

## NONSPECIFIC RESISTANCE TO BACTERIAL INFECTIONS

### Enhancement by Ubiquinone-8

BY LUTZ H. BLOCK,\* APOSTOLOS GEORGOPOULOS, PETER MAYER, AND  
JÜRGEN DREWS

*From the Department of Internal Medicine, Section of Infectious Diseases, Yale University School of Medicine, New Haven, Connecticut, and Sandoz Forschungsinstitut Ges. m.b.H., Vienna, Austria*

The mononuclear phagocyte-system is involved in a variety of pathological and physiological events (1). It is recognized as the major component in the mediation of nonspecific resistance against infectious agents and neoplastic diseases (2, 3). A variety of agents like endotoxin (4), bacille Calmette-Guérin (BCG)<sup>1</sup> (5), *Corynebacterium parvum* (6), and levamisole (7, 8) reportedly enhance the nonspecific resistance of the host to infections and tumors. These increases in resistance can reflect a direct stimulation of macrophages and/or a modulation of macrophage function by lymphokines, produced by lymphocytes after sensitization with specific antigen (9, 10).

Recently Fauve and Hevin (11) demonstrated an increase in nonspecific resistance of mice after treatment with the bacterial phospholipid extracts from various bacteria, which prompted us to initiate further studies of the active principles in bacterial cell wall extracts, which can produce nonspecific resistance. We therefore extracted an apolar lipid fraction from *Escherichia coli* and studied its protective effect against infections with supralethal doses of gram-negative and gram-positive bacteria in mice. The active component of this apolar lipid extract was purified and identified as the benzoquinone ubiquinone-8.

This paper describes the effects of ubiquinone-8 (Q8) in comparison to other Q-analogues (Q4, Q6, and Q10) on host resistance to a variety of pathogens in mice and the possible sites of action of Q8. A method for the preparative purification of Q8 is also described.

### Materials and Methods

**Animals.** Female NMRI mice weighing  $\approx 20$  g were obtained from a commercial breeder, and raised in pathogen-free conditions at a temperature of 20–22°C in the Sandoz Forschungsinstitut (SFI) animal care division. The assay of plaque-forming cells (PFC) was done with spleens of B<sub>6</sub>D<sub>2</sub>F<sub>1</sub> and C<sub>3</sub>H mice. Groups of 10 or more mice were used in individual experiments.

**Bacterial Strains.** *E. coli* 01, *Salmonella typhimurium*, *Erysipelothrix insidiosa*, and *Streptococcus pyogenes* were all obtained from the SFI collection. The doses of infections used were 5–10 times higher than doses of bacteria which can cause death of all control mice. The cultures of bacteria were stored in liquid nitrogen and diluted in isotonic saline just before infection.

\* Present address: Department für Junere Medizin, Universitätsspital Zürich, CH-8091 Zürich Rämistrasse 100, Switzerland.

<sup>1</sup> Abbreviations used in this paper: amu, atomic mass unit; BCG, bacille Calmette-Guérin; b.w., body weight; HPLC, high pressure liquid chromatography; KDO, 2-keto-3-deoxyoctonate; LPS, lipopolysaccharide; PFC, plaque-forming cell; Q8, ubiquinone-8; SRBC, sheep erythrocyte.

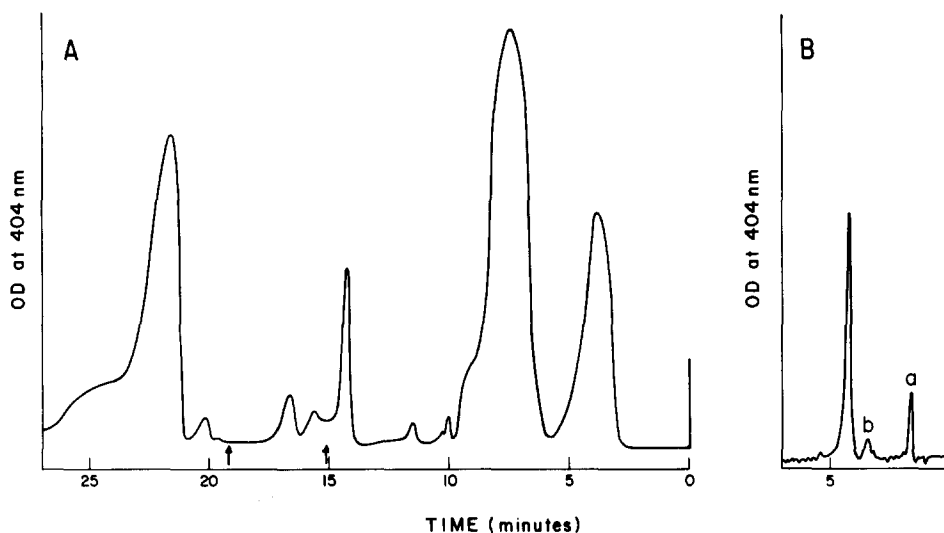


FIG. 1. Chromatograms of ubiquinone extract from *E. coli* separated by silica gel and HPLC. A, Pattern of dried ubiquinone extract on silica gel. Arrows indicate ubiquinone containing fractions which were taken for further purification. B, HPLC of purified ubiquinone peak. a, collected Q8 fraction. b, lower quinones. For condition see Materials and Methods.

**Bacterial Challenge.** Appropriate dilutions of the bacteria were injected into the tail veins of experimental mice, the controls receiving an equivalent volume (0.3 ml/mouse) of isotonic saline. All mice receiving injections of supralethal doses of *E. coli* 01, *S. typhimurium*, and *S. pyogenes* died within 18–24 h after infection, whereas those that were challenged with *E. insidiosus* died by 3–4 days. The animals that survived the infection, as in the case of Q8 treated animals, were kept under observation up to 3 wk. For infectious doses see Table I.

**Bacterial Clearance from Blood and Tissues.** For estimation of bacteria in spleen and liver groups of 30 mice were injected intravenously with  $2 \times 10^8$  *E. coli* 01. At various time intervals the bacterial counts of spleen and liver were performed under sterile conditions according to Pierce et al. (12). For the homogenates of the organs, 1 ml saline/100 mg material was used.

Blood clearance of *E. coli* 01 was estimated by injecting groups of 10 mice intravenously with  $2 \times 10^8$  viable bacteria per animal. At various intervals, the animals were bled from the venous plexus of the eye and bacteria were counted after dilution with saline on blood agar plates.

**Isolation of Q8.** Solvents used for the chromatographic separations were degassed before use; di-isopropyl ether was distilled to eliminate the stabilizers and anti-oxidants. Dry bacteria of *E. coli* B M 1135 (Merck A. G. Darmstadt, W. Germany) were extracted with hexane/ $\text{CHCl}_3$  as described by Friss et al. (13). An average yield of 9.0 g extract/kg dry weight bacteria was obtained. This extract was dissolved in 100 ml hexane and subjected to a first chromatographic separation on a Merck column, type C, filled with silica gel 43–60  $\mu\text{m}$ . The apolar lipids were eluted using the following elution scheme: hexane/diethyl ether 99:1, 98:2, and 97:3 with 1.5 l of each solvent mixture. Monitoring was done with a Schoeffel SF 770 UV detector tuned at 404 nm. The constant flow rate of 18 ml/min was achieved by using an Altex pump (Rainin Instrument Corp., Brighton, Mass.). The ubiquinones were eluted with the 97:3 mixture, evaporated to dryness (average 0.5 g of bright yellow dry material), and dissolved in the mobile phase used in a high pressure liquid chromatographic (HPLC) separation (Fig. 1A). HPLC conditions: stainless steel column 500  $\times$  8.4 mm i.d., slurry packed with LiChrosorb SI 100, 5  $\mu\text{m}$ , (Rainin Instrument Corp.), mobile phase hexane/di-isopropyl ether 95:5 vol/vol, flow rate 12 ml/min, pressure 27 pound per square inch. Fractionation was done by monitoring the effluents with a UV detector at 404 nm. The first eluting peak was collected and consisted of Q8 with a purity >95% as checked by analytical HPLC, (Fig. 1B),  $^1\text{H}$  nuclear magnetic resonance, and mass spectrometry (14). An average yield of  $\approx 100$  mg Q8 (Fig. 2) was obtained. The second peak contained the lower ubiquinones. The more polar lipids remaining on the Merck column were eluted from time to time with 1.5 liters each of  $\text{CHCl}_3$  followed by methanol.

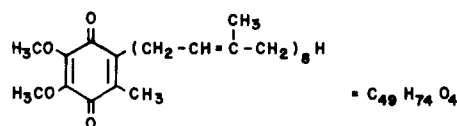


FIG. 2. Q8.

**Physical Properties of Q8.** High resolution mass spectroscopy analysis of Q8 revealed a molecular ion of  $726.562 \pm 0.006$  atomic mass unit (amu) (726.559 theoretical for  $\text{C}_{49}\text{H}_{74}\text{O}_4$ ) and a ubiquinone characteristic fragment ion of  $235.095 \pm 0.002$  amu (235.097 theoretical for  $\text{C}_{13}\text{H}_{16}\text{O}_4$ ). In addition all the characteristic smaller fragments reported by Morimoto et al. (14) for ubiquinone were also observed.

Proton magnetic resonance of Q8 HPLC peak (Fig. 1 B) gave the following expected signals: olefinic protons 5.12 ppm; two phenyl-O-CH<sub>3</sub> at 3.88 ppm and 3.94 ppm; phenyl-CH<sub>3</sub> at 2.16 ppm; the =C-CH<sub>2</sub>-CH<sub>2</sub>-C= group at 1.96-2.10 and the =C-CH<sub>3</sub> moiety at 1.62 ppm.

**Other Ubiquinones.** Q6 and Q10 are commercially available (Sigma Chemical Co., St. Louis, Mo.) Ubiquinone-4 was a gift from Dr. K. Folkers, Institute for Biomedical Research, Austin, Tex.

**Preparation of the Injection Solutions.** The samples were suspended in sterile saline by a short sonication (5-10 s) with a Branson sonifier (Branson Sonic Power Corp., Danbury, Conn.). During this preparation the solution was stored on ice and protected from light. The particle size of the solution was 2-4  $\mu\text{m}$ .

**Lipopolysaccharides.** Lipopolysaccharides (LPS) from *E. coli* O4 (0111/180) were obtained from the Max-Planck-Institut für Immunobiologie, Freiburg, W. Germany and were extracted by the hot phenol-water method (15). Lipid A antigen was purified following the procedure of Galanos et al. (16).

**Macrophage Preparation and Erythrophagocytosis.** Macrophages were harvested from the peritoneal cavities of mice, according to the method of Wellek et al. (17). The cells were extensively washed in medium TC 199 (Grand Island Biological Co., Grand Island, N. Y.) and adjusted at a concentration of  $2 \times 10^6$  viable cells/ml, as determined by Trypan blue exclusion. Phagocytosis was performed in Leighton tubes (Bellco Glass, Inc., Vineland, N. J.). The macrophage suspension (1.0 ml vol) was put into the tubes and was incubated for 60 min at 37°C in 5% CO<sub>2</sub>. Nonadhering cells were washed off with medium TC 199. The remaining cells, about 95% macrophages, formed monolayers on the cover slips. To each tube, antibody-coated sheep erythrocytes (SRBC) (anti-sheep hemolysin 1:6,000) (Behring Diagnostics, American Hoechst Corp., Somerville, N. J.) at a concentration of  $2.0 \times 10^7$ /ml, diluted in 1.5 ml medium TC 199 were added. Phagocytosis was allowed by incubation for 60 min at 37°C and 5% CO<sub>2</sub>. The tubes were rinsed with medium TC 199, and noninternalized SRBC were lysed by using 0.83% ammonium chloride solution. After one more washing step with TC 199, the cover slips were removed, dried, and stained with Wright's stain. Phagocytosis was calculated by counting internalized SRBC within 500 macrophages. Macrophages were divided into four groups containing 0, 1-2, 3-4, and 5 or more erythrocytes/cell. Results were expressed by calculating the phagocytic index (P.I.) according to:

$$\text{P.I.} = \frac{\sum p_i \cdot x_i}{\sum p_i}$$

$p_i$  = number of macrophages in group  $i$   
 $x_i$  = uptake of SRBC on average.

**Spleen Plaque-Forming Cell Assay.** Direct plaque-forming cells (PFC) were assayed by a modification of the hemolytic plaque technique as reported by Plotz et al. (18). Mice were immunized with  $10^8$  SRBC on day 0. On day 4 the animals were killed and a spleen cell suspension was prepared in Hanks' balanced salt solution. 400  $\mu\text{l}$  of 0.5% agarose in 2 ml minimal essential medium (Grand Island Biological Co.) were dispensed in glass tubes and kept at 44°C. 0.01 ml of a 15% suspension of SRBC was added to each tube followed by 100

TABLE I  
Effect of Q8 Treatment of Experimental Infections with Gram-Positive and Gram-Negative Organisms in Mice

Pathogens	Inoculum/mouse i.v.	Controls*	Q8* i.v.
<i>S. pyogenes</i>	$8 \times 10^2$	0/70	42/70
	$3 \times 10^2$	0/70	70/70
<i>E. insidiosa</i>	$10^2$	0/70	70/70
	$1.7 \times 10^5$	0/70	49/70
	$8 \times 10^2$	0/70	70/70
	$3 \times 10^2$	0/70	70/70
<i>E. coli</i> 01	$1 \times 10^9$	0/70	56/70
	$2 \times 10^8$	0/70	70/70
	$1 \times 10^8$	15/70	70/70
<i>S. typhimurium</i>	$1 \times 10^9$	0/70	48/70
	$1 \times 10^8$	0/70	70/70
	$1 \times 10^8$	0/70	70/70

Note: Q8 was injected i.v. (25 mg/kg b.w.) 24 h before bacterial challenge. Bacterial inocula were injected i.v.

\* Survivors represent mice per total number alive at 24 h after infection with *E. coli* 01 and at 4 days after challenge with *E. insidiosa*. These surviving animals continued to live beyond 3 wk after bacterial challenge. Mice shown represent all the animals used in the experiments.

$\mu$ l of a spleen cell suspension. The contents of the tube were mixed, then poured over a glass slide. The slides had previously been coated with a dilute solution of agarose (0.1% wt/vol) and allowed to dry. Subsequently, the slides were incubated on trays in a moist atmosphere at 37°C for 1 h. The slides were then flooded with 1:40 dilution of lyophilized guinea pig complement (Serotherapeutisches Institut, Vienna, Austria) and incubated for another 60 min at 37°C. Plaques were counted under a microscope at a magnification of 8 and with indirect illumination. The number of plaques was related to  $10^7$  spleen cells.

## Results

Because Q8 was isolated from gram-negative organisms it was assayed for contamination with endotoxin. Q8 was found to be free of pyrogenicity by testing in rabbits at different dosages (25–250 mg/kg). As a positive control bacterial endotoxin purified from *Proteus vulgaris* was used at a dose of 0.03  $\mu$ g in sterile saline. While the Q8-treated rabbits showed no significant raise in body temperature (0.1–0.3°C) the endotoxin caused biphasic fever between 0.5 and 1.2°C. In addition, chemical analysis for 2-keto-3-deoxyoctonate (KDO-test, according to Warren, 19) and endotoxin assay with *Limulus polyphemus* (20) were negative in an assay which could detect 1 ng/ml of purified LPS.

When injected intravenously 24 h before infection, Q8 at a dose of 25 mg/kg provided complete protection against 5–10-fold lethal doses of the inoculum. Table I shows the effect of Q8 on experimental infections with inocula of *E. coli* 01 and *S. typhimurium*. The protective effect of Q8 was also observed when the animals were infected with *S. pyogenes* and *E. insidiosa* (Table I).

To demonstrate whether LPS extracts from *E. coli* 04 could reproduce the effect of Q8, nonlethal dosages of *E. coli* 04 lipid A antigen (20–80  $\mu$ g, containing 2–8  $\mu$ g lipid A) were injected into each mouse 24 h before bacterial challenge. 24 h after infection

TABLE II  
*Protective Effect of Q8 against Bacterial Challenge with E. coli 01 and E. insidiosa as a Function of Time of Administration of Q8*

Treatment*	<i>E. coli</i> 01	<i>E. insidiosa</i>
Hours before infection	Surviving animals	
Control‡	0/10	0/10
48	6/10	10/10
24	10/10	5/10
8	5/10	4/10
0	0/10	4/10

Note: Q8 was injected i.v. (25 mg/kg b.w.). Pathogens ( $2 \times 10^8$  *E. coli* 01 and  $8 \times 10^2$  *E. insidiosa*) were injected into mice i.v. at the indicated time intervals after injection of Q8. The data are derived from one representative experiment of seven. Survivors represent mice per total number alive at 24 h after infection with *E. coli* 01 and at 4 days after challenge with *E. insidiosa*. These surviving animals continued to live beyond 3 wk after bacterial challenge.

\* Administration of Q8 at varying points of time before bacterial challenge.

‡ 0.3 ml saline/mouse i.v.

with  $2 \times 10^8$  *E. coli* 01 the survival was 8% (4/50).

The protective action of Q8 was not a transient effect as evidenced by the observation that the protected animals continued to survive beyond 3 wk. Simultaneous injections of Q8 along with *E. coli* 01 did not protect the mice against infections. It was necessary, therefore, to determine the optimal interval between the prior treatment with Q8 and the bacterial infection. Groups of mice were pretreated with Q8 and were infected with *E. coli* 01 at varying time intervals. Table II shows that the optimal time for pretreatment with Q8 for maximal protection against *E. coli* 01 was 24 h. Later intervals, e.g. 48 h, were less effective, presumably because of the drug clearance from the body. The optimal time for treatment against bacterial infections appears to depend on the nature of infection. In animals challenged with *E. insidiosa*, which represents a slowly developing chronic infection, Q8 injected as late as 48 h before infection was most effective. However, even simultaneous administration of Q8 imparted partial protection against infection (Table II).

The protective effect of Q8 injected 24 h before infection on the survival of mice infected with *E. coli* 01 was dose-dependent. As indicated in Fig. 3, the dose-response relationship was not strictly linear. However, when  $2 \times 10^8$  *E. coli* 01 bacteria were used as the inoculum, the ED<sub>50</sub> was in the range of about 4–10 mg/kg and the ED<sub>100</sub> was  $\approx 25$ –30 mg/kg. Because an enhancement of host resistance had previously been described by Casey and Bliznakov (21) for other ubiquinones, the effect of Q8 was compared with that of ubiquinones-4, Q6, and Q10 in experimental infections with *E. coli* 01. All ubiquinones were injected 24 h before infection in equal doses of 25 mg/kg. As demonstrated in Fig. 4, Q8 is 5–10 times more effective, in terms of percent survival at a dose of 25 mg/kg body weight (b.w.) in this experimental model.

The application of BCG and endotoxin results in hypertrophy of the spleen and liver of mice by 48 h (22). We therefore studied the effect of Q8 on both organs. The weights of liver and spleen did not change at 48 h significantly in animals treated with a protective dose of Q8 (25 mg/kg b.w.) (Table III).

The protective action of Q8 appeared to be directed against a variety of pathogens. It could, therefore, be suggested that Q8 stimulates the reticuloendothelial system (RES) and consequently enhances the nonspecific resistance of the host. Stimulation

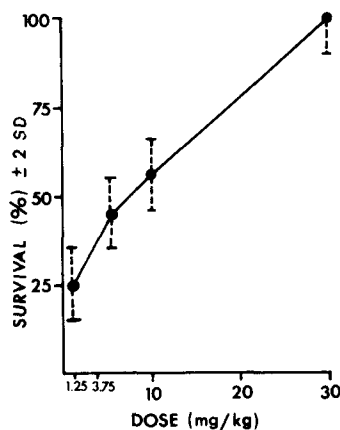


FIG. 3. Dose-response curve for Q8 in experimental sepsis with *E. coli* O1. Groups of 10 mice received different doses of Q8 24 h before i.v. challenge with  $2 \times 10^8$  *E. coli* O1. The data are derived from one representative experiment of seven. Survivors (%) at each dose are statistically significant from other doses ( $P < 0.05$ ).

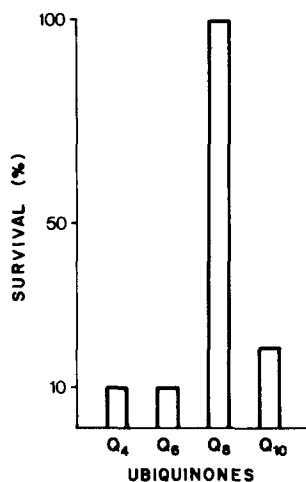


FIG. 4. Protective effect of four different ubiquinones against experimental infection of mice with *E. coli* O1. Groups of mice (10 animals each) received 25 mg/kg b.w. of ubiquinones i.v. 24 h before i.v. challenge with  $2 \times 10^8$  *E. coli* O1. The data are derived from one representative experiment of four.

of the RES would mean both enhanced clearance (from blood) and sequestration of bacterial into the RES as well as destruction of pathogens.

Experiments were carried out to investigate these possibilities. Groups of mice were infected intravenously with  $2 \times 10^8$  *E. coli* O1. Fig. 5 shows the bacteria/ml of blood at varying times after bacterial challenge. 2 h after infection, the bacteremia in Q8 treated mice was nearly 100-fold smaller than in the controls. This could be due to enhanced sequestration of bacteria as well as enhanced destruction of bacteria by the RES. Fig. 6 demonstrates that in control animals, the bacterial counts in the spleens were increased severalfold with time, indicating an uptake of bacteria by splenic tissue. However, in Q8-treated mice the number of bacteria have, in fact, decreased

TABLE III  
Effect of Q8 on the Weight of Livers and Spleens of Female NMRI Mice 48 h after Injection

Group	Treatment i.v.	Tissue	Weight (mean $\pm$ SD)
			mg
1	Q8	Liver	1,170 $\pm$ 110
2	saline	Liver	1,195 $\pm$ 120
3	Q8	Spleen	107 $\pm$ 15
4	saline	Spleen	109 $\pm$ 13

Note: Q8 was injected i.v. at a dose of 25 mg/kg b.w. Controls received 0.3 ml isotonic saline per mouse i.v.

The data are derived from one representative experiment of five.

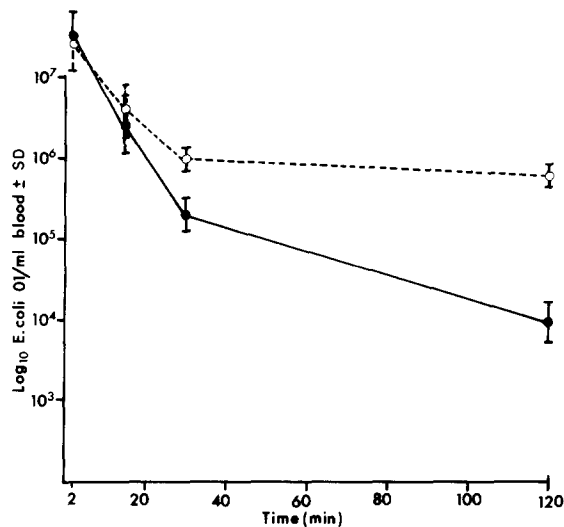


FIG. 5. Blood clearance of *E. coli* O1. Groups of mice (10 animals each) received 25 mg/kg b.w. Q8 i.v. (—●—) or served as controls (---○---). 18 h later all animals were challenged with  $2 \times 10^8$  *E. coli* O1 i.v. and the number of *E. coli* O1 was determined in the blood 2, 15, 30, and 120 min after challenge. The data are derived from one representative experiment of four.

with time. 24 h after i.v. challenge with  $2 \times 10^8$  *E. coli* O1 the Q8-treated animals had approximately 500 times less bacteria than the controls. Comparable data were obtained from livers of control and experimental mice (data pertaining to livers not shown). Livers and spleens of these animals together accounted for the major amount of bacteria injected in control animals at 24 h.

Phagocytosis by macrophages is a necessary condition for sequestration of bacteria by the RES as well as for intracellular digestion of bacteria by macrophages (2). Peritoneal macrophages offer an excellent tool to test the effects of Q8 in vivo and in vitro on the macrophage phagocytosis. Mouse peritoneal macrophages isolated from normal animals and those treated with saline, triolein, and Q8 were compared 24 h after such treatment for their efficacy to internalize SRBC, which were coated with antibody against SRBC. Fig. 7 shows that triolein and Q8 significantly increased phagocytosis of SRBC, the latter being twice as effective as triolein, and four times as effective as the controls. In mice pretreated with Q8 the effect of a single dose on

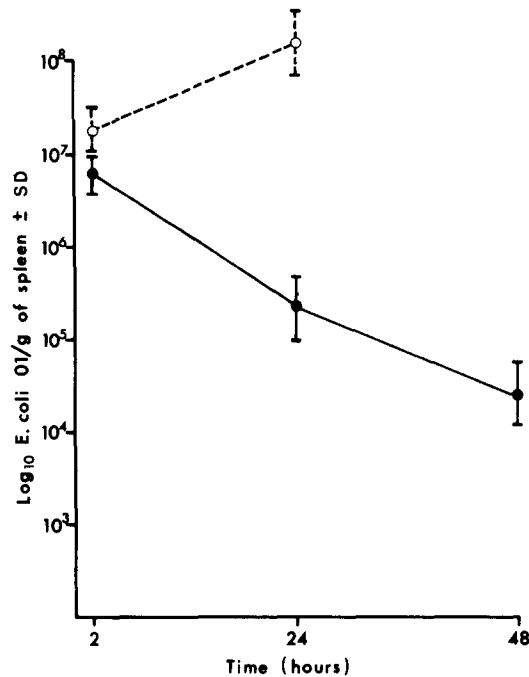


FIG. 6. Number of *E. coli* O1 in the spleens of mice. 30 mice were treated with 25 mg/kg b.w. Q8 i.v. (—) 30 animals received saline instead of ubiquinone-8 (---). 18 h later all animals were challenged with  $2 \times 10^8$  *E. coli* O1 i.v. 2, 24, and 48 h later the number of *E. coli* O1 in the spleens of 10 mice each was determined. The data are derived from one representative experiment of four.

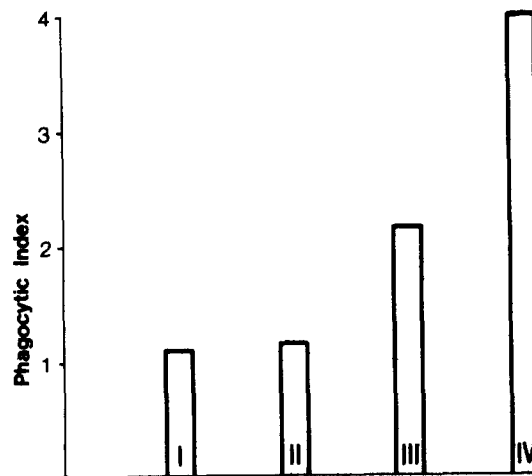


FIG. 7. Erythrophagocytosis of peritoneal mouse macrophages. I, control; II, 0.3 ml saline; III, 30 mg/kg trioleine in 0.3 ml saline; IV, 30 mg/kg Q8 in 0.3 ml saline. The data are derived from one representative experiment out of six.

phagocytosis was maximal at 48 h. A second treatment with Q8 at 72 h was also effective in increasing phagocytosis by macrophages (Fig. 8). Daily injections of Q8 maintained the higher capacity of macrophages to internalize SRBC indefinitely (Fig. 9). Phagocytosis of SRBC by macrophages from normal animals is increased when



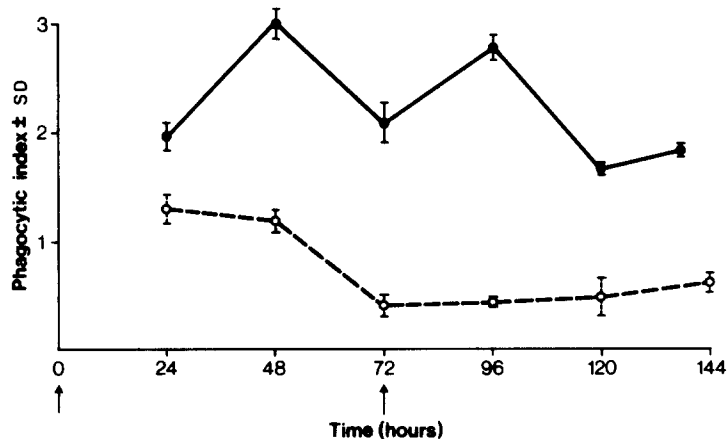


FIG. 8. Phagocytosis of SRBC by peritoneal macrophages of mice after twice treatment with Q8. Different groups of animals, (5 each group) were treated with 25 mg/kg Q8 i.v. at 0 and 72 h. Every 24 h the mice of one group were killed, peritoneal macrophages harvested, and erythrophagocytosis assay performed. Groups of animals were treated with saline instead of Q8 (—) and served as controls (---). The data are derived from one representative experiment of seven.

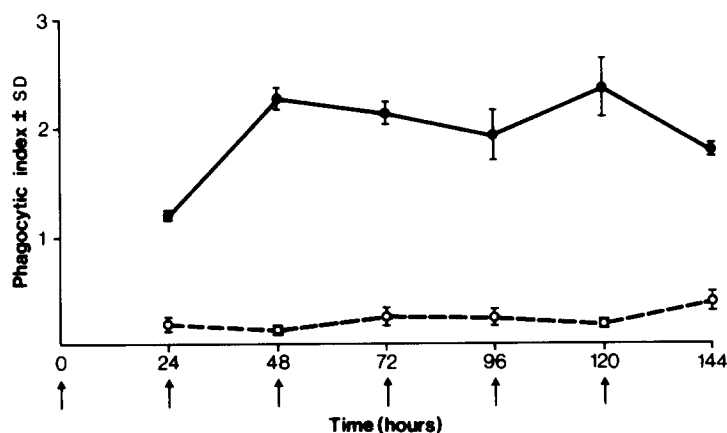


FIG. 9. Phagocytosis of SRBC by peritoneal macrophages of mice after repeated treatment with 25 mg/kg Q8 i.v. at 0, 24, 47, 72, 96, 120, and 144 h. These groups of animals were sacrificed at 24-h intervals (24 h after each injection of Q8), peritoneal macrophages harvested, and the assay of erythrophagocytosis performed. The control groups of animals were correspondingly treated with saline (---) instead of Q8 (—). The data are derived from one representative experiment of five.

the macrophages are first incubated with Q8 *in vitro* for 2 h. Further, this *in vitro* effect of Q8 on phagocytosis could be demonstrated with a variety of substances like paraffin oil (Red Oil O), blue dextran (mol wt  $2 \times 10^6$  daltons), latex particles, etc. (data not shown.)

The direct plaque-forming cell test of Plotz et al. (18) was used to investigate the possible action of Q8 on B lymphocytes. Spleen cells were prepared from two different strains of mice. PFC was assayed 4 days after immunization with SRBC as mentioned above in Materials and Methods. Table IV shows that in both strains of mice Q8 enhanced the proportion of PFC to spleen cells significantly, indicating the enhanced humoral response in presence of Q8.

TABLE IV  
*Effect of Treatment with Q8 on PFC in Vivo of Two Mouse Strains*

Mice strains	Treatment*	PFC/10 <sup>7</sup> spleen cells‡
B <sub>6</sub> D <sub>2</sub> F <sub>1</sub>	Q8	6.848 ± 1.595§
	Control	2.515 ± 919
C <sub>3</sub> H	Q8	22.211 ± 5.716§
	Control	10.005 ± 2.606

\* Mice immunized with 10<sup>8</sup> SRBC were treated 24 and 48 h (twice) before sacrifice with 25 mg/kg b.w. of Q8 i.v. PFC were assayed 4 days after immunization with SRBC.

‡ Each PFC is the arithmetic means (± SD) of 10 mice.

§ Analysis of variance; significant difference between treated and control group (*P* < 0.05). The data are derived from 1 representative experiment of 10.

### Discussion

Stimulation of nonspecific resistance by exogenous agents such as BCG (23, 24) and levamisole (25) is considered to be of potential value in the treatment of infectious diseases and cancer. The observation that the treatment with lipid extracts of a variety of bacteria (11) potentiated nonspecific resistance of the host led us to isolate and purify an apolar lipid component from *E. coli* 01 using HPLC. This isolated compound was capable of enhancing host resistance to a variety of pathogens.

This active compound was identified to be Q8 by nuclear magnetic resonance and mass spectrometry. The isoprene side chain length of the ubiquinone appears to be a critical determinant for its biological activity. It is interesting to note that other Q-analogues (Q10, Q6, and Q4) were also shown to stimulate host resistance (21, 26). Our data confirm these results and further show that the optimal chain length corresponds to that of Q8.

Since Q8 was isolated from gram-negative bacteria, it was of crucial importance to exclude contamination with bacterial endotoxins which are known to be LPS (27). Bacterial endotoxins cause fever and hepatosplenomegaly, effects which have been used as bioassays for endotoxic activities. Our preparations of Q8 were stored in chloroform at 4°C and were repeatedly monitored for lack of pyrogenicity at any dose (25–250 mg/kg b.w.). Further, these Q8 preparations did not cause hepatosplenomegaly in mice or pyrogenic reactions when tested in large amounts of rabbits. Purification of Q8 involved HPLC in apolar solvents. This procedure did not contaminate the samples with endotoxin as evidenced by both a negative KDO test (19) and Limulus assay (20).

The efficacy of Q8 on host resistance to infections appears to be related to the duration of prior treatment and the severity and rapidity of infection. The observation that the protection caused by Q8 against both gram-positive and gram-negative bacteria could be valuable in the therapeutic use of this compound as an agent to stimulate nonspecific host resistance.

The experiments on the bacterial clearance from the blood and bacterial counts in liver and spleen after infection showed that liver and spleen were the major organs for sequestration of bacteria by the RES. Though pretreatment with Q8 enhanced the clearance of bacteria from blood compared to controls, the bacterial counts in spleen and liver were in fact far less in Q8 treated animals. Had Q8 merely increased the

perfusion of liver and spleen, that would account for only the enhanced clearance from blood and not from liver and spleen. This suggested that Q8 indeed potentiated the bacteriocidal and/or bacteriostatic mechanisms of the RES. Q8 had no effect on bacterial growth in vitro and therefore its possible role in enhanced bacteriolysis could be only due to potentiation of the RES function.

Administration of nonlethal dosages of LPS, although minimally effective, could not reproduce the protective effect of Q8. In this regard it is essential to note that RES function of mice 24 h after receiving nonlethal doses of LPS is considerably decreased, although it might be increased after 2–3 days of application (22).

Commercially available LPS is usually extracted by the hot phenol water method or the trichloroacetic acid method. Because of their polarity both solvents could possibly extract Q8 from bacterial cell walls. Therefore, commercial LPS prepared from Q8-rich strains (e.g., *E. coli*) could very well be contaminated by Q8. The fact that both Q8 and LPS are biologically active indicates that former studies performed with crude LPS extracts should be re-examined for possible contaminations with Q8 or its analogues.

The experiments on erythrophagocytosis using macrophages from Q8-treated and control animals indicated that Q8 exerts a potentiating action on phagocytosis in vivo, phagocytosis being a primary event in bacterial sequestration and subsequent destruction by macrophages.

Q8 caused a marked stimulating effect on phagocytosis of macrophages independent of the type of particle internalized and therefore it may be argued that this in vivo effect of Q8 on host resistance to bacterial infection is partially due to enhanced phagocytosis of bacteria.

Triolein treatment of animals is known to enhance phagocytosis in phagocytes when tested in vitro, but has no significant effect on host resistance to pathogens. Further, Q8 had a disproportionately large effect on host resistance compared to other Q-analogues. These observations indicate that Q8 has sites of action other than macrophage phagocytosis which contribute to overall host resistance besides phagocytosis.

The pharmacokinetics of Q8 are apparently different with regard to host resistance in vivo, phagocytosis in vivo and in vitro. This could be explained both in terms of multiple sites of action of Q8 on the host defense mechanisms as well as in terms of the nature of partition and exchange of this coenzyme Q-analogue in various compartments in the body.

A possible site of action of Q8 could also be on the humoral (IgM) response of B lymphocytes. This was tested by the PFC test and the data do support the possibility that Q8 has additional sites of action such as B lymphocytes. Sugimura et al. (28) have reported that Q7 had a comparable effect in stimulating PFC in mice immunized with SRBC.

The molecular mechanism of action of Q8 could not at present be analyzed either in terms of altered membrane function or in terms of its capability to participate in redox reactions. Further, the possible effects on B lymphocytes and bacteriolysis require additional studies by suitable in vitro experimental models. Q8 does, however, offer a useful tool in the potentiation of nonspecific host resistance against a large spectrum of bacteria and is therefore of potential value in the chemotherapy of infectious diseases and cancer.

### Summary

A lipid fraction from *Escherichia coli* was extracted with apolar solvents and was found to protect mice from a number of experimental bacterial infections. The benzoquinone, ubiquinone-8, was isolated from this extract by high pressure liquid chromatography and identified as such by nuclear magnetic resonance and mass spectrometry. At a dose of 25 mg/kg this substance was found to provide complete protection against otherwise lethal infections with gram-negative and gram-positive bacteria in mice. Treatment was most effective when given intravenously 24 h before infection. In comparative studies, ubiquinone-8 had a clearly higher activity than ubiquinones-4, Q6, and Q10. A highly significant increase in the clearance rate of bacteria from the blood by the spleen and the liver of treated animals, correlated well with the protective effect of ubiquinone-8. The compound stimulated the ability of mouse macrophages to incorporate sheep erythrocytes and significantly increased the number of antibody-producing cells in spleens of mice.

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