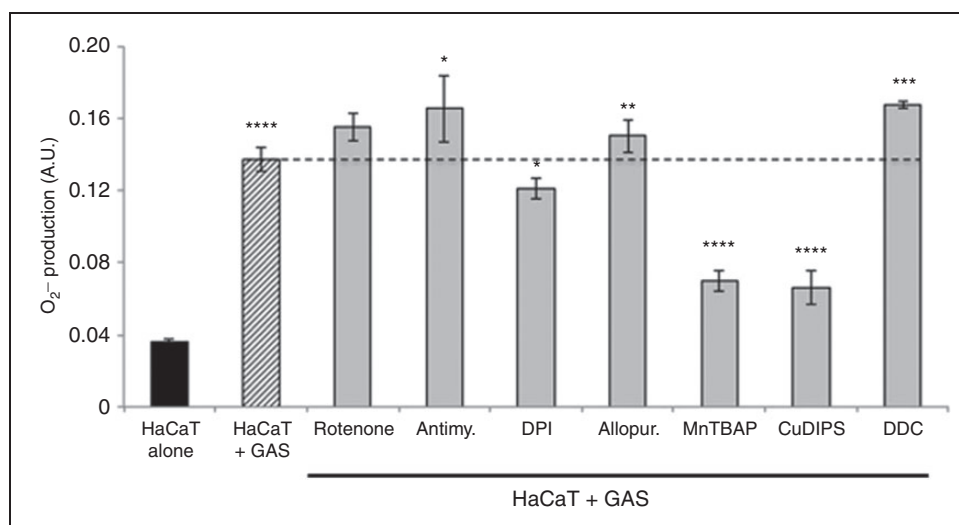


Figure 3. The superoxide anion detoxification pathway is involved in GAS-stimulated keratinocytes. O_2^- was assayed after 6 h of incubation of HaCaT cells alone (black bar) or stimulated with GAS (dashed bar) or previously treated with specific modulators of enzymatic systems involved in ROS metabolism and then stimulated with GAS (strain 6G10, Table I) at an MOI of 10 (gray bars). The concentrations used were as follows: 40 μ M rotenone, 40 μ M antimycin, 40 μ M DPI, 40 μ M allopurinol, 100 μ M manganese [III] tetrakis (5,10,15,20)-benzoic acid porphyrin (MnTBAP), 400 μ M ATZ, 0.8 mM BSO, 400 μ M copper[II]diisopropylsalicylate (CuDIPS) and 2 mM DDC. The values reported are means \pm SD of three independent experiments. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$.

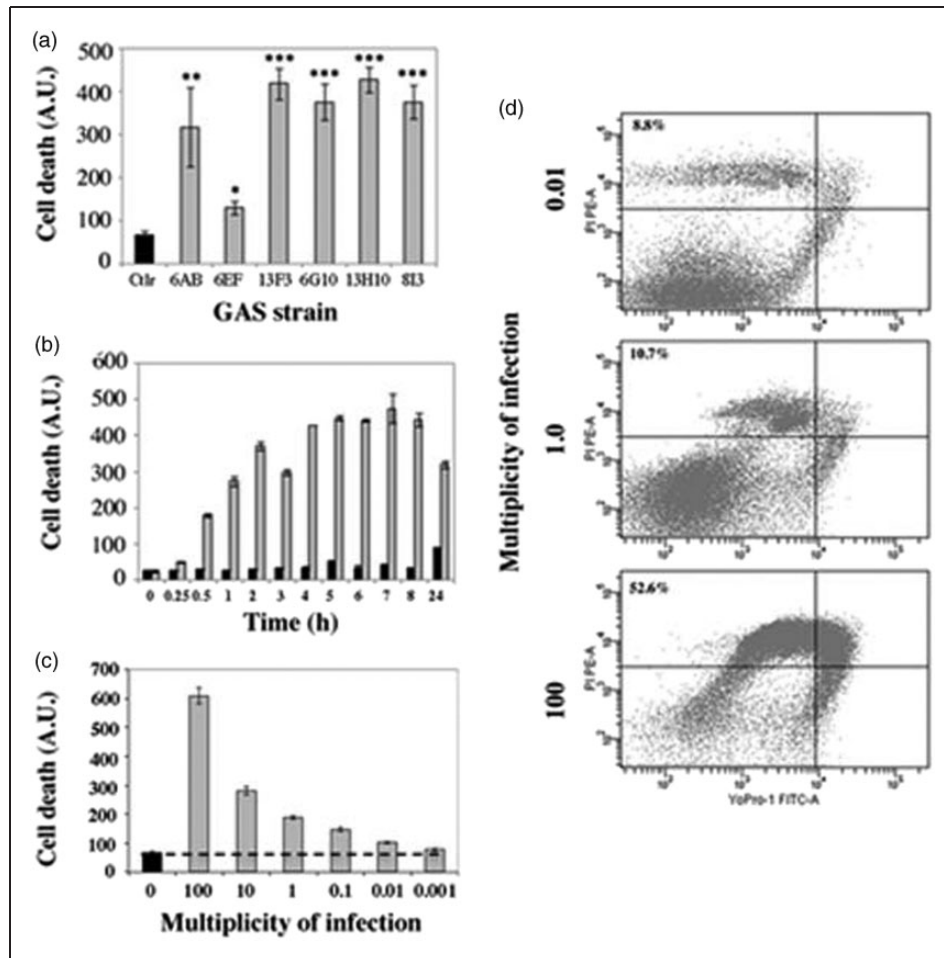


Figure 4. Cellulitis-related GAS strains induce keratinocyte death. HaCaT cells were incubated with GAS (gray bar) or left unstimulated (black bar). Cell death was assessed by spectrofluorimetry, with YO-PRO-1, as described in 'Materials and methods'. (a) Cell death was evaluated in HaCaT cells after 24 h of incubation with six cellulitis-related GAS isolates (Table 1) at an MOI of 10. (b) Cell death kinetics of HaCaT cells incubated with GAS (6G10 strain, Table 1) at an MOI of 10 were followed for 24 h. (c) HaCaT cells were incubated with GAS (strain 6G10, Table 1) for 6 h at MOIs of 100, 10, 1, 0.1, 0.01 and 0.001 to test the dose dependence of the effect. (d) HaCaT cells were incubated for 6 h with GAS (strain 6G10, Table 1) at an MOI of 0.01, 1 or 100, and cell death was evaluated by flow cytometry after staining the samples with YO-PRO-1 and propidium iodide (PI), as described in the 'Materials and methods'. The values shown are the means \pm SD of two independent experiments. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$.

activation of O_2^- detoxification by SOD with MnTBAP or CuDIPS all significantly decreased GAS-induced keratinocyte cell death ($P < 0.001$, $P < 0.01$ and $P < 0.01$, respectively). By contrast, DDC, rotenone and antimycin, all of which increase O_2^- production, increased cell death rates ($P < 0.01$, $P < 0.01$ and $P < 0.001$, respectively) (Figure 5). These results suggest that the high rates of keratinocyte death caused by GAS are at least partly the consequence of inducing the production of O_2^- by the cells themselves.

Role of O_2^- in the defense mechanism of GAS-stimulated keratinocytes

We measured the growth rates of the six GAS strains incubated with keratinocytes. All strains other than 6EF grew significantly more slowly after 18 h of

incubation with keratinocytes in our experimental model (Figure 6a). However, when cultivating GAS strain in HaCaT conditioned media only the bacterial growth was not affected (data not shown). We then studied the effects of various concentrations of chemically generated O_2^- on the survival of both keratinocytes and GAS. The 6G10 strain grew steadily until the O_2^- concentration reached 156 μ M, above which growth was dose-dependently inhibited; the HaCaT cells appeared to be more resistant to this concentration (Figure 6b). We then pretreated the keratinocytes with modulators of O_2^- production: decreasing the amount of O_2^- by blocking the NADPH oxidase (DPI) or activating SOD (MnTBAP) restored GAS growth, at least partly. Conversely, DDC, a SOD inhibitor that increases the amount of O_2^- produced by keratinocytes, decreased GAS growth (Figure 6c).

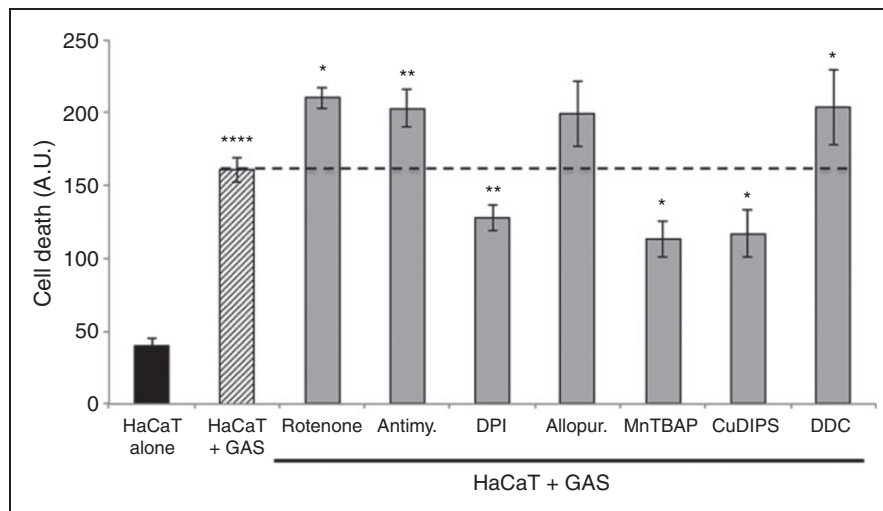
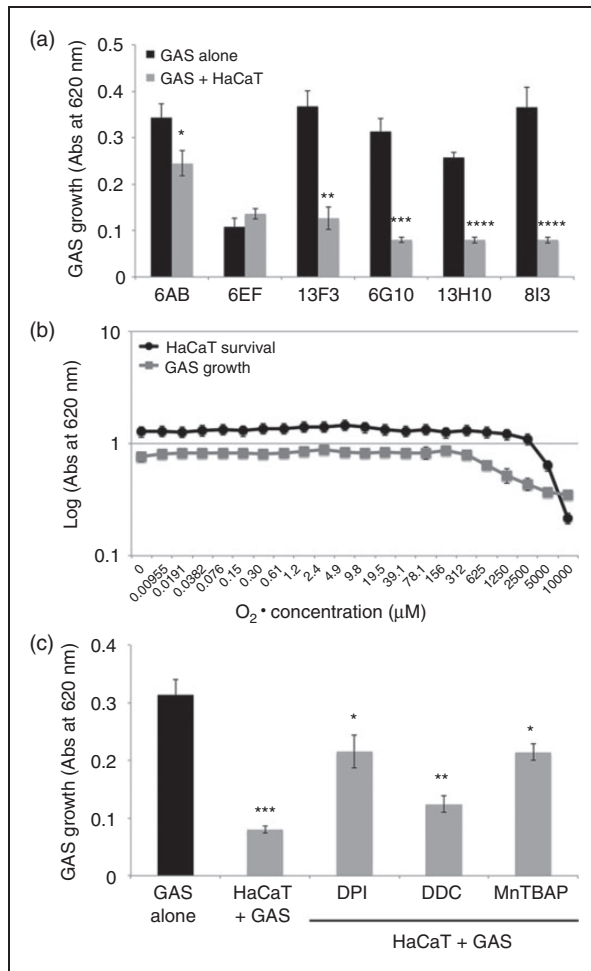


Figure 5. GAS induces cell death by stimulating O_2^- production. HaCaT cells were incubated for 6 h with (dashed bar) or without (black bar) GAS (strain 6G10, Table 1) at an MOI of 10. We also tested HaCaT cells previously treated with the following specific modulators of enzymatic systems involved in ROS metabolism (gray bars): 40 μ M rotenone, 40 μ M antimycin, 40 μ M DPI, 40 μ M allopurinol, 100 μ M manganese [III] tetrakis (5,10,15,20)-benzoic acid porphyrin (MnTBAP), 400 μ M ATZ, 0.8 mM BSO, 400 μ M copper[II]diisopropylsalicylate (CuDIPS) and 2 mM DDC. The values reported are means \pm SD of three independent experiments. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$.

Discussion

GAS is a human pathogen, mostly transmitted by direct contact, which is amplified in overcrowded areas.²⁴ Superficial impetigo is the most common manifestation of dermal GAS infection; these bacteria can spread into and/or penetrate the epidermis through epithelial abrasions and infect deeper tissues. Such secondary infection is characterized by damage to the epithelium, leading to the development of dermal and hypodermal invasion, which is a clinical entity (cellulitis) distinct from impetigo, which remains confined to the superficial layers of the skin.²⁵ We studied GAS strains isolated from patients with cellulitis for their capacity to induce an oxidative burst in an *in vitro* model of infection of keratinocytes. ROS are small, short-lived molecular species, continuously produced in small amounts during the course of normal aerobic metabolism. Mitochondria is the typical site of ROS production: electrons leak from the electron transport chain and react with oxygen to form superoxide, and it has been estimated that 1–3% of oxygen reduced in cells may form superoxide in this manner. However, mitochondria are not the only site of ROS production in the cell. The enzymes NADPH oxidase and xanthine oxidase, located in cytoplasm, can also produce ROS. Several ROS have been described in cells, including O_2^- and H_2O_2 , which react strongly with cellular components.²⁶ Incomplete reduction of O_2 during respiration increases the level of O_2^- and its abundance is determined by multiple enzyme systems, including SOD, which transforms O_2^- into H_2O_2 . Catalase, glutathione S-transferase and thioredoxin are then able to detoxify

H_2O_2 into water. In this study, we show that GAS are also able to induce a massive production of ROS by both primary and immortalized keratinocyte cell lines. Using fluorescent probes, we demonstrate that all the GAS strains tested induced the production of O_2^- by keratinocytes in a time- and dose-dependent manner, arguing for this production being specific. Moreover, we have shown that this ROS production needs live bacteria, suggesting an interaction between cellular receptors and surface components on the GAS strain that may have been altered, thus inhibiting the induction of ROS production. These results are in line with those describing that heat-inactivated GAS are not able to induce cell death as well as the production of pro-inflammatory molecules.²⁷ None of the strains induced substantial H_2O_2 production, although three of the six strains induced NO production. The NO production in keratinocytes seems to be inconsistent; macrophages are able to produce NO when stimulated with GAS, suggesting that innate immune cells rather than keratinocytes are responsible for such production.²⁸ Using specific chemical modulators able to activate or inhibit the production of O_2^- , we confirmed that GAS induced the production of O_2^- and we demonstrated that the production of O_2^- was the result of NADPH oxidase activity. We showed that keratinocytes are able to produce ROS and this finding is consistent with previous work demonstrating that *S. pyogenes* is able to induce the production of ROS by HeLa cells and human leukocytes.^{29,30} Moreover, transcriptional studies with macrophages infected with GAS demonstrated up-regulation of the p47phox gene encoding a component of the NADPH oxidase (*Ncf1*), further



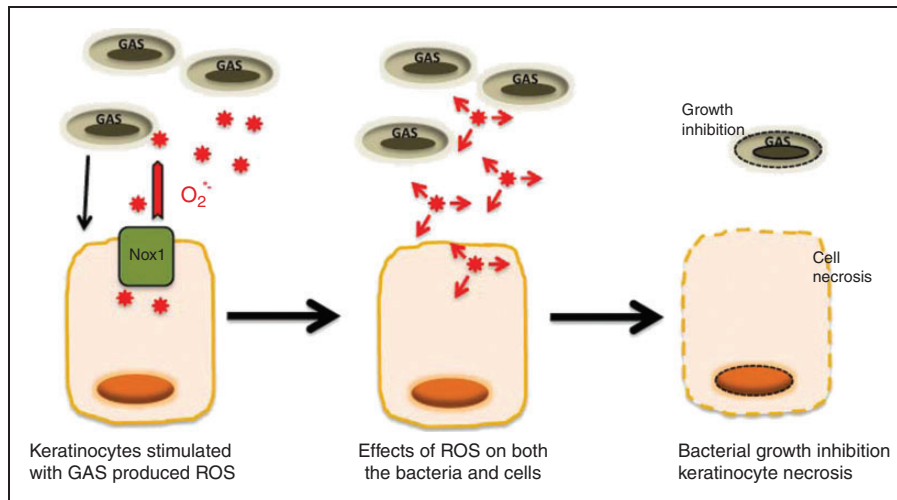


Figure 7. Proposed role of ROS produced by keratinocytes upon stimulation by GAS. Keratinocytes are stimulated by GAS, triggering the production of O_2^- through the NADPH oxidase pathway; this contributes to both keratinocyte necrosis and GAS growth inhibition.

conditions of oxidative stress, protecting GAS against ROS.³⁸ SOD expression in GAS strains is dependent on the superoxide anion concentration, which, in turn, directly interferes with the expression of the surface protein F; this protein binds fibronectin, promoting attachment to the host extracellular matrix and may contribute to protecting the bacteria against O_2^- . However, it has been suggested that *S. pyogenes* may be more susceptible to oxidative stress than other species of the genus *Streptococcus*.³⁹ This observation is interesting. Indeed, cellulitis is characterized by hyperinflammation and necrosis associated with the absence of detection of the bacteria. GAS are able to activate innate immunity triggering the production of pro-inflammatory cytokines and chemokines that recruit macrophages and neutrophils. In addition to this production of pro-inflammatory cytokines, the adhesion of GAS to keratinocytes may also induce the production of ROS, which the cell is unable to detoxify, and consequently impedes bacterial growth as well as contributing to keratinocyte cell death (Figure 7). Further experiments will be necessary to elucidate in depth the molecular mechanisms involved in the keratinocyte and bacteria death.

Overall, our analysis of the oxidative burst response developed by keratinocytes in response to GAS stimulation suggests that innate immune response mediated by keratinocytes may contribute to the inhibition of GAS growth and the strong local inflammatory reaction. In this study, we show that six independent strains of GAS isolated from the blood of patients with cellulitis induce the production of O_2^- by human primary keratinocyte cultures, and that this is associated with substantial cell necrosis.

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Conflict of interest

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