

# Boston University School of Medicine

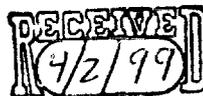
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MAR 22 1999 APR 20 1999

Joseph J. Vitale, Sc.D., M.D.  
Director, Nutrition-Pathology Unit



March 29, 1999

Dr. Lynn Larson  
Director, Programs and Enforcement Policy  
Office of Special Nutritionals  
HFS 455  
FDA 200 C Street, SW  
Washington, D.C. 20204

Dear Dr. Larson:

I am writing you as an interested party, as a Trustee of the Boston International Foundation for Medical Education and on the advice of counsel, notifying you of our intention to market a Structure/Function Claim for a dietary supplement, a lecithin, albeit highly purified, phosphatidyl choline (PC - 95%) with minimum amounts of other phosphatides.

The Claim reads as follows: "**PC-95 has been shown to enhance host defenses and immune functions in both animals and humans**". References to relevant literature, published in well recognized and respected medical journals will be included for the consumer.

For your information, I have enclosed two items related to PC-95, a copy of the most recent Patent and a copy of a publication. As an aside, PC-95 (we have labelled ABVA-1) has the potential for use in several clinical entities (please exam and read various sections of the Patent).

Best wishes.

Sincerely,

Joseph J. Vitale  
Professor of Pathology and Laboratory Medicine  
Professor of Community Medicine

JJV:mgr



US005262406A

**United States Patent** [19]  
**Vitale**

[11] **Patent Number:** **5,262,406**  
[45] **Date of Patent:** **Nov. 16, 1993**

[54] **PREVENTION AND TREATMENT OF MICROBIAL INFECTION BY PHOSPHOGLYCERIDES**

- [75] **Inventor:** Joseph J. Vitale, Weymouth, Mass.
- [73] **Assignee:** Med-Tal, Inc., Weymouth, Mass.
- [21] **Appl. No.:** 863,777
- [22] **Filed:** Apr. 6, 1992

**Related U.S. Application Data**

[63] Continuation of Ser. No. 579,242, Sep. 4, 1990, Pat. No. 4,135,922, which is a continuation of Ser. No. 366,475, Jun. 15, 1989, abandoned, which is a continuation-in-part of Ser. No. 142,522, Jan. 7, 1988, abandoned, which is a continuation-in-part of Ser. No. 75,556, Jul. 20, 1987, abandoned, which is a continuation-in-part of Ser. No. 891,880, Jul. 20, 1986, abandoned, which is a continuation-in-part of Ser. No. 620,886, Jun. 15, 1984, Pat. No. 4,648,080.

- [51] **Int. Cl.<sup>5</sup>** ..... **A61K 31/685**
- [52] **U.S. Cl.** ..... **514/78**
- [58] **Field of Search** ..... 514/78

[56] **References Cited**  
**U.S. PATENT DOCUMENTS**

4,666,893 5/1987 Tsuchiya ..... 514/78

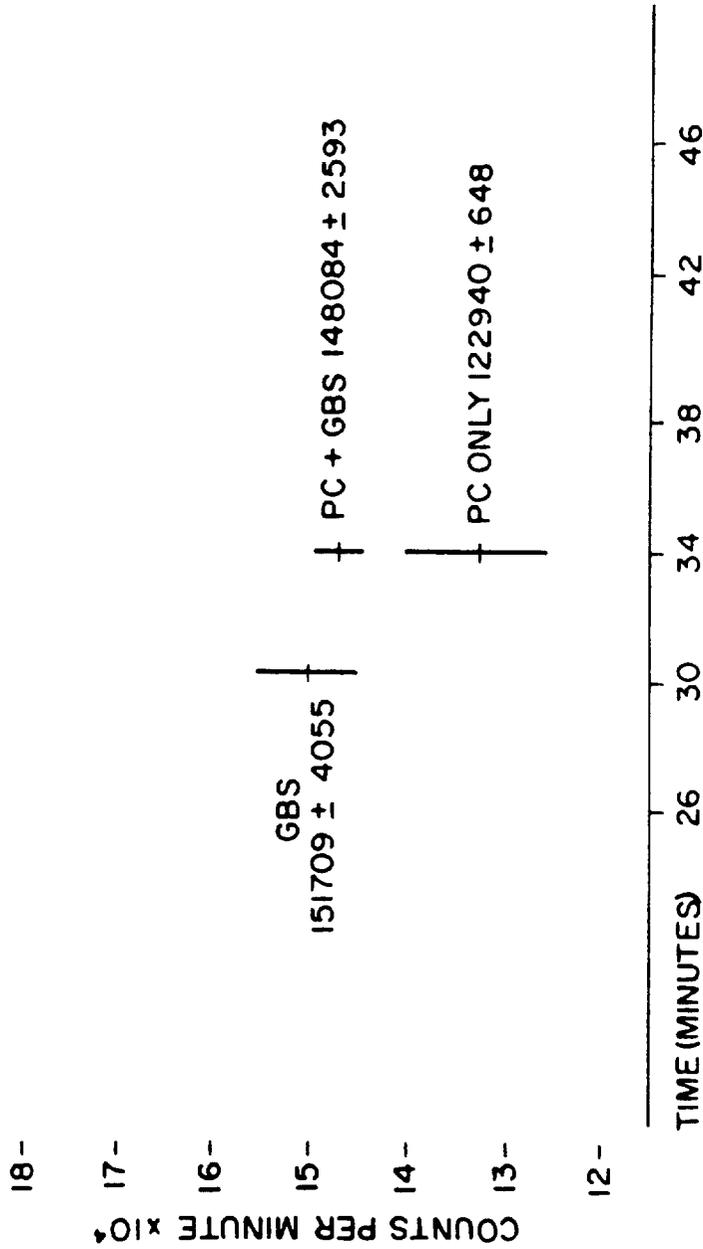
*Primary Examiner*—John W. Rollins  
*Attorney, Agent, or Firm*—Lahive & Cockfield

[57] **ABSTRACT**

Phosphoglycerides are effective therapeutic and prophylactic agents for bacterial, yeast protistan and viral infections. This invention discloses use of these phosphoglycerides in a variety of therapeutic contexts. Therapeutic methods of this invention pertain to use of phosphoglycerides to combat bacterial, yeast protistan, and viral infections. Phosphoglycerides can be used in therapy and in prophylaxis of AIDS patients. A preferred phosphoglyceride is a species of phosphatidylcholine in which linoleic acid is the primary fatty acid constituent.

**8 Claims, 5 Drawing Sheets**

CHEMILUMINESCENCE VALUES FOR RATS INFECTED\* WITH GROUP B STREPTOCOCCUS †



\* 14 DAYS POST INFECTION  
† STRAIN III R

FIG. 1

PROPHYLACTIC EFFECT OF PC GIVEN TO PREGNANT RATS ON GBS INFECTION IN THEIR OFFSPRING

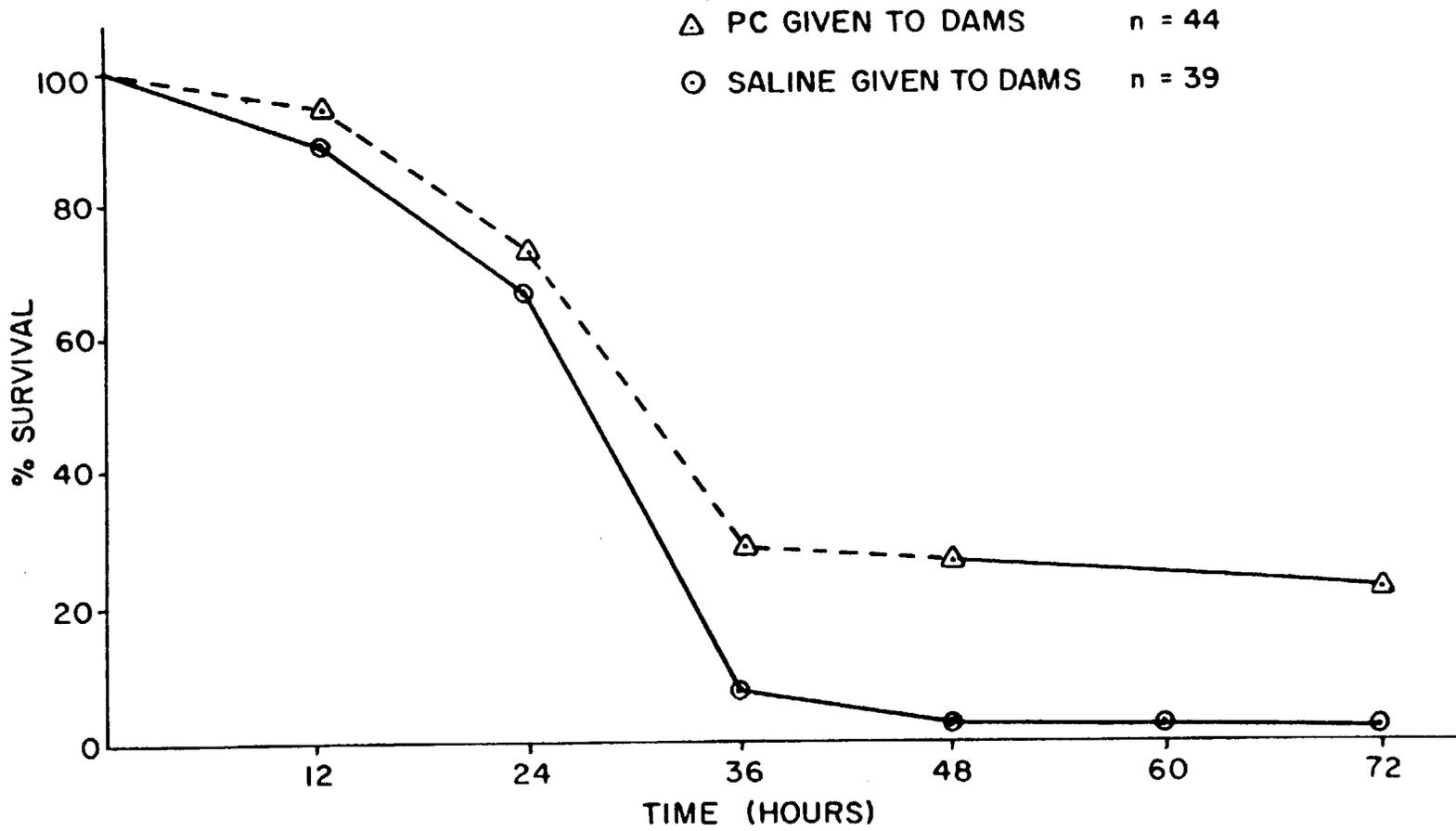


FIG. 2

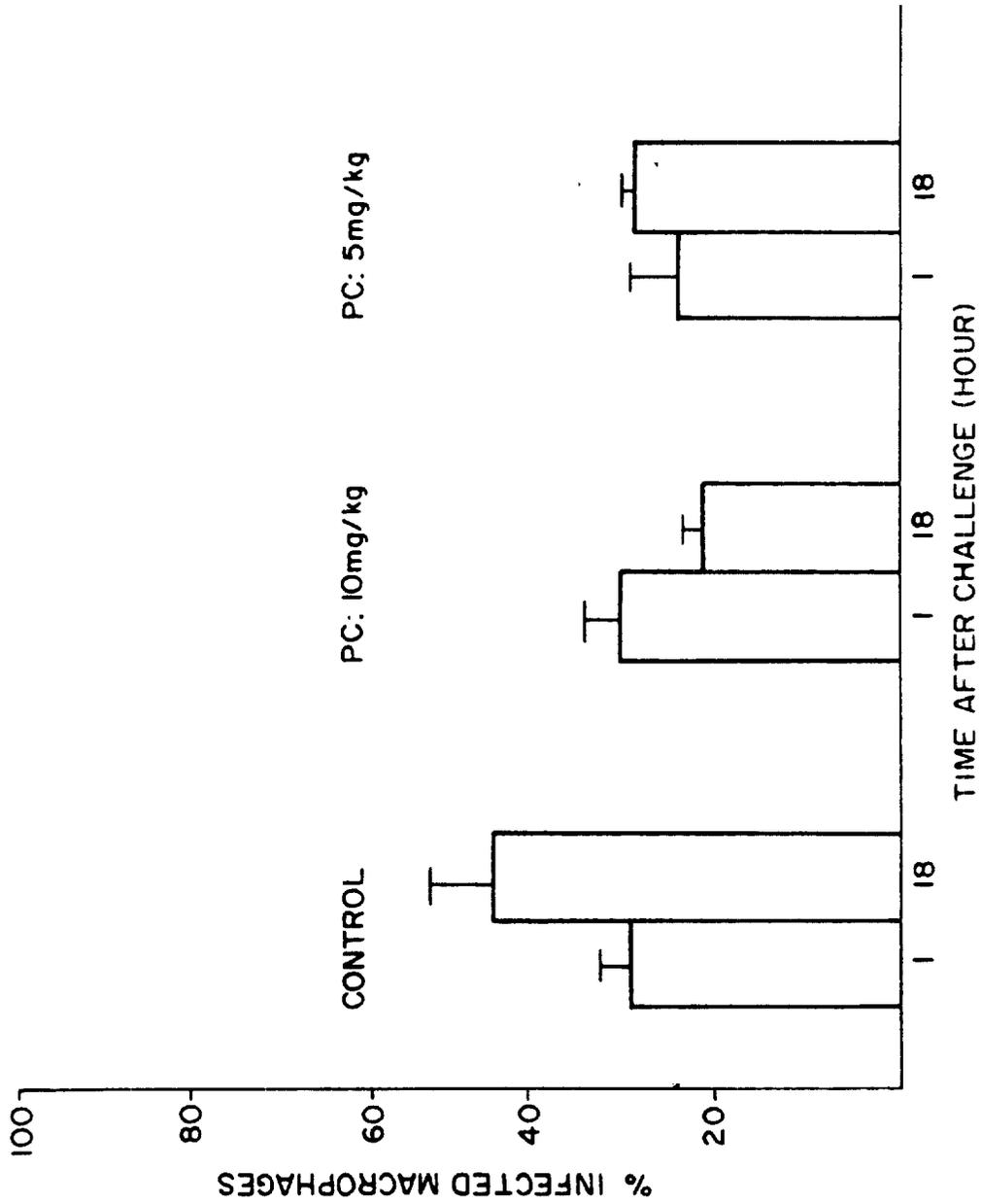


FIG. 3

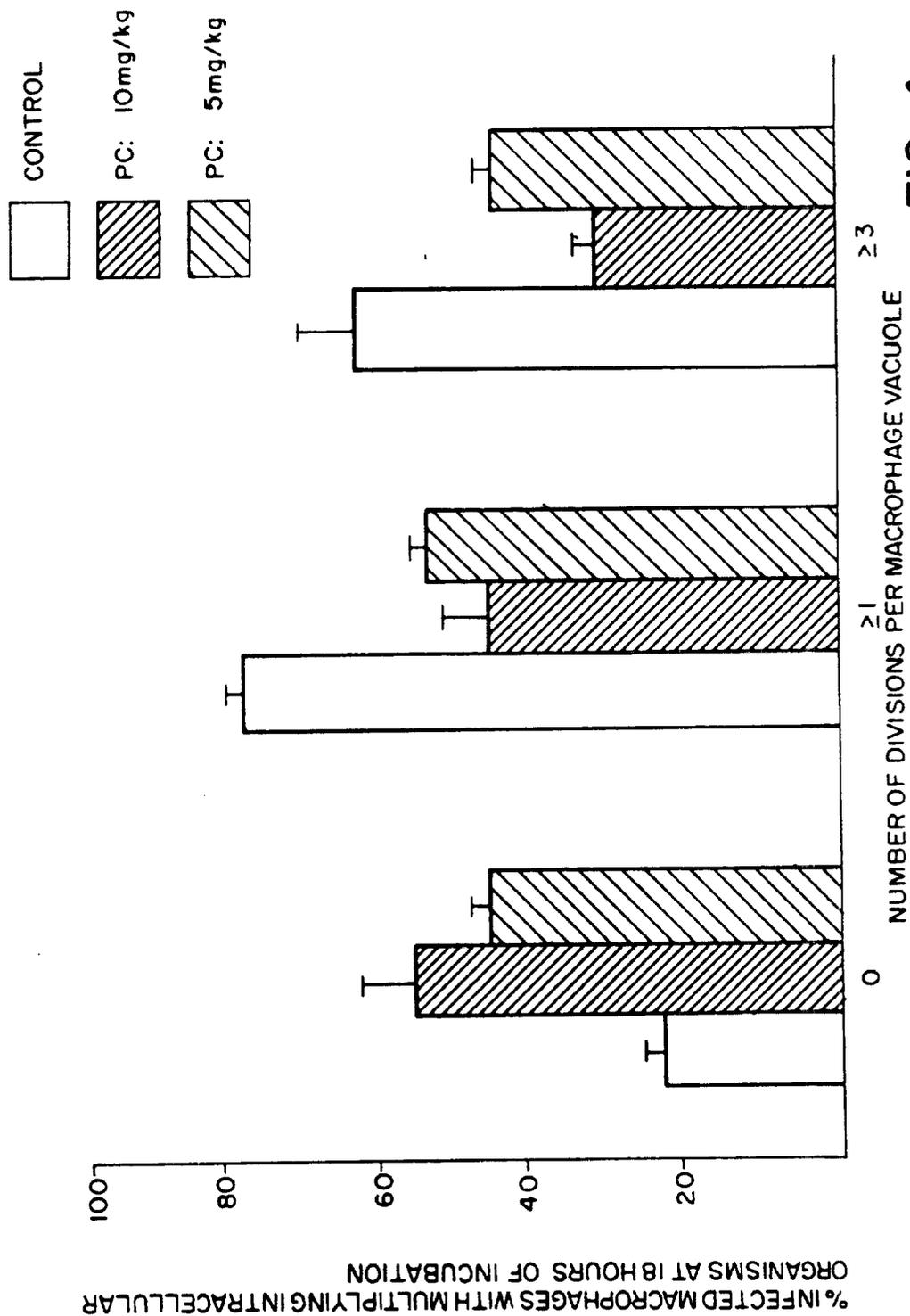
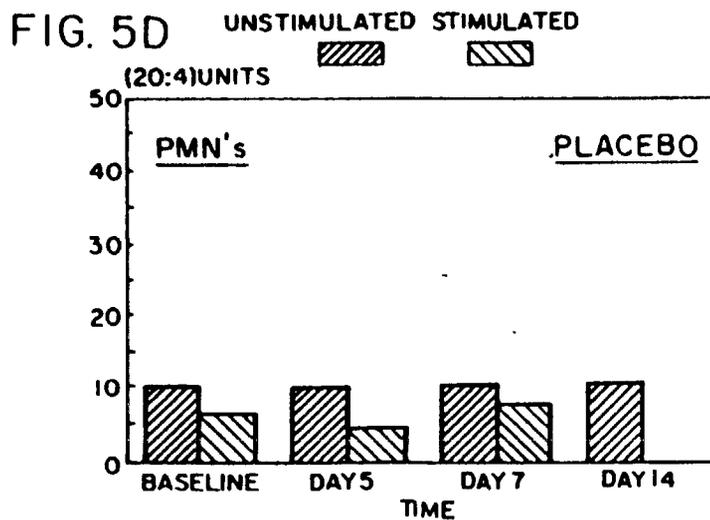
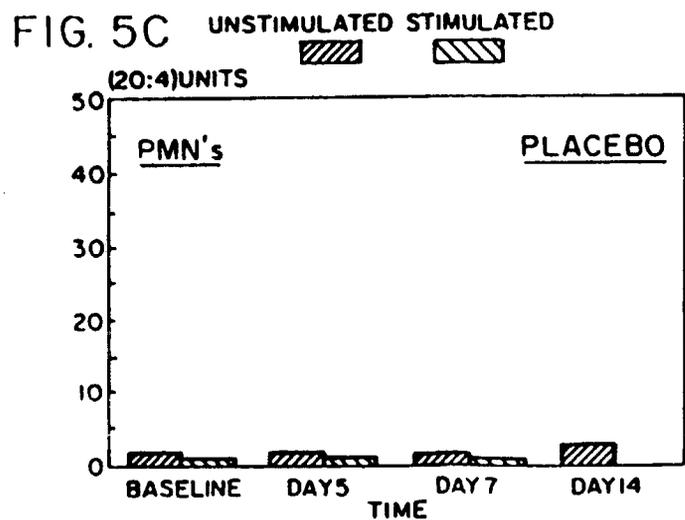
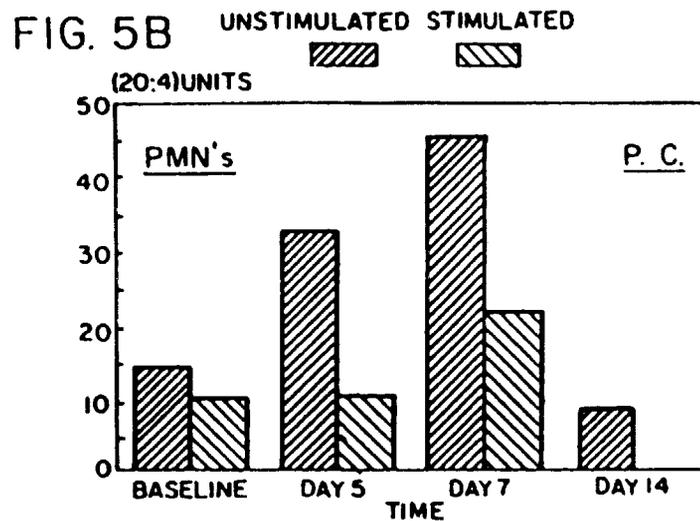
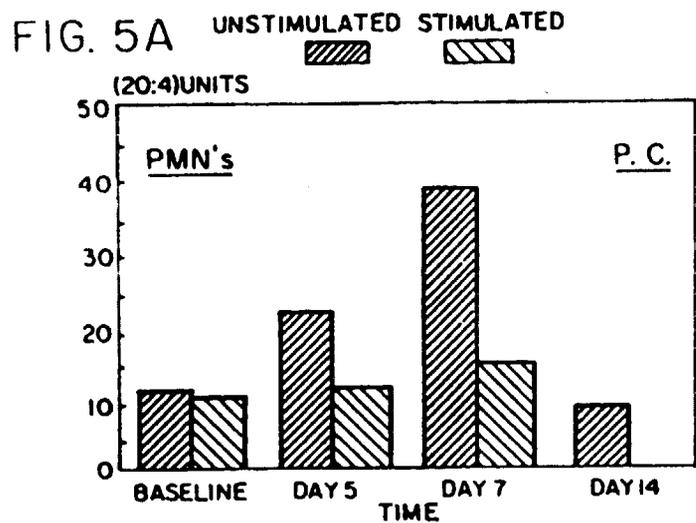


FIG. 4-



## PREVENTION AND TREATMENT OF MICROBIAL INFECTION BY PHOSPHOGLYCERIDES

This is a continuation of application Serial No. 07/579,242, filed Sept. 4, 1990 now U.S. Pat. No. 5,135,922, which is a continuation of U.S. Ser. No. 07/366,475 filed Jun. 15, 1989, now abandoned, which is a continuation in part of U.S. Ser. No. 07/142,522 filed Jan. 7, 1988, now abandoned, which is a continuation in part of U.S. Ser. No. 07/075,556 filed Jul. 20, 1987, now abandoned, which is a continuation in part of U.S. Ser. No. 06/891,880 filed Jul. 20, 1986, now abandoned, which is a continuation in part of U.S. Ser. No. 06/620,886 filed Jun. 15, 1984, now U.S. Pat. No. 4,648,080.

### FIELD OF THE INVENTION

This invention is in the field of anti-microbial chemotherapy and pertains to a method of preventing or treating infectious disease.

### BACKGROUND OF THE INVENTION

Group B Streptococci (GBS) are a major cause of morbidity and mortality in neonates and in other patients with compromised host defense mechanisms. The importance of GBS disease for newborn infants in the United States was underscored in an NIH sponsored workshop on Group B streptococcal Infection. Fisher, G.S. (1983) *J. Infect. Dis.* 148, 163-166.

The incidence of GBS disease is estimated at between 2 to 5 cases per 1000 live births. Pass, M.A. (1979) *J. Pediatr.* 795, 437-443. At highest risk are premature infants, whose birth weight is less than 2500 grams and infants born to mothers with premature rupture of membranes. Fisher, G. W., supra. Susceptibility to and severity of GBS disease has been associated with several risk factors including deficient or altered host defense mechanisms. Hill, H. R. et al. (1979) *Pediatrics* 64, 5787-5794. Studies have shown that human neonates who develop Group B Streptococcal Sepsis usually lack opsonic antibody to their infecting strain. Opsonic antibody is antibody that combines with antigen and facilitates ingestion of the antigen by phagocytes. In addition, these neonates usually have impaired polymorphonuclear leukocyte (PMN) function. Hemming, V. G. et al. (1976) *J. Clin. Invest.* 58, 1379-1387; Shigeoka, A. S. et al. (1979) *J. Pediatr.* 95, 454-460.

Despite aggressive supportive therapy and early institution of appropriate antimicrobial agents, the mortality rate for early-onset Group B Streptococcal Disease continues to be in the 25-75% range. This observation has prompted considerable interest and research in the development and use of adjunctive treatment modalities, which focus on improving the host's immune status, including a vaccine for maternal immunization [Baker, C. J. et al. (1978) *J. Clin. Invest.* 61, 1107-1110]; the use of ante and intrapartum prophylactic antibiotics; and various blood components including: whole blood, polymorphonuclear leukocytes, and immune serum globulins modified for intravenous use. However, despite such investigative work with animal models of disease, the mechanisms of host resistance to GBS have not been clearly defined. Some strains of GBS appear to be more virulent than others. Furthermore, all GBS strains are not uniformly susceptible to the action of antibody and complement.

Susceptibility to GBS disease, like to other infectious diseases, is multi-factorial and impinges on native and acquired immunity which are influenced by the host's genetic constitution, sex, age, and nutritional status. Of the aforementioned factors, there is very little information available as to the role of single or aggregate nutrients on host resistance to GBS disease. Almost any nutritional deficiency or excess may affect adversely one or more components of the immune system. Conversely the same nutrients that impinge on the immune system may also be essential for the multiplication and virulence of certain pathogenic microorganisms.

The need for a safe and effective regimen for preventing GBS infection in newborns was made evident in a recent editorial, "Prevention of Early-Onset Group B Streptococcal Infection in the Newborn" (1984) *Lancet* 1, 1056-1058. In evaluating one potential regimen, antibiotic prophylaxis, the author concluded that firm recommendations as to its use must await further experimentation.

### DISCLOSURE OF THE INVENTION

This invention pertains to a method of therapy or prophylaxis of bacterial, yeast, protozoan and viral infections and to novel compositions comprising essentially pure phosphatidyl choline containing only omega-6 fatty acids, particularly only linoleic acid. The method comprises administering, in either therapeutic or prophylactic amounts, one or more of the phosphoglycerides: phosphatidylcholine (lecithin) preferably phosphatidylcholine containing linoleic acid, phosphatidylethanolamine, phosphatidylserine or phosphatidylinositol.

The method of this invention is founded upon the discovery that the phosphatidylcholine (PC)—an ubiquitous constituent of biomembranes—protects newborn animals against mortality which usually results from Group B hemolytic Streptococcal infection. As explained, this microorganism is particularly virulent in newborns. In an experimental model of neonatal sepsis in which Group B hemolytic streptococci (GBS) was the causative agent, phosphatidylcholine proved an effective therapeutic agent. PC treatment prevented or delayed the death of newborn rats inoculated with GBS. A single injection of PC significantly increased the number of newborn rats which survived GBS inoculation and significantly prolonged the mean survival time of the entire treated group.

PC was also effective in prophylaxis, that is prevention, of GBS infection. When PC was given prophylactically to pregnant rats, PC reduced mortality in their offspring inoculated with GBS. Moreover, PC prevented weight loss in post-natal rats by enhancing host defense mechanisms.

In view of these findings, phosphatidylcholine may have a role as a nutritional supplement or as a practical therapeutic agent, or adjunct, in the treatment and prevention of GBS disease and other infections (e.g. viral, fungal, protistan etc.). In principle, other phosphoglyceride constituents of biomembranes are likely to act similarly. Thus, the phosphoglycerides phosphatidylethanolamine, phosphatidylserine and phosphatidylinositol may also be useful in combating or preventing bacterial infection. Also, because the phosphoglycerides may act by stimulating the hosts immune response to foreign organisms in general, they may be effective therapeutic and prophylactic agents for viral infection.

PC and possibly other phosphoglyceride components of biomembranes are believed to protect against the lethal action of bacteria by promoting the clearance of these disease-causing organisms by the reticuloendothelial system (RES) by altering the fluidity, and consequently the function, of the phagocytic cells (i.e., the polymorphonuclear leukocytes and macrophages) which make up the RES. This theory is supported by a study involving oral ingestion by humans of species of phosphatidylcholine in which linoleic acid was the primary fatty acid constituent (e.g., soy phosphatidylcholine). The study revealed that the composition and the concentration of arachidonic acid in polymorphonuclear leukocytes from these humans had been modified. This alternation in membrane lipid composition was found to occur in conjunction with an increased immune response.

#### BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 illustrates the opsono-phagocytic activity of PC-treated and untreated neonatal rats infected with GBS.

FIG. 2 illustrates the increased survival rate of rat pups whose mothers were treated with PC.

FIG. 3 illustrates the effect of phosphatidyl choline (PC) on infection of murine macrophages.

FIG. 4 illustrates the effect of PC on intracellular multiplication of RH strain tachyzoites within macrophage vacuoles.

FIGS. 5A-5D illustrate the changes in 20:4 of PMNs before (unstimulated) and after (stimulated) exposure to *Candida albicans* in four subjects—two fed PC and two fed placebo.

#### BEST MODE OF CARRYING OUT THE INVENTION

Animal experiments indicate that biomembrane phospholipid phosphatidylcholine (PC) is an effective therapeutic and prophylactic agent for bacterial, yeast and viral infections. The therapeutic efficacy of PC was evaluated in an experimental model of neonatal sepsis. Sepsis was induced in neonatal rats with a type III GBS strain resistant to opsonization. After infection, newborn rat pups were randomly assigned to receive equal volumes of either saline (controls) or 5 mg of PC. For a period of 30 days after administration of the phospholipid, animal mortality was observed. All 35 control animals died. In contrast, six out of thirty-three (18%) of the animals that received PC survived. In addition, the cumulative mean survival time was significantly increased in the PC treated group when compared to the controls (26 hrs vs 12 hrs).

Prophylactic efficacy of PC was evaluated by administering PC to pregnant rats prior to parturition and then inoculating offspring with GBS. The survival rate of pups delivered by PC-treated mothers was greater than pups delivered by mothers that did not receive PC. (23% of pups from PC-treated mothers survived whereas only 2.5% of pups from untreated mothers survived for 72 hours after infection.) PC also prevents vertical transmission of GBS from mother to fetus. Neonates of infected dams treated with PC were less likely to be bacteremic, that is, harbor viable GBS organisms in their tissues.

Because PC is an integral part of biomembranes, two mechanisms of action might be proposed. PC might modulate host immune response by stimulating the phagocytic function of polymorphonuclear leukocytes

(PMNLs) and macrophages. Phagocytosis is probably influenced by membrane fluidity. Because membrane fluidity depends upon its composition, PC, a major membrane component, may modify fluidity, and, in turn, phagocytosis. Together with reticular cells and other phagocytic cells, PMNLs and macrophages, make up the reticulo-endothelial system (RES) a "scavenger" system which traps, ingests and degrades invading organisms. Thus, by stimulating the component cells of the RES, PC may promote clearance of foreign organisms from host tissues by the RES. Countering this hypothesis are some observations that simple lipid complexes (cholesterol, oleate, ethyl stearate, and methyl palmitate) markedly depress the functional activity of the RES.

PC may also act by modifying surface components of the bacteria. These modifications may result in increased phagocytic uptake of invading organisms by host phagocytic cells or they may result in an abatement of the virulence of micro-organisms.

As determined by a chemiluminescence test for assessing opsono-phagocytic activity of phagocytic blood cells, PC-treated animals infected with GBS have a blunted opsono-phagocytic activity. This could mean that PC masks or alters the immunogenic surface receptors of the injected GBS and this results in a diminished type specific antibody response to the organism. Alternatively, PC may have a paradoxical effect of improving PMN function on the one hand and of decreasing B cell function and antibody production on the other hand. A role for PC in the inhibition of lectin mediated blastogenesis has been previously proposed. Chen, S. S. and Keenan, R. M. (1977) *Biochem. Biophys. Res. Comm.* 79, 852-858.

As discussed above, in an experimental model of Group B Streptococcal Disease, phosphatidylcholine offered protection against a virulent Group B Streptococcal strain. Interestingly, these results resemble the results of previous studies employing blood components to protect such animals from Group B Streptococcal Infection. See e.g., Santos, J. I. et al. (1980) *Pediatr. Res.* 14, 1408-1410. However, because PC has no inhibitory effect on GBS in vitro and actually sustains GBS growth in culture, it is reasonable to speculate that PC enhances the immune response of the newborn animal to GBS.

The effects of phosphatidylcholine on the immune system—which is described herein for bacterial and yeast (Example 7) infections—supports the use of phosphoglycerides as a method of therapy and prophylaxis for viral infections, as well.

Phosphatidylcholine belongs to a family of phosphoglycerides which are found almost entirely in cellular membranes as constituents of the lipid bilayer. Phosphoglycerides comprise glycerol phosphate, two fatty acid residues esterified to the hydroxyl groups at carbons 1 and 2 of glycerol and an alcohol component (e.g. choline in phosphatidylcholine) whose hydroxyl group is esterified to the phosphoric acid. Because of the similarity of structure and function of these compounds, the therapeutic and protective action manifested by PC toward bacterial infection may be common to the group. This is believed to be so particularly for the other major phosphoglycerides, phosphatidylethanolamine, phosphatidylserine and phosphatidylinositol.

A preferred embodiment of this invention employs a species of phosphatidylcholine in which linoleic acid (C18:2n-6) is the primary (preferably the only) fatty

acid constituent (e.g. soy phosphatidylcholine). Linoleic acid is an omega-6 fatty acid, as opposed to an omega-3 fatty acid (e.g. marine oils) and is an essential dietary nutrient, because it cannot be synthesized by mammals but must be obtained from plants. Because linoleic acid is the major lipid consumed in conventional Western diets, it is the major fatty acid comprising the phospholipid bilayer of the cell membranes of most people who consume a Western diet. The phosphatidylcholines containing omega-6 fatty acids can be synthesized by adding or replacing fatty acids on carbons 1 and 2 of the glycerol molecule. For example, fatty acids on carbons 1 and 2 of a glycerol molecule can be removed and the phospholipid can be reconstituted in the presence of linoleic acid. In addition, phosphatidylcholines containing omega-6 can be isolated from heterogenous preparations of phosphatidylcholines available from commercial sources.

A study, which is described in greater detail in Example 7, revealed that in response to a dietary supplement providing a minimum of 18 gms of phosphatidylcholine containing linoleic acid as the primary fatty acid constituent, but not in response to a placebo, PMNL phospholipid arachidonic acid (C20:4n-6) content increased. In addition, stimulation of these PMNLs with *Candida albicans* resulted in phospholipid arachidonic acid release which correlated with PMNL killing and phagocytosis of the yeast. Further, when these PMNLs were exposed to a calcium ionophore or N-formyl-methionyl-leucylphenylalanine, the amount of leukotriene B4 (LTB4), an immunosuppressor increased substantially.

In contrast, neutral fats providing equivalent amounts of linoleic acid appeared to have no effect on PMNL function or PMNL fatty acid composition. This difference between linoleic acid contained in phospholipids and linoleic acid contained in neutral fat is likely related to the way the body metabolizes and utilizes linoleic acid from the two different sources. Scrow, R. O., Stein Y. and Stein L., *J. Biol. Chem.*, 242: 4919-24 (1967); Borgstrom B., *Gastroenterology*, 78: 954-64 (1980).

For example, phospholipids, although comprising only 5-9% by weight, form 40-65% of the outer surface of chylomicrons. Davenport, H. W., *Physiology of the Digestive Tract*, p. 218 et seq., Year Book Medical Publishers, Chicago (1982). This enables an easy exchange of phospholipids with the lipophilic cellular membranes with which they contact. Neutral fats, on the other hand, are carried mainly in the triglyceride-rich core and cannot exchange as easily.

The phosphoglycerides may be administered enterally or parenterally. Enteral administration is the preferred route. As the phosphoglycerides are components of many foods, they may be administered conveniently by ordering a diet which will supply the appropriate amount of the phosphoglycerides. Alternatively, the phospholipid may be given alone in bolus doses as a dietary supplement.

In situations of acute need, it may be desirable to give the phosphoglycerides parenterally, preferably by intravenous infusion. Pharmaceutical compositions may be prepared comprising the phosphoglyceride in a physiologically-acceptable vehicle. For example, a sterile emulsion of the phosphoglycerides in phosphate buffered saline is a suitable composition for infusion. In general, dosage regimens may be optimized according to established principles of pharmacokinetics.

As a therapeutic agent, the phosphoglycerides may be administered to a patient who has been determined clinically to have contracted a bacterial, yeast or viral infection. Besides being efficacious therapeutic agents in their own right, the phosphoglycerides may be useful adjuncts to conventional therapeutic regimens. For example, phosphatidylcholine may be administered conjunctively with antibiotics or other anti-microbial agents.

Prophylactic use of the phosphoglycerides is appropriate in patients who are at risk of contracting bacterial infection. Patients especially susceptible to bacterial infection include premature infants, infants born to mothers with prematurely ruptured membranes and persons whose immune system is suppressed or compromised such as certain cancer patients (e.g. leukemics) or cancer patients undergoing chemotherapy, persons suffering from Acquired Immune Deficiency (AIDS) or the aged. In prophylaxis, the phosphoglycerides may be administered alone or as an adjunct to other forms of prophylaxis. In particular, the phosphoglycerides can be used with other prophylaxis forms in the treatment of AIDS. Vitamin D3 or its active metabolites such as the hormonal product, 1,25-(OH)<sub>2</sub>D3, (HD) can inhibit Interleukin-2 production and depress T-cell proliferation when T-cells are exposed to mitogen. Normally, receptors for HD are not present on resting T- and B-cells but are present on monocytes and malignant T-lymphocytes. When resting T-cells are exposed to a mitogen, proliferation and differentiation occurs with expression of HD receptors. The addition of HD in pico-molar concentrations inhibits the proliferation of activated T-cells, presumably by inhibiting the growth promoting lymphokine, IL-2. Macrophages as well as T-cells may be infected with the Human-Immunodeficient Virus (HIV). The macrophage is required for T-cell activation and may have an important role not only in promoting the replication of the virus but also in transmitting virus to resting T-cells. By enhancing macrophage function with, for example, phosphatidylcholine treatment and reducing T-cell proliferation by HD administration, a combined therapeutic regimen of both phosphatidylcholine/HD would be of value in mitigating the morbidity associated with the HIV infection.

A important mode of prophylaxis of neonatal sepsis is treatment of the mother with a prophylactic agent prior to parturition. As described above, maternal administration of PC effectively protects newborns against GBS infection. Thus, the phosphoglycerides may be administered prophylactically late in pregnancy, either alone or in conjunction with other prophylactic agents (e.g. antibiotics) in situations of high risk of neonatal infection.

The use of phosphoglycerides to combat or ward off bacterial infection has several advantages. Because phosphoglycerides are common nutrients, there is minimal risk of toxicity attendant to their administration. In addition, as common components of foods, they can be administered orally and may be given therapeutically by prescribing a diet which provides the desired amount.

From a nutritional point of view, PC, in particular, has the potential for being exploited to the benefit of the host. In mammalian tissue free choline participates in four enzyme-catalyzed pathways: oxidation, phosphorylation, acetylation, and base exchange. See Zeigel, S. H. (1981) *Ann. Rev. Nutr.* 1, 95-121. During periods of rapid growth, large amounts of choline are needed for

membrane and myelin synthesis. The ultimate source of dietary choline is the phospholipid, lecithin. However, the newborn rat is unable to consume choline-containing foods other than its mother's milk. It may be possible to prophylactically increase the plasma choline concentration in the newborn animal by giving a choline-rich diet or by administering PC via parenteral route to the mothers during pregnancy. Although the placenta is incapable of taking up lecithin from maternal blood and transporting it to the fetus, it does possess both passive and active transport mechanisms which result in significant transport of choline from maternal blood into the placenta. Biezanski, J. et al. (1971) *Biochim. Biophys. Acta* 230, 92-97.

The invention is further illustrated by the following examples.

## EXAMPLES

### Example 1

#### Therapeutic Effect of PC in Neonatal Sepsis

##### Preparation of Phosphatidylcholine (PC)

Soy lecithin, 95% pure, (Unilever Inc., Valardinger, Netherlands) was mixed in phosphate buffered saline (PBS) to a concentration of 250 mg/ml. This Lecithin preparation contained 25.84 nanomoles of phosphatidylcholine per 100 ul of mixture as determined by thin layer chromatography.

##### Preparation of Organisms

A human isolate of Group B Streptococci Type III-R, resistant to opsonization by most antibody containing serum was cultured in Todd Hewitt (Difco Laboratories, Detroit) broth overnight at 37° C., washed, concentrated in phosphate buffered saline (PBS) by centrifugation and adjusted to  $1 \times 10^9$  cfu/ml in PBS as previously described. Hemming, V. E., et al. (1976) *J. Clin. Invest.* 58, 1379-1387.

##### Neonatal Rat Infections

Sprague Dawley outbred pregnant female rats (Charles River) and Lewis pregnant female rats were obtained one week before delivery. The neonatal progeny less than 24 hours old were randomly assigned to groups and entered into the study. Animals were inoculated intraperitoneally with 0.1 cc of PBS containing  $10^4$  streptococci. In the protection studies, the animals also received separate equal volume injections of saline or 25.84 nanomoles of phosphatidylcholine immediately after bacterial inoculation. The rat litters were observed at six hour intervals for mortality. Surviving rats were observed for a total of three weeks as previously described. Santos, J. I., et al. (1980) *Pediatr. Res.* 14, 1408-1410.

##### Chemiluminescence Procedure

A chemiluminescence procedure, as modified by Selveraj and co-workers, *J. Retic. Endoth. Soc.* 31 3-16 (1982), for microquantities of whole blood, was employed in assessing the opsono-phagocytic activity of PC-treated and control Lewis rats that survived GBS challenge. Briefly, approximately 0.5 ml of heparinized whole blood was taken from each rat two weeks post infection. Total WBC and differential counts were determined on each blood sample. The assay was conducted in the following manner: to an 8 ml vial, the following reagents were added: 0.8 ml of KRB buffer, 1.5 ml sterile distilled water, 2.5 ml 1 mM luminol (Kodak), 10 ml whole blood, and finally 150 ul of an overnight culture of GBS Type III washed and adjusted to  $1 \times 10^9$  c.f.u. per ml to achieve a PMN: particle ratio of 1:3000. Blood from each rat was studied in duplicate;

thus two samples from each rat were placed in the scintillation counter. Three background samples with the particle and chemiluminescence mixture were run with each assay. The samples were monitored for chemiluminescence using an LKB Rack Beta 1211 Liquid Scintillation counter. Each sample was counted for 5 seconds every 6 minutes. The assay was allowed to run for 30 minutes after which time chemiluminescence activity had peaked and decreased significantly.

##### Statistical Analysis

Comparison of significance of differences between survival values in the animal studies were performed by use of the Fischer exact test.

##### Results

The survival rate in 35 unprotected (controls) neonatal Sprague Dawley rats infected with Type III-R, GBS was 0. In contrast, 6/33 (18%) of the rats that received phosphatidylcholine survived ( $p < 0.01$ ). Moreover, the cumulative mean survival time was also significantly increased in the PC treated group when compared to the controls (26 hours vs. 12 hours;  $p < 0.01$ ).

Unexpectedly, newborn Lewis rats challenged with the same strain of GBS and randomized to receive either saline ( $n=34$ ) or PC ( $n=16$ ) all survived. Differences in animal strain susceptibility were corroborated in several experiments. Sprague Dawley rats were uniformly susceptible and Lewis rats resistant to this GBS strain.

The opsono-phagocytic activity of GBS infected Lewis rats was determined fourteen days post infection using whole blood and un-opsonized live GBS as the particle. Depicted in FIG. 1 are the chemiluminescence (CL) values for rats that were infected and received either saline or PC and uninfected rats that received only PC. Animals that received PC had a lower mean CL response than saline treated controls but both had a greater response than uninfected animals.

### Example 2

#### Prophylactic Effect of PC

The present study was designed to evaluate the prophylactic effect of PC given to pregnant Sprague Dawley dams on GBS infection in their offspring.

Outbred Sprague Dawley rats (Charles River Breeding Laboratory, Wilmington, Mass.) were used in all experiments. Single doses of 250 mg purified phosphatidylcholine dissolved in 1 cc of 0.9% saline were injected intraperitoneally (i.p.) at time intervals ranging from 120 hours (5 days) to 96 hours (4 days) prior to parturition. Control animals received equal volume i.p. injections of saline. After delivery all of the rat pups ranging in age from 24-36 hours were injected i.p. with  $5 \times 10^6$  c.f.u. of GBS and observed for mortality.

The results presented in FIG. 2 indicated that rat pups whose mothers were treated with PC had an increased survival rate of 10/44 (23%) versus 1/39 (2.5%) for pups born to saline-treated controls. ( $P < 0.05$ )

In an additional experiment, nineteen pregnant Sprague Dawley rats were randomized to receive 500 mg PC ( $n=10$ ) or saline ( $n=9$ ) i.p. on day 16 of gestation. All rats were challenged with  $10^9$ - $10^{10}$  CFU of GBS type III, 24-28 hours prior to expected delivery.

17/19 (89%) were documented to be bacteremic 24 hours post inoculation. Two PC treated animals and 4 controls died 24-80 hours post inoculation; GBS was isolated from these animals and their unborn pups.

Seventy-four pups were born to 8 PC-treated animals; fifty-one pups to the 5 remaining controls. Pups were sacrificed and cultured at 4-8 hour intervals following birth. GBS was isolated from liver, spleen, blood or brain in 15/74 (20%) pups in PC-treated group. In contrast, 19/51 (37%) of saline treated groups had positive cultures.

These results suggest that prophylaxis with PC, an important component of cell biomembranes, may interfere with the vertical transmission of GBS. Further investigation of PC interaction with GBS and with maternal immune function may define its role in the antepartum management of GBS infected mothers.

#### Example 3

##### Effect of PC on Postnatal Rats

This Example illustrates that the effect of PC on pre-weaned rats includes weight gains that can be attributed to enhanced host defense mechanisms.

A rat litter (N-13) was randomly assigned to receive either a single i.p. injection of PC soy lecithin (see Example 1, above) or 500 mg/kg of an equivalent control injection of phosphate buffered saline (PBS). Pups were allowed to nurse and weighed every two to three days.

The weight distribution for all animals prior to weaning is given in Table I. All animals had similar weight gain during the first two weeks. Between weeks 2 and 3, however, the PC treated group gained more weight and by 21 days post-injection, the mean weight of the PC-treated group was  $39.1 \pm 1.1$  grams versus  $24.3 \pm 1.2$  grams for the control group.

Differences in weight can be attributed to enhanced host defense mechanisms in the PC-treated group. Although no visible signs of intercurrent illness was noted for the control group, a subclinical infection cannot be ruled out. These results suggest that PC may be used prophylactically as a useful nutritional supplement, not only in rodents, but in humans as well by preventing weight loss during a period in the neonate when host defenses are being established.

#### Example 4

##### Clinical Trials

Clinical studies were conducted on 76 pediatric patients admitted to the hospital with viral and bacterial infections (Table II). Forty randomly selected patients received lecithin over a period of hospital confinement. Thirty-six patients were given placebo treatment over the same period. Blood was drawn on Day 0, 3, and 7. Lecithin (soy bean lecithin) was administered orally for three consecutive days (3 equal doses per day for a total dose of 450-500 mg/kg/day). Longer periods of administration can be tested; however, the rat studies suggested that three days of administration should have some effect. Twenty-six of the experimental group and 25 of the placebo group had bacterial infections; viral infections in each group numbered 12 and 11, respectively. Admissions from other causes received treatment with lecithin. Natural killer cell activity, as measured by  $^{51}$ chromium assay and at three different ratios of effector cell to target cell, showed lecithin to be a potent inducer of NK activity in these patients (Tables III, IV, V). Table VI and VII show the findings of phagocytosis and the percent of phagocytosed bacteria (intracellular killing) "killed" in 85 normal individuals of ages ranging from 2 years to 39 years of age. With this assay, approximately 40% of the bacteria are ingested and 85% of these are "killed". It is clear from

Tables XI and XII that both phagocytosis and "killing" were enhanced by the administration of lecithin. Analysis of Variance indicated a statistically significant effect of lecithin on NK activity and on phagocytosis and "killing" by PMNs compared to the placebo group. A related finding of increased plasma proteins was seen within the treated group.

An additional child admitted for viral bronchopneumonia had a preexisting retinoblastoma and was treated with lecithin. Within 7 days of the treatment his kill activity was seen to increase from 0-7100 with a concomitant rise in WBC from 2320-5600. The child was discharged 10 days post admission with the pneumonia resolved. This isolated case is mentioned anecdotally and not included in the study data. Nonetheless, we were pleased and impressed with both the clinical outcome of this boy's illness and the laboratory data generated by his admission.

Clearly the dramatic effect of lecithin on the clinical course and outcome of both bacterial and viral infections empirically demonstrates the use and value of this and related molecules.

#### Example 5

##### The Effect of PC on Mortality, Bacteremia, White Blood Counts and IgG and IgM in Rats Infected with *Klebsiella Pneumoniae*

Forty-five Wistar rats (five weeks old) were divided into two groups. The experimental group of 25 rats received intraperitoneal injections of PC (800 mg/KG in a 1 ml volume) on Day 0. The control group of 20 rats received equal volume intraperitoneal injections of PBS (pH7.2) at the same time. On Day 4, both the experimental and the control groups received intravenous injections of  $5 \times 10^8$  CFU of *Klebsiella pneumoniae* (K8).

##### Results

At the end of six days, only 10 of the rats who received PBS were alive compared to 18 alive in the PC group.

One day after the administration of *Klebsiella pneumoniae* (K8) the Colony Forming Units ( $\text{Log}_{10}$ ) per ml of blood was approximately 4.2 and slowly fell to 3 by Day 5 in the PBS treated group. The PC group had essentially the same bacterial load on Day 1, but it rapidly decreased to less than one by Day 5. Not only was the bacterial load less in PC treated animals compared to PBS treated rats but so was the clearance. By Day 5, 8 of 9 animals tested in the PC treated group had negative blood cultures compared to 1 of 6 animals in the PBS group.

The neutrophilia induced by K8 increased from about 1000 PMNs/ $\text{mm}^3$  prior to infection to approximately 3000/ $\text{mm}^3$  24 hours later. PC administration resulted in a much greater response and appeared to augment the K8 response by a two and one-half fold increase in circulating PMNs. At the end of six days, there was no difference in PMN counts between the groups (PC vs. PBS). There were no significant changes in IgG or IgM levels in either group during the course of the study. (It should be noted that in a single mouse experiment PC administration resulted in a neutrophilia; PBS and liquid paraffin did not.)

## Example 6

Effect of PC on Response of Macrophages to  
Toxoplasma Gondii Infections

## A. Effect of PC on Infection of Murine Macrophages

For four consecutive days, normal CD-1 female mice (8 weeks-old) were inoculated intraperitoneally with PBS (control), high dose PC (10 mg/kg) or low dose PC (5 mg/kg). The mice were sacrificed seven days later. Adherent peritoneal macrophage monolayers prepared from each group were challenged with RH strain toxoplasma tachyzoites (zero time) at a multiplicity of infection ratio of 1:1 (parasite:macrophage). At 1 and 18 hours after zero time, monolayers were fixed and stained, and then assessed for percent infection (number of infected macrophages/total number of macrophages counted (200)×100).

## Results

The percent infection was not significantly different between groups at 1 hour. At 18 hours, however, the percent infection ± SD in the control group was significantly higher (P 0.05) than in either the high dose (20.25±1.06) or the low dose (28.5±0.0) PC groups. (See FIG. 3.) (The significance of the differences between test and control groups was assessed by chi-square analysis.)

## B. Effect of PC on Intracellular Multiplication of RH Strain Tachyzoites Within Macrophage Vacuoles.

Mice were treated with either PBS (control), high dose PC (10 mg/kg) or low dose PC (5 mg/kg) according to the protocol outlined in 5A. Seven days later, macrophage monolayers were prepared from each group, then infected with RH strain tachyzoites (zero time) at a multiplicity of infection ratio of 1:1 (parasite:macrophage). Eighteen hours after zero time, monolayers were fixed, stained and assessed by light microscopy for evidence of intracellular multiplication of toxoplasma within macrophage vacuoles. Data are expressed as the percent of infected macrophages with 0, 1 and 3 parasite divisions per vacuole for each group.

## Results

The control group revealed significantly (P 0.01) fewer percent infected macrophages ± SD with no parasite divisions (22.5±0.71) than either the high dose PC group (54.5±6.36) or the low dose PC group (45.5±0.71). However, the control group had significantly (P 0.05) more percent injected macrophages with 1 (77.5±0.71) or 3 (63.00±8.49) parasite divisions compared with the respective values for the high dose PC group (1:45.5±6.36; 3:28.5±3.54) and the low dose PC group (1:54.5±0.71; 3:45.5±2.12). (See FIG. 4.) (The significance of the differences between test and control groups was determined by the t-test for summary data.)

## Summary of Example 6

Peritoneal macrophages obtained from CD-1 mice 7 days after treatment with PC either at high dose or low dose exhibited: (a) significantly reduced infection rates 18 hours after in vitro challenge with RH strain toxoplasma tachyzoites compared with the controls, and (b) significantly reduced intracellular replication of the parasite 18 hours after in vitro infection compared with the controls.

These data suggest that in vitro treatment of normal mice with PC has significant direct or indirect effects on enhancement of macrophage microbicidal activity for the parasite in vitro. Since macrophages are the single most crucial element in the cell-mediated immune response to this parasite, the data also suggest that PC

treatment may significantly affect the course of acute toxoplasma injection in the normal host as well.

## Example 7

5 The Effect of "Local" Versus "Systemic" PC or PBS  
on NBT Reduction by Isolated Rat PMNs and  
Macrophages

10 "Local": Polyvinyl sponges treated with either PBS or PC were implanted under the skin. After 18 hours, the sponges were removed and PMNs isolated. Just prior to sacrifice, PMNs and peritoneal macrophages were isolated from blood. NBT (Nitroblue tetrazolium) Reduction was used to assess microbicidal activity.

15 "Systemic": Rats were treated with PC or PBS once 4 days prior to sacrifice. Then, 18 hours prior to sacrifice, peritoneal macrophages were isolated by 10 mls of PBS lavage.

## Results

20 Table VIII indicates that circulating PMNs that migrated to the sponge were not affected by the PC and that if PC leaked out of the sponge, it had no effect on PMNs isolated from blood. No differences in NBT reduction by PMNs or macrophages were observed between PC and PBS treatments.

25 Table IX demonstrates that rats pretreated with PC had significantly more active PMNs and macrophages compared to the PBS treated animals.

30 Furthermore, as the results are based on a known cell quantity, in PC treated animals the PMNs isolated from the sponge showed significantly greater activity than those isolated from blood.

The same observation holds true for PMNs isolated from the sponge versus those isolated from the blood in 35 PBS treated animals. The difference between the two groups is also statistically significant suggesting that (a) PMNs responding to a foreign substance may not be from the same pool as those circulating in blood; and/or that (b) more active PMNs (and possibly macrophages) are attracted to the site of a foreign body (sic. infection); 40 (c) PC affects different pools of PMN; and (d) PC is acting on PMN precursors.

## Example 8

45 The Effect of Ingested PC by Normal Healthy Young  
Adults on PMN Function

Seven healthy young adults ages 23-35 were fed 27 grams of PC or a placebo per day in three divided doses for 3 days in a cross-over study. Normally, these individuals would ingest approximately 4 to 6 grams of phospholipids per day, not all of which would be PC derived from soy. Tables X and XI describe briefly the phagocytosis and "killing" assays.

50 Bloods were drawn on Days 0, 3, 7 and 14 and PMNs isolated according to accepted procedures. Phagocytized *Candida albicans* yeast particles were also determined according to accepted procedures.

Initially, four individuals were fed the PC and three, the placebo. A two week period of non-testing was observed at which time the groups were reversed and those who initially were given PC were given the placebo, and, conversely, the initial placebo-treated group was given PC.

## Results

65 The results for the first part of the study are shown on Tables XII, XIII and XIV. As was anticipated from the Klebsiella experiment, the effect of PC on phagocytosis

and "killing" was observed up to 4 days post-ingestion. By Day 14, the enhanced phagocytosis and "killing" was inconsistent. These experiments are being continued to determine more precisely the effects of one feeding versus several, how long the effect lasts, etc.

In summary, PC increases PMN phagocytosis and the percent of phagocytized *Candida albicans* killed.

#### Effect of PC on Arachidonic Acid Concentrations in PMNs Before and After Exposure to *Candida Albicans* and Relation to Phagocytosis/Killing

Since subjects were fed 4 to 5 times the average intake of lecithin (a lipid), and since lipid is the most abundant material on all cell membranes, it was important to determine if any changes in total lipid or fatty acids occurred in the PMNs of the subjects. The phospholipid used in these studies is PC with a fatty acid composition shown in Table XV. It contains principally linoleic acid (18:2 6) and no arachidonic acid (20:4 6).

Initial studies on screening the fatty acid composition of PMNs isolated from subjects who had ingested PC revealed a notable increase in 20:4 with minimal changes in other fatty acids. Since arachidonic acid plays an essential role in the production of several substances, such as vasoactive substances and immune modulators (i.e., prostaglandins and leukotrienes), we decided to assay the 20:4 levels in PMNs of the seven healthy adult subjects fed PC before and after exposure to *Candida albicans* and correlate changes, if any, in 20:4 with the phagocytic and "killing" results (previous section). Assays were done on Day 0 (baseline) and on Days 3 or 4, 7 or 8 and 13 or 14. The subjects reported on in the section entitled "The Effect of Ingested PC by Normal Healthy Young Adults on PMN Function" provided the PMNs making it possible to correlate the changes in phagocytosis and "killing" by PMNs with levels of 20:4 before and after PMN exposure to *Candida albicans*. Fatty acids were extracted from PMNs and assayed by GLC according to published methods.

FIG. 5 depicts the concentration (Units, relevant to a standard) of 20:4 in PMN before (unstimulated) and after (stimulated) exposure to *Candida albicans* ( $5 \times 10^6$  PMN to  $10 \times 10^6$  *C. albicans*) on Day 0 (baseline), 5, 7 and 14 from four subjects: two fed PC and two fed placebo for only three days. Fatty acid analysis of PMNs showed a 2 fold and a 4 to 5 fold increase in arachidonic acid on Days 5 and 7, respectively, from baseline values in PC group. No changes were noted when the individuals were ingesting the placebo. When these PMNs from PC fed individuals were exposed to *Candida albicans* the arachidonic levels returned to baseline levels. Additional data on seven other subjects is shown in Table XVI. Those results suggested that the enhanced microbicidal effects of PC may be partly mediated through this increase in arachidonic acid which is a precursor of a leukotriene, LTB<sub>4</sub>, a potent chemoattractant. There is a significant increase in LTB<sub>4</sub> release from PMNs isolated from subjects fed PC compared to normal individuals ingesting amounts found in the average American diet. No changes in arachidonic acid were noted in platelets; however, there is a need to look at other cell types (e.g., T and B cells).

Table XVII illustrates the association between the decrease (change) in PMN 20:4 levels after "stimulation" with *Candida* and the percent of phagocytized *Candida* killed, the latter data from the previous section (Table XIV).

#### Example 9

##### Effects of Soy-Lecithin (PC-95) on Neutrophil (PMN) Killing of *Candida Albicans* in AIDS Subjects

These subjects included 3 males and 4 females who ranged in age from 25 to 45 years old, and who had a history of intravenous drug abuse. At the time of study, 4 subjects were receiving methadone treatment and three subjects were methadone-free. Four of the seven had a documented history of oral candidiasis. The experimental design consisted of the oral administration of 18 grams of PC-95 to subjects in a single dose. PC-95 is a mixture of 95% phosphatidyl choline and 5% phosphatidyl ethanolamine. Blood samples were drawn pre-supplementation, and at 48 hours and 72 hours post-supplementation for analysis of PMN killing of *C. albicans*. The results of assays showed that PMN killing increased significantly over baseline (Table XVIII). In three of four methadone treated and one of three methadone-free subjects, the candidicidal activity increased 48 hours post-supplementation with the remainder of the study population showing the increase in PMN killing capacity 72 hours post-supplementation. PMN killing of *Candida* increased between 200 and 300 percent in each subject (Table XVIII). In four additional patients not shown in Table XVIII, comparable increases in neutrophil killing of *C. albicans* were observed. These results suggest significant clinical improvement may be obtained by PC-95 supplementation. Given the high recurrence rate of opportunistic infections in AAC and AIDS patients requiring repeated and costly physician visits and hospitalizations, PC-95 may be a promising treatment both for producing clinical improvement and reducing the financial strain on the medical care system.

#### EXAMPLE 10

##### Effects of Oral Soy Phosphatidylcholine Containing Linoleic Acid as the Fatty Acid Constituent on Polymorphonuclear Leukocyte (PMNL) Phagocytosis and Killing of *Candida albicans*

###### Subjects

Eight healthy adult volunteers, six males and two females, 23-35 years of age, supplemented their normal diets with either a noodle soup (placebo) or noodle soup to which 27 grams of soy phosphatidylcholine (PC) was added. The PC was 95% phosphatidylcholine and 5% lysophosphatidylcholine; its fatty acid composition is indicated in Table XIX. The placebo or PC was given in three divided servings daily for three days in an open crossover design. Initially, five individuals were fed PC and three placebo.

Following a two week period without supplementation, the groups were reversed. The PC provided approximately 18 grams of linoleic acid and 368 kcal/day. The placebo provide no linoleic acid and 125 kcal/day. Blood was drawn prior to the ingestion of the supplements and than at four, seven and fourteen days after initiation of the supplements. In four of these same subjects, after a two week hiatus, 18 grams of PC were administered in a single feeding and blood was drawn prior to ingestion and at 48 hours post-ingestion for leukotriene B<sub>4</sub> (LTB<sub>4</sub>).

Separate experiments were done in an open design in sixteen healthy adult volunteers after informed consent. Five males and three females, 23-63 years of age, sup-

plemented their normal diets with a single feeding of 18 grams of PC added to noodles.

Six males and two females, 23-63 years of age, supplemented their normal diets with a single feeding of 15 grams of safflower or soybean oil added to noodles. The noodles with PC provided 12 grams of LA and 287 kcal whereas the triglycerides, safflower or soybean oil, provided 12 grams of LA and 260 kcal. Blood was drawn at baseline and at 48 hours post-ingestion.

#### Analysis

PMNLs were isolated by the method of Boyum, A., *Scand J Clin Invest* 21: 77-89 (1968), and PMNL phagocytosis was measured by the method of Bridges, C. G., et al., *NEED JOURNAL* 42: 226-33 (1979), which involves inhibition of H-uridine uptake. PMNL killing of *Candida albicans* was determined by the method of Levitz, S. M., and Diamond, R. D., *J. Infect Dis*, 152(5): 938-45 (1985), utilizing the reduction of tetrazolium salt. PMNL lipids were extracted by the Folsch technique, Folsch, J., et al., *J. Biol Chem*, 226: 497-509 (1957). The addition of an internal standard, La-di-heptadeconylphosphatidylcholine (Avanti Polar Lipids, Birmingham, Ala.) to the Folsch extract enabled the quantitative analysis of the phospholipid fatty acids. Gravimetric analysis of lipid concentration was determined on an automatic electrobalance (Cahn/Ventron, Cerritos, Calif.) following solvent evaporation. The PMNL lipid extract was then streaked onto a 20×20 cm silica gel-G thin layer plate (Analtech, Inc. Newark, N.J.). Thin layer chromatography was performed in a hexane:diethyl ether: glacial acetic acid (70:30:1) solvent system, which separated the major lipid classes, phospholipids (origin), unesterified cholesterol (rF-0.25), free fatty acids (rf-0.45), triglycerides (rf-0.68) and cholesterol esters (rf-0.085). The phospholipid silica band was then scraped for the transmethylation process, Morrison, W., Lloyd, D. *J. Lipid Res*, 5: 600-8 (1976). The sample was subjected to 0.5 ml of boron trifluoride reagent (Supelco, Bellefonte, Pa.); 0.5 ml of fresh methanol (Fisher Scientific, Medford, Mass.) and then mixed with a Vortex mixer under nitrogen. This solution was heated in a steam bath at 80° C. for one hour to complete transesterification, Metcalfe, L. D., Schmitz A. A., *Anal. Chem.*, 33: 363-4, (1961). To stop the reaction, 1 ml of H<sub>2</sub>O was added. The fatty acid methyl esters were extracted with 5 ml of hexane (Fisher Scientific, Medford, Mass.). The hexane was then evaporated under nitrogen in preparation for gas liquid chromatographic (GLC) analysis. Phospholipid fatty acid methyl esters from PMNLs were separated using a Varian Model 3700 capillary gas-liquid chromatograph and a Varian DS-651 chromatographic workstation (Varian Instruments), Palo Alto, Calif.). The GLC was equipped with a Supelco 2330 fused silica capillary column (30 meters×0.25 mm I.D., 0.2 micron film) and a flame ionization detector (Varian Instruments). The optimal detection conditions were helium carrier gas at a linear velocity of 20 cm/second and a split ratio of 25:1. The capillary column temperature was 190° C. with injector and detector temperatures at 250° C. LTB<sub>4</sub> release was measured after exposure of PMNLs to the calcium ionophore, A21387, and to N-formyl-methionylleucylphenylalanine (fMLP) by H radioimmunoassay (Advanced Magnetics, Inc., Cambridge, Mass.) according to the method of Salmon, et al., *A Radioimmunoassay for Leukotriene B<sub>4</sub>. Prostaglandins*, 24(2): 225, (1982), Palmer, R. M. J., Salmon, J. A., *Immunology*, 50: 65-73, (1983).

Statistics were done utilizing Student t-tests, analysis of variance for four repeated measures and two independent groups, and Pearson correlations, Colton, T., *Statistics in Medicine*. Boston: Little-Brown, (1974).

#### Results

##### Neutrophil Function

Phagocytosis by PMNLs from subjects provided 27 grams of PC for three days increased 1.6 fold ( $p < 0.01$ ) at four days, peaked at 2.0 fold ( $p < 0.01$ ) times baseline seven days post-ingestion, and returned to baseline values at day fourteen (Table XX). Similarly, PMNL killing increased 2.6 fold ( $p < 0.01$ ) to peak by day four in the PC group, and returned nearly to baseline at fourteen days post-ingestion (Table XX). Compared with the subjects provided placebo, the increase in phagocytosis by PMNLs from the PC group was highly significant at days four and seven whereas killing was significantly increased at days four, seven and fourteen post-ingestion.

The results observed on study day four, one day after the last of three days of feeding 27 grams of PC per day, raised the issue as to whether the effects of three days of feeding PC were required for the observed effects or whether similar results in PMNL function could be observed after a single feeding. Also, were an effect observed after a single feeding, the question was raised as to the minimal time interval for an effect after a single dose.

In a preliminary experiment in which multiples of 9 grams of PC were given to eight subjects and bloods drawn at 24 hour intervals, it was found that 18 grams of PC providing 12 grams of LA was the minimal dose and 48 hours was the minimal time interval in which an increase in PMNL killing was observed (Table XXI).

##### Arachidonic Acid Composition of Neutrophil Phospholipids

The phospholipid arachidonic acid (PL-AA) content of PMNLs from the group fed 27 grams of PC increased 2.9 to 3.7 fold at four and seven days, respectively, post-PC ingestion (Table XX). A smaller, albeit significant, increase of 1.3 fold, was noted 48-hours post-ingestion of 18 grams of PC (Table XXI). Compared with the groups fed placebo or an equal amount of linoleic acid as safflower or soy oil the increases in PL-AA of PMNLs from the PC groups were highly significant at 48 hours (Table XXI). No significant changes were noted in the triglyceride fed groups at four or seven days post ingestion (Table XX).

##### Arachidonic Acid Release

The PL-AA release from PMNLs stimulated with *Candida albicans* increased 3.2 times that at baseline ( $p < 0.01$ ) at day four post PC ingestion, with a peak release of 5.3 times baseline ( $p < 0.001$ ) at day seven (Table XX). Forty-eight hours after a single 18 grams dose of PC, PL-AA release increased 1.5 times baseline (Table XXI). There were no significant changes in PL-AA release from PMNLs of placebo treated (Table XX) or safflower and soy oil treated subjects (Table XXI). Both PMNL phagocytosis and killing of *Candida albicans* were highly correlated ( $r = 0.872$  and  $r = 0.932$ , respectively), with PMNL PL-AA release.

##### Generation of the 5-Lipoxygenase Metabolite LTB<sub>4</sub>

In response to calcium ionophore, A21387, LTB<sub>4</sub> generation from PMNLs of the subjects fed the 18 grams of PC increased from 14.5 to 56.2 pg/(10<sup>5</sup>

PMNL) at 48 hours. In response to fMLP, LTB<sub>4</sub> increased from <0.05 pg/(10<sup>5</sup> PMNL) to 98.75 pg/(10<sup>5</sup> PMNL), (Table XXII). The PMNLs from one nonsupplemented subject showed no increase in LTB<sub>4</sub> generation when stimulated with either A21387 or fMLP.

### Conclusions

The results of these studies on humans and animals indicate that the administration or ingestion of PC (1) has long lasting effects, at least up to 4 days or longer post feeding or administration; (2) has beneficial effects in both children with bacterial and viral infections and in animals made septic; (3) is effective in the prevention and treatment of neonatal morbidity and mortality due to *T. gondii*, *K. pneumoniae*, *C. albicans* and streptococcal infections and thus to other bacterial, viral and protozoan infections; (4) increases the level of PMN arachidonic acid, presumably in the membrane, and that the enhancement of microbicidal activity of PMNs and macrophages seen in animals and humans fed PC is likely due to an increase in the precursor, arachidonic acid, of the leukotriene, LTB<sub>4</sub>; and (5) increases PMN and macrophage microbicidal activity as well as cellular and humoral responses. Dietary lipids and/or cholesterol in various quantitative and qualitative levels can alter the composition of the lipid membrane of all cells resulting in changes in structure and function. Knowing the rate at which cells differentiate, proliferate and depending on their half-life, the lipid composition of host defense cells can be altered by proper dosing (feeding) with PC, qualitatively and quantitatively, causing them to be more active resulting in lessening the morbidity and mortality of bacterial and viral infections in animals and humans. Furthermore, the data demonstrates that we can enhance the production of a leukotriene, LTB<sub>4</sub>, as well as enhance phagocytosis and intracellular killing by polymorphonuclear leucocytes and macrophages.

As with most intracellular pathogens—including HIV (AIDS virus)—replication, and thus severity of disease, occurs within the cell by altering receptor sites, rigidity (or fluidity) and function of cells. For example, the malaria parasite replicates within the red cells by causing perturbations in the lipid membrane of the RBC both in a qualitative and quantitative sense as does *Toxoplasma gondii* in macrophages. Macrophages play a key role in amplifying both cell mediated and humoral responses to infection and in disposing of foreign and infected cells.

These studies suggest that PC can diminish/suppress the conversion of AIDS positive individuals to clinical disease.

### Equivalents

Those skilled in the art will recognize, or be able to ascertain, using no more than routine experimentation, numerous equivalents to the specific substances and procedures described herein. Such equivalents are considered to be within the scope of this invention, and are covered by the following claims.

TABLE I

WEIGHT GAIN IN NEWBORN WISTAR RATS TREATED WITH PHOSPHATIDYL-CHOLINE (MEAN ± S.D. GRAMS)			
DAY	TREATMENT (SINGLE DOSE ip)		
	PBS CONTROL (n:6)	P.C. 95 (n:7)	p
0	8.6 ± 0.3	9.1 ± 0.4	NS

TABLE I-continued

WEIGHT GAIN IN NEWBORN WISTAR RATS TREATED WITH PHOSPHATIDYL-CHOLINE (MEAN ± S.D. GRAMS)			
DAY	TREATMENT (SINGLE DOSE ip)		
	PBS CONTROL (n:6)	P.C. 95 (n:7)	p
2	9.8 ± 0.3	10.4 ± 0.6	NS
5	11.9 ± 0.4	12.4 ± 0.4	NS
7	14.9 ± 0.5	15.4 ± 0.4	NS
9	17.9 ± 0.8	18.7 ± 0.7	NS
11	20.5 ± 0.7	21.1 ± 0.8	NS
14	20.2 ± 1.0	31.2 ± 0.7	0.001
16	21.9 ± 1.1	35.8 ± 1.1	0.001
19	24.3 ± 1.2	39.1 ± 1.1	0.001
50	123.0 ± 6.5	151.0 ± 16.4	0.01

TABLE II

CLINICAL AND LABORATORY CHARACTERISTICS OF STUDY GROUPS ON ADMISSION		
Characteristics	Protocol	
	Lecithin	Placebo
No. of Patients	40	36
Male	21	19
Female	19	17
Age (months)		
Mean ± S.E.	66 ± 11.3	40 ± 9.13
Range	5-192	6-180
Fever: mean °C. ± S.E.	33.31 ± 0.14	38.53 ± 0.14
Total WBC ± S.E.	12,203 ± 1,490	15,668 ± 1,453
Infections:		
Bacterial	26	25
Viral	12	11
Other	2	0

TABLE III

NATURAL KILLER CELL ACTIVITY IN 60 CHILDREN WITH INFECTION (Effector: Target* ratio 50:1)			
Group:	% Killing ± S.E.		
	Day 0	Day 3	Day 7
Placebo n = 30	19.78 ± 1.10	18.33 ± 1.18	20.09 ± 1.16
PC n = 30	18.89 ± 1.09	26.01 ± 1.15	28.78 ± 1.52
Control n = 30		20.67 ± 1.69	

\*Target K562 16-hour <sup>51</sup>Cr release assay.

TABLE IV

NATURAL KILLER CELL ACTIVITY IN 60 CHILDREN WITH INFECTION (Effector: Target* ratio 25:1)			
Group:	Killing ± S.E.		
	Day 0	Day 3	Day 7
Placebo n = 30	13.42 ± 0.89	13.19 ± 0.75	14.03 ± 0.80
PC n = 30	13.61 ± 0.76	20.96 ± 1.33	22.49 ± 1.29
Control n = 30		13.85 ± 1.35	

\*Target K562 16-hour <sup>51</sup>Cr release assay.

TABLE V

NATURAL KILLER CELL ACTIVITY IN 60 CHILDREN WITH INFECTION (Effector: Target* ratio 12.5:1)			
Group:	Killing ± S.E.		
	Day 0	Day 3	Day 7
Placebo n = 30	8.95 ± 0.84	8.56 ± 0.88	10.12 ± 1.04

TABLE V-continued

Group	Killing $\pm$ S.E.		
	Day 0	Day 3	Day 7
PC n = 30	8.91 $\pm$ 0.78	14.89 $\pm$ 1.30	15.44 $\pm$ 1.16
Control n = 30		10.64 $\pm$ 1.19	

\*Target K562 16-hour  $^{51}\text{Cr}$  release assay

TABLE VI

No. of Subjects	Percent $\pm$ S.E.	
	Phagocytosis	Intracellular Killings
n = 85	43.73 $\pm$ 1.73	85.61 $\pm$ 1.94

\* $^3\text{H}$ -Uridine Assay in Mexican study population. *Staphylococcus aureus*: PMN ratio, 5:1

TABLE VII

Group	Day 0	% Phagocytosis $\pm$ S.E.	
		Day 3	Day 7
Placebo n = 36	33.45 $\pm$ 2.85	30.63 $\pm$ 2.55	28.82 $\pm$ 1.67
PC n = 40	31.98 $\pm$ 2.48	42.40 $\pm$ 2.31	36.02 $\pm$ 2.58
Control n = 85		46.73 $\pm$ 1.73	

\**Staphylococcus aureus*: PMN ratio, 5:1 AB serum used Mexican study population

TABLE VIII

White Cell	Source	NBT Reduction (O.D.) ( $1.5 \times 10^6$ cells)	
		PC* (6 rats)	PBS* (6 rats)
PMNs	Blood	.065 (.011)	.052 (ns) (.011)
PMNs	Sponge	0.083 (.019)	.047 (ns) (.012)
Macrophages	Peritoneal	0.238 (.043)	.244 (ns) (.062)

\*0.5 ml PC (5 mg) PBS/sponge (implanted - 18 hrs)

\*\*Peritoneal macrophages obtained prior to sacrifice with 10 ml PBS lavage (i.p.)

TABLE IX

White Cell	Source	NBT Reduction (O.D.)	
		PC-i.p.	PBS
PMNs	Blood	.074 (.012)	.038 sig. (.010)
PMNs	Sponge	0.132 (.015)	.079 sig. (.009)
Macrophages	Peritoneal	0.160 (.011)	.112 sig. (.011)

NBT Reduction - Mean ( $\pm$  S.E.) ( $1.5 \times 10^6$  cells)

PC (1 ml) 800 mg/Kg or PBS (1 ml) on day -4; Sponge implanted - 18 hrs

Peritoneal cells obtained prior to sacrifice with 10 ml of PBS lavage (i.p.)

P = &lt; .05

TABLE X

	PHAGOCYTOSIS	
	PMN ( $2.0 \times 10^5$ )	<i>C. Albicans</i> ( $5 \times 10^5$ )
5	Blanks (2)	PMNs & <i>C. Albicans</i>
	a) media	
	b) PMNs	Incubate 1 hour (add $^3\text{H}$ -Uridine (30') Harvest cells count
10	<1% CMP	
	Phagocytized <i>C. Albicans</i> do not take up label and do not adhere	

TABLE XI

PMN ( $2.5 \times 10^5$ )	"KILLING" ASSAY	
	PMN and Heat "killed" <i>C. Albicans</i>	Viable <i>C. Albicans</i>
15		
	Incubate 1 hour Wash (3x) 300 ul H <sub>2</sub> O	
20	Centrifuge-Resuspend(NTB)-(incubate 3 hrs)-Centrifuge Pellet - add 400 ul HCl/Isopropyl alcohol Mix - read at O.D. 570	
	(live <i>C. Albicans</i> converts NTB - blue color)	

TABLE XII

Days	Phagocytosis (%) of <i>Candida Albicans</i> * by PMNs from Healthy Adults Fed Phosphatidyl Choline for Three Days**		
	0	3-4	7-8
30			
PC (4)	34.5 (23-56)	43.5 (32-55)	60.0 (52-74)
Placebo (3)	30.7 (22-32)	32.3 (31-33)	21.7 (16-27)

\*PMN/*C. Albicans* - ( $2.5 \times 10^6/5 \times 10^6$ )

\*\*27 g PC/day/3 days

TABLE XIII

Days	Killing (%) of <i>Candida Albicans</i> * by PMNs from Healthy Adults Fed Phosphatidyl Choline for Three Days**		
	0	3-4	7-8
40			
PC (4)	19 (15-22)	40.2 (28-60)	38.2 (31-43)
Placebo (3)	35 (10-52)	24.7 (11-46)	31.7 (21-44)

\*PMN/*C. Alb.* -  $2.5 \times 10^6/5 \times 10^6$ 

\*\*27 g PC/day/3 days

TABLE XIV

Days	Percent of Phagocytized <i>C. Albicans</i> * Killed by PMNs Isolated from Healthy Adults Fed Phosphatidyl Choline for Three Days**		
	0	3-4	7-8
50			
PC (4)	6.3 (3.9-8.4)	17.8 (11.9-31.8)	22.8 (18.0-29.6)
Placebo (3)	10.4 (3.2-16.7)	8.0 (3.6-15.2)	8.4 (4.8-10.2)

\*PMN/*C. Albicans* -  $2.5 \times 10^6/5 \times 10^6$ 

\*\*27 g PC/day/3 days

TABLE XV

FATTY ACID	SOY LECITHIN (% weight)	
65		
16:0		12.8
16:1w7		0.2
18:0		2.9
18:1w6		-
18:1w9		10.6
18:2w6		65.9

TABLE XV-continued

FATTY ACID COMPOSITION OF SOY LECITHIN	
FATTY ACID	SOY LECITHIN (% weight)
18:3w3	6.5
20:1w9	0.2
20:4w6	—
20:5w3	—

TABLE XVI

Effect of Oral PC on Arachidonic Acid (20:4) Levels of PMNs and After Exposure to <i>Candida Albicans</i>			
Days	0	3-4	7-8
PC (4)			
Before	6.4 (3.1-9.0)	10.4 (3.8-20.6)	17.3 (5.7-29.1)
20:4 (Units)			
Change	-3.9 (2.3-6.2)	-8.6 (2.7-15.9)	-15.3 (4.8-28.6)
Placebo (3)			
Before	4.1 (3.2-5.8)	2.6 (1.8-3.3)	2.6 (1.9-3.7)
20:4 (Units)			
Change	-2.6 (1.4-4.1)	-1.8 (1.3-1.5)	-1.9 (1.5-2.5)

TABLE XVIII

THE EFFECTS OF SOY-LECITHIN (PC-95) ON NEUTROPHIL KILLING OF <i>CANDIDA ALBICANS</i> IN SEVEN HIV POSITIVE PATIENTS			
DAYS	BASELINE		
	48 HOURS	72 HOURS	
SUBJECT 1	10.4%	22.2%	16.5%
SUBJECT 2	16.3%	32.1%	20.4%
SUBJECT 3	11.8%	36.6%	18.3%
SUBJECT 4	8.0%	24.9%	14.5%
SUBJECT 5	19.6%	20.3%	55.7%
SUBJECT 6	47.9%	53.2%	57.1%
SUBJECT 7	31.6%	25.1%	56.7%

\*percent killing of *C. albicans* by MTT reduction

TABLE XIX

THE FATTY ACID COMPOSITION OF SOY PHOSPHATIDYLCHOLINE	
FATTY ACID	PERCENT
Palmitic (16:0)	12.8
Palmitoleic (16:1 n7)	0.2
Stearic (18:0)	2.9
Oleic (18:1 n9)	10.6
Linoleic (18:2 n6)	65.9
Linolenic (18:3 n3)	6.5
Eicosenoic (20:1 n9)	0.2
Arachidonic (20:4 n6)	—
Eicosapentaenoic (20:5 n3)	—

TABLE XX

EFFECTS OF ORAL PHOSPHATIDYLCHOLINE ON NEUTROPHIL FUNCTION AND ARACHIDONATE IN NORMAL ADULTS*								
Days	Phagocytosis of <i>Candida albicans</i> (%)		Killing of <i>Candida albicans</i> (%)		Arachidonic acid levels (ng/10 <sup>5</sup> PMNL)		Arachidonic acid release	
	Placebo	PC	Placebo	PC	Placebo	PC	Placebo	PC
	0	25.8 ± 4.1	29.3 ± 4.5	15.6 ± 2.7	16.0 ± 1.2	35 ± 8	38 ± 11	25 ± 8
4	32.1 ± 2.9	48.0 ± 5.4 <sup>+S</sup>	20.2 ± 4.9	42.1 ± 4.9 <sup>+S</sup>	29 ± 11	109 ± 25 <sup>+S</sup>	17 ± 8	74 ± 16 <sup>+S</sup>
7	27.4 ± 4.0	59.4 ± 5.2 <sup>+S</sup>	19.7 ± 4.3	39.9 ± 3.7 <sup>+S</sup>	19 ± 3	142 ± 26 <sup>+ + II</sup>	10 ± 3	123 ± 26 <sup>+ + II</sup>
14	31.4 ± 3.4	33.0 ± 3.9	11.0 ± 1.4	18.6 ± 1.4 <sup>S</sup>	17 ± 3	42 ± 7	8 ± 3	17 ± 3

\*N = 8; PC, soy phosphatidylcholine. Values expressed as mean ± SEM PMNL/*Candida albicans* = 2.5 × 10<sup>6</sup> × 10<sup>6</sup>

<sup>+S</sup>P < 0.01 vs. baseline

<sup>+ +</sup>P < 0.001 vs. baseline

<sup>S</sup>P < 0.01 vs. placebo

<sup>..</sup>P < 0.001 vs. placebo

TABLE XXI

EFFECTS OF PHOSPHATIDYLCHOLINE OR TRIGLYCERIDE (SAFFLOWER OR SOYBEAN OIL) ON NEUTROPHIL KILLING OF <i>Candida albicans</i> AND ARACHIDONATE LEVELS*							
	Killing of <i>Candida albicans</i> <sup>+</sup> (%)		Arachidonic acid levels (ng/10 <sup>5</sup> PMNL)		Arachidonic acid release		
	SO	PC	SO	PC	SO	PC	
	Baseline	24.6 ± 4.4	21.2 ± 4.1	28 ± 6	29 ± 3	20 ± 8	21 ± 7
48 hours	18.2 ± 1.8	36.8 ± 3.7 <sup>+ +</sup>	22 ± 3	39 ± 3 <sup>+ +</sup>	19 ± 3	31 ± 4 <sup>+ +</sup>	

\*Both phosphatidylcholine and triglycerides provided 12 grams linoleic acid; N = 8 normal adults, SO, safflower oil or soybean oil, PC, phosphatidylcholine. Values expressed as mean ± SEM.

<sup>+</sup>PMNL/*C. albicans* = 2.5 × 10<sup>6</sup>/5 × 10<sup>6</sup>

<sup>+ +</sup>P < 0.01 vs. baseline

TABLE XVII

Association Between Δ20:4 and Percent of Phagocytized <i>Candida Albicans</i> Killed (% P/K)			
Days	0	3-4	7-8
PC (4)			
% P/K	6.3	17.8	22.8
Δ20:4	-3.9	-8.6	-15.3
Placebo (3)			
% P/K	10.4	8.0	8.4
Δ 20:4	-2.6	-1.8	-2.6

TABLE XXII

GENERATION OF LEUKOTRIENE B <sub>4</sub> BY NEUTROPHILS IN RESPONSE TO CALCIUM IONOPHORE, A21387, AND fMLP*		
	A21387 (n = 4)	fMLP (n = 4)
Baseline	14.5 ± 5.6	<0.05

5

10

15

20

25

60

65

TABLE XXII-continued

PC - 48 hours	56.2 ± 21.4	98.75 ± 33.9
---------------	-------------	--------------

\*fMLP, N-formyl-methionyl-leucyl-phenylalanine. PC, phosphatidyl-choline provided as a single dose 18 grams. Values expressed as mean ± SEM.

P < 0.01 vs baseline

P < 0.01 vs baseline

I claim:

1. A method of treating Group B Streptococcal infection in human neonates comprising administering to a human neonate having Streptococcal infection an effective therapeutic amount of phosphatidylcholine.

2. A method of prophylaxis of Group B Streptococcal infection in human neonates comprising administering to a human neonate susceptible to Group B Streptococcal infection an effective prophylactic amount of phosphatidylcholine.

3. A method of preventing bacterial infection in animal neonates comprising administering to the animal mother having a bacterial infection or susceptible to a bacterial infection prior to parturition, an effective amount of a phosphoglyceride selected from the group consisting of phosphatidylcholine, phosphatidyletha-

nolamine, phosphatidylserine and phosphatidylinositol, or mixtures thereof.

4. A method of claim 3, wherein the phosphoglyceride is phosphatidylcholine.

5. A method of preventing vertical transmission of bacteremia from human mother to fetus comprising administering to the human mother an effective amount of a phosphoglyceride selected from the group consisting of phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine and phosphatidylinositol, or mixtures thereof.

6. A method of claim 5, wherein the phosphoglyceride is phosphatidylcholine.

7. A method of preventing vertical transmission of bacteremia from human mother to fetus comprising administering to the human mother an effective amount of a phosphoglyceride containing linoleic acid as the primary fatty acid constituent.

8. A method of treating bacterial infection in human neonates comprising administering to a human neonate having bacterial infection an effective therapeutic amount of phosphoglyceride containing linoleic acid as the primary fatty acid constituent.

\* \* \* \* \*

25

30

35

40

45

50

55

60

65

# Effects of oral soy phosphatidylcholine on phagocytosis, arachidonate concentrations, and killing by human polymorphonuclear leukocytes<sup>1-3</sup>

Peter W Jannace, Robert H Lerman, Jose Ignacio Santos, and Joseph J Vitale

**ABSTRACT** A dietary supplement of linoleic acid (LA) as soy phosphatidylcholine (PC) or as triglyceride on polymorphonuclear leukocyte (PMNL) functions, arachidonate (AA) concentrations, AA release, and leukotriene B<sub>4</sub> (LTB<sub>4</sub>) generation was studied in normal adults. Study 1: Eight subjects were fed PC (27 g) or placebo for 3 d in a blinded crossover experiment with PMNL assays at baseline and 4, 7, and 14 d. Study 2: Subjects were fed equal quantities of LA as PC (18 g, *n* = 8), safflower (SF, *n* = 4), or soybean oil (SY, *n* = 4) with PMNL assays at baseline and 48 h. Study 1: PC increased PMNL phagocytosis and killing of *Candida albicans* twofold (*P* < 0.001) and PMNL phospholipid AA content threefold (*P* < 0.001); AA release after *Candida albicans* stimulation increased 5.3-fold, correlating with PMNL killing (*r* = 0.932) and phagocytosis (*r* = 0.872). Study 2: PC, but not SF or SY, produced changes similar to those of study 1. With PMNL exposure to calcium ionophore A23187 or *N*-formyl-methionyl-leucyl-phenylalanine, PC increased LTB<sub>4</sub> generation. Phospholipid LA, in contrast to triglyceride LA, enhanced PMNL phospholipid AA, phagocytosis, and killing. *Am J Clin Nutr* 1992;56:599-603.

**KEY WORDS** Polymorphonuclear leukocyte, *Candida albicans*, linoleic acid, arachidonic acid, phagocytosis, killing, leukotriene B<sub>4</sub>, humans

## Introduction

Linoleic acid (LA, 18:2n-6), an ω-6 fatty acid, is an essential dietary nutrient. It is the principal polyunsaturated fatty acid in safflower oil (SF) and soy bean oil (SY), which have been shown to be immunosuppressive in studies involving both humans and animals (1-9). It is reasonable to assume that the effects of these oils on immune functions are mediated through LA.

Most of the studies that have examined the effects of dietary lipids on immune functions or nonspecific host defenses (eg, response to mitogen or phagocytosis) have used neutral fats (ie, triglycerides or oils) as the major or sole component of the lipid used. However, in addition to oils, a significant source of dietary fat consumed in the conventional Western diet is in the form of phospholipids. Thus, it was of interest to study the relative effects of an oral supplement of LA as a component of major sources of dietary fat: ie, phospholipids as phosphatidylcholine (PC) and neutral fats as SF and SY on polymorphonuclear leukocyte (PMNL) functions (phagocytosis and killing), PMNL arachidonic acid (AA) content and release, and on leukotriene B<sub>4</sub> (LTB<sub>4</sub>) generation from stimulated PMNLs.

## Subjects and methods

### Subjects

**Study 1.** Eight healthy adult volunteers, six males and two females, 23-35 y of age, supplemented their normal diets for 3 d with either a noodle-soup placebo (PL) or noodle soup to which 27 g soy PC was added. The PC was 95% PC choline and 5% lysophosphatidylcholine; its fatty acid composition is indicated in Table 1. PL or PC was given in three divided servings daily for 3 d in an open crossover design. Initially, five individuals were fed PC and three PL. After a 2-wk period without supplementation, the groups were reversed.

The PC provided ≈18 g LA and 1.5 kJ/d. PL provided no LA and 0.5 kJ/d. A fasting blood sample was drawn before the ingestion of the supplements and then at 4, 7, and 14 d after initiation of the supplements. In four of the above subjects and after a 2-wk hiatus, 18 g PC was administered in a single feeding and blood was drawn before ingestion and at 48 h postingestion for LTB<sub>4</sub> generation from stimulated PMNLs.

**Study 2.** Five males and three females, 23-63 y of age, supplemented their normal diets with a single feeding of either 18 g PC or 15 g SF (*n* = 4) or SY (*n* = 4), which were added to noodles. The 18 g noodles to which were added PC, SF, or SY provided 12 g LA and ≈1.2 kJ/serving. In this experiment blood was drawn at baseline and at 48 h postingestion. The results from the blood samples taken from the four individuals fed SF and the four fed SY were pooled because they were not statistically different (*P* > 0.2) from each other.

### Experimental protocol

Signed informed-consent forms were obtained from all subjects in accordance with the ethical standards of the Institutional Review Board for Human Research, Boston University Medical Center.

<sup>1</sup> From the Department of Pathology and Nutrition Pathology Unit, Mallory Institute of Pathology; the Clinical Nutrition Unit, Evans Memorial Department of Clinical Research and Preventive Medicine, University Hospital; and the Boston University School of Medicine, Boston.

<sup>2</sup> Supported in part by the Boston University School of Medicine.

<sup>3</sup> Reprints not available.

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TABLE 1  
The fatty acid composition of soy phosphatidylcholine

Fatty acid	Composition	
	%	
Palmitic (16:0)	12.8	
Palmitoleic (16:1n-7)	0.2	
Stearic (18:0)	2.9	
Oleic (18:1n-9)	10.6	
Linoleic (18:2n-6)	65.9	
Linolenic (18:3n-3)	6.5	
Eicosenoic (20:1n-9)	0.2	
Arachidonic (20:4n-6)	—	
Eicosapentaenoic (20:5n-3)	—	

### Procedures

PMNLs were isolated by the method of Boyum (10) and PMNL phagocytosis was measured by the method of Bridges et al (11), which involves inhibition of [<sup>3</sup>H]uridine uptake. PMNL killing of *Candida albicans* was determined by the method of Levitz and Diamond (12) by using the reduction of tetrazolium salt. PMNL lipids were extracted by the Folch et al technique (13). The addition of an internal standard, L- $\alpha$ -di-heptadecanoyl-phosphatidyl-choline (Avanti Polar Lipids, Birmingham, AL) to the Folch extract enabled the quantitative analysis of the phospholipid fatty acids. Gravimetric analysis of lipid concentration was determined on an automatic electrobalance (Cahn/Ventron, Cerritos, CA) after solvent evaporation. The PMNL lipid extract was then streaked onto a 20 × 20-cm silica gel-G thin-layer plate (Analtech, Inc, Newark, NJ). Thin-layer chromatography was performed in a hexane:diethyl ether:glacial acetic acid (70:30:1, by vol) solvent system, which separated the major lipid classes: phospholipids (origin), unesterified cholesterol ( $rF = 0.25$ ), free fatty acids ( $rF = 0.45$ ), triglycerides ( $rF = 0.68$ ), and cholesterol esters ( $rF = 0.85$ ). The phospholipid silica band was then scraped for the transmethylation process (14). The sample was subjected to 0.5 mL boron trifluoride reagent (Supelco, Bellfonte, PA) and 0.5 mL fresh methanol (Fisher Scientific Co, Medford, MA) and then mixed under nitrogen with a Vortex-Genie mixer (Fisher

Scientific Co). This solution was heated in a steam bath at 80 °C for 1 h to complete transesterification (15). To stop the reaction, 1 mL H<sub>2</sub>O was added. The fatty acid methyl esters were extracted with 5 mL hexane (Fisher Scientific).

The hexane was then evaporated under nitrogen in preparation for gas-liquid-chromatographic (GLC) analysis. Phospholipid fatty acid methyl esters from PMNLs were separated with a Varian Model 3700 capillary gas-liquid chromatograph and a Varian DS-651 chromatographic workstation (Varian Instruments, Palo Alto, CA). The GLC was equipped with a Supelco 2330 fused silica capillary column (30 m × 0.25 mm ID, 0.2- $\mu$ m film) and a flame ionization detector (Varian Instruments). The optimal detection conditions were helium carrier gas at a linear velocity of 20 cm/s and a split ratio of 25:1. The capillary column temperature was 190 °C with injector and detector temperatures at 250 °C. AA release from isolated PMNLs was measured after their exposure to *Candida albicans*.

LTB<sub>4</sub> release was measured after exposure of PMNLs to the calcium ionophore A23187 and to *N*-formyl-methionyl-leucyl-phenyl-alanine (fMLP) by <sup>3</sup>H radioimmunoassay (Advanced Magnetics, Inc, Cambridge, MA) according to published methods (16, 17).

### Statistical analysis

Statistics were done by using Student's *t* tests, analysis of variance for four repeated measures and two independent groups, and Pearson correlations (18).

## Results

### Neutrophil function

Phagocytosis by PMNLs from subjects provided 27 g PC for 3 d increased 1.6-fold ( $P < 0.01$ ) at 4 d, peaked at 2.0-fold ( $P < 0.01$ ) times baseline 7 d postingestion, and returned to baseline values at day fourteen (Table 2). Similarly, PMNL killing increased 2.6-fold ( $P < 0.01$ ) to peak by day four in the PC group and returned to baseline at 14 d postingestion (Table 2) by analysis of variance. Compared with the subjects provided PL, the increase in phagocytosis by PMNLs from the PC group was highly significant at days four and seven whereas killing was

TABLE 2  
Effects of oral soy phosphatidylcholine (PC) on neutrophil function and arachidonate in normal adults\*

Day	Phagocytosis of <i>Candida albicans</i>		Killing of <i>Candida albicans</i> †		Arachidonic acid concentration		Arachidonic acid release	
	Placebo	PC	Placebo	PC	Placebo	PC	Placebo	PC
	%		%		ng/10 <sup>5</sup> PMNL	ng/10 <sup>5</sup> PMNL	ng/10 <sup>5</sup> PMNL	ng/10 <sup>5</sup> PMNL
0 (baseline)	25.8 ± 4.1	29.3 ± 4.5	15.6 ± 2.7	16.0 ± 1.2	35 ± 8	38 ± 11	25 ± 8	23 ± 7
4	32.1 ± 2.9	48.0 ± 5.4‡§	20.2 ± 4.9	42.1 ± 4.9‡§	29 ± 11	109 ± 25‡§	17 ± 8	74 ± 16‡§
7	27.4 ± 4.0	59.4 ± 5.2‡§	19.7 ± 4.3	39.9 ± 3.7‡§	19 ± 3	142 ± 26¶¶	10 ± 3	123 ± 25¶¶
14	31.4 ± 3.4	33.0 ± 3.9	11.0 ± 1.4	18.6 ± 1.4§	17 ± 3	42 ± 7	8 ± 3	17 ± 3

\*  $\bar{x} \pm SE$ ;  $n = 8$ . PMNL, polymorphonuclear leukocyte.

† PMNL/*Candida albicans* =  $2.5 \times 10^6/5 \times 10^6$ .

‡|| Significantly different from baseline (ANOVA): ‡ $P < 0.01$ , || $P < 0.001$ .

§¶ Significantly different from placebo (ANOVA): § $P < 0.01$ , ¶ $P < 0.001$ .

TABLE 3

Effect of phosphatidylcholine (PC) or triglyceride [safflower or soybean oil (SO)] on neutrophil killing of *Candida albicans* and arachidonate concentrations\*

	Killing of <i>Candida albicans</i> †		Arachidonic acid concentration		Arachidonic acid release	
	SO	PC	SO	PC	SO	PC
	%	%	ng/10 <sup>5</sup> PMNL	ng/10 <sup>5</sup> PMNL	ng/10 <sup>5</sup> PMNL	ng/10 <sup>5</sup> PMNL
Baseline	24.6 ± 4.4	21.2 ± 4.1	28 ± 6	29 ± 3	20 ± 8	21 ± 7
48 h	18.2 ± 1.8	36.8 ± 3.7‡§	22 ± 3	39 ± 3‡§	19 ± 3	31 ± 4‡§

\*  $\bar{x} \pm SE$ ;  $n = 8$  normal adults. Both PC and triglycerides provided 12 g linoleic acid. PMNL, polymorphonuclear leukocyte.† PMNL/*Candida albicans* =  $2.5 \times 10^6/5 \times 10^6$ .‡ Significantly different from baseline,  $P < 0.01$  (ANOVA).§ Significantly different from SO,  $P < 0.01$  (ANOVA).

significantly increased at days four, seven, and fourteen post-ingestion.

The results observed on study day four, 1 d after the last of 3 d of feeding 27 g PC/d, raised the question as to whether the effects of 3 d of feeding PC were required for the observed effects or whether similar results in PMNL function could be observed after a single feeding.

In a preliminary experiment in which multiples of 9 g PC were given to eight subjects and blood samples drawn at 24-h intervals, it was found that 18 g PC providing 12 g LA was the minimal dose and that sometime between 24 and 48 h was the minimal time interval in which an increase in PMNL killing was observed. Thus, 48 h postingestion of 12 g LA in PC, PMNL killing of *Candida albicans* increased 1.7-fold whereas no increase in PMNL killing occurred after the ingestion of an equal amount of LA in SF or SY (Table 3), by analysis of variance.

#### AA composition of PMNL phospholipids

The phospholipid AA content of PMNLs from the group fed 27 g PC for 3 d increased 2.9- to 3.7-fold at 4 and 7 days, respectively (Table 2). A smaller albeit significant increase of 1.3-fold was noted 48-h postingestion of 18 g PC (Table 3). The increases in phospholipid AA of PMNLs from the PC-fed subjects were highly significant at 48 h (Table 3) whereas those fed an equal amount of LA as SF or SY were not. No significant changes were noted in the PL-fed subjects at 4 or 7 d postingestion (Table 2).

#### AA release

The phospholipid AA release from PMNLs stimulated with *Candida albicans* increased 3.2 times that at baseline ( $P < 0.01$ ) at day four post PC ingestion with a peak release of 5.3 times baseline ( $P < 0.001$ ) at day seven (Table 2). Forty-eight hours after a single 18 g dose of PC, phospholipid AA release increased 1.5 times baseline (Table 3). There were no significant changes in phospholipid AA release from PMNLs of PL-fed (Table 2) or SF- and SY-fed subjects (Table 3). Both PMNL phagocytosis and killing of *Candida albicans* were highly correlated ( $r = 0.872$  and  $r = 0.932$ , respectively) with PMNL phospholipid AA release.

#### Generation of the 5-lipoxygenase metabolite LTB<sub>4</sub>

In response to calcium ionophore A23187, LTB<sub>4</sub> generation from PMNLs of the eight subjects fed 18 g PC increased from

14.5 to 56.2 pg/10<sup>5</sup> PMNLs at 48 h ( $P < 0.01$ ) (Table 4). In response to fMLP, LTB<sub>4</sub> increased from  $< 0.05$  to 98.8 pg/10<sup>5</sup> PMNLs ( $P < 0.001$ ).

#### Discussion

The results of this study have demonstrated that a dietary lipid, soy PC, can modify the composition and concentration of a major fatty acid of the PMNL, ie, AA (19). Added to a normal unrestricted diet, PC enhanced neutrophil phagocytosis and killing of *Candida albicans* in an ex vivo system. The mechanism of action may be related to alterations in the membrane lipid composition of the PMNL leading to release of vasoactive eicosanoids, bioregulators of immune responses (20–26). Specifically, the oral ingestion of PC, which contains no AA, resulted in increased concentrations of AA in the PMNLs. In addition, LTB<sub>4</sub>, a potent chemotactic agent for PMNLs (27, 28), which also stimulates aggregation (29), degranulation (30), and hexose transport of PMNLs (31), was generated in response to fMLP and A23187 by PMNLs from subjects fed PC.

In contrast, neutral fats such as SF or SY, which provided equivalent amounts of the precursor fatty acid LA, had no effect on PMNL function or PMNL fatty acid composition in this study. The possibility that an equivalent amount of choline, glycerol, or phosphate added to an SF or SY preparation would have resulted in the same effects as produced by PC feeding seems unlikely. PC was added to the routine diet, which was assumed to contain more than adequate amounts of choline, phosphorus, and glycerol, albeit in different forms.

TABLE 4

Generation of leukotriene B<sub>4</sub> by neutrophils in response to calcium ionophore A23187 and fMLP\*

	A23187	fMLP
	ng/10 <sup>5</sup> cells	
Baseline	14.5 ± 5.6	<0.05
PC, 48 h	56.2 ± 21.4†	98.8 ± 33.9‡

\*  $\bar{x} \pm SE$ ;  $n = 8$ . fMLP, *N*-formyl-methionyl-leucyl-phenylalanine; PC, phosphatidylcholine provided as a single 18-g dose.†‡ Significantly different from baseline (ANOVA): † $P < 0.01$ , ‡ $P < 0.001$ .

The possible difference between neutral fat and phospholipid LA may be related to differences in their absorption, metabolism, or utilization (32–34). Phospholipids, although composing only 5–9% by weight, form 40–65% of the outer surface of chylomicrons (35). This may facilitate an exchange of phospholipids with the lipophilic cellular membranes. Neutral fats, on the other hand, are carried mainly in the triglyceride-rich core and cannot exchange easily with the lipid components of cellular membranes.

The 48-h, or less, hiatus and the duration of the observed PC effect on PMNL phagocytosis and killing, extending to  $\geq 7$  days, is of interest. Because PMNLs have a short survival period in the circulating blood, PC would not be expected to have a significant impact on PMNL plasma membranes. Also, it is unlikely that the absorption of the PC, and its subsequent metabolic by-products, is delayed beyond 12–24 h. A reasonable explanation for the hiatus in the PC effect is that it occurs in hematopoietic sites with the LA from the PC being incorporated, desaturated, and elongated to AA in progenitor cells. The increase and incorporation of eicosatrienoic and eicosapentaenoic acids in phospholipids of PMNLs and other cell types after feeding these fatty acids to humans and animals is delayed, suggesting that short-lived circulating cells such as PMNLs do not exchange lipids in their membranes freely or easily (36–39).

In conventional Western diets the major lipids are of the  $\omega$ -6 (eg, LA) and not of the  $\omega$ -3 (eg, marine oils) class. As a result, the major fatty acids of the phospholipid bilayer of the cell membranes of individuals consuming Western diets are principally of the  $\omega$ -6 series. Because  $\omega$ -3 and  $\omega$ -6 fatty acids compete for the enzymes involved in desaturation and elongation, depending on the quantity of the lipid source fed, either  $\omega$ -3 or  $\omega$ -6 fatty acids will predominate on the membrane. Therefore, the ingestion of large amounts of marine oils such as eicosapentaenoic acid (C20:5 n-3) can lead to displacement of phospholipid AA on many cell types, including PMNLs, and thus may effect the metabolic processes and function of these cells (37, 40–44).

PMNLs, which are the first cells to invade sites of infection, can produce chemotactic or chemoattractive metabolites as can infectious agents (45, 46). These metabolites serve a beneficial purpose in mitigating against infections. In response to bacteria, or any agent that perturbs the cell membrane, there is a concomitant increase in the activity of phospholipase A2 and release of AA resulting in an increased production of the potent chemotactic and chemoattractant eicosanoid, LTB<sub>4</sub> (28). Displacement of  $\omega$ -6 with  $\omega$ -3 fatty acids on cell membranes results in the generation of the leukotriene LTB<sub>5</sub>, a weak chemotactic and chemoattractant analogue (40). In any case, no attempt was made to measure the leukotriene LTB<sub>5</sub>, notwithstanding that a small concentration of linolenic acid ( $\omega$ -3), < 10% of the concentration of LA (an  $\omega$ -6 fatty acid), is present in PC. A review of several studies supports the view that the leukotriene LTB<sub>5</sub> is not generated until the concentration of  $\omega$ -3 fatty acids equals or exceeds the  $\omega$ -6 fatty acids in any dietary fat (47–49).

One of the major functions of LA is related to its elongation, desaturation, and conversion to AA, which is the usual eicosanoid precursor of the prostaglandins, synthesized by way of the cyclooxygenase pathway and the leukotrienes, formed via the lipoxygenase pathways. These vasoactive substances are not considered integral parts of the T- and B-cell network, although they have been shown to modulate immune cell activity as well as the inflammatory responses to infectious agents (20–24).

In several of the subjects studied, no effects of PC were noted on T-cell responses to the mitogen phytohemagglutinin (JJ Vitale, E Levy, unpublished observations, 1989). Possibly, the effects of PC noted in PMNLs may not occur or may be significantly delayed in lymphocytes or other cell types with much slower turnover rates. It is likely that LA or other dietary lipids may effect the lipid membrane fatty acid composition and/or function of other cell types if given over a more prolonged period.

The effects of PC on PMNL phagocytosis and killing are in keeping with results from other studies (50–52). These studies report that the administration of PC protects against morbidity and mortality from experimental *Klebsiella pneumoniae* infection in neonatal rats (50), against *Staphylococcus aureus* infections in mice (51), and against morbidity and mortality of animals infected with herpes simplex virus Type-II (52). In addition, it has been reported that the administration of lysophosphatidylcholine can activate peritoneal macrophages or PMNLs leading to increased superoxide activity associated with increased phagocytosis and killing (53). The present study is the first to demonstrate a possible mechanism for the previous studies, ie, the relatively long lasting effect of PC on membrane AA concentrations and the generation of LTB<sub>4</sub>.

Future studies using other sources of PC (eg, egg PC and hydrogenated soy PC) are planned to confirm the critical role of LA in the lipid moiety. Clearly, the present study demonstrates a distinct difference in PMNL function when LA is fed as an integral part of a phosphoglyceride or as a component of a neutral fat.

Coupled with data from animal experiments, these results suggest a potential immunomodulatory role for PC in humans who have increased risks of bacterial and/or fungal infections. It should be stressed from the present study that the quality of the fat rather than the quantity may be important in how dietary lipids effect host defenses and immune functions. **□**

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