INHIBITION OF BACTERIAL DISSEMINATION

Bacterial dissemination is inhibited by administering a substance which accelerates recruitment of neutrophils to the peritoneal cavity and thereby inhibits bacterial dissemination from the peritoneal cavity lipids are of particular interest. Substances are preferably screened in normal and/or CDN deficient mice.
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INHIBITION OF BACTERIAL DISSEMINATION

Mention of Government Grant

The development of this invention was supported in part by a grant from the National Institutes of Health, grant no. NIH AI23859, to Sanna M. Goyert. The U.S. Government has certain rights in the invention.

Cross-Reference to Related Patents and Applications


Goyert, WO97/00081 discloses a method for inhibiting bacteremia and bacterial dissemination which involves administration of a CD14 antagonist.

The use of soluble CD14 in treating sepsis, and a baculovirus expression system for CD14, are disclosed in PCT/US93/03154, filed 6 April 1993, published as WO93/19772.

A transgenic nonhuman animal model for developing and testing therapies to treat sepsis, e.g., a mouse which expresses human CD14, is disclosed in PCT/US92/07031 filed August 21, 1992.

WO97/00081 discloses transgenic mice which express human CD14 but not mouse CD14, as well as mice which are human and mouse CD14 deficient.

BACKGROUND OF THE INVENTION

Field of the Invention

Description of the Background Art

Following infection, bacteria or their products stimulate the production of proteins (chemoattractants or chemokines) by locally residing cells including macrophages, fibroblasts and/or endothelial cells. These chemokines stimulate the mobilization of neutrophils and monocytes from the blood (and eventually bone marrow) to the site of the infection; the monocytes (2-6% of peripheral blood leukocytes) and neutrophils (60-70% of peripheral blood leukocytes) aid in clearance of the bacteria by phagocytosis and killing. Under normal conditions, a small, local
infection is easily resolved by this mechanism; however, sometimes infection is not resolved and can lead to a systemic dissemination. Alternately, damage to a blood vessel and contact with bacteria (as can occur in various types of trauma) can lead directly to a systemic infection which may lead to septic shock.

Bacterial infection occurs when a pathogenic bacterium enters the host and multiplies inside the body. Most infections begin on the mucous membranes of the respiratory, alimentary, or genitourinary tracts, or in tissues exposed by wounds. After initial entry, the bacteria may remain localized, or they may spread through the blood and lymph systems, resulting in the infection of diverse tissues.

This bacterial dissemination is undesirable.

Dissemination may be inhibited either by interfering with the transfer of the bacteria from the local seat of infection to the systemic circulation, or by speeding the clearance of the bacteria from the blood and lymph systems.

If bacteria are allowed to disseminate into the circulation, and are not cleared quickly enough, the bacterial levels in the bloodstream will become higher than normal, a condition known as "bacteremia".

Gram-negative bacteria produce lipopolysaccharides (LPS) as part of the outer layer of their cell walls. When released, such as by lysis of the bacteria, they have a toxic effect, and hence are referred to clinically as "endotoxins".

If bacteremia persists, the patient may develop "septicemia". This is a life-threatening condition attributable to the body's reaction to high levels of bacterial endotoxins.

Cytokines, such as interleukin-6 (IL-6) and tumor necrosis factor (TNF-α) are known to promote the invasiveness of pathogenic bacteria, particularly gram-negative bacteria. It is known that IL-6 and TNF-α, as well as other inflammatory mediators, are released by the body in response to activation of the immune system by LPS endotoxins. Tracey, et al., Ann. Rev. Med. 45: 491-503
(1994); Akira, et al., Adv. Immunol. 54: 1-78 (1993); Bone, R.C., Ann. Intern. Med. 115: 457 (1991). Activation of the immune system by endotoxin/LPS can result in a cascade of reactions including the production of pro-inflammatory cytokines such as IL-6 and TNF-α. Activation of the immune system by endotoxin/LPS can result in a cascade of reactions including the production of proinflammatory cytokines such as IL-6, IL-1 and TNF-α. This initial production of cytokines may lead to a cascade of events which can include some or all of the following: bacteremia and bacterial dissemination, procoagulant activity, acute respiratory distress syndrome and death. This activation is mediated in part by interactions of various molecular receptors with foreign antigen. Several monocyte/macrophage surface markers that possess receptor and signal transduction functions have been identified. Many of them are cell differentiation markers, i.e., characteristically present in defined stages of development, especially the end stages of cells of defined lineage and function.

into the bloodstream involves interference with the body's normal clearing mechanisms to remove bacteria. Some bacteria, such as E. coli and Salmonella spp; produce endotoxins that interfere with the body's bacterial clearance mechanisms that depend largely on fixed macrophages lining the sinusoids of a number of parenchymatous organs, especially the liver and spleen. Interference with cellular defenses that otherwise would destroy large numbers of bacteria in the bloodstream enables bacteria to rapidly disseminate, and can lead to death.

The peritoneal cavity can be an important pathway for bacterial dissemination. The parietal peritoneum is a membrane lining the walls of the abdominal and pelvic cavities. The visceral peritoneum is a similar membrane investing the contained viscer a. Together the two membranes define an enclosed space known as the peritoneal cavity.

Bacteria can enter the peritoneal cavity as a result of perforation of the GI tract, infection of an intraabdominal organ, and direct contamination from an external source, such as by trauma, burns or surgery. Once bacteria enter the peritoneal cavity, dissemination is rapid. Within 6 minutes of intraperitoneal inoculation of bacteria in dogs, thoracic lymph is culture-positive; within 12 minutes, there is bacteremia (bacteria at elevated levels in the bloodstream).

Peritonitis is an inflammation of the peritoneum, often attributable to a severe local infection. It may be caused by a number of pathogenic microorganisms, and can result from gastrointestinal trauma, including surgery or peritoneal dialysis. Typical medical treatments for the prevention of peritonitis include antibiotic therapy, especially prior to surgical procedures, radio therapy or chemotherapy. This approach is hindered, however by the multiple drug resistance of many of the bacteria known to cause peritonitis. Moreover, since peritonitis may be caused by both gram-positive and gram-negative microorganisms, the choice of antibiotic may not be sufficient.

Also, antibiotic treatment is non-specific, eliminating
many non-pathogenic indigenous microorganisms which normally prevent bacterial disease through bacterial antagonism, especially in the gastrointestinal tract. When broad spectrum antibiotics, such as tetracyclines, are given in large doses for many days, growth of most of the bacteria that thrive in the intestinal tract is suppressed. As a result, antibiotic resistant strains of pathogenic microorganisms, normally held in check by the antagonistic action of the coliforms and other organisms, multiply freely and can actually foster peritonitis, rather than prevent it.

Gram-negative sepsis resulting in sepsis syndrome and endotoxin shock is a potentially lethal consequence of Gram-negative bacteremia that can occur in spite of adequate antibiotic therapy (Bone, 1991; Rangel-Frausto, 1995). Death from sepsis is currently one of the leading causes of death in ICU units. Overall, it ranks number 13 as the cause of mortality in the US (MMWR 1990). Sepsis and endotoxin shock occur most commonly in immunosuppressed patients, surgery patients, patients in intensive care, and patients with severe injury following trauma or burns. The incidence of sepsis is predicted to increase further, as the number of patients with impaired immune systems secondary to medical treatment and the number of elderly patients with multiple disorders is expected to increase rapidly. The mortality rates of patients with sepsis and shock are between 40 and 70% and have not changed significantly over the past decade, despite the introduction of potent antibiotics and sophisticated intensive support procedures (MMWR 1990).

Gram-negative or endotoxin shock occurs when endotoxin (lipopolysaccharide, LPS) is liberated from the outer membrane of gram-negative bacteria and enters the circulation (Rietschel 1984; 1992); this release can occur either as a result of the host's cellular response to the bacteria or from antibiotic therapy. Once released, LPS is able to activate many cell types including monocytes-macrophages, neutrophils and endothelial cells (Morrison 1987), causing them to release many factors, including tumor necrosis factor α (TNFα), interleukins, oxygen-free radicals...
and activators of the complement cascade; the clinical symptoms of endotoxin shock are typically fever, hypotension, and diffuse intravascular coagulation. Activation of this cascade can lead to death with or without multiorgan failure.

The pathophysiologic effects of endotoxin shock are mediated by a molecule known as CD14 which is a receptor for endotoxin (LPS), and is found on the surface of monocytes and macrophages. CD14 was first cloned by Dr. Sanna Goyert, [see U.S. Patent No. 5,543,303]. The preeminent role of CD14 in the induction of endotoxin shock is demonstrated by the fact that mice which have been genetically engineered to lack CD14 are resistant to the lethal effects of LPS and Gram-negative bacteria (Haziot 1996).

In the course of studying these CD14-deficient mice, Drs. Goyert and Haziot observed that not only were they resistant to the lethal effects of Gram-negative (E. coli) bacteria but they also were able to clear E. Coli much more rapidly and efficiently than normal mice. This accelerated clearance was observed not only in blood but also in other tissues. At the time when these observations were made and published the mechanism for this accelerated clearance was not known. The studies reported here have led to a better understanding of this process and how it can be exploited for therapeutic purposes.

Ribi, USP 4,866,034 discloses that a refined detoxified endotoxin obtained from Enterobacteriaceae is useful as an adjuvant. This RDE was shown to activate mitogenesis of B-cells, stimulate IL-1 production by macrophages, stimulate phagocytosis by mycrophage, and augment the immune response to SRBCs. Ribi, USP 4,844,894 suggested that monophosphoryl lipid A obtained from Enterobacteriaceae, especially S. typhimurium and E. coli, could be useful in inhibiting the onset of septicemia. This MPLA was equated with the RDE of the '034 patent. The mechanism of action of MPL was not known.

Myers, USP 4,912,094 describes certain modified enterobacterial liposaccharides, and in particular modified
MPLA. The only disclosed utility was as an adjuvant. Other derivatives of MPLA are disclosed in Myers, USP 4,987,237, which likewise contemplates adjuvant use.

Christ, USP 5,612,476 and 5,530,113 disclose the use of various analogues of lipid A in the treatment or prevention of LPS-mediated disorders by antagonizing LPS-mediated target cell activation. The analogs inhibited LPS-induced production of TNF. They are considered useful in the prevention of septic shock triggered by bacterial endotoxins.

Kamireddy, USP 5,593,969 discloses a variety of lipid A analogues. They are said to be useful for inhibiting the binding of lipid A to lipid A receptors, and other purposes.

Kodama, USP 5,654,289 discloses a disaccharide derived from Bacteroides LPS which has immunopotentiating activity but does not substantially induce production of TNF.

None of the foregoing references teaches that it is desirable to use a substance which accelerates neutrophil recruitment in inhibiting bacterial dissemination, and in protecting against bacteremia or septicemia.
SUMMARY OF THE INVENTION

Bacterial infections and the physiological responses to such infections are a continuing major problem especially in the hospital setting and among individuals who have been immuno-compromised for various reasons (e.g. chemotherapy for cancer, immunosuppression in the treatment of autoimmune diseases). The current method for dealing with bacterial infections is the use of antibiotics; however, with the rapid increase in antibiotic-resistant bacteria this method for dealing with bacterial infections is becoming increasingly difficult. Furthermore, in many instances antibiotic therapy not only does not prevent but can actually exacerbate the body's deleterious response to the killing of bacteria by antibiotics; this deleterious response is often referred to as systemic inflammatory response syndrome (SIRS) and frequently leads to death. Currently, there is no effective method to enhance the clearance of bacteria by the body's own immune mechanisms or to inhibit the spread (dissemination) of bacteria from one site to another.

Hence, there is a need for improved methods of inhibiting bacterial dissemination, and hence in the prevention and treatment of bacteremia and septicemia. Such a method will be particularly useful in the prevention and treatment of severe local infections, such as those associated with peritonitis, pneumonia, gastrointestinal colitis, dysentery, severe cellulitis, urinary tract infections, bacterial translocation from the gastrointestinal tract, inflammatory bowel disease, hemorrhagic shock, burn infections, etc.

The present invention relates to, inter alia:

1. A method for assessing the ability of a compound to enhance bacterial clearance and inhibit bacterial dissemination.


3. Specific substances capable of enhancing bacterial
clearance and inhibiting bacterial dissemination.


5. The identification of the "receptor" to which these molecules bind that activates the bacterial clearance mechanism.


7. A method for ameliorating the symptoms of SIRS.

We have identified a series of compounds that, when administered prior to bacterial infection, not only enhances the clearance of bacteria but also prevents them from spreading from one site to another. Administration of such compounds prior to bacterial infection can not only prevent death due to the infection (Fig. 5) and decrease the spread of bacteria (Fig. 6) but can also ameliorate many of the symptoms that characterize SIRS (Figs. 7-12).

The rapid identification of such compounds was made possible by the development of a novel assay. Compounds identified by this novel assay function by activating a previously unknown mechanism for bacterial clearance. They are active at very low concentrations and do not display any known toxicity. These compounds would be used for the prevention of bacteremia and SIRS in situations such as in peritonitis, bowel surgery, urinary tract infections, and burns, and other situations where local infections occur.

More particularly, the present invention relates, in one aspect, to the use of substances which accelerate recruitment, i.e., elicit early recruitment, of neutrophils to the peritoneal cavity, in the inhibition of bacterial dissemination. In another aspect, it relates to the in vitro and in vivo screening of substances for such activity and utility. Finally, it relates to novel compounds found to have such activity. The substances of the present invention may be, but need not be, CD14 antagonists.
BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Infiltration of neutrophils in the peritoneal cavity after injection of E. coli. CD14-deficient and control C57BL6/J mice were injected (i.p.) with 1 x 10^7 E. coli 0111:B4 and, after various times, the cellularity in the peritoneal cavity was analyzed. Methods: CD14-deficient mice of C57BL6/J or BALB/c genetic background (sixth backcross) and the appropriate control mice were used. Bacteria were prepared and injected as described (3). After lavage of the peritoneal cavity the cells present in the peritoneal cavity were counted and the percent of neutrophil was determined by morphological analysis of Wright-Giemsa stained cytopsin slides.

Figure 2. Time course analysis of the infiltration of neutrophils in the peritoneal cavity after injection of LPS. CD14-deficient and control C57BL6/J mice were injected (i.p.) with 0.5 μg/gbw of protein-free LPS from E. coli K235 and, after various times, the cellularity in the peritoneal cavity was analyzed.

Figure 3. Dose response analysis of the influx of neutrophils in the peritoneal cavity after injection of LPS. CD14-deficient and control C57BL6/J mice were injected (i.p.) with increasing doses of LPS from E. coli K235 and after a 6 h incubation, the cellularity in the peritoneal cavity was analyzed. CD14 deficient, open square; control, closed circle.

Figure 4. Clearance of E. coli in neutrophil-depleted mice. CD14-deficient and control BALB/c mice were treated with cyclophosphamide as described (8) or with vehicle (pyrogen-free mannitol in saline) and were infected (i.p.) with 3 x 10^7 E. coli 0111:B4 bacteria (3). Eight hours later, bacterial counts in the blood were determined.

Figure 5. MPLA protects mice from a lethal dose of E. coli. Mice were pretreated with MPLA or saline and after 2 hr a lethal dose of E. coli was injected. Survival over time was measured.

Figure 6. MPLA accelerates bacterial clearance. Saline or MPLA was administered at two different doses and
after 3 hr a lethal dose of E. coli was administered to each mouse. Four hours later the number of bacteria in the blood was measured.

Figure 7. MPLA protects mice from the symptoms of SIRS (diarrhea). Phosphate-buffered saline or MPLA was administered and after 2 hr a sub-lethal dose of E. coli was administered. Symptoms of SIRS (diarrhea) were evaluated on a continuous basis for 6 days.

Figure 8. MPLA protects mice from the symptoms of SIRS (prostration). Phosphate-buffered saline or MPLA was administered and after 2 hr a sub-lethal dose of E. coli was administered. Symptoms of SIRS (prostration) were evaluated on a continuous basis for 6 days.

Figure 9. MPLA protects mice from the symptoms of SIRS (reactivity). Phosphate-buffered saline or MPLA was administered and after 2 hr a sub-lethal dose of E. coli was administered. Symptoms of SIRS (reactivity to tactile stimulation) were evaluated on a continuous basis for 6 days.

Figure 10. MPLA protects mice from the symptoms of SIRS (eye exudate). Phosphate-buffered saline or MPLA was administered and after 2 hr a sub-lethal dose of E. coli was administered. Symptoms of SIRS (degree of eye exudate) were evaluated on a continuous basis for 6 days.

Figure 11. MPLA protects mice from the symptoms of SIRS (weight loss). Phosphate-buffered saline or MPLA was administered and after 2 hr a sub-lethal dose of E. coli was administered. Symptoms of SIRS (amount of weight loss) were evaluated on a continuous basis for 6 days.

Figure 12. MPLA protects mice from the symptoms of SIRS (ruffled fur). Phosphate-buffered saline or MPLA was administered and after 2 hr a sub-lethal dose of E. coli was administered. Symptoms of SIRS (degree of ruffled fur) were evaluated on a continuous basis for 6 days.

Figure 13. This figure shows the chemical structures of the compounds initially screened for activity.

These compounds are identified in the figure by Roman numerals. The compounds are described in more detail in the
table of compounds, below. The structure of compound XIII is not given in Fig. 13, but is described in general terms in the Table of Compounds. The structure shown for compounds XVII and XVIII is a general one; they differ primarily in the nature and number of "n" residues but also differ in some of the other "R" components.

Figure 14 shows the results of an in vivo assay (example 102) for compounds I, II, V, VII and VIII.

Figure 15 Resistance to infection with Listeria monocytogenes in normal mice treated with lipid A. BALB/c mice (n=3) were infected i.v. with 3.7 x 10^3 CFU/gbw L. monocytogenes and treated i.v. with MPLA (180 ng/gbw) or PBS 3 h after the infection. Bacterial counts were determined at 14 h after inoculation, both in the blood (Fig. 15A) and in the spleen (Fig. 15B) (*, p=0.05, Mann-Whitney test).

Figure 16 compares inducement of neutrophil infiltration into the peritoneal cavity by MPLA, PBS, palmitic acid, and myristoyl alcohol.
DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS OF THE INVENTION

It was observed that when CD14-deficient mice were injected with Gram-negative bacteria intraperitoneally a massive infiltration of neutrophils occurred within a very short period (2 hr) of time (Fig. 1). No similar infiltration of neutrophils into the peritoneal cavity was observed in control mice injected with E. Coli. Furthermore, a similar infiltration of neutrophils could be induced with purified LPS (Fig. 2). This infiltration of neutrophils can be shown to be responsible for the accelerated clearance of E. Coli in CD14-deficient mice since mice that are depleted of neutrophils prior to injection of E. Coli are no longer able to rapidly clear them (Fig. 4). Based on these observations we hypothesized that there was an additional receptor for LPS that was not CD14 and that triggering of that receptor somehow resulted in neutrophil infiltration and the rapid clearance of E. Coli. In addition, we hypothesized that the triggering of CD14 by LPS, which occurred in normal mice but not CD14-deficient mice, somehow inhibited this process. We further speculated that if these hypotheses were correct there might be substances that could trigger the receptor that led to neutrophil infiltration without triggering CD14.

Accordingly, we established an efficient procedure for identifying such substances and went about screening for them. The procedure consisted of analyzing the ability of small amounts (<1 ng/gram body weight) of these substances to cause neutrophil infiltration in CD14-deficient mice followed by testing them in normal mice. Only those substances that were able to cause a significant infiltration of neutrophils in both CD14-deficient and control mice within 4 hr were deemed to be useful. In addition, each substance was tested on normal monocytes to make certain it did not trigger CD14 and cause the release of TNFα which is responsible for some of the pathophysiologic effects of endotoxin.

We have now identified at least six different molecules
that can cause rapid infiltration of neutrophils. The therapeutic utility of these substances was demonstrated by showing that pretreatment of mice with one of them mitigates many of the pathophysiologic effects of endotoxin.

These substances would be useful in the treatment of conditions where inflammatory processes are mediated or initiated by Gram-negative bacteria or endotoxin.

Similarly, we have observed that certain lipids improve resistance to infection with gram-positive bacteria.

In general, the drugs of interest are substances which accelerates recruitment of neutrophils to the peritoneal cavity, i.e., elicit an early and intense recruitment of neutrophils thereto, and, consequently, inhibit bacterial dissemination and its sequelae.

Patients/Subjects

The terms "patients" and "subjects" are used interchangeably. The term "animal" includes "humans". The subject may be any animal in which bacterial dissemination may be inhibited by administration of a substance which directly or indirectly promotes the early recruitment of neutrophils to the peritoneal cavity. The subject is preferably a mammal, especially of the orders primata (humans, apes, monkeys), artiodactyla or perissodactyla (esp. cows, pigs, goats, sheep, horses), rodenta or lagomorpha (esp. rats, mice, rabbits, hamsters), or carnivora (esp. cats and dogs). It is especially preferable that the subject be human, but the subject may be a nonhuman mammal.

While it is possible that the substances of the present invention may be useful in treating an established infection, it is believed that they will be most useful in preventing a nascent infection from becoming well established. This is because the principal difference seen in the comparison of CD14-deficient and normal mice is in the early recruitment of neutrophils to the peritoneal cavity. Hence, it is believed that it is desirable that the substances be administered prophylactically. As a matter of
good medical practice, the subjects most suitable for prophylaxis are those believed to be either unusually susceptible to bacterial infection, or those who, if infected, are more likely to be severely affected. Thus, the subjects may be patients who are about to undergo, are undergoing, or have undergone, surgery, radiotherapy, or immunotherapy; subjects with compromised immune systems, such as infants, the elderly, drug addicts, and AIDS patients; subjects in environments where exposure to bacterial infection is unusually pronounced, such as subjects in war or other emergency zones, or in regions where certain bacterial diseases are epidemic or endemic, and subjects with severe and prolonged viral infections, where there is concern with regard to bacterial superinfections. However, the invention is not limited to the treatment of these subjects.

**Bacteria**

The bacteria whose dissemination is inhibited are pathogens of humans or other animals. They may be obligate or opportunistic pathogens. They may be gram-negative or gram-positive bacteria. The gram-negative bacteria include bacteria of the families Pseudomonadaceae, Enterobacteriaceae, Vibrionaceae, Bacteroidaceae, Neisseriaceae and Veillonellaceae, and some Bacillaceae, and the order Chlamydiales. The gram-positive bacteria include bacteria of the families Micrococcaceae, Streptococcaceae, Peptococcaceae, some Bacillaceae, and Lactobacillaceae, and the order Rickettsiales. There are also genera of uncertain affiliation, of which the gram-negative Brucella, Bordetella, Francisella, Chromobacterium, Haemophilus, Pasteurella, Actinobacillus, Cardiobacterium, Streptobacillus, and Calymmatobacterium, and the gram-positive Listeria Erysipelothrix, and Corynebacterium, are worthy of note.

Among the gram-negative bacteria, the Enterobacteriaceae (Escherichia, Edwardsiella, Citrobacter, Salmonella, Shigella, Klebsiella, Enterobacter, Hafnia,
Serratia, Proteus, Yersinia, and Erwinia) are of particular interest.

Neutrophils

Neutrophils are granular polymorphic leukocytes. They account for about 40-75% of blood leukocytes, but are only rarely found in the peritoneal cavity of normal subjects. Neutrophils may be recognized and distinguished from other leukocytes by any art-recognized technique, including, e.g., their exhibition of fine purple granules in a pink cytoplasm after staining with a Wright-Romanovsky stain.

A preferred method of identification of neutrophils is by Wright's-Giemsa staining. Neutrophils are smaller than monocytes/macrophages, and have a deep-staining nucleus which often is shaped as the letter E, Z or S. Frequently there appear to be several separate nuclei. Less mature neutrophils have a band-shaped nucleus. The cytoplasm (light pink in color) contains many neutrophilic lilac-colored granules. Neutrophils are easily distinguished from the larger monocytes with a bluish-gray cytoplasm and a U-shaped nucleus. These cells are distinguished from lymphocytes which have a round nucleus and very little cytoplasm.

Basophils (distinguished by their dark blue large granules) and eosinophils (distinguished by large red granules) are not seen in the peritoneal cavity.

Recruitment of Neutrophils

The term "recruitment of neutrophils" refers to a net influx of neutrophils into the indicated compartment of the body, such as the peritoneal cavity, leading to an increase in the total population of those neutrophils in that compartment. It is presumed that one or more recruitment factors are released, eliciting migration of neutrophils into the compartment in question. These recruitment factors may be substances released by the bacteria as a result of their dissemination and proliferation in the body, or they may be released by cells of the body as a result of the
interaction of those cells with the bacteria, or with cells damaged by the bacteria.

In our experience, bacterial challenge of CD14-deficient mice leads to a rapid and dramatic increase in the neutrophil count in the peritoneal cavity. However, there was not a substantial change in the neutrophil count in the blood. It therefore appears that surveillance of the peritoneal cavity is more useful, both in assaying substances for potential utility, and in monitoring the effect of the therapy using active substances, than is blood monitoring. However, blood monitoring may serve other useful clinical purposes in the prevention and treatment of bacterial infections.

Nor did we observe a substantial change in recruitment of other leukocytes. However, monitoring of other leukocytes may likewise serve other useful clinical purposes.

The substances of the present invention desirably accelerate the recruitment of neutrophils. A substance "accelerates the recruitment of neutrophils to the peritoneal cavity" if (1) a given level L of neutrophils is reached at a significantly earlier time (e.g., one hour earlier) than would be the case in the absence of the substance, or (2) at a given time T the level of neutrophils is significantly higher (e.g., at least 50% higher) than would be the case in the absence of the substance. Preferably, the acceleration effect is manifested within the first 6 hours, more preferably the first 4 hours, still more preferably, the first three hours, after the substance is administered and the subject is challenged with suitable bacteria.

The recruitment should occur sufficiently early, and with sufficient intensity, to substantially inhibit bacterial dissemination.

Preferably, according to a suitable assay, and preferably according to one or more of the assays specifically disclosed herein, there is at least a two-fold, more preferably at least a three-fold, still more preferably
at least a four-fold, even more preferably at least a five-fold, even more preferably at least a five-fold increase, most preferably at least an eight fold increase in neutrophil levels in the peritoneal cavity within an "early period" after protective administration and bacterial challenge (whichever comes later). For this purpose, the "early period" may be the first one, two, three, four, five or six hours.

Preferably, significant recruitment occurs within four hours, more preferably within two hours, of bacterial entry into the peritoneal cavity.

Preferably, according to a suitable assay, and preferably according to one or more of the assays disclosed herein, the degree of inhibition of bacterial dissemination which is achieved is at least 50%, more preferably at least 80%, still more preferably at least 90%, most preferably at least 90%, as measured by the decrease in bacterial load. Preferably, there is an observable and statistically significant protective effect against one or more symptoms of SIRS.

These drugs preferably cause a 2 to 10 fold increase in neutrophils within 6 hours at the site of administration. This should result in accelerated bacterial clearance preferably resulting in a 5 to 20 fold decrease in bacterial load after 6 hours.

One example of a suitable in vitro assay is given in example 101, while an example of an in vivo assay appears in example 102. The invention is not limited to any particular means of assaying for the recruitment of neutrophils in the peritoneal cavity.

The assays may be used: (1) to determine the utility of a substance, (2) to determine whether a patient is likely to benefit from the therapy (i.e., the patient, in the absence of treatment, shows slow recruitment of neutrophils to the peritoneal cavity after bacterial exposure), (3) to determine the effectiveness of the therapy once it is initiated, and (4) to determine whether bacteria are proliferating, and hence whether other anti-infective
therapies should be initiated or escalated.

The drugs of the present invention, while they must accelerate recruitment of neutrophils to the peritoneal cavity, may differ in terms of their interaction with CD14. A first class of such drugs is one which do not substantially interact with the receptor at all, i.e., do not induce TNF production by a CD14-mediated mechanism.

A second class of such drugs is one where the drugs are CD14 antagonists, i.e., they competitively inhibit the binding of LPS to CD14.

A third class of such drugs are drugs which at certain dosages are CD14 agonists, i.e., they cause TNF production. While such production is generally undesirable, at low dosages the drugs may accelerate recruitment of neutrophils without activating CD14. Activating CD14 ultimately inhibits recruitment of neutrophils by perhaps 12-24 hours. Or the drug may be used in conjunction with a CD14 antagonist.

Preferably, the compounds of the first class have an affinity for CD14 which is less than $10^{-6}$ liters/mole, more preferably less than $10^{-4}$ liters/mole.

In a preferred embodiment, the substances in question do not interact with CD14. This can be demonstrated by showing that the substances recruit neutrophils in CD14-deficient mice.

The drugs of interest include drugs which do not have substantial adjuvanting activity, as well as those which do.

**Selection of Compounds for Screening**

There is no restriction on the chemical class of substances which may be screened for activity. However, since lipopolysaccharides are now shown to elicit early recruitment of neutrophils in CD14-deficient mice, it follows that substances which comprise lipid and/or carbohydrate moieties are especially likely to exhibit similar behavior. In preliminary screening of 23 LPS analogues, 16 elicited a positive response (induction of neutrophil dissemination). 17 and 18 were isolated from 2
different strains of bacteria and differ primarily in the carbohydrate moieties of their O-antigen. These are real
differences since they confer specific antigenicity; the
other parts of the LPS are more conserved (i.e. their lipid
A).

5 and 21 were isolated from 2 different bacterial
strains of Re mutants. 5 is from a special mutant which is
only hexaacyl while 21 is from an Re mutant which is a
mixture of hexaacyl and heptaacyl suggesting that the
heptaacyl interferes with the response.

4 and 23 are from 2 different bacterial strains. 4 is
an Re mutant from E. coli; thus it contains KDO and lipid A
but no other inner core carbohydrate. 23 is an Rd-LPS
isolated from Salmonella minnesota. Rd-LPS contains a small
amount of inner core carbohydrate. Also, the lipid A of the
E. coli mutant is only hexaacyl while that of S. Minnesota
is a mixture of hexaacyl and heptaacyl.

The compounds of potential interest include fatty
acids, glycerides, phosphoglycerides, sphingolipids,
glycolipids, gangliosides, slycosylsacacyl, glycerols,
ceramides, cerebrosides, steroids, terpenes, prostaglandins,
lipoproteins, monosaccharides, oligosaccharides,
polysaccharides, sugar alcohols, sugar acids, sugar
phosphates (including DNAs); amino sugars, peptidoglycans,
liroarabinomannans, and bacterial cell wall components (some
of the above categories are overlapping).

The lipid-chains may be saturated or unsaturated, and,
in the latter case, may be mono-, di- or polyunsaturated.
The chains are preferably composed of an even number of
carbon atoms in length.

Preferably, in the unsaturated lipid chains, the double
bonds are separated by at least one methylene group.
Preferably, the first double bond is between C-9 and C-10;
preferably any additional double bonds are between C-10 and
the -CH, terminal of the chain. For unsaturated fatty acid
chains, the cis configuration is preferred.

Preferred saturated lipid chains are lauric (12),
myristic (14), palmitic (16), stearic (18), arachidic (20),
behenic (22), and lignoceric (24) acid chains; preferred
unsaturated lipid chains are palmitoleic (16), oleic (18),
linoleic (18), linolenic (18) and arachidonic (20) acid
chains. The myristic and palmitic chains are especially
preferred saturated lipid chains.

The substance may include both saturated and
unsaturated fatty acid chains.

Preferably, the compound presents 1-6 lipid chains.

The lipid chains may be attached to a carbohydrate.

The carbohydrate may be a mono-, di, tri-, tetra- or higher
oligosaccharide. The individual saccharide units may be
aldoses or ketoses, and may be composed of a chain of 3, 4,
5, 6, 7, 8 or more carbon atoms. Hexoses are of particular
interest. Aldoses and Ketoses, and rings of different

sizes, may be used in the same molecule.

The lipid chains may be attached to any desired point
of attachment on the sugar, e.g., O-linked to C-1, C-2, C-3,
C-4, C-5, etc., or N-linked to an amino nitrogen attached to
C-2.

The active substances were all lipids, with 3-7 lipid
chains of up to 17 carbons. Most of the compounds were
glycolipids, in which these lipid chains have been attached
directly to a two disaccharide unit, with the exception of
compounds XV (no sugars) and XVI (monosaccharide). Other
sugars may be present, as in IV (a tetrasaccharyl compound),
and X and XII (polysaccharides attached). There may be
zero, one or two phosphate groups present. Thus,
phosphoglycolipids are considered to be of particular
interest. The foregoing structures are not considered to be
limiting features.

Of the unsuccessful structures, compound 17/18 and 19
are quite complex. Compound 20 is a phosphoglycolipid with
2 phosphates, 4 sugar units, and 6 lipid chains. Compound
21 has 2 phosphates, 2 sugar units, and 6 lipid chains.
Compound 22 has 2 phosphates, 4 sugar units, and 2 lipid
chains. Compound 23 has no phosphates, 4 sugar units, and 6
lipid chains.

Compounds 2 (active) and 21 (inactive) appear to differ
solely in that a hydroxyl group of 2 is changed to a phosphate in 21.

Peptidoglycan (PCN) makes up about 40% of the cell wall of gram-positive bacteria. It has endotoxin-like properties in that it can stimulate macrophages to produce cytokines. It is a polysaccharide polymer composed of unbranched, beta-linked (1->4) chains containing alternating subunits of N-acetylglucosamine. Pentapeptide side chains are linked to the muramic acid residue and are cross-linked by a pentaglycine bridge attached to L-lysine of one chain and D-alanine on the other chain. PGN (500 ng/animal=25 ng/gbw [gram weight]) induces slightly lower PMN infiltration than mPLA (8 to 10 times background) giving approximately 6 to 8 times over background. Background is approximately 0.2 x 10^5 PMN (total in the peritoneal cavity) compared to 1.9 to 2.0 x 10^6 PMN induced within 6 hours by mPLA, 1.6 to 1.8 x 10^6 induced by soluble peptidoglycan (obtained from R. Drizarski).

Lipoarabinomannan, a lipo-arabinose-galactose-mycolic acid polymer which is a major component of the cell wall of *Mycobacterium;* it has endotoxin-like properties in that it can induce cytokine production. (Zhang Y et al. J. Clin. Invest. 91:2076-2083, 1993). In our studies, araLAM induces neutrophil infiltration in normal mice; manLAM (which is "mannose capped" by short (alpha 1-2)-linked mannoopyranose) does not. This infiltration is equivalent to the infiltration induced by mPLA and is 8 to 10 times over background (induction by administration of PBS). Chatterjee D et al. Glycobiology, 1993, 3:497-506. (Structure of LAM).

araLAM (isolated from rapidly growing attenuated H37Ra *Mycobacterium* strain) is a lipoglycan with arabinofuranosyl (araLAM)-terminal repeats as compared to the mannose-capped version (ManLAM) isolated from the Erdman strain of *Mycobacterium.*

It is believed that it would be desirable to screen some simpler carbohydrates, lipids and glycolipids, to more readily correlate structure to activity and thereby further focus further screening efforts.
The methods of combinatorial chemistry may also be useful. Controlled randomness may be introduced vis-a-vis (a) the choice of sugars which are reacted, (b) the number and manner of joining the sugars, (c) the number of phosphate groups, and (d) the number, length, saturation, and point of attachment of the lipid chains.


The drugs may or may not be the LPS analogues disclosed in the background references. In some but not all embodiments, the present method excludes any prior art method of using these previously disclosed analogues which would have inherently inhibited bacterial dissemination by accelerating recruitment of neutrophils.

Pharmaceutical Compositions and Methods

The pharmaceutical composition of the present invention comprise one or more drugs as previously defined, and are effective, when administered according to an effective pharmacological schedule to a patient, of providing "protection".

"Protection", as used herein, is intended to include prevention, suppression, and treatment. Prevention involves administration of the protective composition prior to the induction of the disease. Treatment involves administration of the protective composition after the appearance of the disease. It will be understood that in medicine, it is not always possible to distinguish between preventing and suppressing, since the ultimate inductive event or events may be unknown, latent, or the patient is not ascertained until well after the occurrence of the event or events.

Therefore, it is common to use the term protection as distinct from treatment to encompass both preventing and suppressing as defined herein. The term protection, as used
herein, is meant to include prophylaxis.

It should also be understood that to be useful, the protection provided need not be absolute, provided that it is sufficient to carry clinical value. An agent which provides protection to a lesser degree than do competitive agents may still be of value if the other agents are ineffective for a particular individual, if it can be used in combination with other agents to enhance the overall level of protection, or if it is safer than competitive agents.

The treatment method may be applied prior to, during or after other medical procedures, such as, for example, surgery, especially gastrointestinal surgery, radiotherapy, chemotherapy or peritoneal dialysis. The present method may also be applied to a patient thought to be at risk of bacteremia to thereby prevent dissemination of bacteria.

The composition may be administered parentally or orally, and, if parentally, either systemically or topically. Parenteral routes include subcutaneous, intravenous, intradermal, intramuscular, intraperitoneal, intranasal, transdermal, or buccal routes. One or more such routes may be employed. Parenteral administration can be, e.g., by bolus injection or by gradual perfusion over time. Alternatively, or concurrently, administration may be by the oral route.

It is understood that the suitable dose of a composition according to the present invention will depend upon the age, sex, health and weight of the recipient, kind of concurrent treatment, if any, frequency of treatment, and the nature of the effect desired. However, the most preferred dosage can be tailored to the individual subject, as is understood and determinable by one of skill in the art, without undue experimentation. This typically involves adjustment of a standard dose, e.g., reduction of the dose if the patient has a low body weight.

Prior to use in humans, a drug is first evaluated for safety and efficacy in laboratory animals. In human clinical trials, one begins with a dose expected to be safe
in humans, based on the preclinical data for the drug in question, and on customary doses for analogous drugs, if any. If this dose is effective, the dosage may be decreased to determine the minimum effective dose, if desired. If this dose is ineffective, it will be cautiously increased, with the patients monitored for signs of side effects. See, e.g., Berkow et al., eds., The Merck Manual, 15th edition, Merck and Co., Rahway, N.J., 1987; Goodman et al., eds, Goodman and Gilman's The Pharmacological Basis of Therapeutics, 8th edition, Pergamon Press, Inc., Elmsford, N.Y. (1990); Avery's Drug Treatment: Principles and Practice of Clinical Pharmacology and Therapeutics, 3rd edition, ADIS Press, LTD., Williams and Wilkins, Baltimore, MD (1987); Ebadi, Pharmacology, Little, Brown and Co., Boston (1985), which references and references cited therein are entirely incorporated herein by reference.

The total dose required for each treatment may be administered in multiple doses (which may be the same or different) or in a single dose, according to a pharmacological schedule, which may be predetermined or ad hoc. The schedule is selected so as to be pharmaceutically effective, i.e., so as to be sufficient to elicit a response which protective is in itself or which enhances the protection provided by other agents. The doses adequate to accomplish this are defined as "therapeutically effective doses." (Note that a schedule may be effective even though an individual dose, if administered by itself, would not be effective, and the meaning of "therapeutically effective dose" is best interpreted in the context of the schedule.) Amounts effective for this use will depend on, e.g., the peptide composition, the manner of administration, the stage and severity of the disease being treated, the weight and general state of health of the patient, and the judgment of the prescribing physician.

The appropriate dosage form depends on the status of the disease, the composition administered, and the route of administration. Dosage forms include tablets, capsules, lozenges, dental pastes, suppositories, inhalants,
solutions, ointments, and parenteral depots. See, e.g., Berker, supra, Goodman, supra, Avery, supra and Ebadi, supra, which are entirely incorporated herein by reference, including all references cited therein.

In one embodiment, the drug is dissolved or suspended in an aqueous carrier. A variety of aqueous carriers may be used, e.g., water, buffered water, 0.9% saline, 0.3% glycine, hyaluronic acid and the like. These compositions may be sterilized by conventional, well known sterilization techniques, or may be sterile filtered. The resulting aqueous solutions may be packaged for use as is, or lyophilized, the lyophilized preparation being combined with a sterile solution prior to administration. The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions, such as pH adjusting and buffering agents, tonicity adjusting agents, wetting agents and the like, for example, sodium acetate, sodium lactate, sodium chloride, potassium chloride, calcium chloride, sorbitan monolaurate, triethanolamine oleate, etc.

For solid compositions, conventional nontoxic solid carriers may be used which include, for example, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharin, talcum, cellulose, glucose, sucrose, magnesium carbonate, and the like. For oral administration, a pharmaceutically acceptable nontoxic composition is formed by incorporating any of the normally employed excipients, such as those carriers previously listed, and generally 10-95% of active ingredient, that is, one or more peptides of the invention, and more preferably at a concentration of 25%-75%.

For aerosol administration, the drugs are preferably supplied in finely divided form along with a surfactant and propellant. Typical percentages of drugs are 0.01%-20% by weight, preferably 1%-10%. The surfactant must, of course, be nontoxic, and preferably soluble in the propellant. Representative of such agents are the esters or partial esters of fatty acids containing from 6 to 22 carbon atoms,
such as caproic, octanoic, lauric, palmitic, stearic, linoleic, linolenic, olesteric and oleic acids with an aliphatic polyhydric alcohol or its cyclic anhydride. Mixed esters, such as mixed or natural glycerides may be employed. The surfactant may constitute 0.1%-20% by weight of the composition, preferably 0.25-5%. The balance of the composition is ordinarily propellant. A carrier can also be included, as desired, as with, e.g., lecithin for intranasal delivery.

In addition to the drugs of the invention, a pharmaceutical composition may contain suitable pharmaceutically acceptable carriers, such as excipients, carriers and/or auxiliaries which facilitate processing of the active compounds into preparations which can be used pharmaceutically.

As an alternative to a pharmaceutical composition comprising the drug of the present invention, per se, when the drug is a peptide or protein, the pharmaceutical composition may instead comprise a vector comprising an expressible gene encoding such drug. In the case of genes encoding naturally occurring proteins, or peptide fragments thereof, one may, but need not, use the DNA sequence which encodes the proteins or peptides in nature.

The pharmaceutical composition and method would then be chosen so that the vector was delivered to suitable cells of the subject, so that the gene would be expressed and the drug produced in such a manner as to elicit a protective effect. A preferred vector would be a Vaccinia virus. Alternatively, a nonpathogenic bacterium could be genetically engineered to express the drug. The drug must, of course, either be secreted, or displayed on the outer membrane of the bacterium (or the coat of a virus) in such a manner that it can interact with the appropriate receptor.

The specific amount of drug administered for the inhibition of bacterial dissemination or prevention of bacteremia or peritonitis can be determined readily for any particular patient according to recognized procedures and based on the expertise and experience of the skilled
practitioner. Precise dosing for a patient can be determined according to routine medical practice.

The term "patient" is used herein to mean an animal, including humans and other mammals. Thus, the present method is useful in veterinary medicine as well as in the treatment of humans.

Treatment of a patient with a pharmaceutically effective amount of a drug of the present invention is carried out for a period of time required to inhibit bacterial dissemination, etc. The treatment regimen will vary depending on such factors as the particular condition to be treated, e.g., peritonitis or gastroenteritis, the medical condition underlying the risk of bacteremia, e.g., the presence of a localized bacterial infection or trauma, such as an invasive medical treatment or surgery, particularly to the area of the gastrointestinal or respiratory tract, or other predisposing medical condition, the overall health of the patient, the route of administration, etc. For example, the present method may be applied to a patient thought to be at risk of bacterial dissemination into or from the blood stream from such underlying medical conditions as peritonitis, physical injury resulting in intestinal perforation, diverticulitis, appendicitis, acute pancreatitis, other trauma (incl. surgery, i.v. lines, or invasive diagnostic procedures) immunosuppression as a result of chemotherapy, preparation for transplant, or HIV infection, pneumonia, gastroenteritis, colitis, dysentery, severe cellulitis, urinary tract infection, inflammatory bowel disease, hemorrhagic shock, burn infection, endocarditis, meningitis, tuberculosis, etc. Typically, use of the present method to prevent bacteremia includes application of the method at least once per day until the risk of bacteremia is assessed to be over.

A preferred dose for mice is 18 mg/gbw; if a human weighs 75kg, and metabolism for this drug is analogous, the equivalent human dose is 1.35 mg. A preferred human dose is between 0.2 and 2.0 mg/adult human. This material can be
delivered either by spray or a gel or other routine delivery methods or it may be possible to use 10 fold less if the material is sprayed into the peritoneal cavity. 

Material would be given either 2 to 6 hours before surgery or 2 to 6 hours prior to the end of surgery. Material might also be used on recent open wounds by spray. Material might also be given to individuals who have undergone trauma which makes them susceptible to bacterial infection (i.e., resulting in breaking of the skin barrier). 

Material might also be given to individuals 2 to 6 hours before undergoing invasive procedures.

The drugs of the present invention may be administered in conjunction (simultaneously or sequentially) with other agents for the prevention and/or treatment of bacterial infections, including immunogens (vaccines), adjuvants, antibiotics and CD14 antagonists. 

These drugs might be used in conjunction with antibiotics as well as with other drugs that enhance the formation and proliferation of neutrophils such as GCSF (granulocyte-colony stimulating factor) and drugs that block the activation of macrophages by LPS and E. coli (e.g. CD14 antagonists). The drugs of the present invention may, however, be used independently, e.g., not in conjunction with a vaccine or CD14 antagonist. In particular, they may be used when the drug does not have a substantial adjuvanting effect on a previously, simultaneously or subsequently administered vaccine immunogen.

Two drugs are administered in "conjunction" if their times of administration are sufficiently close so that (1) one drug alters the biological response to the other drug, or (2) both drugs have a protective effect on the subject at the same time.

Example 1

Here we show that in CD14-deficient mice an early and intense recruitment of neutrophils to the peritoneal cavity follows the injection of Gram-negative bacteria or LPS; in contrast, a similar treatment of mice expressing CD14
results in a delay of neutrophil infiltration by 24 hours. This inhibition of the influx of neutrophils in normal animals correlates with bacterial dissemination of Gram-negative bacteria.

To determine the mechanism of the enhanced clearance of Gram negative bacteria by CD14-deficient mice, the cellularity in the peritoneal lavage fluid after i.p. injection of E. Coli 0111:B4 was analyzed quantitatively and qualitatively. Surprisingly (Fig. 1), CD14-deficient mice had dramatically higher numbers of neutrophils in the peritoneal cavity at early time points than control mice. Two hours after infection, $3.2 \times 10^6$ neutrophils were recovered from the peritoneal cavity of CD14-deficient mice representing a 6-fold increase over normal levels. Microscopic analysis of the cells harvested from CD14-deficient mice showed bacteria attached to and/or phagocytosed by PMN.

Example 2

To determine the nature of the bacterial component that elicits this early PMN influx in CD14 deficient mice, mice were injected i.p. with highly purified, protein depleted LPS (4) (0.5 μg/gbw of body weight) and the number of neutrophils recruited in the peritoneal cavity was counted at various time points. CD14-deficient mice rapidly responded to LPS at early time points with a peak of $1.7 \times 10^6$ neutrophils 6 h after the infection whereas control mice had no detectable neutrophils at this timepoint (Fig. 2). The response of control mice slowly increased with time, peaking 24 h after the injection of LPS. These studies indicate that the neutrophil infiltration induced by LPS and E. coli is not dependent on CD14; moreover, responsiveness to LPS via CD14 strongly delays the influx of neutrophils.

Example 3

To determine the sensitivity of this response to LPS, increasing doses of LPS were injected (i.p.) into CD14-deficient and control mice and neutrophils were counted in
the peritoneal lavage fluid 6 h later. As shown in Fig. 3, neutrophil recruitment was strongly induced in CD14-deficient mice with a dose of LPS as low as 0.5 ng/gbw. A moderate response (0.6 x 10^6 neutrophils) was induced with a dose of LPS of 5 pg/gbw. Interestingly, in control mice, a small increase in neutrophil influx into the peritoneal cavity was observed with low doses of LPS (5 to 50 pg/gbw), however, as the amount of LPS increased, neutrophil influx was inhibited. This shows that the infiltration of neutrophils is elicited by very low doses of LPS via a CD14-independent pathway. These doses of LPS (5 pg/gbw) are 100-fold lower than those needed to induce endotoxic shock in sensitized animals where the response is largely CD14-dependent. The emergent influx of neutrophils at low doses of LPS in control mice may therefore result from the lack of responsiveness via the CD14 pathway at these low doses of LPS.

Example 4

Neutrophils represent the first line of cellular defense in the elimination of bacteria. An increased and early influx of neutrophils at the site of infection in CD14-deficient mice may explain the improved clearance of Gram-negative bacteria observed in these mice. However, recent studies suggested a direct role for CD14 in the phagocytosis of Gram-negative bacteria. To examine the role of neutrophils in the clearance of Gram-negative bacteria in CD14-deficient mice, the ability of neutropenic animals to clear the bacteria was determined. As shown in Figure 4, neutrophil depletion by treatment with cyclophosphamide abrogated the improved clearance of CD14-deficient mice. The bacterial load in the blood and in the liver (not shown) of neutrophil depleted mice was similar in wild-type and CD14-deficient mice. This result indicates that neutrophils participate in the clearance of Gram-negative bacteria in CD14-deficient mice and that an early influx of neutrophils correlates with the reduced dissemination of bacteria. This observation does not corroborate a major direct role of CD14
in the phagocytosis of Gram negative bacteria in vivo.

Conclusions from Examples 1-4

Studies in CD14-deficient mice have revealed the crucial role played by CD14 in responsiveness to Gram-negative organisms. CD14 is essential for the production of shock associated cytokines (TNFα, IL-6, IL-1). We show here that CD14 is not required for the influx of neutrophils induced by Gram negative bacteria. In fact, the CD14-dependent cellular activation by LPS considerably delays this infiltration, thus giving the bacteria an opportunity to disseminate.

Recently, we described an additional pathway of response to LPS which is CD14-independent and which results in the production of acute phase proteins (APP). LPS induction of APP is distinct from induction of neutrophil recruitment since induction of the APP pathway requires 1000 times more LPS.

In conclusion, the influx of neutrophils elicited by LPS results from the activation of a pathway that does not involve CD14; this influx correlates with enhanced bacterial clearance in CD14-deficient mice. CD14 responsiveness strongly hinders the recruitment of neutrophils, facilitating the dissemination of Gram negative bacteria.

Example 5

In further work, described in the descriptions for Figures 5-12, it was shown that MPLA (compound I) protects mice from a lethal dose of E. coli (Fig. 5), accelerates bacterial clearance (Fig. 6), and protects mice from various symptoms of SIRS.

Example 101

In vitro assay for substances that induce neutrophil infiltration

Mouse cells (fibroblast cell line L929, ATCC or lung endothelial cell line LE-II,) were seeded in 24-well plates at 2.5 x 10⁵ per well in a volume of 0.5 ml of DMEM
supplemented with 10% fetal calf serum. After culturing overnight at 37°C under 5% CO₂, the medium was replaced with 0.6 ml of fresh medium. The substances to be tested were then added to a final concentration of 2-10 ng/ml. After a 3 hour incubation at 37°C under 5% CO₂, 0.5 ml of medium was collected from each well and centrifuged 3 min at 16,000 x g. The supernatants were distributed in new 24-well plates and cell culture inserts (3.0 μm pore size, Falcon 3096) containing 1 x 10⁵ bone marrow neutrophils in 0.2 ml DMEM-10% FCS were placed into the wells. As a positive control for the migration of neutrophils, wells containing fMLP (10⁻⁶ M final) were included in the assay. Bone marrow neutrophils were obtained from ICR mice by centrifugation of femoral bone marrow cells over a cushion of Ficoll-Hypaque (d=1.077) and collection of the pelleted cells.

After a 1 hour incubation at 37°C under 5% CO₂, the inserts were removed and the cells in the 24-well plate were resuspended by pipetting. The number of cells in each well was then estimated by counting the number of cells in 5 optical fields (magnification 250 x) using a 10 x 10 division counting reticle.

**Example 102**

*in vivo* assay for substances that induce neutrophil infiltration

Normal mice (C57BL/6J, BALB/c or ICR) or CD14-deficient mice were anesthetized by inhalation of Metofane (Pittman-Moore, Mundelein, IL) and injected intra-peritoneally, using a 25G5/8 needle, with 0.2 ml of a sterile PBS (< 0.03 endotoxin units/ml) solution containing the substance to be tested that provides a dose ranging from 500 ng/g of body weight to 0.005 ng/g of body weight. After 2-6 hours, the mice were sacrificed by inhalation of CO₂ and lavage of the peritoneal cavity was performed by injecting 10 ml of cold RPMI-HEPES supplemented with 1% FCS. The total number of cells in the lavage fluid was measured and the percentage of neutrophils was determined by Wright-Giemsa staining. Normally, there are no neutrophils in the peritoneal cavity.
of mice although the trauma induced by injection of saline alone can induce infiltration of some neutrophils. The extent of neutrophil infiltration induced by several of the substances at a dose of 180 ng/g body weight is shown in the figure below although similar degrees of neutrophil infiltration can be induced using doses as low as 18 ng/g body weight for some of the substances.

Example 201

Administration of MPLA following infection with Gram-positive bacteria enhances bacterial clearance.

To determine if MPLA could also improve the resistance to infection with Gram-positive bacteria, mice (BALB/c) were infected i.v. with Listeria monocytogenes (4x10^3/gbw) and treated i.v. with MPLA or PBS 3 h later. Treatment with MPLA induced a significant decrease of bacterial load in the blood (Figure 15A) and in the spleen (Figure 15B) collected 14 h after the infection with a four-fold reduction in the number of organisms in the spleen (p=0.05). Significantly lower bacterial counts were still found 48 h after infection. At that time, MPLA treated mice had markedly reduced signs of infection such as diarrhea and eye exudate. Furthermore, ICR mice infected i.v. with Listeria monocytogenes (3.3 x 10^3/gbw) and treated with MPLA 3 and 24 hours after the inoculation had no abscess in the liver at the 40th hour of the infection, in strong contrast to PBS-treated mice which had numerous liver abscesses (10-15 per liver).

These results show that the recruitment of neutrophils induced by MPLA following infection with Gram-positive bacteria is associated with an improved bacterial clearance. Furthermore, as shown, MPLA can be administered several hours following infection.

Example 202

Neutrophil infiltration induced by single chain lipids.

Mice (n=3) were injected (i.p.) with MPLA (0.18 µg/gbw), myristoyl alcohol (0.03 µ/gbw), palmitic acid (0.03
µg/gbw) [dissolved in PBS containing DMSO (1 mg/ml)] or PBS supplemented with DMSO. The number of PMN in the peritoneal cavity was determined 2 h following injection.

These results show that single chain lipid containing 14 (Myristoyl) or 16 (Palmitic) carbons can induce neutrophil infiltration to a similar extent as MPLA and the other molecules previously listed.
### Table 1. Table of compounds

<table>
<thead>
<tr>
<th>Compound</th>
<th>Lab name</th>
<th>Activity*</th>
<th>Bacterial Source</th>
<th>Chemical treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>I  Monophosphorylated lipid A (MPLA)</td>
<td></td>
<td>yes 8-20x</td>
<td><em>Salmoneilla minnesota</em>, Re 595 (Re mutant) (mixture of hexaacyl and heptaacyl)</td>
<td>Acid hydrolysis</td>
</tr>
<tr>
<td>II Monophosphorylated Lipid A</td>
<td>504</td>
<td>yes 20x</td>
<td><em>Escherichia coli</em>, F515 (Re mutant) (hexaacyl)</td>
<td>Acid hydrolysis</td>
</tr>
<tr>
<td>III Dephosphorylated Lipid A (hexaacyl)</td>
<td>503</td>
<td>yes 4-5x</td>
<td><em>Escherichia coli</em>, F515 (Re mutant)</td>
<td></td>
</tr>
<tr>
<td>IV Dephosphorylated Re-LPS (hexaacyl Lipid A)</td>
<td>KK1, 95</td>
<td>yes 3-4x</td>
<td><em>Escherichia coli</em>, F515 (Re mutant)</td>
<td>Hydrogen fluoride hydrolysis</td>
</tr>
<tr>
<td>V  Diphosphorylated lipid A (hexaacyl)</td>
<td>506, KK1, 283</td>
<td>yes 10x</td>
<td><em>Escherichia coli</em>, F515 (Re mutant)</td>
<td>Mild acid hydrolysis</td>
</tr>
<tr>
<td>VI Diphosphorylated pentaacyl lipid A</td>
<td>UZVII, 130</td>
<td>yes 4-5x</td>
<td><em>Escherichia coli</em>, F515 (Re mutant)</td>
<td>Produced by chemical modification</td>
</tr>
<tr>
<td>VII Diphosphorylated pentaacyl lipid A</td>
<td>109A, #2b, UZVII, 238</td>
<td>yes 8-10x</td>
<td><em>Escherichia coli</em> mutant containing pentaacyl Lipid A</td>
<td></td>
</tr>
<tr>
<td>VIII Monophosphorylated pentaacyl lipid A</td>
<td>MHI, 65 III [3.1 and 3.2]</td>
<td>yes 20x</td>
<td><em>Escherichia coli</em>, F515 (Re mutant)</td>
<td></td>
</tr>
<tr>
<td>IX Diphosphorylated tetraacyl lipid A</td>
<td>KKI, 282</td>
<td>yes 3-4x</td>
<td><em>Escherichia coli</em>, F515 (Re mutant)</td>
<td></td>
</tr>
<tr>
<td>X  LPS</td>
<td></td>
<td>yes 5x</td>
<td><em>Rhodobacter sphaeroides</em></td>
<td></td>
</tr>
<tr>
<td>XI Diphosphorylated lipid A</td>
<td></td>
<td>yes 2-3x</td>
<td><em>Rhodobacter sphaeroides</em></td>
<td></td>
</tr>
<tr>
<td>XII LPS</td>
<td></td>
<td>yes 3-4x</td>
<td><em>Bacteroides fragilis</em></td>
<td></td>
</tr>
<tr>
<td>XIII Dephosphorylated Lipid A</td>
<td></td>
<td>yes 4x</td>
<td><em>Rhodospirillum fulvum</em></td>
<td></td>
</tr>
<tr>
<td>XIV Tetraacyl LPS</td>
<td></td>
<td>yes 4x</td>
<td><em>Escherichia coli</em> LCD25 (K12 strain)</td>
<td>Partially deacylated using acyloxyacyl hydrolase (AOAH)</td>
</tr>
<tr>
<td>XV 4(S)-[3(R)-Hydroxy-tetradecanoylamido]-5-[3(R)-tetradecanoyloxy-tetradecanoyloxy]-pentaacid, Tris-(hydroxymethyl)-aminomethan salt (C&lt;sub&gt;18&lt;/sub&gt;H&lt;sub&gt;31&lt;/sub&gt;N&lt;sub&gt;2&lt;/sub&gt;O&lt;sub&gt;3&lt;/sub&gt;)</td>
<td>MLR 280, SDZ280, 961</td>
<td>yes 2x</td>
<td><em>Escherichia coli</em> LCD25 (K12 strain)</td>
<td>Synthesized</td>
</tr>
<tr>
<td>XVI Monophosphorylated Monosaccharide triacyl lipid A analog (C&lt;sub&gt;18&lt;/sub&gt;H&lt;sub&gt;31&lt;/sub&gt;N&lt;sub&gt;2&lt;/sub&gt;O&lt;sub&gt;3&lt;/sub&gt;^2)</td>
<td>MLR 953</td>
<td>yes 2x</td>
<td><em>Salmoneilla minnesota, wild type</em></td>
<td>Synthesized</td>
</tr>
<tr>
<td>XVII LPS</td>
<td></td>
<td>no</td>
<td><em>Salmoneilla minnesota, wild type</em></td>
<td></td>
</tr>
<tr>
<td>XVIII</td>
<td>LPS</td>
<td>no</td>
<td>Escherichia coli, K235</td>
<td></td>
</tr>
<tr>
<td>------</td>
<td>------</td>
<td>----</td>
<td>-----------------------</td>
<td></td>
</tr>
<tr>
<td>XIX</td>
<td>LPS</td>
<td>no</td>
<td>Neisseria meningitidis</td>
<td></td>
</tr>
<tr>
<td>XX</td>
<td>Re LPS</td>
<td>no</td>
<td>Salmonella minnesota, Re 595 (Re mutant)</td>
<td></td>
</tr>
<tr>
<td>XXI</td>
<td>Diphosphorylated lipid A</td>
<td>no</td>
<td>Salmonella minnesota, Re 595 (Re mutant)</td>
<td></td>
</tr>
<tr>
<td>XXII</td>
<td>Diphosphorylated diacyl Re-LPS</td>
<td>SPI, 98, 165.3</td>
<td>no</td>
<td>Escherichia coli, F515 (Re mutant)</td>
</tr>
<tr>
<td>XXIII</td>
<td>Dephosphorylated Rd-LPS</td>
<td>Rz1</td>
<td>no</td>
<td>Salmonella minnesota, Rd mutant</td>
</tr>
</tbody>
</table>
The activity (induction of granulocyte infiltration) was tested on normal mice. All of those which did not induce granulocyte infiltration in normal mice were found to induce it in CD14-deficient mice except N. Meningitidis which was not tested due to lack of material.

Activity is quantified as the ratio of the activity (neutrophil count) in normal mice treated with the compound relative to mice injected with PBS. Most measurements were made at 6 hours, but a few (XI, XIII, III) were made at 2 hours.

In CD14-deficient mice, activities were 9x(XV), 2x(XVI), 6-7x(XVII), 2-3x(XXI), 9x(XXII), and 6x(XXIII). Clearly, the "negative" compounds XVII, XXI, XXII and XXIII activate CD14 and thereby inhibit neutrophil recruitment in normal mice.

In the case of XV the CD-14-mediated inhibition is outweighed by the non-CD14-mediated acceleration.

soluble peptidoglycan in normal mice = 6-7x
Ara LAM in normal mice = 7x
manLAM in normal mice = 0
All references cited herein, including journal articles or abstracts, abandoned or pending (whether or not published) U.S. or foreign patent applications, issued U.S. or foreign patents, or any other references, are entirely incorporated by reference herein, including all data, tables, figures, and text presented in the cited references. Additionally, the entire contents of the references cited within the references cited herein are also entirely incorporated by reference.

Reference to known method steps, conventional method steps, known methods or conventional methods is not in any way an admission that any aspect, description or embodiment of the present invention is disclosed, taught or suggested in the relevant art.

The foregoing description of the specific embodiments will so fully reveal the general nature of the invention that others can, by applying knowledge within the skill of the art (including the contents of the references cited herein), readily modify and/or adapt for various applications such specific embodiments, without undue experimentation, without departing from the general concept of the present invention. Therefore, such adaptations and modifications are intended to be within the meaning and range of equivalents of the disclosed embodiments, based on the teaching and guidance presented herein. It is to be understood that the phraseology or terminology herein is for the purpose of description and not of limitation, such that the terminology or phraseology of the present specification is to be interpreted by the skilled artisan in light of the teachings and guidance presented herein, in combination with the knowledge of one of ordinary skill in the art.

Any description of a class or range as being useful or preferred in the practice of the invention shall be deemed a description of any subclass or subrange contained therein, as well as a separate description of each individual member or value in said class or range.

Where a set of preferred embodiments are recited for particular elements of the invention, any combination of a first set of preferred embodiments for a first element, with a second set of preferred elements for a second element, shall also be considered a preferred embodiment, and so forth for higher combinations including additional elements.
References Cited


CLAIMS

1. A method of inhibiting bacterial dissemination in a subject which comprises administering to the subject a pharmaceutically effective amount of a substance which accelerates recruitment of neutrophils to the peritoneal activity and thereby inhibits bacterial dissemination from the peritoneal cavity.

2. The method of claim 1 in which the substance is a lipid.

3. The method of claim 1 in which the substance is a carbohydrate.

4. The method of claim 3 in which the substance is a glycolipid.

5. The method of claim 4 in which the substance is a phosphoglycolipid.

6. The method of claim 1 in which the substance does not substantially bind CD14.

7. The method of claim 1 in which the substance is not an adjuvant.

8. The method of claim 1 in which, if the substance is administered in conjunction with a bacterial vaccine, the amount is insufficient to adjuvant the immune response to the vaccine.

9. The method of claim 1 in which the substance is a CD14 antagonist.

10. The method of claim 9 in which the amount is insufficient for substantial CD14 antagonist activity.

11. The method of claim 1 where the substance is a CD14 agonist, but either (a) the amount is insufficient to substantially activate CD14, or (b) the substance is administered in conjunction with a CD14 antagonist.

12. The method wherein the substance is any one of compounds I-XVI, or is a peptidoglycan or a lipoarabinomannan, or is myristoyl alcohol or palmitic acid.

13. A method of screening for substances which inhibit bacterial dissemination which comprises determining the ability of the compound to accelerate recruitment of neutrophils to the peritoneal cavity.
14. The method of claim 13 which is an in vitro assay.
15. The method of claim 13 which is an in vivo assay.
16. The method of claim 13 which further comprises determining whether the substance is a CD14 agonist or antagonist.
17. The method of claim 1 where the dissemination is of gram-positive bacteria.
18. The method of claim 1 where the dissemination is of gram-negative bacteria.
19. The method of claim 17 in which the dissemination is of Listeria.
20. The method of claim 18 in which the dissemination is of Enterobacteriaceae.
21. The method of claim 20 in which the dissemination is of Escherichia coli.
22. The method of claim 1 in which the subject is human.
23. The method of claim 1 in which the subject is a rodent.
24. The method of claim 2 in which the lipid is a single chain lipid.
25. The method of claim 2 in which the lipid comprises not more than six lipid chains.
26. The method of claim 2 in which the lipid chains are saturated.
27. The method of claim 26 in which the lipid chains are lauric, myristic, palmitic, stearic, arachidic, behenic or lignoceric.
28. The method of claim 13 in which neutrophil levels are measured about 2-6 hours after challenge infection.
29. The method of claim 13 in which the screening is carried out in normal mice.
30. The method of claim 13 in which the screening is carried out in CD14-deficient mice.
31. Use of a substance which accelerates recruitment of neutrophils to the peritoneal cavity in the manufacture of a composition for inhibiting bacterial dissemination from the peritoneal cavity in a subject.
Figure 3

- CD14-deficient
- Control

PMN (×10⁶)

LPS (ng/g)
Figure 4
Figure 5

![Graph showing survival (%) over time after infection (hr) with two lines: one for MPLA and one for saline.](image)

- **MPLA**
- **Saline**

The graph illustrates the decline in survival percentage over time for animals infected with MPLA compared to those infected with saline.
Figure 6

![Bar Graph](image)

- **Y-axis:** CFU/ml
- **X-axis:** ng/g of mouse
- Comparison of CFU/ml for saline, 1.8, and 18 treatments.
Figure 7

Diarrhea

Symptom intensity

Time after infection (d)

PBS
MPLA
Figure 8

Prostration

Symptom intensity

Time after infection (d)

PBS
MPLA
Figure 9

Reactivity

Symptom intensity

0.6
0.5
0.4
0.3
0.2
0.1
0.0

Time after infection (d)

0 1 2 3 4 5 6

PBS
MPLA
Figure 10

Eye exudate

Symptom intensity

Time after infection (d)

- O --- PBS
- ■ --- MPLA
Figure 11

Weight loss

Percent weight loss

Time after infection (d)

- ○ Saline
- ■ MPLA
Figure 12

Fur

Symptom intensity

Time after infection (d)

- PBS
- MPLA
Figure 13b

IV

V

VI

VII

VIII

IX
Figure 13C

\[ \text{O-antigen} \]

\[ \text{Polysaccharide} \]

\[ (Kdo)_k \]

X

XI
Figure 13b

Polysaccharide

XII
XIV

XVI

\[ \text{NH}_2C\left(\text{CH}_2\text{OH}\right)_3 \]
Figure 3&

XVII/XVIII

O-Antigen Repeat
n = 1-40

Outer Core

Inner Core

Lipid A

Chemotypes
Ra
Rb
Rc
Rd1
Rd2
Re

R^2: Kdo, Rha and/or P-Etn
R^3: P or P-P-Etn
R^4: P
R^5: Hep
R^6: Hep or GlcNAc

Proposed Partial Substituents
R^1: P
Figure 15

A. CFU per ml blood

B. CFU per g of tissue (x 10^7)

Treatment

PBS MPLA

PBS MPLA
Figure 16

![Bar chart showing data for MPLA, PBS, Palmitic Acid, and Myristyl Alcohol.](chart_image)