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Group B streptococci persist inside macrophages

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SUMMARY

Group B streptococci (GBS) are an important cause of neonatal sepsis, pneumonia and meningitis. In the early phase of infection, macrophages and polymorphonuclear cells (PMN) are the first immune cells that interact with GBS. In this *in vitro* study, to gain insight into GBS–macrophage interaction in the absence of type-specific antibodies, we examined the features of GBS survival in thioglycollate-elicited murine peritoneal macrophages and the effect of GBS on the protein kinase C (PKC)-dependent transduction pathway. Our results demonstrate that type Ia GBS, strain 090 (GBS-Ia) and type III GBS strain COH 31r/s (GBS-III), after *in vitro* phagocytosis survive and persist intracellularly in macrophages for up to 24 and 48 hr, respectively. However, macrophage activation by interferon-γ (IFN-γ) and lipopolysaccharide from *Escherichia coli* (LPS) caused a significant reduction in the time of intracellular persistence. Macrophage activation by IFN-γ and LPS seems to be a multifactorial event involving multiple intracellular signal pathways also including PKC. Since PKC is one of the components in the signal network leading to macrophage activation and an important target for several intracellular micro-organisms, we wondered whether PKC could have a role in intracellular GBS survival. Both PKC depletion by treatment with phorbol 12-myristate 13-acetate (PMA) for 18 hr and PKC inhibition by Calphostin C rendered macrophages more permissive for the intracellular GBS survival. Furthermore, GBS-infected macrophages were unable to respond to PMA and LPS, activators of PKC, by inducing antimicrobial activity. The ability of GBS to impair PKC-dependent cell signalling was also demonstrated by the reduced c-*fos* gene expression in GBS-infected macrophages with respect to control macrophages, after LPS stimulation. In conclusion, our results indicate that GBS survive in macrophages and impairment of PKC signal transduction contributes to their intracellular survival.

INTRODUCTION

Group B streptococci (GBS) are the major cause of pneumonia, sepsis and meningitis in neonates and a serious cause of mortality or morbidity in immunocompromised adults.[1,2] The main virulence factor of GBS is thought to be the capsular polysaccharide because of its antiphagocytic properties.[3,4] In resistance to GBS infection, a central role is played by antibodies to the type-specific capsular polysaccharide and complement which potentiate *in vitro* phagocytosis and GBS killing by phagocytic cells[5–8] and confer *in vivo* protection.[9–14] However, the phagocyte functionality is also important and correlates with the susceptibility or resistance of neonates to GBS infection.[15,16]

The discovery that macrophages can phagocytose GBS in the absence of immune serum by C3-dependent binding[17] and C3-independent binding using complement receptor type three (CR3)[18] suggests that there is also a potential role for antibody-independent mechanisms in resistance to GBS infection. However, the recent demonstration that type III GBS phagocytosed by a macrophage-like line J774 in the absence of type-specific antibodies survived within its host cell,[19] seems to indicate that macrophages, in the absence of opsonins, are not very effective in GBS killing. Furthermore, this also suggests that GBS, like intracellular micro-organisms, could have evolved some strategies to survive successfully within the host's phagocytic cells. It is well known that intracellular pathogens, to survive inside macrophages, deactivate some important cell effector functions,[20–25] many of which are regulated by protein kinase C (PKC), one of the components in the signal transduction network involved in macrophage activation.[26–30]

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Therefore, the PKC signal transduction pathway is a useful intracellular target to ensure survival of several pathogens inside macrophages.[31–34] To better understand GBS–macrophage interaction we studied the survival of type Ia GBS, strain 090 (GBS-Ia) and type III GBS, strain COH 31r/s (GBS-III) inside the murine peritoneal macrophages after antibody-independent phagocytosis and the possible role of PKC in this phenomenon.

The results show that both GBS strains can survive inside the macrophages for more than 24 hr after phagocytosis and that an impairment of PKC-associated events contributes to intracellular survival of this micro-organism.

MATERIALS AND METHODS

Animals

Outbred CD-1 mice of both sexes, 8–10 weeks old, were obtained from Charles River Breeding Laboratories, Calco, Milan, Italy.

Micro-organisms

GBS type Ia mouse-passed prototype strain 090 (GBS-Ia) was kindly provided by Dr J. Jelinkova (Institute of Hygiene and Epidemiology, Prague, Czech Republic). GBS type III, strain COH 31r/s (GBS-III), clinically isolated from a diabetic foot ulcer in an adult and rendered resistant to rifampicin and streptomycin, and its isogenic transposon-induced unencapsulated mutant COH 31-15 (GBS-III$^-$) were kindly provided by Dr M. Wessels (Channing Laboratory, Boston, MA).

Micro-organisms were grown in Todd–Hewitt broth (THB, Unipath SpA, Garbagnate Milanese, Italy) at 37° and aliquots were stored at −70° until used. For both *in vitro* assays and experimental infections, the organisms were grown to stationary phase in THB overnight. On the day of experiment, a 400 µl volume of stationary-phase broth culture was brought to log phase in 10 ml of THB by incubating at 37° in a shaking bath. The optical density at 600 nm was approximately 0·4 [mild-log phase or equivalent to approximately 10^8 colony-forming units (CFU)/ml]. The bacteria were washed and resuspended in RPMI-1640 medium (Flow Laboratories, McLean, VA). The number of live bacterial cells was confirmed by counting the CFU on Islam agar (Unipath) plates containing 5% inactivated horse serum, incubated under anaerobic conditions.

Coagulase-positive *Staphylococcus aureus* strain Cowan I (NCTC; National Collection Type Culture, London, UK) was grown at 37° on tryptone soya broth (TSB, Unipath) for 24 hr. Bacteria were harvested by centrifugation, washed extensively and diluted in serum-free RPMI-1640 medium to the desired number of CFU/ml. The inoculum size was estimated turbidimetrically at 540 nm in a Beckman DU-68 spectrophotometer (Beckman Instruments, Fullerton, CA) and the number of live bacterial cells was confirmed by counting CFU on mannitol salt agar (MSA, Unipath) plates.

Candida albicans, strain CA-6, was isolated from a clinical specimen.[35] It was grown at 28° with mild agitation in low-glucose Winge medium as previously described.[35] Under these conditions cultures yielded 10^8 cells/ml and the organism grew as an essentially pure yeast-phase population. After 24 hr culture, yeast cells were washed twice in saline by low-speed centrifugation (600 ***g***) and diluted to the desired concentrations in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS).

Preparation of peritoneal macrophages

Mice were injected intraperitoneally (i.p.) with 1 ml of a 10% solution of Bacto Brewer thioglycollate medium (thioglycollate broth, Difco Laboratories, Detroit, MI). After 4 days, peritoneal exudate cells were harvested by washing the peritoneal cavity with 10 ml cold RPMI-1640 medium containing 5 U/ml heparin and aspirating the exudate with a syringe. The cells were washed three times in cold antibiotic-free RPMI-1640 medium with 5% FCS (complete medium) and cell viability was evaluated by the dye exclusion method.

Murine peritoneal macrophages were plated at a concentration of 1×10^6 cells/ml (100 µl/well) in 96-well tissue culture dishes in complete medium. After 2 hr incubation at 37° in 5% CO_2, the non-adherent cells were removed, and the resulting adherent monolayers (>98% macrophages evaluated by non-specific esterase staining) were washed with warm complete medium before use. Giemsa-stained macrophage monolayers were examined by light microscopy to confirm uniform density of intact adherent macrophages.

Assay for GBS intracellular survival in macrophages

The macrophage monolayers were infected with GBS at a cell:micro-organism ratio of 1:15 in complete medium. Microplates were incubated for 2 hr at 37° in 5% CO_2 to obtain GBS phagocytosis. After this time (time 0 of the assay), the culture supernatants of infected macrophages were removed by aspiration, and monolayers were washed three times with antibiotic-free medium. Evaluation of Giemsa-stained infected cells by light microscopy indicated that 2 hr after exposure to antibiotics, approximately 8–12% of macrophages were infected with GBS-Ia and 35–45% with GBS-III.

To kill extracellular bacteria, the cultures of infected macrophages were further incubated in RPMI medium containing 5% FCS and antibiotics (100 U/ml penicillin and 100 µg/ml gentamicin). To quantify the number of intracellular GBS, at different times postinfection (2, 5, 8, 24, 48 and 72 hr), the supernatants containing antibiotics were removed, the cells were washed and then lysed with Triton-X-100 at a final concentration of 0·1% (v/v) in sterile distilled water. Serial dilutions of lysate from each well were prepared and 0·1 ml of each dilution was plated on Islam agar. The number of CFU was determined after 24 hr incubation under anaerobic conditions. Exposure of GBS-Ia or GBS-III to 100 U/ml penicillin and 100 µg/ml gentamicin for 2 hr was sufficient to kill 100% of micro-organisms.

Electron microscopy

Macrophage monolayers in 90-mm diameter plates were infected with GBS-Ia and GBS-III and treated as described in the intracellular survival assay. At different times, monolayers were washed with cacodylate buffer (pH 7·4) and fixed for 2 hr in 2·5% glutaraldehyde in 0·1 M cacodylate buffer (pH 7·4). After washing in buffer, the cells were postfixed in 2% osmium tetroxide plus 1% potassium ferricyanide in cacodylate buffer, dehydrated through a graded alcohol series, embedded in Epon, thin-sectioned and finally stained with uranyl acetate and lead citrate.

Activation of macrophages

Mouse recombinant interferon-γ (rmIFN-γ) and lipopolysaccharide from *Escherichia coli* (LPS) were purchased from Boehringer Mannheim (Indianapolis, IN) and Sigma Chemical Co. (St. Louis, MO), respectively.

The macrophage monolayers were preincubated with rmIFN-γ (100 U/ml) and LPS (1 μg/ml) for 18 hr before infection. Macrophages preincubated with RPMI for 18 hr were used as controls. Assays for intracellular GBS survival in controls and activated macrophages were performed as described above.

Controls of macrophage activation were performed by evaluating the growth inhibition of coagulase-positive *S. aureus* strain Cowan I and the yeast form of *C. albicans* as previously reported.[36]

Depletion of PKC and intracellular survival of GBS

Macrophage monolayers were preincubated with 100 ng/ml of phorbol 12-myristate 13-acetate (PMA, Sigma) or with RPMI overnight prior to infection with GBS-Ia or GBS-III for 2 hr. It has been reported that this treatment specifically depletes PKC activity.[37,38] The PMA was maintained throughout the course of the experiment. A second method of inhibiting PKC was based on the use of Calphostin C (0·02 μM, 0·2 μM and 0·6 μM, PKC $IC_{50}=0{\cdot}05$ μM) purchased from Sigma. This inhibitor was added to macrophages 15 min prior to infection with GBS and maintained during the course of the experiment. Macrophages preincubated for 15 min with RPMI were used as controls. Assays for GBS intracellular survival were performed as described above. Doses of the inhibitor were chosen taking account of its specificity and cytotoxicity.

Stimulation of macrophages

Peritoneal murine macrophages were infected for 2 hr with GBS-Ia or GBS-III at an infection ratio of 1:15. At time 0, the infected cells, after repeated washings, were incubated with RPMI or stimulated with PMA (1 μg/ml) or LPS (1 μg/ml) for 2 hr in medium containing antibiotics. The culture supernatants were then removed and the monolayers were washed. At different times, after addition of medium containing antibiotics, the number of intracellular GBS was evaluated as described in the intracellular assay. In cultures of macrophages infected with GBS-Ia, washed and stimulated with LPS (1 μg/ml) for 30 min in presence of antibiotics, c-*fos* mRNA expression was analysed.

Northern blot analysis

Total cellular RNA was isolated from uninfected and GBS-Ia-infected macrophages, after 30 min LPS (1 μg/ml) stimulation, using a single-step phenol/chloroform extraction procedure.[39] Twenty micrograms of total mRNA was denatured by heating for 10 min at 65° in a solution of 20 mM MOPS buffer, pH 7·0, containing 6·5% formaldehyde, 50% formamide, 5 mM sodium acetate and 1 mM ethylenediaminetetraacetic acid (EDTA). Separation of mRNA by electrophoresis was performed using a 1% agarose/2·2 M formaldehyde gel with 80 volts of applied current. RNA was then transferred to a nitrocellulose filter membrane (Schleicher & Schuell, Dassel, Germany) by Northern capillary blotting using 10 × SSC (1 × SSC is 0·15 M NaCl, 0·015 M sodium citrate, pH 7·2). The filter was baked for 2 hr at 80° and then prehybridized for 4 hr at 42° in 0·1 M phosphate buffer (pH 6·8), 50% formamide, 5 × SSC, 5 × Denhardt's solution (0·1% Ficoll, 0·1% polyvinylpyrrolidone, 0·1% bovine serum albumin), 0·2% sodium dodecyl sulphate (SDS) and 100 μg/ml denatured salmon sperm DNA (Sigma). Hybridization was carried out for 18 hr in the same solution by adding a specific ^{32}P-labelled cDNA probe. The ^{32}P-labelled probe was obtained using the Nick translation kit (Boehringer Mannheim Biochemicals), according to the procedure suggested by the manufacturer. The specific activity of the labelled probe was 2×10^8–5×10^8 d.p.m./μg DNA. After hybridization, the filter was washed in 2 × SSC, 0·1% SDS for 20 min at room temperature and then in 0·2 × SSC, 0·1% SDS for 30 min at 60°. The filter was blotted dry and exposed to Kodak X-ARS film (Eastman-Kodak Company, Rochester, NY) for 24 hr at −80° in the presence of an intensifying screen. The cDNA probe for murine c-*fos* was obtained from American Type Culture Collection (Rockville, MD).

To ensure that equal amounts of RNA were analysed, blots were stripped and reprobed with β-actin cDNA. The intensities of radiographic signals were quantified using laser densitometry (Ultrascan XL, LKB Instruments, Houston, TX).

Statistical analysis

Experiments of intracellular survival in macrophages were repeated six times. Data are presented as the means ± standard deviations (SD) of six individual experiments performed in triplicate. The data for each experiment were analysed by the Student's *t*-test.

The Northern blot and electron microscopy analysis was repeated three times and data reported are those of a typical experiment.

RESULTS

Intracellular survival of GBS in macrophages

To analyse the time–course of intracellular survival of GBS in macrophages, GBS were allowed to invade phagocyte monolayers for 2 hr, then the infected cells, after repeated washings, were exposed to antibiotics and at different time-points after antibiotic addition, the number of viable intracellular GBS was determined by quantitative plating. The results show that the number of internalized GBS was different for the two GBS strains. In fact, 2 hr after infection, $9{\cdot}8\times10^3$ CFU of GBS-Ia and $5{\cdot}2\times10^4$ CFU of GBS-III were recovered from monolayers of 10^5 macrophages (Fig. 1, time-point 2 hr). However, both GBS strains survived inside the macrophages for a long time. Even if a progressive decrease in the number of intracellular GBS was observed in time, viable GBS-Ia were found inside the macrophages up to 24 hr and GBS-III up to 48 hr postinfection (Fig. 1, time-points from 5 to 48 hr).

To confirm the intracellular persistence of GBS, transmission electron microscopy (TEM) analysis of macrophages was performed at various times after infection. Electron micrographs show that at early postinfection times, intact GBS-Ia and GBS-III were present in the phagosomes of macrophages with normal morphology (data not shown). Furthermore, at 24 hr for GBS-Ia (Fig. 2a) or 48 hr for GBS-III (Fig. 2b), it was possible to detect macrophages with normal morphology,

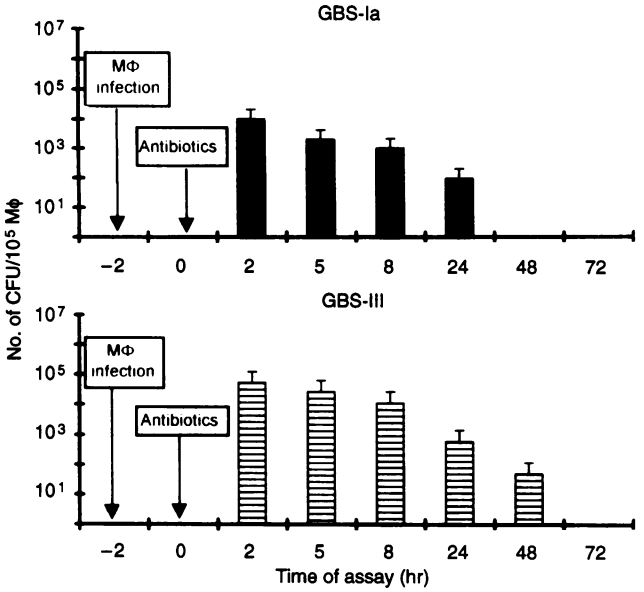

Figure 1. Time–course of GBS-Ia and GBS-III intracellular survival in peritoneal macrophages. Thioglycollate-elicited peritoneal murine macrophages were infected *in vitro* at an infection rate of 15 micro-organisms per cell. After 2 hr, time 0 of assay, antibiotics were added to kill extracellular bacteria. Then, at different times postinfection (2 to 72 hr) the number of intracellular GBS was evaluated by viable plate count of CFU as described in the Materials and Methods. Data are presented as the means ± SD of six independent experiments performed in triplicate.

where the intracellular GBS seemed well adapted to the intracellular environment.

Effect of the capsule on intracellular survival of GBS-III

Since the capsule is known to be the major GBS virulence factor,[4,5] we examined its role in intracellular survival. To this end, macrophages were infected with GBS-III or with the corresponding unencapsulated mutant (GBS-III$^-$) and intracellular survival in macrophages was then evaluated. At 2 hr postinfection, the number of internalized GBS-III$^-$ was higher than that of GBS-III but no significant differences in intracellular survival time between the two strains were observed (Table 1). Therefore, the capsule is not responsible for GBS intracellular survival.

Effect of macrophage activation on intracellular survival of GBS-Ia and GBS-III

Since activated macrophages become more effective in killing micro-organisms,[40] we evaluated whether macrophage activation reduced or abolished the intracellular survival of GBS.

Macrophages were cultured for 18 hr with rmIFN-γ (100 U/ml) and LPS (1 μg/ml)[41] or with medium alone and were then infected with either GBS-Ia or GBS-III. The number of intracellular GBS-Ia in activated macrophages, after 2 hr infection was significantly decreased and after 8 hr infection, micro-organisms were no longer detectable, while in control macrophages GBS-Ia persisted for up to 24 hr (Fig. 3). A similar trend was observed for GBS-III which survived for up

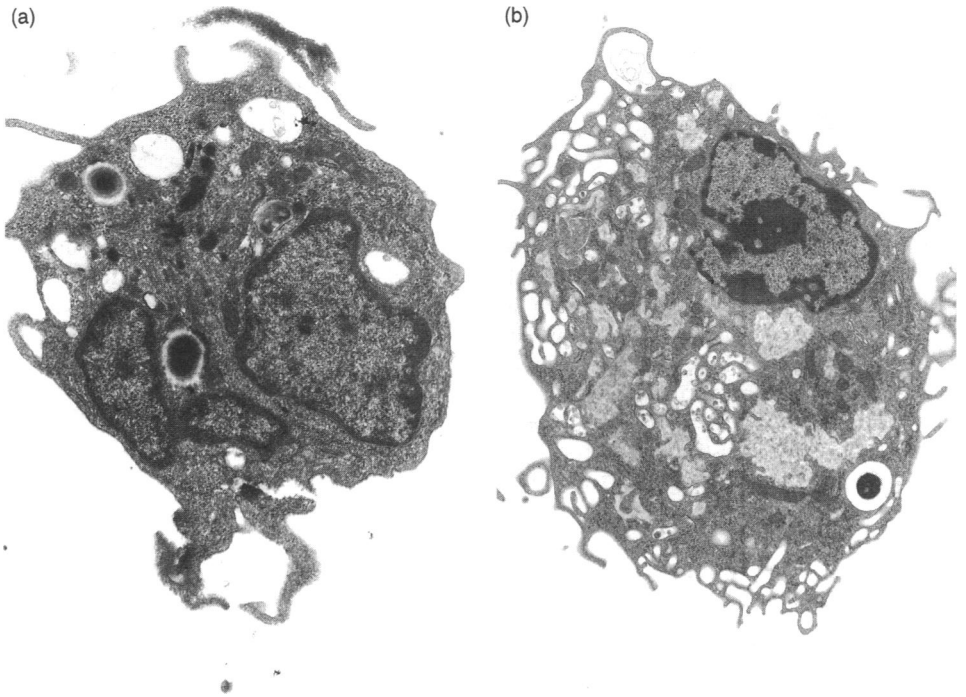

Figure 2. Transmission electron micrographs of peritoneal macrophages infected with GBS-Ia and GBS-III. (a) GBS-Ia cells inside a macrophage with normal morphology observed 24 hr after infection; magnification × 4550. (b) Intact GBS-III cells in a macrophage with a normal appearance detected at 48 hr after infection; magnification × 3185.

Table 1. Effect of the capsule on intracellular survival of GBS-III in macrophages

Time of assay*	Number of CFU/10^5 macrophages†	
	GBS-III‡	GBS-III⁻§
2 hr	$4{\cdot}7 \times 10^4$ ($\pm 0{\cdot}54$)	$7{\cdot}5 \times 10^4$ ($\pm 0{\cdot}68$)
5 hr	$2{\cdot}5 \times 10^4$ ($\pm 0{\cdot}27$)	$4{\cdot}1 \times 10^4$ ($\pm 0{\cdot}50$)
8 hr	$9{\cdot}6 \times 10^3$ ($\pm 0{\cdot}80$)	$1{\cdot}9 \times 10^4$ ($\pm 0{\cdot}24$)
24 hr	$5{\cdot}1 \times 10^2$ ($\pm 0{\cdot}52$)	$8{\cdot}1 \times 10^2$ ($\pm 0{\cdot}65$)
48 hr	$4{\cdot}7 \times 10^1$ ($\pm 0{\cdot}53$)	$8{\cdot}5 \times 10^1$ ($\pm 0{\cdot}75$)
72 hr	0	0

*Thioglycollate-elicited peritoneal macrophages were infected *in vitro* with GBS-III or GBS-III⁻ at an infection rate of 15 micro-organisms per cell for 2 hr. Then, antibiotics were added to kill extracellular bacteria and infected monolayers at the indicated times treated as described in the Materials and Methods.

†Number of intracellular GBS was evaluated by viable plate count of CFU. Data are presented as the means ± SD of six individual experiments performed in triplicate.

‡Encapsulated type III GBS.

§Unencapsulated type III GBS.

to 8 hr in activated macrophages and 48 hr in control macrophages (Fig. 3).

Appropriate controls of macrophage activation, performed with *S. aureus* and *C. albicans*,[36] show that the microbicidal activity of activated macrophages was significantly higher than that of control macrophages (data not shown).

Effect of PKC inhibition on intracellular survival of GBS

Since PKC is one of the components involved in the pathway leading to macrophage activation[26–30] and intracellular survival of several micro-organisms appears to be associated with inhibition of several effector functions of phagocytic cells,[31] we explored the potential role of PKC in the intracellular survival of GBS in macrophages. To this end, peritoneal macrophages, prior to infection with GBS-Ia or GBS-III, were treated with PMA (100 ng/ml) for 18 hr. It is reported that this treatment specifically depletes PKC activity in murine macrophages.[37,38] As shown in Table 2 the number of internalized GBS-Ia or GBS-III at 2 hr postinfection was similar in control and PMA-treated macrophages. However, at 5 hr postinfection, the PMA-treated macrophages contained about 50% more GBS-Ia or GBS-III than non-treated macrophages. A further increase in the number of intracellular GBS in PMA-treated macrophages with respect to non-treated macrophages was observed at 8 and 24 hr postinfection (Table 2). To show better the role of PKC on GBS intracellular survival we used Calphostin C, a potent and specific inhibitor of PKC. Peritoneal macrophages, prior to infection with GBS-Ia or GBS-III, were treated with different doses of Calphostin C for 15 min. The results in Fig. 4 show that at 5, 8 and 24 hr after infection, macrophages treated with Calphostin C, 0·2 μM and 0·6 μM, contained an increased number of intracellular GBS-Ia or GBS-III with respect to non-treated macrophages. In contrast, Calphostin C 0·02 μM had no effect on the GBS intracellular survival.

Treatment of normal uninfected macrophages with the

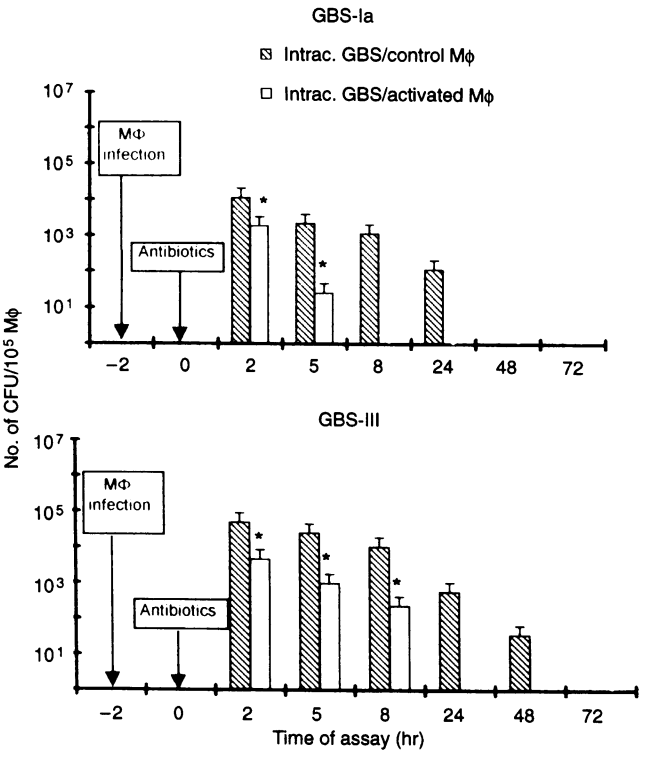

Figure 3. Effect of macrophage activation on *in vitro* intracellular survival of GBS-Ia and GBS-III in macrophages. Thioglycollate-elicited peritoneal murine macrophages were incubated for 18 hr with medium or with medium plus rmIFN-γ (100 U/ml) and LPS (1 μg/ml). Then, in the same experiment control and activated macrophages were infected *in vitro* with GBS-Ia or GBS-III at an infection rate of 15 micro-organisms per cell. After 2 hr, time 0 of assay, antibiotics were added to kill extracellular bacteria. Then, at different times postinfection (2 to 72 hr) the number of intracellular GBS was evaluated by viable plate count of CFU as described in the Materials and Methods. Data are presented as the means ± SD of six independent experiments performed in triplicate. *$P < 0{\cdot}01$ (GBS infection of macrophages activated with rmIFN-γ and LPS versus GBS infection of macrophages incubated in medium).

above indicated concentrations of inhibitors over the same period of time did not result in a loss of viability as determined by trypan blue assay. Furthermore, neither inhibitor had an effect on GBS growth (data not shown).

GBS alteration of PKC activation

The results obtained show that an impairment of the PKC-mediated signal transduction pathway favours intracellular survival of GBS, suggesting that integrity of PKC-dependent cell signalling could be important for combating intracellular GBS infection. To determine whether GBS altered PKC-associated events, we analysed the ability of infected macrophages to respond to activating signals for PKC, by examining the expression of c-*fos*, a gene induced early during macrophage activation,[42,43] and the induction of antimicrobial activity. As shown in Fig. 5, a significant reduction in c-*fos* mRNA level was observed in response to LPS (1 μg/ml for 30 min) in GBS-Ia-infected macrophages, with respect to uninfected macrophages. Besides, the results reported in Table 3

Table 2. Effect of protein kinase C depletion on intracellular survival of GBS in macrophages

Time of assay‡	Number of CFU/10^5 macrophages§			
	GBS-Ia†		GBS-III	
	Medium	PMA	Medium	PMA
2 hr	$1{\cdot}1 \times 10^4$ ($\pm 0{\cdot}13$)	$1{\cdot}0 \times 10^4$ ($\pm 0{\cdot}15$)	$4{\cdot}8 \times 10^4$ ($\pm 0{\cdot}63$)	$5{\cdot}0 \times 10^4$ ($\pm 0{\cdot}65$)
5 hr	$2{\cdot}0 \times 10^3$ ($\pm 0{\cdot}32$)	$3{\cdot}9 \times 10^3$ ($\pm 0{\cdot}52$)*	$2{\cdot}2 \times 10^4$ ($\pm 0{\cdot}30$)	$4{\cdot}5 \times 10^4$ ($\pm 0{\cdot}60$)*
8 hr	$1{\cdot}1 \times 10^3$ ($\pm 0{\cdot}18$)	$3{\cdot}1 \times 10^3$ ($\pm 0{\cdot}45$)*	$9{\cdot}8 \times 10^3$ ($\pm 0{\cdot}75$)	$2{\cdot}9 \times 10^4$ ($\pm 0{\cdot}34$)*
24 hr	$1{\cdot}2 \times 10^2$ ($\pm 0{\cdot}60$)	$5{\cdot}6 \times 10^2$ ($\pm 0{\cdot}58$)*	$4{\cdot}6 \times 10^2$ ($\pm 0{\cdot}53$)	$2{\cdot}8 \times 10^3$ ($\pm 0{\cdot}41$)*
48 hr	0	0	$3{\cdot}1 \times 10^1$ ($\pm 0{\cdot}40$)	$1{\cdot}1 \times 10^2$ ($\pm 0{\cdot}22$)*
72 hr	0	0	0	0

†Thioglycollate-elicited peritoneal macrophages were incubated in the same experiment with PMA (100 ng/ml) or medium for 18 hr, then infected with GBS-Ia or GBS-III at an infection rate of 15 micro-organisms per cell for 2 hr.

‡Infected monolayers were recovered at the indicated times after addition of antibiotics as described in the Materials and Methods.

§Number of intracellular GBS was evaluated by viable plate count of CFU. Data are presented as the means ± SD of six individual experiments performed in triplicate.

*$P < 0{\cdot}01$ (GBS infection of macrophages treated with PMA versus GBS infection of macrophages incubated in medium).

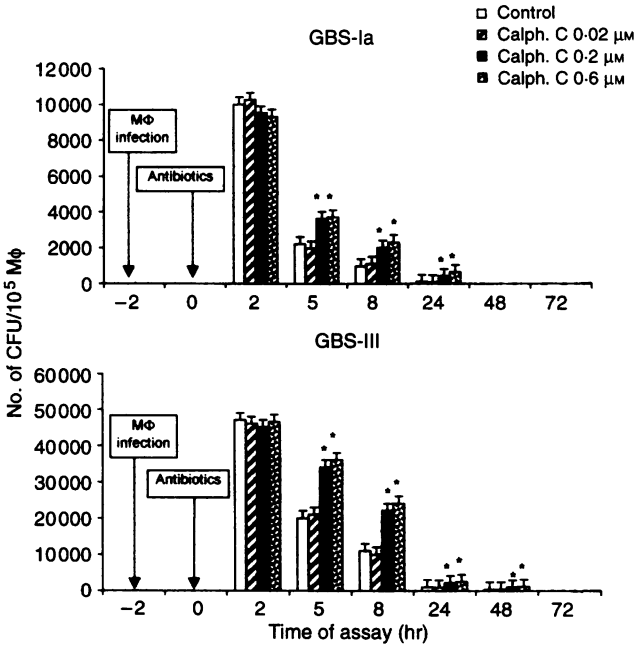

Figure 4. Effect of Calphostin C on intracellular survival of GBS-Ia and GBS-III in macrophages. Thioglycollate-elicited peritoneal murine macrophages were incubated in the same experiment for 15 min with Calphostin C, 0·02 μM, 0·2 μM and 0·6 μM or medium before infection *in vitro* with GBS-Ia or GBS-III at an infection rate of 15 micro-organisms per cell. After 2 hr, time 0 of assay, antibiotics were added to kill extracellular bacteria. Then, at different times postinfection (2 to 72 hr) the number of intracellular GBS was evaluated by viable plate count of CFU as described in the Materials and Methods. Data are presented as the means ± SD of six independent experiments performed in triplicate. *$P < 0{\cdot}01$ (GBS infection of macrophages treated with Calphostin C versus GBS infection of macrophages incubated in medium).

show that in macrophages infected with GBS-Ia or GBS-III and stimulated with PMA or LPS for 2 hr, the number of intracellular micro-organisms was similar to that in unstimulated macrophages at all times tested (Table 3). Collectively, these findings support the hypothesis that GBS infection rendered macrophages unresponsive to PKC activators and this is consistent with a direct impairment of the PKC-associated signal transduction pathway.

DISCUSSION

GBS are a major cause of bacterial sepsis and pneumonia during the neonatal period.[1,2] Protection against this disease is based on type-specific antibodies and complement.[9] There is evidence that effective uptake and killing of GBS requires opsonization by specific antibodies and/or complement.[5–8,44] It is also known that macrophages can phagocytose GBS in the absence of opsonins by C3-dependent binding[17] and by C3-independent binding using the complement receptor type three (CR3) to interact directly with GBS.[18] This suggests that prior to development of specific anti-GBS immunity, in the presence of little or no type-specific antibodies, opsonin-independent mechanisms could play an important role in determining macrophage-dependent clearance of GBS.[15,18,45] Indeed, macrophages, together with PMN, are the first immune cells which interact with GBS at the start of infection and in susceptible individuals this interaction occurs in the presence of little or no type-specific antibodies.[6,8,16,44,46] However, the recent demonstration that GBS-III after opsonin-independent phagocytosis survive in the macrophage-like cell line J774 indicates that macrophages in the absence of immune serum are not very effective in GBS killing.[19] This observation and the reported ability of GBS to invade and survive in respiratory epithelial,[47,48] and endothelial cells,[49] suggest that GBS, known as exclusively extracellular pathogens, have evolved some strategies to survive for some time within various cell types as an intracellular micro-organism. This could be a mechanism to avoid host defences and disseminate infection. However, it is not paradoxical that GBS can persist inside mononuclear phagocytes even if they are considered a potent barrier against infections on account of their bactericidal enzymes and production of destructive oxygen radicals,[40,50] because several bacterial species are able to survive intracellularly in macrophages.[31,51]

In this study to further understand macrophage–GBS interaction in the absence of opsonin antibodies, we first

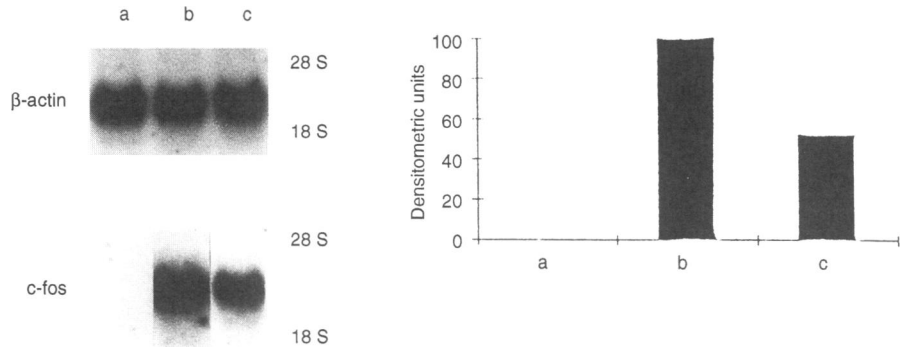

Figure 5. Effect of GBS-Ia infection on LPS-induced c-*fos* mRNA expression in macrophages. Uninfected macrophages (lane b) and macrophages infected with GBS-Ia for 2 hr (lane c) were stimulated with LPS (1 μg/ml) for 30 min. Uninfected macrophages not stimulated with LPS are indicated in lane a. Northern blot analysis was performed on 20 μg of total cellular RNA. Results of densitometry analysis are presented as a histogram after c-*fos* levels were relative to β-actin levels and expressed as densitometric units. Lane designations are identical for both blots and histogram.

Table 3. Lack of effect of PMA or LPS, added to macrophages after infection, on intracellular survival of GBS

Time of assay†	Number of CFU/10^5 macrophages‡					
	GBS-Ia*			GBS-III		
	Unstimulated	PMA	LPS	Unstimulated	PMA	LPS
2 hr	$1{\cdot}1 \times 10^4$ ($\pm 0{\cdot}12$)	$9{\cdot}5 \times 10^3$ ($\pm 0{\cdot}30$)	$9{\cdot}9 \times 10^3$ ($\pm 0{\cdot}75$)	$5{\cdot}2 \times 10^4$ ($\pm 0{\cdot}58$)	$4{\cdot}9 \times 10^4$ ($\pm 0{\cdot}56$)	$5{\cdot}1 \times 10^4$ ($\pm 0{\cdot}59$)
8 hr	$1{\cdot}2 \times 10^3$ ($\pm 0{\cdot}16$)	$1{\cdot}1 \times 10^3$ ($\pm 0{\cdot}21$)	$1{\cdot}0 \times 10^3$ ($\pm 0{\cdot}18$)	$1{\cdot}1 \times 10^4$ ($\pm 0{\cdot}21$)	$9{\cdot}8 \times 10^3$ ($\pm 0{\cdot}66$)	$9{\cdot}8 \times 10^3$ ($\pm 0{\cdot}75$)
24 hr	$9{\cdot}8 \times 10^1$ ($\pm 0{\cdot}70$)	$1{\cdot}2 \times 10^2$ ($\pm 0{\cdot}12$)	$9{\cdot}5 \times 10^1$ ($\pm 0{\cdot}62$)	$6{\cdot}1 \times 10^2$ ($\pm 0{\cdot}68$)	$5{\cdot}8 \times 10^2$ ($\pm 0{\cdot}64$)	$5{\cdot}5 \times 10^2$ ($\pm 0{\cdot}65$)

*Thioglycollate-elicited peritoneal macrophages were infected with GBS-Ia or GBS-III at an infection rate of 15 micro-organisms per cell for 2 hr and then stimulated with PMA (1 μg/ml) or LPS (1 μg/ml) for 2 hr in presence of antibiotics.

†After removal of stimuli, the infected monolayers were recovered immediately or after further incubation in medium containing antibiotics as described in the Materials and Methods.

‡Number of intracellular GBS was evaluated by viable plate count of CFU. Data are presented as the means ± SD of six individual experiments performed in triplicate.

analysed the features of the intracellular survival of GBS-Ia and GBS-III in murine peritoneal macrophages. The results clearly demonstrate that different GBS serotypes, namely, GBS-Ia and GBS-III, when phagocytosed *in vitro* by macrophages, can survive and persist inside these cells for 24 and 48 hr, respectively. Electron micrographs show that in both the early phase of infection and at 24 hr for GBS-Ia and 48 hr for GBS-III, macrophages containing intact intracellular bacteria had a normal morphology.

Since the capsule is the major virulence factor of GBS,[4,5,52] we wondered whether it had a role in GBS intracellular survival. We observed that the unencapsulated GBS-III mutant strain can survive intracellularly in phagocytes like the capsulated wild-type strain. However, the amount of polysaccharide present on the cell surface seems to have a remarkable effect on the phagocytosis of the micro-organisms. GBS-Ia which has a large capsule[53] is phagocytosed to a lesser degree than GBS-III.[3] These results are in agreement with those obtained by Gibson *et al.*[49] who showed that the acapsular mutants of type III GBS were taken up more efficiently by epithelial cells than the encapsulated wild-type strain. Therefore, the capsule is important in the phagocytosis of GBS but does not influence intracellular survival. We are unable to explain why in our *in vitro* model the capsule played no role in intracellular killing.

Since microbicidal activity of macrophages is strongly enhanced by IFN-γ and LPS,[40,41] we investigated whether macrophage activation by these signals affected the intracellular survival of GBS. The results demonstrate that macrophage activation is relevant in determining the fate of phagocytosed GBS. In fact, a more rapid reduction in the number of intracellular GBS-Ia and GBS-III was observed in macrophages activated with IFN-γ and LPS. This observation seems to be consistent with our previous *in vivo* data which demonstrated that immunostimulated mice were able to resist a lethal challenge with GBS-Ia.[54] The effect of macrophage activation on GBS intracellular survival is very interesting if we consider that the phagocytic cells of neonates and preterm babies frequently have a reduced microbicidal activity[15,16,55–57] and are therefore more susceptible to GBS infection.[58]

Similar results on intracellular persistence in normal or activated macrophages were also obtained with GBS-VI (data not shown). A similar trend of *in vitro* intracellular survival for GBS-Ia, GBS-III and GBS-VI was observed with peritoneal resident macrophages thus also demonstrating that non-inflammatory macrophages are susceptible to GBS infection (data not shown).

The results of this study demonstrating the presence of intact and viable GBS within macrophages are not at odds with the *in vivo* observation that macrophages from lungs of infants who succumbed to GBS infection contain disrupted

GBS.[59] On the contrary, under our *in vitro* experimental conditions, the absence of an important macrophage-activating factor, such as IFN-γ and of its complex co-operative interactions with other cytokines, would not favour an effective macrophage activation. In fact, the ability of lung macrophages to kill phagocytosed GBS could be due to the macrophage activation by the cytokines produced during *in vivo* immune response to GBS. The importance of macrophage activation in GBS infection is suggested by the reduced GBS survival in macrophages activated *in vitro* with IFN-γ and LPS.

Multiple signal transduction pathways are involved in macrophage activation by LPS and IFN-γ. In fact, biochemical pathways in response to LPS include the activation of PKC,[26,29,30,60] tyrosine phosphorylation[61] and changes in cytoskeletal structure[60] while IFN-γ induces direct activation of the JAK-STAT pathway[62,63] and can also modulate PKC activity.[29,64]

It is well known that micro-organisms, which successfully establish persistent and productive infections within mononuclear phagocytes, have evolved mechanisms which by altering intracellular signalling impair the important effector functions of their host cells.[20–25,31] Since PKC is one of intracellular pathways leading to activation of effector macrophage functions,[26–30] several micro-organisms, to survive intracellularly, affect the integrity of PKC-dependent cell signalling.[31–34] These knowledges led us to investigate whether GBS persistence in macrophages is also linked to impairment of the PKC-associated signal transduction pathway. The data of this study provide evidence that a defect in PKC-dependent events contributes to the survival of both GBS strains within murine peritoneal macrophages. It was found that inhibition of the PKC-pathway by pretreatment with PMA for 18 hr or with Calphostin C for 15 min, a potent and specific inhibitor of PKC, rendered macrophages more permissive for GBS intracellular survival. Furthermore, infection with GBS altered the ability of macrophages to respond to second external activating signals. In fact, stimulation of infected macrophages with PMA or LPS did not affect the intracellular survival of GBS. The observation that GBS-infected macrophages have a decreased c-*fos* mRNA level, after stimulation with LPS for 30 min, with respect to non-infected macrophages, provides further evidence that GBS impair the PKC-dependent pathway because the rapid and transient expression of the c-*fos* gene induced by LPS in macrophages, is mediated through PKC.[65]

Although the strains used do not represent true human pathogens because they were not isolated from infected infants, some hypothesis about GBS pathogenicity can be made from the findings of this study. Intracellular localization of GBS in macrophages could protect micro-organisms from the more effective microbicidal activity of PMN, the main effector cells of anti-GBS resistance,[55] and from the action of antimicrobials. Moreover, macrophages could carry GBS to body sites where the antibiotic concentration is too low to eradicate the infection. Knowledge of selective impairment of the PKC-dependent signal pathway may therefore represent a new strategy for identifying targets for treating GBS infection.

In conclusion, our findings provide additional evidence of the survival of GBS in macrophages in the absence of antibodies and suggest that impairment of the PKC-dependent signal transduction pathway contributes to intracellular survival of GBS. Further studies by using human macrophages and infecting GBS strains are needed to gain insight into the validity of these results for human pathogenesis.

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