Antioxidant supplementation enhances bacterial peritonitis in mice by inhibiting phagocytosis

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Antioxidants are known to exhibit numerous health benefits including anti-ageing, anti-apoptotic and immuno-stimulatory effects. However, we present the data showing counterproductive effects of therapeutically relevant antioxidants on bacterial clearance by the immune system in a murine peritonitis model. The antioxidants ascorbic acid, glutathione and N-acetylcysteine augmented morbidity and mortality in mice carrying Escherichia coli-induced acute bacterial peritonitis. Treatment of peritonitic mice with antioxidants significantly increased their bacterial load in the range of 0.3–2 logs. Antioxidant administration to peritonitic mice resulted in decreased numbers of macrophages, B-cells and dendritic cells at the primary site of infection and increased neutrophil infiltration. Serum TNF-α levels were also decreased in antioxidant-treated peritonitic mice. In vitro experiments showed that antioxidants reduced the phagocytic efficiency of peritoneal macrophages by ~60–75% and also decreased E. coli-induced oxidative burst in macrophages cells. Taken together, our data indicate that the antioxidants increased the severity of peritonitis by decreasing the phagocytic efficiency, oxidative burst, and TNF-α production, and increasing neutrophil infiltration. Based on these results, we propose that antioxidant supplementation during the course of bacterial infection is not recommended as it could be detrimental for the host. In addition, the present study underlines the importance of timing and context of antioxidant administration rather than indiscriminate usage to gain the best possible therapeutic advantage of these redox compounds.

INTRODUCTION

Intraperitoneal (i.p.) infections are one of the prominent reasons for morbidity and mortality in humans, despite significant advances in critical care and antimicrobial therapy (May et al., 2000; Herzog et al., 2010). Among diverse known pathogens, Escherichia coli has emerged as the predominant causative agent for intraperitoneal infections (Hau, 1990; May et al., 2000). Establishment of peritoneal bacterial infection is termed ‘bacterial peritonitis’ and accounts for a number of deaths worldwide (Lopez et al., 2011). An appropriate immune response, including local destruction of bacteria through phagocytosis, leukocyte infiltration, cytokine production and subsequent activation of humoral immunity, is required for successful clearance of bacterial peritonitis (Hau, 1990). Antibiotic therapy prevents the spread of infection and augments recovery from bacterial peritonitis (Pavlidis, 2003; Blot & De Waele, 2005). However, due to a large innovation gap in the discovery of newer antibiotics (Fischbach & Walsh, 2009) and the rapid emergence of antibiotic resistance (Foucault & Brouqui, 2007), the best possible use of the available therapeutic resources for management of infections is absolutely vital in the fight against bacterial infections.

Reactive oxygen species (ROS) play a vital role in many physiological processes including progression of bacterial infections (Miller & Britigan, 1997; Nathan & Shiloh, 2000) and diverse aspects related to antibiotic-mediated bacterial clearance (Albesa et al., 2004; Goswami et al., 2006; Kohanski et al., 2010). ROS are reactive by-products formed by the partial reduction of molecular oxygen (Pomposiello & Dempile, 2000). According to our earlier findings (Goswami et al., 2006) and a recently emerging scientific paradigm, many of the classical antibiotics kill bacteria by stimulating the formation of ROS (Dwyer et al., 2009; Kohanski et al., 2010). Redox cycling of the antibiotic could affect the level of ROS produced by cells during the oxidation process (Butler & Hoey, 1993). Fluoroquinolones
are reported to induce the formation of singlet oxygen (\( ^1{\text{O}}_2 \)) and superoxide anion (\( \mathrm{O}_2^- \)), which are responsible for their phototoxic effect (Umezawa et al., 1997). In addition, the two prominent side effects of aminoglycoside antibiotics, ototoxicity and nephrotoxicity, are believed to be mediated through ROS (Mingeot-Leclercq & Tulkens, 1999; Wu et al., 2002), and antioxidant therapy has been projected to mitigate the adverse effects associated with their usage (Sha & Schacht, 2000; Kadkhodae et al., 2005).

We previously demonstrated that the presence of antioxidants such as glutathione (GSH) or ascorbic acid (Asc) eliminated ciprofloxacin- and streptomycin-induced killing of \( E. \ coli \) (Goswami et al., 2006, 2007). Subsequently, \( \text{N-acetylcysteine} \) (NAC) was found to exhibit similar inhibitory activity (Goswami & Jawali, 2010). These findings suggested that some of the common dietary and cellular antioxidants adversely affect the action of therapeutic antibiotics. Our earlier studies strengthened the hypothesis that antioxidants may interfere with the clearance of bacterial infections in vivo (Goswami et al., 2007; Goswami & Jawali, 2010). However, to understand the consequence of antioxidant supplementation on antibiotic therapy, it is imperative to comprehend the effect of antioxidants per se on progression of bacterial infection. The importance of the present study is also underlined by the fact that ascorbic acid (vitamin C) and cysteine-rich foodstuffs are either prescribed or inadvertently taken during bacterial infection and antibiotic therapy. This could have an important bearing on the overall progression of the disease. Similarly, use of NAC as a mucolytic agent in patients having a lower respiratory tract infection may affect the outcome of the antibiotic therapy (Goswami & Jawali, 2010). Therefore, in the light of above-mentioned facts, we used an \( E. \ coli \)-induced murine peritonitis model to investigate the effects of therapeutically relevant antioxidants on clearance of bacteria by the immune system. This was undertaken by examining the survival, body weight, visible pathophysiological changes, local bacterial load, plasma cytokine levels and leukocyte infiltration in acute peritonitic mice. We further investigated the effect of antioxidants on phagocytic efficacy and oxidative burst in murine peritoneal macrophages challenged with \( E. \ coli \).

**METHODS**

**Experimental animals.** Swiss albino mice aged 6–8 weeks belonging to either sex were acquired and housed in the Bhabha Atomic Research Centre animal house facility. They were maintained at 22 °C and fed rodent chow and water ad libitum. This experimental study was approved and performed in accordance with guidelines of the Institutional Animal Ethics Committee.

**Studies in a mouse model with acute bacterial peritonitis.** Mice were injected intraperitoneally with non-pathogenic \( E. \ coli \) K-12 strain MG1655 to induce peritonitis as described previously (Tan et al., 2005). MG1655 is an \( E. \ coli \) strain with fimbiae (pili), which are required for successful attachment and adhesion to the host tissues (Krogfelt et al., 1990; Müller et al., 2009). Therefore, in spite of being non-pathogenic, it is an excellent model system to simulate bacterial infection in animal studies (Billips et al., 2007). A minimum of three animals were used in all the experiments performed. For infection purposes, \( E. \ coli \) cells grown overnight in Luria–Bertani (LB) medium were isolated by centrifugation and resuspended in sterile normal saline at 4 °C. About \( 3 \times 10^8 \) c.f.u. (determined by plating) were injected i.p. into mice in a volume of 400 μl. One of the antioxidant solutions (GSH/Asc/NAC) in a final volume of 400 μl was injected by the i.p. route in mice, 3 to 4 h after challenge with bacterial inoculum. The antioxidant solutions were always prepared fresh before injections to avoid the possibility of auto-oxidation. The concentration of antioxidants used for injection was 500 mg (kg body weight)\(^{-1}\) in accordance with previous scientific literature (Bump et al., 1992; Rocksen et al., 2000; Chen et al., 2006). The concentrations of antioxidants were chosen carefully based on earlier scientific reports investigating their effect in different model hosts (primarily mice) against bacterial endotoxin challenge through the i.p. route (Tajimi, 1982; Rocksen et al., 2000; Chen et al., 2006). These levels of antioxidants did not cause any apparent toxicities in the mice (Yoda et al., 1986). Nevertheless, in our studies, we ensured that the antioxidants per se were not toxic to the host. Importantly, as a mucolytic agent, NAC was used at much higher concentrations in the range of 400–1200 mg (kg body weight)\(^{-1}\) (Aitio, 2006).

In order to investigate the effect of antioxidant administration (GSH/Asc/NAC) on bacterial peritonitis, the following five treatment groups were used: (1) control; (2) \( E. \ coli \); (3) \( E. \ coli + \text{Asc} \); (4) \( E. \ coli + \text{GSH} \); and (5) \( E. \ coli + \text{NAC} \).

The first group comprised normal healthy control mice, whilst in the remaining four groups, peritonitis was induced experimentally by injecting \( E. \ coli \) cells (these groups were referred to as peritonitic mice). In groups 3–5, each of the antioxidants was then administered in the peritonitic mice. After the injections, the mice were housed for 24 h before collection of the different datasets. Separate experimental groups were used for collection of different datasets.

**Survival and pathophysiological observations.** The survival and total body weight of mice belonging to different experimental groups along with the control group were determined before and 24 h after the first injection was given, and they were subsequently monitored on a daily basis for the next 30 days. The reduction in weight gave an indication of treatment-induced weight loss. The mice were also monitored for diarrhoea-associated symptoms.

**Quantification of bacterial counts in the peritoneal cavity.** At 24 h after the first bacterial injection, the animals were sacrificed and their peritoneal cavity was flushed using 5.0 ml chilled saline. The recovered peritoneal flush (~3.5 ml) was serially diluted 10-fold and plated in duplicate on eosin methylene blue agar, followed by overnight incubation at 37 °C for enumeration of c.f.u. The bacterial counts obtained in this way were compared across different treatments to assess the effect of antioxidants on bacterial clearance from the given host.

**Effect of antioxidants on the phagocytic activity of peritoneal macrophages.** The phagocytic activity of peritoneal macrophages isolated from healthy Swiss albino mice was estimated using FITC-labelled \( E. \ coli \) K-12 cells (commercially available; Sigma Aldrich). Peritoneal macrophages were obtained by injection of 5 ml ice-cold RPMI 1640 into the peritoneal cavity, and viable cells were counted using trypan blue dye exclusion. Macrophage cells were mixed with FITC-labelled \( E. \ coli \) at an effect-to-target ratio of 1:10 (1 × 10\(^5\) macrophages: 1 × 10\(^5\) \( E. \ coli \) cells) and centrifuged for 15 s to increase their interaction. As phagocytosis and the related oxidative burst are kinetic processes, macrophages were treated with the antioxidants in two different ways. For pre-incubation assays, the antioxidants were added to the macrophages 1 h prior to the addition of \( E. \ coli \) cells. For co-incubation assays, the antioxidants and \( E. \ coli \) cells were added together to the macrophage population. The mixtures were incubated at 37 °C for 30 min. Ice-cold PBS was added to stop phagocytosis. The cells were
acquired in a flow cytometer, immediately, with or without crystal violet to distinguish the fluorescence of surface-bound E. coli cells from phagocytosed bacteria. A total of 20,000 cells were acquired for each sample in a flow cytometer (Partec PAS III). The fraction of cells that had phagocytosed bacteria was calculated using FlowJo software (Tree Star).

**Effect of antioxidants on oxidative burst in peritoneal macrophages.** Changes in intracellular ROS levels induced by bacteria were studied using two fluorescent dyes: 2',7'-dichlorofluorescein diacetate (H2DCF-DA) and dihydroethidium (DHE). The bacterial cells were opsonized with normal human serum by incubating at 37 °C for 30 min in the dark. Freshly isolated peritoneal macrophages were stained with fluorescent probes as described elsewhere (Checker et al., 2010; Khan & Poduval, 2011). Bacterial cells were mixed with freshly isolated macrophage cells at a 1:10 ratio (1 × 10⁶ macrophages : 1 × 10⁶ bacterial cells). After 15 min of incubation, the increase in fluorescence resulting from oxidation of H2DCF to 2',7'-dichlorofluorescin (DCF) or DHE to ethidium was measured using a spectrofluorometer at excitation wavelengths of 488 and 535 nm and emission wavelengths of 535 and 610 nm, respectively. Antioxidant treatment was applied in two ways: for pre-incubation assays, antioxidants were added to macrophages 30 min prior to the addition of opsonized bacteria; for co-incubation assays, the antioxidants were added along with the addition of macrophages to the opsonized bacteria.

**Effect of antioxidants on leukocyte infiltration.** The frequency of different leukocytes (CD3+, CD11b+, CD11c+, CD14+, CD19+ and Gr-1+) in the peritoneal flush from mice subjected to E. coli and antioxidant administration was examined by staining with fluorochrome-conjugated antibodies and flow cytometry as described previously (Sharma et al., 2012). In brief, peritoneal flush containing peritoneal exudate cells (PECs) was recovered 24 h after bacterial challenge. One million PECs were stained with phycoerythrin (PE)-Cy5–CD3, PE–CD14, PE–CD19, FITC–CD11b, FITC–CD11c and FITC–Gr-1 antibody and fixed with 1 % formaldehyde. The cells were washed, resuspended in PBS and stained with Hoechst 33342 (5 μg ml⁻¹), and 50,000 cells were acquired in a flow cytometer (Partec CyFlow Space). The data were analysed using FlowJo software.

**Measurement of serum cytokine levels.** The mice were bled from their retro-orbital sinuses 24 h after bacterial infection. The blood samples were allowed to coagulate and then centrifuged at 1000 g to isolate the serum. The serum was immediately frozen and stored at −80 °C. The levels of pro-inflammatory cytokines (IL-1β, IL-6, TNF-α and IFN-γ) were measured using commercial ELISA kits (Opt EIA ELISA kits; BD Biosciences Pharmingen) according to the manufacturer’s instructions.

**Statistical analysis.** Statistical analysis was carried out using Student’s t-test with Microcal Origin 8.0 software. Unless indicated otherwise, * denotes P<0.05 as compared with the control and ‘#’ indicates P<0.01 compared with the E. coli-treated group. All experiments were repeated at least twice to confirm the reproducibility of the results, and a representative result from a single experiment is used for representation and analysis purposes. The number of replicates (n) for given samples is indicated in the figure legends.

**RESULTS**

**Effect of antioxidants on survival and pathophysiological features of mice with acute bacterial peritonitis**

The survival of mice with acute bacterial peritonitis was monitored for all the treatment groups and the results are shown in Fig. 1(a). Like the control group, mice administered with E. coli did not show any mortality during 30 days of observation period. However, the peritonitic groups administered with antioxidants (Asc/GSH/NAC) showed significant increases in mice mortality. The most profound effect was seen after the administration of NAC, which resulted in 100 % mortality by day 3. In GSH- and Asc-treated peritonitic mice, the survival was reduced to 50 % (Fig. 1a).

The mean body weight of control mice was 25.8 ± 0.5 g (mean ± SEM). The i.p. administration of bacterial cells led to the initiation of diarrhoea in the mice within 2–6 h. The dehydration caused by the diarrhoea probably contributed towards weight loss in the animals. The animals from the control group exhibited a steady weight gain, whereas all of the peritonitic animal groups (with or without antioxidant administration) lost ~5–8 % of their body weight over a 30-day observation period (Fig. 1b). In the antioxidant control groups (Asc/GSH/NAC administered in healthy mice), no mortality was observed till the end of day 5 (data not shown). In contrast, a maximum decline in animal survival was observed by day 5 among all three antioxidant-administered peritonitic mice groups (Fig. 1a).

**Quantification of bacterial load after antioxidant administration in peritonitic mice**

The effect of antioxidant administration on clearance of peritoneal bacterial load was investigated by quantifying the bacterial counts in the peritoneal flush acquired from animals belonging to different experimental groups (Fig. 1c). As shown in Fig. 1(c), the peritoneal cavity of mice in the control group was free of any bacterial infection. The mice challenged with E. coli cells had more than 10⁶ c.f.u. ml⁻¹ in the peritoneal flush 24 h after the bacterial challenge. It was interesting to note that administration of any of the antioxidants after E. coli injection (i.e. the E. coli+Asc, E. coli+NAC or E. coli+GSH group) resulted in substantial augmentation of peritoneal bacterial load in comparison with the E. coli group. In particular, NAC and Asc were found to be remarkably effective in terms of increasing the bacterial load of the animals by ~2 logs (P<0.01). GSH also increased the peritoneal bacterial load of the mice, albeit to a comparatively lesser extent of 0.3 logs (P<0.05).

**Effect of antioxidants on the phagocytosis of FITC-labelled E. coli by peritoneal macrophages**

As bacterial peritonitis was found to be further intensified after antioxidant administration, we investigated the effect of antioxidants on phagocytic activity of peritoneal macrophages ex vivo. This represents the natural ability of the given host to fight bacterial infection. The effect of antioxidant co-incubation and pre-incubation on phagocytic activity of peritoneal macrophages was evaluated by flow cytometry and is shown in Fig. 2. Peritoneal macrophages incubated with FITC-labelled E. coli showed an increase of about 80 % in intracellular bacteria compared with the control group or the group where peritoneal macrophages were not exposed to FITC-labelled E. coli (Fig. 2a). In contrast, both antioxidant
pre-incubation and co-incubation resulted in a significant decrease of intracellular bacteria, suggesting that presence of these antioxidants inhibited bacterial phagocytosis. Of the total macrophage population, about 3–7 % was found to be FITC labelled when NAC/GSH/Asc was co-incubated with macrophages and E. coli (Fig. 2b). In contrast, pre-incubation of peritoneal macrophages with these antioxidants resulted in 6–14 % FITC-labelled cells. Therefore, this indicates that the presence of antioxidants is inhibitory for bacterial phagocytosis.

**Effect of antioxidants on intracellular ROS levels in peritoneal macrophages in vitro**

Upon exposure to bacteria, phagocytes produce high levels of ROS by a process called oxidative burst. The bacteria-induced alterations in intracellular ROS levels of murine peritoneal macrophages were measured in the presence or absence of exogenously added antioxidants. Two ROS-sensitive probes, namely, DCF-DA and DHE, were used to measure the extent of oxidative burst. Optimum oxidative burst was seen between 5 and 15 min after E. coli were added to DCF-DA-labelled or DHE-labelled peritoneal macrophages (data not shown). Hence, the effects of both antioxidant co-incubation and pre-incubation on oxidative burst in peritoneal macrophage were measured 10 min after incubation with E. coli cells. Exposure of peritoneal macrophages to E. coli cells led to increased ROS levels, as evidenced by augmented DCF and DHE fluorescence (Fig. 3). DCF fluorescence was significantly decreased in the presence of all three antioxidants during both antioxidant

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**Fig. 1.** Survival, body weight and peritoneal bacterial load after antioxidant administration in peritonitic mice. (a) Survival of the mice given different treatments over a period of 30 days. The mice were challenged with ~3×10⁸ E. coli cells and 500 mg (kg body weight)⁻¹ of one of the antioxidants (GSH/Asc/NAC) through the i.p. route (n=6 mice per group). (b) Changes in body weight of the mice during 30 days after injection of E. coli and one of the antioxidants (n=6 mice per group). Each data point represents mean ± SEM. (c) Changes in peritoneal bacterial load of mice subjected to different combinations of E. coli and antioxidant treatments (n=4 mice per group). *P<0.05 compared with the control; #P<0.05 compared with the E. coli-treated group.
Effect of antioxidants on bacterial peritonitis

Immune cells like macrophages, neutrophils, dendritic cells and lymphocytes (B-cells and T-cells) are known to play an important role in clearance of bacterial infection. Accordingly, the infiltration of these cells in response to bacterial peritonitis and antioxidant administration was examined in vivo. Using antibody staining and flow cytometry, we enumerated the frequencies of \( \text{CD3}^+ \), \( \text{CD14}^+ \), \( \text{Gr-1}^+ \), \( \text{CD19}^+ \), \( \text{CD11b}^+ \) and \( \text{CD11c}^+ \) cells in the peritoneal flush. In Fig. 4, the overlaid histograms show the effect of bacterial challenge and antioxidant administration on different immune cells in the peritoneal flush, with the frequency of gated cells shown in the bar charts. The \( \text{E. coli} \)-challenged peritonitic mice had a significantly lower frequency of \( \text{CD11b}^+ \) cells and \( \text{CD19}^+ \) cells and a higher frequency of \( \text{CD14}^+ \) cells compared with that in peritoneal flush from healthy control mice (Fig. 4a, c, d). Treatment of control as well as peritonitic mice with any of the antioxidants resulted in significantly decreased frequency of \( \text{CD11b}^+ \) cells and \( \text{CD19}^+ \) cells in the peritoneal flush (Fig. 4a, d). In addition, NAC treatment decreased the frequency of \( \text{CD11c}^+ \) cells in the peritonitic mice (Fig. 4b). As there was a decrease in the two major subpopulations (\( \text{CD11b}^+ \) and \( \text{CD19}^+ \) cells) of PECs after bacterial challenge and antioxidant treatment, we investigated the phenotype of other infiltrating cells in the peritoneal flush. For this purpose, the PECs were stained with \( \text{Gr-1} \) antibody and total \( \text{Gr1}^+ \text{FSC}^{\text{high}} \) (large-sized granulocytes) neutrophils and \( \text{Gr1}^+ \) cells were gated using FlowJo software. The gating for \( \text{Gr1}^+ \text{FSC}^{\text{high}} \) and total \( \text{Gr1}^+ \) cells is shown in representative flow cytometric zebra plots in Fig. 5(a, b) and overlaid histograms in Fig. 5(c, d) respectively. The peritonitic mice had an almost fivefold increase in total \( \text{Gr1}^+ \) cells and a fourfold increase in the \( \text{Gr1}^+ \text{FSC}^{\text{high}} \) neutrophils. The antioxidants also increased infiltration of \( \text{Gr1}^+ \) cells and neutrophils in control as well as peritonitic mice (Fig. 5b, d).

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col-\( \text{coli}\)-induced increase in DHE fluorescence was significantly inhibited by both antioxidant co-incubation and pre-incubation. The decrease in \( \text{E. coli}\)-induced ROS production was more prominent in macrophages co-incubated with antioxidants compared with those pre-incubated with antioxidants.

Effect of antioxidant administration on infiltration of leukocytes in animals with acute bacterial peritonitis

Co-incubation and pre-incubation. Similarly, the bacterial-induced increase in DHE fluorescence was significantly inhibited by both antioxidant co-incubation and pre-incubation. The decrease in \( \text{E. coli}\)-induced ROS production was more prominent in macrophages co-incubated with antioxidants compared with those pre-incubated with antioxidants.

**Effect of antioxidant administration on infiltration of leukocytes in animals with acute bacterial peritonitis**

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Plasma levels of pro-inflammatory cytokines after antioxidant administration in animals with acute bacterial peritonitis

In order to examine the effect of antioxidant administration on *E. coli*-induced inflammatory response in mice, the plasma levels of the pro-inflammatory cytokines IL-1β, IL-6, TNF-α and IFN-γ were measured. The plasma levels of IL-1β, IL-6 and TNF-α increased significantly (*P* < 0.01) in mice challenged with bacterial cells compared with the control group (Fig. 6). After 24 h starting from the point of bacterial challenge, none of the antioxidants increased the plasma level of the above-mentioned pro-inflammatory cytokines in mice with bacterial peritonitis. In contrast, significantly decreased TNF levels (~12–25%) were observed after administration of antioxidants in comparison with the group that was challenged with *E. coli* (Fig. 6a). Similarly, IL-1β levels were reduced by GSH or NAC administration in mice that already had bacterial peritonitis (Fig. 6b). However, IL-6 levels were brought down only by Asc administration (Fig. 6c). The alterations in plasma levels of IFN-γ were found to be insignificant (*P* > 0.05) after exposure to *E. coli* or any of the antioxidants evaluated (Fig. 6d).

**Fig. 3.** Effect of antioxidants on *E. coli*-induced oxidative burst in peritoneal macrophages. Freshly isolated peritoneal macrophages were labelled with DCF-DA or DHE and treated with GSH, NAC or Asc. The cells were incubated with *E. coli* (1:10, cell:*E. coli* ratio) and fluorescence was measured using a spectrofluorometer. (a, c) Results obtained with co-incubation of antioxidants and macrophages. (b, d) Pre-incubation of antioxidants and macrophages for 30 min before exposure to bacteria. Each bar represents the mean fluorescence intensity ± SEM from three replicates. *P* < 0.05 compared with the control. AU represents mean fluorescence intensity value of the given probe.
Effect of antioxidants on bacterial peritonitis

Fig. 4. Effect of antioxidant administration on infiltration of leukocytes in the peritoneum of *E. coli*-infected mice. Mice were injected with *E. coli* strain MG1655 (~3×10⁸ cells per mouse i. p.) and were administered GSH, NAC or Asc [500 mg (kg body weight)i.p.] after 3 h. The mice were sacrificed on next day and PECs were isolated. The cells were stained with different antibodies and a minimum of 50,000 cells in each group were acquired in a flow cytometer. Overlaid flow cytometric histograms in the right panel show CD11b⁺ (a), CD11c⁺ (b), CD14 (c) and CD119⁺ (d) cells in the PECs isolated from the primary site of infection from animals in different treatment groups. The frequency of gated cells is shown in the respective bar diagrams on the left. Data points show means ± SEM; n=3 mice per group. *P<0.05 compared with the control untreated group.
DISCUSSION

Our previous studies established that antioxidants such as GSH, Asc and NAC modulate the antibacterial activity of therapeutic antibiotics in vitro (Goswami & Jawali, 2007, 2010). Here, we report the in vivo effects of antioxidant administration on clearance of acute bacterial peritonitis in Swiss albino mice. In order to test the effect of antioxidants on bacterial infection in vivo, we used a murine peritonitis model. The mice were injected with E. coli strain MG1655 (~3×10^8 cells per mouse i.p.) and were administered GSH, NAC or Asc [500 mg (kg body weight)^{-1} i.p.] after 3 h. The mice were sacrificed the next day and PECs were isolated. The cells were stained with FITC-conjugated Gr1 antibody and a minimum of 50,000 cells in each group were acquired in a flow cytometer. The data were analysed using FlowJo software. (a) Representative flow cytometric zebra plots indicating Gr1^+ FSC^high neutrophils in different treatment groups. (b) Bar diagram showing the frequency of neutrophils. (c) Flow cytometric histograms showing total Gr1^+ cells in PECs isolated from the primary site of infection in different treatment groups. (d) The frequency of total Gr1^+ cells is shown in bar diagram. Data points show means±SEM; n=3 mice per group. *P<0.05 compared with the control untreated group.
The present study conceivably proposes that antioxidant administration in animals with bacterial infection could be harmful for the host physiology. This statement is supported by our data that antioxidant administration caused increased bacterial load in the peritoneal cavity, ultimately leading to decreased survival. Introduction of any of the antioxidants after bacterial exposure leading to either total or partial mortality in these groups of mice implies that antioxidant treatment actively potentiates the detrimental effects of bacterial infection. The possibility of direct antioxidant toxicity contributing to the reduced animal survival was ruled out by using a corresponding antioxidant control. Dehydration-induced weight loss in mice with bacterial peritonitis revealed that such animals experienced compromised host physiology (Fig. 1b).

The quantification of peritoneal bacterial load in different experimental groups of mice illustrated that the peritoneal cavity of control group was free from E. coli infection. The mouse group that was injected with bacteria showed the presence of live bacterial cells in the peritoneal cavity, albeit the number of cells obtained from the peritoneal flush was 2 logs lower in comparison with the original inoculum used for infection purposes. This decrease in the number of bacterial cells could be attributed mainly to phagocytosis-mediated killing by the host immune system. Phagocytosis is performed by peritoneal macrophages and polymorphonuclear leukocytes, which are primary cells for immunological defence against bacterial peritonitis (Dunn et al., 1985; Kim et al., 1996). During phagocytosis, both macrophages and polymorphonuclear leukocytes produce a mixture of diverse ROS and reactive nitrogen species such as O₂⁻, H₂O₂, ·OH, HOCl and NO*, which together play...

Fig. 6. Effect of antioxidant administration on plasma cytokine levels in E. coli-infected mice. Mice were injected with E. coli strain MG1655 (~3×10⁸ cells per mouse i.p.) and were administered GSH, NAC or Asc [500 mg (kg body weight)⁻¹ i.p.] after 3 h. The concentrations of the pro-inflammatory cytokines TNF-α (a), IL-1β (b), IL-6 (c) and IFN-γ (d) were measured at 24 h in plasma isolated from peritonitic mice 24 h after treatment with different antioxidants. The data points represent means ± SEM values for different treatment groups; n=3 mice per group. *P<0.05, compared with the control.
an important role in antimicrobial host defence (Nathan & Shiloh, 2000). Moreover, $O_2^-$ and NO$^\cdot$ react together at a diffusion-controlled rate (McLean et al., 2010) to yield peroxynitrite (ONOO$^-$), which plays a key role in bacterial killing (Brunelli et al., 1995). The antimicrobial effect of these intermediates could be due to depletion of thiols, inhibition of ATP and DNA synthesis (Nathan & Hibbs, 1991), oxidation of thiols, DNA damage (Wink et al., 1991; Hogg et al., 1994) and oxidation of vitamin C and other key cellular enzymic activities (Castro et al., 1994; Hogg et al., 1994). Apart from the major role played by the phagocytosis process, complement-mediated killing (Joiner et al., 1984; Rooijakkers & van Strijp, 2007) and bacterial ozonolysis (Wentworth et al., 2002) further assist in eliminating bacterial infection. We hypothesized that the antioxidant-induced increase in bacterial load in peritonitic mice could be due to reduced efficacy of bacterial phagocytosis and killing. This proposition was corroborated by our findings showing that antioxidant supplementation eliminated intracellular ROS generation and bacterial phagocytosis performed by the peritoneal macrophages (Figs 2 and 3). The antioxidant-mediated decrease in phagocytosis highlights the fact that the presence of antioxidants at the site of infection creates a reducing environment that is not favourable for clearance of bacteria due to decreased phagocytosis and oxidative burst (Kim et al., 2004). Increased inhibition of bacterial phagocytosis and oxidative burst in peritoneal macrophages where antioxidant and bacteria were co-incubated in comparison with their pre-incubation counterparts underscores the importance of timing of administration of antioxidants during bacterial peritonitis.

The increased infiltration of neutrophils in the peritoneum of E. coli-challenged mice showed the normal course of the immune response involved in the clearance of bacteria (Fig. 5). All three antioxidants increased neutrophil infiltration in the peritoneum, even in the absence of any bacterial infection, indicating that a change in extracellular redox state in the peritoneum leads to recruitment of neutrophils. The increased neutrophil infiltration may clear bacteria but would also injure the surrounding tissue leading to increased host mortality (Walley et al., 1997). Neutrophils require complement receptor 3 (CD11b/CD18) for effective phagocytosis of bacteria (Ehlers, 2000). The concurrent decrease in CD11b expression in PECs after antioxidant administration (Fig. 4a) would impair the phagocytic efficiency. Thus, the antioxidant-mediated decrease in phagocytosis and oxidative burst would disarm the host immune response and thus contribute to the increased bacterial load, leading to higher host mortality. Our proposal is further supported by an independent recent report showing a curcumin-mediated compromised immunological response against invading pathogens in a murine typhoid model (Marathe et al., 2013). Similarly, increased progression of Chlamydia trachomatis infection in the presence of GSH and NAC has been reported previously (Lazarev et al., 2010). Moreover, the presence of supplementary antioxidants at the site of infection could also interfere with the antibody-mediated bacterial killing via the ozonolysis pathway, thereby inhibiting the formation of neutrophil extracellular traps, which facilitate the clearance of bacterial infection from the host’s body (Brinkmann et al., 2004). However, these propositions are yet to be validated experimentally.

Increased plasma levels of pro-inflammatory cytokines such as IL-1$\beta$, IL-6 and TNF-$\alpha$ in animals challenged with bacterial cells signify the activation of immune effector mechanisms for clearance of infection. These findings are in agreement with earlier reports of cytokine production during bacterial peritonitis or polymicrobial sepsis (Montravers et al., 1997; Sterns et al., 2005). Specifically, IL-1$\beta$ and IL-6 are known to be involved in initiating the cascades of downstream immunological mediators (Jiang et al., 2000; Steinhauser et al., 2000) and controlling the propagation of bacterial peritonitis by induction of fever in the host (Jiang et al., 2000). The relatively small increase in TNF-$\alpha$ after bacterial challenge suggests that its plasma levels are much more tightly regulated in the host. However, the fact that there was no significantincrease in IFN-$\gamma$ levels in peritonitic mice may be attributed to the lack of a T-helper 1 response during infection by an obligate extracellular microbe like E. coli. Decreased plasma levels of pro-inflammatory cytokines after antioxidant administration in animals carrying bacterial peritonitis could be a reflection of elimination of immune effector mechanisms by these antioxidants.

On the whole, the results of our present study reveal for the first time that antioxidant administration in animals having acute bacterial peritonitis leads to overall deterioration of host conditions. This is the fallout of antioxidant-mediated modulation of three important immunological parameters involved in antibacterial defence: (i) phagocytic efficacy and oxidative burst; (ii) neutrophil infiltration and Gr1 expression; and (iii) expression of CD11b, which is a component of complement receptor CR3. This is an initial but significant step towards understanding the in vivo role of antioxidant supplementation during clearance of bacterial infection. Importantly, we previously established that antioxidants eliminate antibiotic-induced bacterial killing (Goswami et al., 2006, 2007), and our current study indicates that they can also promote bacterial infection by decreasing the capacity of immune cells. Therefore, the knowledge obtained from our previous and present studies strengthens the hypothesis that antioxidants may interfere with the overall clearance of bacterial infections in vivo. In addition, the current study also highlights the importance of timing and context of antioxidant supplementation as opposed to indiscriminate usage of these redox-active compounds for improved therapeutic management of bacterial infections.
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