USE OF VITAMIN E TOCOTRIENOLS FOR THE INHIBITION OF INTRACELLULARLY OBLIGATE PATHOGEN CHLAMYDIA

Cross-Reference To Related Applications

This application claims priority upon United States Provisional Patent application Serial No. 60/673,837 filed on April 22, 2005 and United States Provisional Patent application Serial No. 60/778,432 filed on March 1, 2006, the contents of which are all herein incorporated by this reference in their entireties. The contents of co-pending United States Patent Application Serial No. 10/823043 filed on April 12, 2004 and United States Patent Application Serial No. 10/821679 filed on April 8, 2004 are all herein incorporated by this reference in their entireties. All publications, patents, patent applications, databases and other references cited in this application, all related applications referenced herein, and all references cited therein, are incorporated by reference in their entirety as if restated here in full and as if each individual publication, patent, patent application, database or other reference were specifically and individually indicated to be incorporated by reference.

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Field of the Invention

The invention is on the use of Vitamin E to inhibit, prevent, and disrupt the developmental cell cycle and infection of *Chlamydia*, and its use to alleviate the effects of *Chlamydia*-related diseases.

Background Of The Invention

Chlamydia is an obligate intracellular pathogen known to be associated with several diseases that are common today. *Chlamydia trachomatis* is the primary cause of bacterial sexually transmitted disease (STD), and can lead to ectopic pregnancies and infertility. *Chlamydia pneumoniae* causes respiratory tract infections including bronchitis, pneumonia, sinusitis, and pharyngitis. In addition, it is linked to numerous pathologies, including Alzheimer's disease, multiple sclerosis, atherosclerosis, coronary heart disease, and asthma. Three recent *Chlamydia* findings are 1) various strains of clinically important *Chlamydia* species are associated with caveolin, a molecule important to cholesterol homeostasis, 2) apoptosis (programmed cell death) is downregulated among infected cells (Byrne *et al.*, 2004), and 3) treatment of chlamydial infections with antibiotics drives the pathogens into a resistant and persistent state. Clinical persistence is an essential element of chlamydial pathogenesis, where the inability of the host to eliminate the pathogen leads to a state of chronic infectivity along with attendant tissue injury.

Delta tocotrienol is a vitamin E compound also available as dietary supplement. It has been shown to lower cholesterol in a controlled fashion, and to induce apoptosis in cancer cells, reducing tumors by as much as 70%. Since *Chlamydia* species enter cells via cholesterol-rich lipid raft domains involved in cholesterol trafficking, tocotrienols in general and hypocholesterolemic delta tocotrienol in particular will reduce infection by *Chlamydia*. Mouse macrophages (J774A.1), human mammary tumor cells (MCF-7, TMX2-28), human epithelial cells (Hep-2), and human B-lymphocytes (JY) were incubated with delta tocotrienol at concentrations of 10-30 μmol/L for 6 hours prior to infection by *C. trachomatis* serovar K, a subspecies of *Chlamydia* that is the primary cause of bacteria-initiated STD. Infections were detected by immunofluorescence staining followed by either microscopy or quantitative flow cytometric analysis. Infection levels in cells

pretreated with delta-tocotrienol were decreased by >50%, with concomitant aberrant pathogen development observed with confocal microscopy. The number of large and small inclusions in the delta tocotrienol-versus-control cells was decreased by 3- and 2-fold, respectively. Flow cytometry showed that chlamydial inhibition in JY cells was at least 2-fold for an infection period of 72 hours, with a 2.6-fold maximum inhibition at 36 hours. The impact of dietary delta tocotrienol on *Chlamydia* infection in hyperlipidemic patients is being examined in a clinical study. Cholesterol-suppressive delta tocotrienol may have the potential to reduce *Chlamydia* infection in humans.

Chlamydia is an obligate intracellular pathogen, which means that it has to invade cells first in order to successfully survive inside the host. This infection is initiated by the so-called chlamydial elementary bodies (EBs). Once inside host cells, chlamydial replication occurs within a segregated, membrane-bound compartment called an inclusion that progressively enlarges due to metabolically active *Chlamydia* replication within the host cell.

Species of the genus *Chlamydiaceae* that are frequently associated with chronic human diseases are *Chlamydiophila pneumoniae* and *Chlamydia trachomatis*. *C. pneumoniae* causes 5-10% of respiratory tract infections in adults and children including bronchitis, pneumonia, sinusitis, and pharyngitis. Additionally, it has been linked to chronic diseases like late onset Alzheimer's, multiple sclerosis, reactive arthritis, atherosclerosis, and asthma. Infection by *C. pneumoniae* has been suggested to induce autoimmunity. In multiple sclerosis (MS), 73% of the patients were shown to be *Chlamydia*-positive compared to 22% of controls. In addition, *C. pneumoniae* is involved in atherosclerotic processes such as cellular oxidation of LDL and macrophage foam cell formation. By age 20, 50% of the population exhibits evidence of past infection by *C. pneumoniae* (shown by serological tests), and re-infection is common. Additionally, in a study examining a normal blood donor population for chlamydial infection levels, 25% of the population was found to be positive by immunostained blood smear. Therefore, it is possible that serological tests (e.g., ELISA) overestimate immunostaining of blood tests (e.g., actual WBCs) by $\geq$ two-fold. *C. trachomatis*, on the other hand, is the world's leading cause of preventable infectious blindness, and the most common cause of STD. In

females, infection with *C. trachomatis* initially affects mucosal membranes and leads to continual inflammation of tissue in the genital tract, which results in scarring and eventual infertility. After invasion of cells, *Chlamydia* aggregate in vacuole-like structures called inclusions, and can escape many of the first line host defenses of the immune system. Another feature of the *Chlamydia* is that they prevent apoptosis of infected cells (Byrne, *et al.*, 2004). This effect is protective for the pathogen because it can complete a full replicative cycle within the single host cell (Figure 1). Since chlamydial inclusions inside a single infected host cell require incubation to give rise to 200-1,000 new infectious units, inhibition of host cell apoptosis by *Chlamydia* is advantageous to the pathogen and supports the establishment of a prolonged infection. Therefore, one aspect of the invention is that tocotrienols will inhibit chlamydial growth inside host cells by causing the infected host to undergo apoptosis. Another aspect of the invention is that tocotrienols will prevent the progression of the incubative cell cycle of the pathogen.

Delta tocotrienol is a vitamin E compound. Cancer studies with this compound have shown that it induces apoptosis of the tumor cells, but does not harm the surrounding healthy cells. Learning that delta tocotrienol has this ability, at least for cancer cells, triggered the idea that the compound may also up-regulate apoptosis of cells infected by *Chlamydia*. Since chlamydial inhibition of host cell apoptosis is important to the pathogen's success, such an effect by delta tocotrienol could have a profound impact as treatment against *Chlamydia*. Another delta tocotrienol characteristic of importance is its cholesterol-lowering effect (Pearce *et al.*, 1992). *Chlamydia* enters the host cell via cholesterol-rich lipid-rafts or caveolae, which are comprised of lateral assemblies of cholesterol and sphingolipids that float in the glycerophospholipid membrane, and are impaired with removal of plasma membrane cholesterol. Therefore, cells that are exposed to delta tocotrienol could remodel the lipid rafts, and thus interfere with pathogen entry into host cells directly or indirectly. Since *Chlamydia* is metabolically inactive outside the host, it is unable to survive outside the cell. A potential strategy is to inhibit cholesterol synthesis or availability in order to contain or eliminate *Chlamydia*.

In this invention, we tested delta tocotrienol's effect on chlamydial infection in numerous normal white blood cells, cancer cells, and buffy coats of *Chlamydia*-positive human blood samples.

Summary Of The Invention

The invention relates to uses of Vitamin E to inhibit and prevent infection by *Chlamydia*. Additionally, the present disclosure relates to the mechanism of action by Vitamin E, especially delta-tocotrienol, to interrupt the infection process of a *Chlamydia*.

In one example the present disclosure is drawn to a method of using Vitamin E tocochromanol to inhibit, prevent, and disrupt the developmental cell cycle and infection of *Chlamydia*. In an example the present disclosure is drawn to a method of using tocotrienol to inhibit, prevent, and disrupt the developmental cell cycle and infection of *Chlamydia*. In another example the present disclosure is drawn to a method of using delta-tocotrienol to inhibit, prevent, and disrupt the developmental cell cycle and infection of *Chlamydia*.

In one example the present disclosure is drawn to a method of using Vitamin E tocochromanol to alleviate the effects of *Chlamydia*-related diseases. In an example the present disclosure is drawn to a method of using tocotrienol to alleviate the effects of *Chlamydia*-related diseases. In another example the present disclosure is drawn to a method of using delta-tocotrienol to alleviate the effects of *Chlamydia*-related diseases.

In one aspect of the invention, there is provided the use of a tocotrienol for the preparation of a medicament for the treatment of a *Chlamydia* infection, wherein said tocotrienol is administered daily for at least one month to a mammal or avian to treat *Chlamydia*-associated disease selected from the group consisting of cardiovascular disease, hypertension, atherosclerosis, COX-I- and COX-II-induced inflammation, a sexually transmitted disease, a genital tract infection, arthritis, prediabetes, metabolic syndrome, diabetes, polycystic ovarian syndrome (PCOS), a respiratory tract infection, pneumonia, an ocular infection, a neurological disease, Alzheimer's disease, and multiple sclerosis.

In one example the present disclosure is drawn to a method of using Vitamin E tocochromanol to reduce the quantity of chlamydial inclusions. In an example the present disclosure is drawn to a method of using tocotrienol to reduce the quantity of chlamydial inclusions. In another example the present disclosure is drawn to a method of using delta-tocotrienol to reduce the quantity of chlamydial inclusions.

In one example the present disclosure is drawn to a method of using Vitamin E tocochromanol to impede and suppress the initial infection by chlamydial EBs of host cells. In an example the present disclosure is drawn to a method of using tocotrienol to impede and suppress the initial infection by chlamydial EBs of host cells. In another example

the present disclosure is drawn to a method of using delta-tocotrienol to

impede and suppress the initial infection by chlamydial EBs of host cells.

In one example the present disclosure is drawn to a method of using 5 μmol/L delta-tocotrienol to inhibit chlamydial development within the host cell. In an example the present disclosure is drawn to a method of using 10 μmol/L delta-tocotrienol to inhibit chlamydial development within the host cell. In another example the present disclosure is drawn to a method of using 20 μmol/L delta-tocotrienol to inhibit chlamydial development within the host cell. In another example the present disclosure is drawn to a method of using 30 μmol/L delta-tocotrienol to inhibit chlamydial development within the host cell. In another example the present disclosure is drawn to a method of using

40 μmol/L delta-tocotrienol to inhibit chlamydial development within the host cell.

In one example the present disclosure is drawn to a method of using delta-tocotrienol several times to reduce the level of infection by *Chlamydia.* In an example the present disclosure is drawn to a method of using delta-tocotrienol 3 to 10 times to reduce the level of infection by *Chlamydia.* In another example the present disclosure is drawn to a method of using delta-tocotrienol more than 10 times to reduce the level of infection by *Chlamydia.* In another example the present disclosure is drawn to a method of using

delta-tocotrienol repeatedly to reduce the level of infection by *Chlamydia.*

In one example the present disclosure is drawn to a method of using delta-tocotrienol several times to inhibit re-infection of the host cell by chlamydial EBs. In an example the present disclosure is drawn to a method of using delta-tocotrienol 3 to 10 times to inhibit re-infection of the host cell by chlamydial EBs. In another example the present disclosure is drawn to a method of using delta-tocotrienol more than 10 times to inhibit re-infection of the host cell by chlamydial EBs. In another example the present disclosure is drawn to a method of using delta-tocotrienol repeatedly to inhibit re-infection

of the host cell by chlamydial EBs.

In one example the present disclosure is drawn to a method of using tocotrienol to inhibit inclusion formation by 5%. In an example the present disclosure is drawn to a method of using tocotrienol to inhibit inclusion formation by 10%. In another example the present disclosure is drawn to a method of using tocotrienol to inhibit inclusion

formation by 15%. In another example the present disclosure is drawn to a method of using tocotrienol to inhibit inclusion formation by 20%. In another example the present disclosure is drawn to a method of using tocotrienol to inhibit inclusion formation by 25%. In another example the present disclosure is drawn to a method of using tocotrienol to inhibit inclusion formation by 30%. In another example the present disclosure is drawn to a method of using tocotrienol to inhibit inclusion formation by 40%. In another example the present disclosure is drawn to a method of using tocotrienol to inhibit inclusion formation by 45%. In another example the present disclosure is drawn to a method of using tocotrienol to inhibit inclusion formation by 50%. In another example the present disclosure is drawn to a method of using tocotrienol to inhibit inclusion formation by 60%. In another example the present disclosure is drawn to a method of using tocotrienol to inhibit inclusion formation by 75%. In another example the present disclosure is drawn to a method of using tocotrienol to inhibit inclusion formation by 90%.

In one example the present disclosure is drawn to a method of using Vitamin E tocochromanol. In one example the present disclosure is drawn to a method of using tocotrienol for at least one day. In another example the present disclosure is drawn to a method of using tocotrienol for at least three days. In another example the present disclosure is drawn to a method of using tocotrienol for more than three days.

In one example the present disclosure is drawn to a method of using tocotrienol is effective in humans against *Chlamydia* as represented in circulating WBCs, including neutrophils, monocytes, lymphocytes, eosinophils, and basophils.

In one example the present disclosure is drawn to a method of using tocotrienol to reduce the cholesterol level and chlamydial infection in hyperlipidemic patients, and rendered the chlamydial infection status negative.

In one example the present disclosure is drawn to a method of using an agent that restricts cholesterol/lipids to restrict *Chlamydia* infection and growth.

In one example the present disclosure is drawn to a method of using tocotrienol to reduce *Chlamydia* infections in cancer patients.

In one example the present disclosure is drawn to a method of using tocotrienol to ward off pathogenic infections including *Chlamydia* via activation of antigen-presenting dendritic cells and T-lymphocytes.

In one example the present disclosure is drawn to a method of using tocotrienol to resist opportunistic chlamydial infection of hypertensive patients, and thereby reduce the patient's blood pressure.

In one example the present disclosure is drawn to a method of using tocotrienol to reduce cardiovascular risk factors associated with metabolic syndrome, and thereby reduce the risk of diabetes and concomitant or subsequent infection by *Chlamydia*.

In one example the present disclosure is drawn to a method of application of tocotrienol by aerosol sprays (to reach respiratory tract airways), oral ingestion via softgels, tablets or capsules (to reach vascular-organistic systems), topical creams, douches, and lotions (to reach genital sites), and topical liquid drops (to reach ocular sites).

In another aspect of the invention, there is provided the use of a tocotrienol Vitamin E tocochromanol in the manufacture of a medicament for the treatment of an *Chlamydia* infection, wherein said medicament is to be administered daily for at least one month to a mammal in need of treatment; wherein the amount of the tocotrienol is a dose between 10 mg and 1000 mg per day, and wherein the tocotrienol treats the *Chlamydia* infection.

In another example the present disclosure is drawn to a method of application of tocotrienol by oral ingestion via softgels, tablets or capsules, and topical creams, douches, and lotions. In another example the present disclosure is drawn to a method of application of tocotrienol by oral ingestion via softgels, tablets or capsules. In one example of the present disclosure is drawn to a method of administering delta tocotrienol in a range from 10 to 1000 mg per day. In another example the present disclosure is drawn to a method of administering delta tocotrienol in a range from 20 to 500 mg per day. In an example the present disclosure is drawn to a method of administering delta tocotrienol in a range from 50 to 150 mg per day. In one example of the present disclosure is drawn to a method of treatment with delta tocotrienol of administering daily for one month. In an example of the present disclosure is drawn to a method of treatment with delta tocotrienol of administering daily for six months. In another example of the present disclosure is drawn to a method of treatment with delta tocotrienol of administering daily for one year. In another example of the present disclosure is drawn to a method of treatment with delta tocotrienol of administering daily until infection and inflammation due to *Chlamydia* is cleared.

In one example the present disclosure is drawn to a method of using tocotrienol for respiratory tract infections by using an aerosol spray at a dosage of 1 spray per day. In an example the present disclosure is drawn to a method of using tocotrienol for respiratory tract infections by using an aerosol spray at a dosage of 2 sprays per day. In another example the present disclosure is drawn to a method of using tocotrienol for respiratory tract infections by using an aerosol spray at a dosage of 4 sprays per day.

In one example the present disclosure is drawn to a method of using tocotrienol for genital tract infections by using an aerosol spray at a dosage of 1 application per day. In an example the present disclosure is drawn to a method of using tocotrienol for genital tract infections by using an aerosol spray at a dosage of 2 applications per day. In another example the present disclosure is drawn to a method of using tocotrienol for genital tract infections by using an aerosol spray at a dosage of 4 applications per day.

In one example the present disclosure is drawn to a method of using tocotrienol to reduce inflammation of vascular-organistic systems due to chlamydial infection by oral ingestion of 1 dose per day. In an example the present disclosure is drawn to a method of using tocotrienol to reduce inflammation of vascular-organistic systems due to chlamydial infection by oral ingestion of 2 doses per day. In another example the present disclosure is drawn to a method of using tocotrienol to reduce inflammation of vascular-organistic systems due to chlamydial infection by oral ingestion of 4 doses per day.

In one example the present disclosure is drawn to a method of using tocotrienol to reduce ocular infections and conjunctivitis due to chlamydial infection by applying liquid eye drops with 1 application per day. In an example the present disclosure is drawn to a method of using tocotrienol to reduce ocular infections and conjunctivitis due to chlamydial infection by applying liquid eye drops with 2 applications per day. In another example the present disclosure is drawn to a method of using tocotrienol to reduce ocular infections and conjunctivitis due to chlamydial infection by applying liquid eye drops with 4 applications per day.

In one example the present disclosure is drawn to a method of using tocotrienol where the delta-to-gamma ratio of tocotrienols is 1:100 to 100:1. In an example the present disclosure is drawn to a method of using tocotrienol where the delta-to-gamma ratio of

tocotrienols is 1:25 to 25:1. In another example the present disclosure is drawn to a method of using tocotrienol where the delta-to-gamma ratio of tocotrienols is 1:10 to 10:1. In another example the present disclosure is drawn to a method of using tocotrienol where the delta-to-gamma ratio of tocotrienols is 1:5 to 5:1. In another example the present disclosure is drawn to a method of using tocotrienol where the delta-to-gamma ratio of tocotrienols is 1:1.

In one example the present disclosure is drawn to a method of using tocotrienol comprising a mixture of annatto extract and a natural extract that is an appropriate spectrum. In an example the present disclosure is drawn to a method of using tocotrienol where more than 50% of the tocotrienols are delta-T3 and gamma-T3. In another example the present disclosure is drawn to a method of using tocotrienol where more than 50% of the tocotrienols are delta-T3. In another example the present disclosure is drawn to a method of using tocotrienol where it is tocopherol-free.

In one example the present disclosure is drawn to a method of using tocotrienol where the C5 unsubstituted tocotrienols are >60%, and tocopherols are <15%. In an example the present disclosure is drawn to a method of using tocotrienol where the C5 unsubstituted tocotrienols are >70% C5 unsubstituted tocotrienols and <10% tocopherols. In an example the present disclosure is drawn to a method of using tocotrienol where the C5 unsubstituted tocotrienols are >80% C5 unsubstituted tocotrienols and <5% tocopherols.

In one example the present disclosure is drawn to a method of using tocotrienol where the method of using tocotrienol is tocopherol-free with >98% tocotrienols, and tocotrienols are predominantly delta-T3 and gamma-T3. In an example the present disclosure is drawn to a method of using tocotrienol where the tocotrienol is tocopherol-free with >98% tocotrienols and tocotrienols are predominantly delta-T3.

In one example the present disclosure is drawn to a method of using tocotrienol comprising annatto extract where C5 unsubstituted tocols inhibit surface chemotactic bioactive materials (CBM). In an example the present disclosure is drawn to a method of using tocotrienol comprising annatto extract where annatto C5 unsubstituted T3 inhibit surface chemotactic bioactive materials. In another example the present disclosure is

drawn to a method of using tocotrienol comprising annatto extract where annatto C5 unsubstituted T3 inhibit surface chemotactic bioactive materials and prevent the tether or adhesion of circulating monocytes and leucocytes onto stationary endothelia. In another example the present disclosure is drawn to a method of using tocotrienol comprising annatto extract where annatto C5 unsubstituted T3 inhibit surface chemotactic bioactive materials and prevent the tether or adhesion of circulating monocytes and leucocytes onto stationary endothelia that cause the loss of vasculature integrity. In another example the present disclosure is drawn to a method of using tocotrienol comprising annatto extract where annatto C5 unsubstituted T3 inhibit surface chemotactic bioactive materials and prevent the tether or adhesion of circulating monocytes and leucocytes onto stationary endothelia that cause the loss of vasculature integrity, and prevent micro- and macro-vascular diseases, and atherosclerosis. In another example the present disclosure is drawn to a method of using tocotrienol comprising annatto extract where annatto C5 unsubstituted T3 inhibit CBM and prevent pathological events selected from the group consisting of chemotaxis, vasoconstriction, hypercoagulation, glycoxidation and oxidized LDL by via HDL elevation.

In yet another aspect of the invention, there is provided the use of tocotrienol for preparation of a medicament for the treatment of an infection by *Chlamydia*, wherein said tocotrienol is administered in combination with at least one agent selected from the group consisting of a statin, a bioflavonoid, a polyphenolic, a polymethoxylated flavone, a plant sterol, a oryzanol, a policosanol, a B vitamin, Coenzyme Q10 (CoQ10), an omega 3 fatty acid, a lecithin, garlic, a gugul lipid, an insoluble fiber, a soluble fiber, a soy protein, a chitosan, a red yeast rice, and a mineral.

In one example the present disclosure is drawn to a method of using tocotrienol where the tocotrienol is combined with other nutrients. In another example the present disclosure is drawn to a method of using tocotrienol were the tocotrienol is combined with other nutrients, and the tocotrienol contains geranyl geraniol. In another example the present disclosure is drawn to a method of using tocotrienol where the nutrient is selected from the group consisting of phytosterols, oryzanols, policosanols, pantethine, red yeast rice (Monascus), oat bran, garlic, gugul lipids, chitosan, soy protein (e.g., oligo- and poly-peptides, hydrolysates), CoQ10, carnitine, magnesium, chromium, potassium, calcium, D-tyrosine, fibers (insoluble and soluble types, including beta-glucans), omega-3s (DHAs and EPAs, ALAs), and lecithin.

In one example, the present disclosure is drawn to a method of using geranyl geraniols and tocotrienols and it increases the de novo biosyntheses of all subsequent intermediate isoprenoid pool and distal products.

In one example, the present disclosure is drawn to method of using tocotrienol containing geranyl geraniols that anabolically increases the endogenous de novo synthesis of CoQ10 via geranyl geraniols elongation/prenylation of side chain and conversely CoQ10 catabolically increases the endogenous de novo synthesis of geranyl geraniols via CoQ 10 beta-oxidation.

In one example, the present disclosure is drawn to a method of supplementation, comprising the administering of a tocotrienol or a tocotrienol and geranyl geraniol, and reducing Chlamydia-induced blindness.

Detailed Description of the Preferred Embodiment

In one embodiment the method administers a tocotrienol, where the tocotrienol contains delta-tocotrienol and gamma-tocotrienol, and where the delta-to-gamma ratio of tocotrienols is 1:100 to 100:1. In a preferred embodiment the method administers an tocotrienol, where the tocotrienol contains delta-tocotrienol and gamma-tocotrienol, and where the delta-to-gamma ratio of tocotrienols is 1:25 to 25:1. In a more preferred embodiment the method administers an tocotrienol, where the tocotrienol contains delta-tocotrienol and gamma-tocotrienol, and where the delta-to-gamma ratio of tocotrienols is 1:10 to 10:1. In a more preferred embodiment the method administers an tocotrienol, where the tocotrienol contains delta-tocotrienol and gamma-tocotrienol, and where the delta-to-gamma ratio of tocotrienols is 1:5 to 5:1. In a more preferred embodiment the method administers an tocotrienol, where the tocotrienol contains delta-tocotrienol and gamma-tocotrienol, and where the delta-to-gamma ratio of tocotrienols is 1:1.

In one embodiment a method administers a tocotrienol, where the method of using tocotrienol is a mixture of an annatto extract and a natural extract, and where the mixture has standardized low levels of tocopherols. In a preferred embodiment the method administers a tocotrienol, where the method of using tocotrienol is a mixture of an annatto extract and a natural extract, and where the standardized level of tocopherols is $\leq 50\%$. In a more preferred embodiment the method administers a tocotrienol, where the method of using tocotrienol is a mixture of an annatto extract and a natural extract, and where the standardized level of tocopherols is $\leq 20\%$. In a more preferred embodiment the method

administers a tocotrienol, where the method of using tocotrienol is a mixture of an annatto extract and a natural extract, and where the standardized level of tocopherols is ≤ 10%. In a more preferred embodiment the method administers a tocotrienol, where the method of using tocotrienol is a mixture of an annatto extract and a natural extract, and where the standardized level of tocopherols is ≤ 1%. In more preferred embodiment the method administers a tocotrienols, where the natural extract is selected from the group consisting of a vegetable oil of rice bran, palm, cranberry seed, and litchi seed.

In one embodiment a method administers a mixture of annatto extract and a natural extract that is an appropriate spectrum. In a preferred embodiment the method administers a mixture of annatto extract and a natural extract, and more than 50% of the tocotrienols are delta-T3 and gamma-T3. In a more preferred embodiment the method administers a mixture of annatto extract and a natural extract, and more than 50% of the tocotrienols are delta-T3. In a most preferred embodiment the method administers a mixture of annatto extract and a natural extract, and it is tocopherol-free.

In one embodiment a method administers an >60% C5 unsubstituted tocotrienols and <15% tocopherols. In a preferred embodiment a method administers an >70% C5 unsubstituted tocotrienols and <10% tocopherols. In a more preferred one embodiment a method administers an >80% C5 unsubstituted tocotrienols and <5% tocopherols.

In one embodiment a method administers an annatto extract and the method of using tocotrienol is tocopherol-free with >98% tocotrienols, and tocotrienols are predominantly delta-T3 and gamma-T3. In a more preferred embodiment the method administers an annatto extract and is tocopherol-free with >98% tocotrienols and tocotrienols are predominantly delta-T3.

In one embodiment a method administers an C5 unsubstituted tocols inhibit surface chemotactic bioactive materials. In a more preferred embodiment a method administers an C5 unsubstituted tocols, where the C5 unsubstituted tocols are C5 unsubstituted T3 and the C5 unsubstituted T3 inhibit surface chemotactic bioactive materials. In a more preferred embodiment a method administers an C5 unsubstituted tocols, where the C5 unsubstituted tocols are C5 unsubstituted T3 and the C5 unsubstituted T3 inhibit surface chemotactic bioactive materials and prevent the tether or adhesion of circulating monocytes and

leucocytes onto stationary endothelia. In a more preferred embodiment a method administers an C5 unsubstituted tocols, where the C5 unsubstituted tocols are C5 unsubstituted T3 and the C5 unsubstituted T3 inhibit surface chemotactic bioactive materials and prevent the tether or adhesion of circulating monocytes and leucocytes onto stationary endothelia that cause the loss of vasculature integrity. In a more preferred embodiment a method administers an C5 unsubstituted tocols, where the C5 unsubstituted tocols are C5 unsubstituted T3 and the C5 unsubstituted T3 inhibit surface chemotactic bioactive materials and prevent the tether or adhesion of circulating monocytes and leucocytes onto stationary endothelia that cause the loss of vasculature integrity, and prevent micro- and macro-vascular diseases, and atherosclerosis. In a more preferred embodiment a method administers an C5 unsubstituted tocols, where the C5 unsubstituted tocols are C5 unsubstituted T3 and the C5 unsubstituted T3 inhibit surface chemotactic bioactive materials and prevent pathological events selected from the group consisting of chemotaxis, vasoconstriction, hypercoagulation, glycoxidation and oxidized LDL by via HDL elevation.

In one embodiment a method administers an annatto extract and the annatto extract is combined with other nutrients. In a preferred embodiment a method administers an annatto extract and the annatto extract is combined with other nutrients, and the annatto extract contains tocotrienol and geranyl geraniol. In a more preferred embodiment a method administers an annatto extracts and where the nutrient is selected from the group consisting of phytosterols, oryzanols, policosanols, pantethine, red yeast rice (Monascus), oat bran, garlic, gugul lipids, chitosan, soy protein (e.g., oligo- and poly-peptides, hydrolysates), CoQ10, carnitine, magnesium, chromium, potassium, calcium, D-tyrosine, fibers (insoluble and soluble types, including beta-glucans), omega-3s (DHAs and EPAs, ALAs), and lecithin. In another embodiment a method administers an annatto extract and a nutrient, and the nutrient is selected from the group consisting of banaba extract (e.g., corosolic acid), lipoic acids (all isomeric forms), chromium, and the B vitamins including niacin.

In one embodiment, a method administers an geranyl geraniols and tocopherol-free C-5 unsubstituted tocotrienols. In a more preferred embodiment, the method administers an

geranyl geraniols, tocopherol-free C-5 unsubstituted tocotrienols, and inactive and/or active ingredients.

In one embodiment, a method of using tocotrienol containing geranyl geraniols treats a disease of the nervous system. In a preferred embodiment, the method of using tocotrienol containing geranyl geraniols treats a disease of the nervous system, where the disease is selected from the group consisting of chronic Alzheimer's, Parkinson's, Familial Dysautonomia, Muscular Sclerosis, and Muscular Atrophy.

Some embodiments of the present invention are described with reference to the numbered paragraphs below:

1. A method of treating an infection by *Chlamydia*, comprising administering a Vitamin E tocochromanol to a mammal in need of treatment.

2. The method of paragraph 1, where the treatment inhibits the developmental cell cycle and infection of *Chlamydia*.

3. The method of paragraph 1, where the treatment prevents the developmental cell cycle and infection of *Chlamydia*.

4. The method of paragraph 1, where the treatment disrupts the developmental cell cycle and infection of *Chlamydia*.

5. The method of paragraph 1, further comprising administering a geranyl geraniol.

6. The method of paragraph 1, where the mammal has a condition selected from the group consisting of elevated intracellular calcium, increased caveolae expression, increased vasoconstriction, hypertension and primary pulmonary hypertension.

7. The method of paragraph 1, where the Vitamin E tocochromanol is selected from the group consisting of a natural tocopherol, a synthetic tocopherol, a natural tocotrienol and a synthetic tocotrienol.

8. The method of paragraph 7, where the tocotrienol is an isomer of a tocotrienol.

9. The method of paragraph 8, where the isomer of tocotrienol is selected from the group consisting of alpha, beta, gamma, delta, desmethyl, and didesmethyl.

10. The method of paragraph 7, where the tocopherol is an isomer of a tocopherol.

11. The method of paragraph 10, where the isomer of tocopherol is selected from the group consisting of alpha, beta, gamma, delta, desmethyl, and didesmethyl.

12. The method of paragraph 1, where the *Chlamydia* is selected from the group consisting of *Chlamydia trachomatis, Chlamydia suis, Chlamydia muridarum, Chlamydiophila pneumoniae, Chlamydiophila psittaci, Chlamydiophila pecorum, Chlamydiophila abortis, Chlamydiophila felis,* and *Chlamydiophila caviae.*

13. The method of paragraph 1, where the tocotrienol stunts the growth and inhibits *Chlamydia* maturation of reticulate bodies into elementary bodies, preventing its progression and development.

14. The method of paragraph 1, where the tocotrienol reduces chlamydial infection of a white blood cell.

15. The method of paragraph 14, where the white cell is selected from the group consisting of neutrophils, monocytes, lymphocytes, eosinophils, and basophils.

16. The method of paragraph 7, where the tocotrienol lowers cholesterol in hypercholesterolemic patients to inhibit chlamydial infection.

17. A method of treating an infection by *Chlamydia,* comprising administering an agent that restricts cholesterol.

18. The method of paragraph 17, where the agent that restricts cholesterol is selected from the group consisting of a statin, a bioflavonoid, a polyphenolic, a polymethoxylated flavone, a plant sterol, a oryzanol, a policosanol, a B vitamin, CoQ10, an omega 3 fatty acid, a lecithin, garlic, a gugul lipids, an insoluble fiber, a soluble fiber, a soy protein, a chitosan, a red yeast rice, and a mineral.

19. The method of paragraph 18, where the bioflavonoid is selected from the group consisting of citrus bioflavonoid and polymethoxylated flavone.

20. The method of paragraph 1, where mode of application of the Vitamin E tocochromanol is selected from the group consisting of aerosol spray, oral ingestion, creams, douches, lotions, and eye drops.

21. The method of 20, comprising administering a dose of tocotrienol between 10 mg and 1000 mg per day.

22. The method of 21, comprising administering a dose of tocotrienol between 20 mg and 500 mg per day.

23. The method of 22, comprising administering a dose of tocotrienol between 50 mg and 150 mg per day.

24. A method of treating an infection by *Chlamydia*, comprising administering a combination of a tocotrienol and at least one agent selected from the group consisting of a statin, a bioflavonoid, a polyphenolic, a polymethoxylated flavone, a plant sterol, a oryzanol, a policosanol, a B vitamin, CoQ10, an omega 3 fatty acid, a lecithin, garlic, a gugul lipids, an insoluble fiber, a soluble fiber, a soy protein, a chitosan, a red yeast rice, and a mineral.

25. A method of treating an infection by *Chlamydia*, comprising administering a combination of a polymethoxylatyed flavone and at least one agent selected from the group consisting of a tocotrienol, a statin, a bioflavonoid, a polyphenolic, a polymethoxylated flavone, a plant sterol, a oryzanol, a policosanol, a B vitamin, CoQ10, an omega 3 fatty acid, a lecithin, garlic, a gugul lipids, an insoluble fiber, a soluble fiber, a soy protein, a chitosan, a red yeast rice, and a mineral.

26. A method of treating *Chlamydia*-associated diseases, comprising administering a hypocholesterolemic agent to a mammal or avian to treat a *Chlamydia*-associated disease selected from the group consisting of cardiovascular disease, hypertension, atherosclerosis, COX-I- and COX-II-induced inflammation, a sexually transmitted disease, a genital tract infection, arthritis, prediabetes, metabolic syndrome, diabetes, polycystic ovarian syndrome (PCOS), a respiratory tract infection, pneumonia, an ocular infection, a neurological disease, Alzheimer's disease, and multiple sclerosis.

27. The method of Paragraph 26, where the hypocholesterolemic agent is a tocotrienol.

28. The method of Paragraph 27, where tocotrienol inhibits intracellular calcium, $[Ca^{2+}]$, and caveolae expression, and thereby reduces vasoconstriction and primary pulmonary hypertension (PPH), and therefore inhibits chlamydial infection associated with hypertension.

29. A method to improve immunity to *Chlamydia*, comprising administering a tocotrienol to a mammal to potentiate an antigen-presenting dendritic cell and improve immunity against *Chlamydia*.

30. A method to inhibit progression of a cancer, comprising administering a tocotrienol to a mammal to potentiate an antigen-presenting dendritic cell and inhibit progression of a cancer.

Other Embodiments

It is to be understood that while the invention has been described in conjunction with the detailed description thereof, the foregoing description is intended to illustrate and not limit the scope of the invention, which is defined by the scope of the appended claims. For example, although the above description relates to human cells, various aspects of the invention might also be applied to cells from other animals (e.g., mammals, avians, fish, crustaceans, and domestic and farm animals) by making appropriate modifications to the described methods. Other aspects, advantages, and modifications are within the scope of the following claims.

Brief Description Of The Several Views Of The Drawing

Figure 1 illustrates the Chlamydial Developmental Cycle. In step (1), elementary bodies (EBs) infect host cells by a process similar to receptor-mediated endocytosis, and form a vacuole-like structure called an inclusion (2). The EBs then transform into non-infectious reticulate bodies (RBs), in which state they replicate and push the nucleus to the side of the cell (2→3). The inclusion enlarges, the RBs transform back to EBs (3), and the inclusion eventually lyses the cell (4). The freed infectious EBs are now able to re-infect surrounding host cells.

Figure 2 illustrates the Molecular and Chemical Structure of Delta Tocotrienol. Delta tocotrienol is a Vitamin E compound with a chromanol nucleus (site of antioxidant activity, typical for all Vitamin E compounds), and an isoprenoid tail (farnesyl tail). The farnesylated tail downregulates the rate-limiting cholesterol biosynthesis enzyme, 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase (Parker *et al.*, 1993). This cholesterol inhibition by delta tocotrienol is unique to farnesylated Vitamin E compounds. Among the known isomers of tocotrienol are alpha, beta, gamma, and delta tocotrienol. The potency of cholesterol inhibition by these isomers is delta>gamma>beta>alpha

tocotrienol. Apparently, desmethyl tocotrienols are more active, especially in the absence of a methyl group at C5 on the benzene ring (see arrow). Delta tocotrienol is monomethylated at position 8 of the benzene ring, making it the least substituted, and therefore the most potent isomer of the four common tocotrienol compounds (Tan, 2005).

Figure 3 illustrates *Chlamydia*-infected mouse macrophage cells treated with delta tocotrienol (400X magnification). Mouse macrophages (J774A.1 cells) were pretreated with a 30 μmol/L concentration of delta tocotrienol in culture for 4 hours and infected with *C. trachomatis* SVK for 48 hours. Arrows indicate inclusions. Cells were viewed and digitally documented using a Zeiss LSM 510 Meta Confocal System at a magnification of 400X. White arrows point out characteristic inclusions (Scale indicates 50 μm).

Figure 4 illustrates *Chlamydia*-infected mouse macrophage cells treated with delta tocotrienol (630X magnification). White arrows point to representative cells. Note numerous very small, unfused inclusions in A (Scale indicates 50 μm).

Figure 5 illustrates flow cytometry on *Chlamydia*-infected human B-lymphocytes treated with delta tocotrienol. Human B-lymphocyte (JY) cells were treated with a 30 μmol/L concentration of delta tocotrienol in culture for 4 hours, infected with *C. trachomatis* SVK for 24-72 hours. Immunofluorescent *Chlamydia*-positive cells were detected by flow cytometry.

Figure 6 illustrates detection and quantification of *Chlamydia*-infected white blood cells in hyperlipidemic patients with delta-tocotrienol supplementation. Buffy coats of hyperlipidemic patients were immunostained, and for each sample, aliquots of 10,000 cells were assessed. A dotplot of a hyperlipidemic patient prior to supplementation with delta-tocotrienol is shown in the left panel, and a dotplot of a hyperlipidemic patient after 2 months of delta-tocotrienol supplementation (100 mg/day) is shown in the right panel.

Figure 7 illustrates *Chlamydia*-infected human mammary tumor cells treated with delta tocotrienol (630X magnification). Human mammary tumor cells (TMX2-28) were infected with *Chlamydia* for 72 hours and treated with a 15 μmol/L concentration of delta tocotrienol (Figure 7A). Controls were left untreated (Figure 7B).

Detailed Description Of The Invention

The developmental cycle of *Chlamydia* consists of the infectious, but metabolically inactive elementary body (EB) that will initiate cell entry, and the metabolically active, but non-infectious reticulate body (RB), which replicates within the cell and differentiates back to EBs prior to release from the infected cell [Figure 1].

Microbes usurp normal host cell endocytic pathways to gain entry. Several infectious agents, including viruses and intracellular parasites, were found to enter host cells via caveolae or rafts. Many mechanisms for the entry of *Chlamydia* into host cells are proposed. These include the passage-like caveolae, cavity-like clathrin, and/or other pathways. Since only the EB form of *Chlamydia* is infectious and the EB at 300 nm is about 3-fold larger than either caveolae or clathrin (both ca. 100 nm), it seems unlikely that *Chlamydia* would enter host cells via these mechanisms. *Chlamydia* entry into host cells and subsequent infection requires lipid rafts, especially those that are rich in cholesterol content. Severe sequestration of cholesterol by cholesterol precipitation/chelation (Stuart *et al.*, 2003), as well as, inhibition of cholesterol synthesis using antihyperlipidemic drugs (Yamazaki *et al.*, 2006) interferes with host cell endocytosis of *Chlamydia*. Therefore, *Chlamydia* is more likely to enter cells via lipid rafts than some form of caveolar enclosure (invagination). In addition, statins were found to disrupt caveolae in human vascular endothelial cells, thus providing an alternate route of Chlamydia inhibition (Ling and Bundey, 2006), hitherto unknown. Such route of Chlamydia inhibition may further be supported by reduction of cholesterol with statin. The raft-mediated endocytosis may utilize a zipper-type process, whereby the lipids fuse or coalesce onto the EB to facilitate entry. Such fusion of cholesterol materials onto the EB is plausible, since *Chlamydia* cannot synthesize its own lipids, and is known to obtain cholesterol from its host. This cholesterol from the host may be from *de novo* synthesis in the host cell or from the systemic circulation *ex vivo* where it was transported from the liver. *Chlamydia* obtains cholesterol preferentially from extracellular sources by trafficking from the Golgi apparatus, which is then found in large amounts in the chlamydial inclusion membrane (Carabeo *et al.*, 2003). *De novo*-synthesized cholesterol is mediated via lipid-rich transport intermediates by energy- and temperature-dependent transport to the plasma membrane.

In a 2-year clinical study on hyperlipidemic patients positive for *C. pneumoniae*, the cholesterol-lowering drug pravastatin did not induce a significant decrease in serum total- and LDL-cholesterol (1-2% drop), but reduced carotid atherosclerosis by 12%, significantly (Sawayama *et al.*, 2003). This study is instructive in that if these patients were not *Chlamydia*-positive, cholesterol levels might have decreased significantly by administration of pravastatin, and carotid atherosclerosis might have been reduced by more than 12%. Another study found that *Chlamydia*-infected cells showed an enhanced uptake of LDL in macrophages, thereby contributing to foam cell and eventual atherogenic lesion formation. Put together, these studies imply that a relationship exists between *Chlamydia* infection, cholesterol, and carotid atherosclerosis. Earlier pathological studies have shown a positive association between prior or current infection with *C. pneumoniae* and coronary artery disease (Grayston *et al.*, 1990). Interestingly, in a 4-year study, tocotrienol caused a regression of human carotid atherosclerosis without a significant cholesterol drop (Kooyenga *et al.*, 2001).

Tocotrienols [Figure 2] belong to the same group of vitamin E as tocopherols, and their ring structure gives these compounds antioxidant properties. Tocotrienol specifically inhibits *de novo* synthesis of cholesterol via the HMG-CoA reductase pathway. It is expected that the tocotrienol, while lowering systemic LDL, will compromise the constitution of the cholesterol-rich lipid rafts in host cell outer membranes. Importantly, tocotrienol inhibits monocyte-endothelial cell adhesion, which in turn aids a more uniform distribution in the membrane bilayer, and greatly reduces the risk of developing atherosclerotic lesions. Therefore, it is further expected that tocotrienol inhibits the infection of pathogen to host by adhesion of *Chlamydia* to the host.

Once chlamydial entry into host cells has occurred, the bacteria have an ongoing requirement for cholesterol to continue pathogen development. This cholesterol is specifically derived from the membrane of host cells (Azenabor, 2005), and it has been shown that this process is mediated via the Golgi organelles (Carabeo *et al.*, 2003). The depletion of cholesterol from host cell membrane occurs because the *Chlamydia* inclusion hijacks cholesterol the host cell produces, making it unavailable for the host cell membranes. This results in membrane fluidity, increased adherence of macrophages to

endothelial cells, and subsequently the risk of developing atherogenic lesions. Tocotrienol is expected to inhibit intracellular synthesis of cholesterol, thus reducing cholesterol hijacking and Golgi trafficking of cholesterol by *Chlamydia*. Therefore, tocotrienol is expected to inhibit the progression or development of *Chlamydia* from RB to EB, and concomitantly limits the enlargement process of chlamydial inclusion formation. Finally, this would reduce or retard bacterial replication in infected cells that support subsequent infection of uninfected host cells, which occurs following lysis of *Chlamydia*-infected host cells [Figure 1, steps 4→1]. It also may be possible that lipid raft-mediated chlamydial entry is not required for infection (Gabel *et al.*, 2004). Therefore, tocotrienol may inhibit chlamydial infection in mechanism(s) besides the cholesterol reduction route. Nonetheless, tocotrienol inhibits chlamydial infection. However, the cholesterol reduction route may still account for the inhibition of chlamydial progression.

Therefore, the summary of this invention is that delta tocotrienol inhibits cholesterol *de novo* synthesis, disrupting or inhibiting lipid raft formation, although its involvement in restricting lipid rafts may not be necessary. By this invention, tocotrienol restricts or halts the entry (endocytosis) via pathogen-host adhesion and the progression of the various stages of the chlamydial developmental cycle. Delta tocotrienol may have a dual purpose of simultaneous prevention of infection and arrest of normal chlamydial developmental progression that would lead to formation of new infectious EBs. Therefore, this invention describes that the inhibition of cholesterol in the cholesterol-rich lipid raft impedes the progression of *Chlamydia* infection in all of its developmental stages.

Definitions

Assorted Nutritional Supplements – Plant sterols, oryzanols, corosolic acid, policosanols, B vitamins (e.g., pentathine, niacin, carnitine, alpha-lipoic acid, taurine), CoQ10, omega 3 fatty acids (e.g., DHAs, EPAs, alpha linoleic acid), lecithins (e.g., phosphotidyl-choline, serine, ethanolamine, inositol), garlic, gugul lipids, insoluble and soluble fibers, soy protein (e.g., oligo- and poly-peptides, hydrolysates), chitosan, red yeast rice (*Monascus*), and minerals (e.g., magnesium, calcium, chromium, potassium).

Caveolae -- Small pockets, vesicles, caves, or recesses communicating with the outside of a cell and extending inward, causing indents in the cytoplasm and cell membrane. Such caveolae may be pinched off to form free vesicles within the cytoplasm. They are considered sites for uptake of materials into the cell, and are one of the routes *Chlamydia* takes to enter host cells. Caveolae are also sites of expulsion of materials from the cell, or sites of addition or removal of cell (unit) membrane to or from the cell surface.

Chlamydia -- Bacteria belonging to the order of *Chlamydiales*, and are contained in the family of obligate intracellular organisms, which includes other obligate intracellular bacteria, viruses, and parasites, as well as the different species of *Chlamydia* such as *Chlamydia trachomatis, Chlamydiophila pneumoniae, Chlamydiophila pecorum, Chlamydiophila psitacci, Chlamydiophila abortus, Chlamydiophila felis, Chlamydiophila caviae, Chlamydia suis,* and *Chlamydia muridarum.*

***Chlamydia* Infection** – Initial infection of host cells by *Chlamydia*. This occurs with the infectious morphological form of *Chlamydia*, the elementary body (see Figure 1).

***Chlamydia* Progression** – Development of *Chlamydia* within host cells. This involves transformation of the infectious elementary body to the non-infectious reticulate body, transformation back to the infectious elementary body form, and growth of the *Chlamydia*-containing inclusion up to the point of cell lysis (see Figure 1).

***Chlamydia*-Reinfection** – At completion of the chlamydial developmental cycle, the infected host cell undergoes cell lysis, setting free infectious elementary bodies (see Figure 1). These infectious elementary bodies then infect neighboring host cell.

***Chlamydia*-Related Diseases** -- Cardiovascular disease, hypertension, atherosclerosis, COX-I- and COX-II-induced inflammation, sexually transmitted disease and genital tract infection, arthritis, respiratory tract infection and pneumonia, ocular infection, neurological diseases, including Alzheimer's disease and multiple sclerosis.

Clathrin-Coated Pits – Involved in internalization of receptor-bound ligands by receptor-mediated endocytosis. This is one of the pathways *Chlamydia* uses to enter a cell.

Cholesterol-Reducing Drugs -- Statins (e.g., lovastatin, simvastatin, pravastatin), citrus bioflavonoids, or specifically polymethoxylated flavones (e.g., tangeretin, nobiletin, hesperidin, rutin), polyphenolics (e.g., EGCG, catechins, resveratrol).

Lipid Rafts – Domains high in sphingolipids and cholesterol in the cell membrane. These domains are detergent-insoluble glycolipid-rich domains and move within the fluid bilayer. These lipid rafts are one of the pathways by which *Chlamydia* enter host cells.

Tocochromanol – Vitamin E. This includes all individual isomers of tocopherol and tocotrienol, tocotrienol-rich fractions from natural sources such as palm, rice, and annatto, and various spectrum vitamin E (e.g., full and appropriate spectra).

Examples

Example 1

Chlamydial Strains: Stocks of *C. trachomatis* serovar K/VR887 were grown in J774A.1 cells without centrifuge assistance. Infected cells were lysed, this stock aliquoted and frozen down in SPG freeze medium (75.0 g sucrose, 0.52 g potassium phosphate, 1.22 g sodium phosphate dibasic, 0.72 g glutamic acid, diluted in 100 ml ddH_2O). These aliquots were stored in liquid N_2 or at –80° C, and later thawed for use to infect monolayers.

Cell Lines Used: Mouse macrophages (J774A.1), human mammary tumor cells (MCF-7), and human epithelial cells (Hep-2), were obtained from the American Type Culture collection. Human mammary tumor cells (TMX2-28) were a kind gift from Dr. Arcaro, and human B-lymphocytes (JY) were a kind gift from Dr. Eric Martz. All cell lines were maintained in Richter's improved MEM insulin (IMEMZO, Irvine Scientific, Santa Ana, CA) with 5% fetal bovine serum (FBS, Atlanta Biologicals, Norcross, GA). Cells were grown to 80% confluence on 12 mm coverslips in 12 well plates (Becton Dickinson Labware, Franklin Lakes, NJ). A dilution of 1:125 or 1:200 of the *C. trachomatis* serovar K stock was made using the standard complete cycloheximide overlay media (Bio-Whittaker, Walkersville, Md.) containing 10% FBS, and 1X L-glutamine (CCOM). This was layered onto the coverslip containing monolayers, and incubated for 24-48 hours at 37° C with 5% CO_2. Coverslips with the cell monolayers were harvested, rinsed with phosphate buffered saline (PBS), fixed with 70% cold methanol, stored and subsequently immunostained.

Delta Tocotrienol Treatment: A stock of delta tocotrienol (98% purity, American River Nutrition, Hadley, MA) was diluted to 1 mg/1 ml in absolute ethanol [EtOH]. Confluent monolayers of J774A.1, MCF-7, TMX2-28, JY, or Hep-2 cells were treated with 5, 10, 20, 30, or 40 μmol/L concentrations of delta tocotrienol. Treatment occurred 5 hours prior to infection with *C. trachomatis* serovar K, at point of infection, or three hours post-infection.

Immunostaining: Briefly, infected cells were immunostained with a 1:2 dilution of guinea pig anti-chlamydia polyclonal antibody (Biomeda, Foster City, CA), or a 1:125 dilution of rabbit anti-chlamydia EB whole serum for 1 hour at 37° C. Following PBS rinses X3, the bound antibodies were detected using a 1:100 dilution of FITC-conjugated donkey anti-guinea pig or a 1:125 dilution of TRITC-conjugated goat anti-rabbit secondary antibodies (Jackson ImmunoResearch, West Grove, PA). Following incubation for 1 hour at room temperature and 3 rinses with PBS, coverslips were mounted onto slides using Fluoromount-G (Southern Biotechnology Associates Inc., Birmingham, AL) or Vectashield® Mounting Medium with DAPI (Vector Laboratories, Inc., Burlingame, CA), and were then sealed. Initially, slides were examined at 400X using a Nikon LABPHOT-2. For photography, slides were assessed in the Massachusetts Central Microscopy Facility using a Zeiss LSM 510 Meta Confocal System and 630X magnification. Images were captured, and as relevant, merged using the Confocal Assistant™ version 4.02 Image Processing Software.

At this magnification, the differences in the number of inclusions present in the tocotrienol-treated sample (Figure 3A) and control (Figure 3B) were readily visualized. As the white arrows point out, no evidence of large, mature inclusions was found with tocotrienol-treatment, whereas untreated cells (3B) exemplify a high level of infection by *Chlamydia*, as shown by the large number and size of the inclusions present.

Quantitatively, immunofluorescence staining showed that the number of chlamydial inclusions was decreased significantly in tocotrienol-treated cells (Figure 3, Table 1). This showed that the initial infection by chlamydial EBs of host cells was impeded and suppressed (Figure 1, step 1).

Example 2

The methods of Example 1 were used in this study. Cells in culture were treated with delta tocotrienol concentrations of 5, 10, 20, 30, and 40 μmol/L, where the viscous Vitamin E compound is diluted in 100% EtOH without cytotoxicity. Controls incubated with the corresponding amounts of EtOH, as well as controls incubated with delta tocotrienol diluted in EtOH showed no difference in cell number when compared to cells grown in culture medium. At 630X magnification, the difference in size and morphology of inclusions was seen when comparing the tocotrienol-treated sample (Figure 4A) to the control (Figure 4B). Inclusions in (4A) were small, and did not fuse to the morphology of mature inclusions. In (4B), inclusions were mature, large, and solidly stained. Optical sections through the Z-axis (third dimension) demonstrate that these untreated cells (4B) were three-fold thicker than the tocotrienol-treated cells (4A). Therefore, the chlamydial inclusion volumes would be expected to be even larger (see Example 3).

Chlamydial inclusions in cells that were treated with higher concentrations (20-40 μmol/L) of delta tocotrienol were immature, small, and less round when visualized by *Chlamydia*-specific immunofluorescence staining (Figure 4). This observation showed that chlamydial development within the host cell was grossly inhibited (Figure 1, step 2→3).

Example 3

Table 1. Summary of Inclusion and Mouse Macrophage Cell Count

Inclusions (per field of view)	First Infection		Second Infection		Third Infection	
	Treated	Control	Treated	Control	Treated	Control
Large (15-20 μm)	0.7	2.6	1.4	2.6	0.7	1.2
Small (≤10 μm)	4.5	10.0	0.6	3.2	1.1	2.1
Total	5.2	12.6	2.0	5.8	1.8	3.3
Total/Cell	0.058	0.111	0.032	0.073	0.016	0.041
Percent Inhibition	52.3%		43.8%		39.0%	

* Controls were cells that were infected, but never treated with delta tocotrienol

The methods of Example 1 were used in this study. Counts on all coverslips with experimental conditions as in Figure 3 and 4 were done for the average of 10 fields of view. Cells were treated with 30 μmol/L concentrations of delta tocotrienol. Re-infectability was studied, where transfer of the supernatant containing infectious elementary bodies (EBs) from infected, tocotrienol-treated cells to uninfected, tocotrienol-treated cells (Table 1).

This observation suggested that repeated use of delta tocotrienol significantly reduces the level of infection by *Chlamydia*, and showed that delta tocotrienol inhibited chlamydial EBs from re-infecting host cells (Figure 1, step 4→1). Therefore, tocotrienol helped the host cells to resist the repeated attempts of infection by *Chlamydia*.

The percent inhibition of inclusion formation in tocotrienol-treated as compared to untreated cells was approximately 40-50% (Table 1). It also should be noted that large inclusions of the control cells were larger (20 μm; more like Figure 1, step 2→3) than large inclusions in the tocotrienol-treated cells (10-15 μm; more like Figure 1, step 1→2). For example, tocotrienol-treated chlamydial inclusions were typically 2 to 3-fold smaller (X-Y axes) and 3-fold thinner (Z-axis; Example 2) than inclusions in control cells. The chlamydial inclusion volume thus calculated (2x3 to 3x3) was 6-9-fold smaller; suggesting that tocotrienol effectively inhibited the normal chlamydial cell cycle (Figure 1; steps 1→2 and 2→3).

The overall effect of delta tocotrienol on *Chlamydia*-infected cells was that it reduced infection by influencing the pathogen's infectivity and by inhibiting its normal development (Table1, Figures 3 and 4), thus compromising the pathogen's entire developmental cycle (Figure 1). This study showed that repeated doses of tocotrienol combat *Chlamydia* infection and progression.

It is likely that control and eradication of *Chlamydia* in humans will require tocotrienol supplementation for an extended period. In light of this, tocotrienols and other agents (such as tangeretin, nobiletin, EGCG, and resveratol; more in Example 7) in monotherapies or in combination are superior because of their lack of toxicity. In contrast, other drugs (such as statins) have sustained toxicities with chronic usage. This invention highlights the use of safe natural products for fighting infections.

Example 4

The methods of Example 1 were used in this study. Samples were analyzed for carriage of *Chlamydia*-infected cells by FACScan (Becton, Dickinson and Company, Franklin Lakes, NJ).

For each period, infection was decreased with delta-tocotrienol treatment. Chlamydial inhibition in JY cells was at least 2-fold over an infection period of 72 hours, with a 2.6-fold maximum inhibition at 36 hours (Figure 5).

Clearly, chlamydial inhibition was effective in as short a period as 1 day, and following one single tocotrienol treatment, the effect was persistent for at least 3 days. This study provides showed that at least one-time dose of tocotrienol is effective against *Chlamydia*.

Example 5

Flow Cytometry Assessment of WBC: Flow cytometry (FC) quantification of *Chlamydia*-infected peripheral WBC used PBS rinsed buffy coat (BC) from HP samples. The BC cells were fixed and permeabilized (1% paraformaldehyde and 1% Triton X-100, 10 min. at RT; Aldrich Chemical Company, Inc., Milwaukee, WI). BCs were separately incubated for 1 hour with a 1:200 dilution of rabbit anti-*Chlamydia* primary antibody (Biodesign International, Saco, ME) followed by a 1 hour incubation with a 1:150 dilution of FITC-conjugated goat anti-rabbit IgG (H+L) (Jackson ImmunoResearch, West Grove, PA), PBS rinsed X3, and mono-dispersed by passage through a nylon mesh filter (Lab-Line Instruments Inc, Melrose Park, IL). Sufficient WBCs were added to obtain 10,000 cells/tube and samples were analyzed for carriage of *Chlamydia*-infected cells by FACScan (Becton, Dickinson and Company, Franklin Lakes, NJ).

In this patient (Figure 6), tocotrienol consumption reduced the granularity and amount of *Chlamydia*-infected cells, which is represented by the *Chlamydia*-negative population in the lower left quadrant of the panel.

Since flow cytometry samples a larger cell population [~10,000 cells], this human study further supported and corroborated the chlamydial inhibition observed in the

microscopic studies of *Chlamydia* infected cells treated *in vitro* (Figures 3 and 4) and shown in the earlier four examples.

This study shows that tocotrienol is effective in humans against *Chlamydia* as represented in circulating WBCs, including neutrophils, monocytes, lymphocytes, eosinophils, and basophils.

Example 6

Table 2. Comparison of LDL Levels and Chlamydial Infection Status in Hyperlipidemic Patients with Delta Tocotrienol Supplementation

	Patient 1	Patient 2	Patient 3
% Decrease in LDL	20%	7%	25%
Chlamydial Infection Status before Delta-Tocotrienol Supplementation	negative	positive	strong positive
Chlamydial Infection Status after Delta-Tocotrienol Supplementation	negative	negative	negative

Detection of *Chlamydia*-Infected White Blood Cells (WBC): Smears of whole blood from each10 ml hyperlipidemic HP blood sample, average donor age 54 years, were fixed with 70% MEOH for subsequent staining. Immunostaining used a 1:125 dilution of rabbit polyclonal anti-*Chlamydia* EB antibody followed by a 1:125 dilution of TRITC-conjugated goat anti-rabbit antibody (H+L) (Jackson ImmunoResearch, West Grove, PA). Slides were incubated at room temperature for 1 hour with each diluted antibody, rinsed, mounted and sealed as described above. Digital images of optical sections through these samples were acquired with a Zeiss LSM 510 Meta Confocal System.

In a clinical study, buffy coats from blood samples drawn both before and after delta-tocotrienol supplementation were immunostained for detection of *Chlamydia*-infected cells. The samples were tested for LDL levels both before and after delta-tocotrienol supplementation.

Tocotrienol lowered cholesterol in patients with or without prior chlamydial infection. Apparently, a cholesterol drop was more significant when the hyperlipidemic

patient was *Chlamydia*-negative (Patient 1 vs. 2). As discussed earlier (Sawayama *et al.*, 2003), pravastatin only lowered cholesterol marginally in *Chlamydia*-positive patients albeit that the carotid plaques were significantly reduced. This is consistent with the four-year clinical study described earlier, wherein patients who took tocotrienols had progressive carotid arteriosclerosis regression, but their cholesterol did not drop until the fourth year (Kooyenga *et al.*, 2001).

However, in patient 2, the chlamydial infection status reversed from positive to negative with tocotrienol supplementation. When chlamydial inhibition responded to tocotrienol supplementation strongly (Patient 3), the cholesterol drop was also larger, similar to the patient that was *Chlamydia*-negative. Since tocotrienol is known to lower cholesterol, such cholesterol synthesis restriction inhibited the cholesterol-requiring growth of *Chlamydia* (Carabeo, 2003), seen in patients 2 and 3.

Tocotrienol supplementation reduced the cholesterol level and chlamydial infection in hyperlipidemic patients, and rendered the chlamydial infection status negative.

Example 7

A corollary of example 6 is that any agent that restricts cholesterol synthesis would therefore prevent or retard *Chlamydia* from hijacking cholesterol. Such agents include statins (e.g., lovastatin, simvastatin, pravastatin), citrus bioflavonoids, or specifically polymethoxylated flavones (e.g., tangeretin, nobiletin, hesperidin, rutin; English, 2004), polyphenolics (e.g., EGCG, catechins, resveratrol; Yamazaki, 2005), and an assortment of nutritional supplements (e.g., plant sterols, B vitamins, omega 3 fatty acids, insoluble and soluble fibers, red yeast rice; Strum, Faloon, 2005). This list is not meant to be limiting, but to exemplify the effect of cholesterol-lowering agents on restriction of *Chlamydia* infection. Other non-limiting examples for cholesterol-lowering, lipid-lowering, and cardiovascular support agents have recently been widely published (English, 2005; Granato, 2003 & 2005; Myers, 2006; Naguib, 2004; Srejic, 2006).

This study shows that any agent that restricts cholesterol/lipids also restricts *Chlamydia* infection/growth. In other words, any agent that restricts cholesterol restricts *Chlamydia*.

Example 8

The methods of Example 1 were used in this study. Untreated cancer cells showed evidence of solid large inclusion (>20 μm; Figure 7B) compared to intermediate size inclusions (20 μm) in non-cancerous murine macrophages (Figure 4B) and even smaller inclusions in tocotrienol treated cells, both murine macrophages (Figure 4A) and mammary tumor cells (10-15 μm; Figure 7A).

The cholesterol content of cancer cells is very high because the rate-limiting HMG-CoA reductase enzyme for cholesterol synthesis in these cells is aberrantly elevated and the enzyme is resistant to sterol feedback regulation. Unequivocally, tocotrienol has been shown to inhibit the reductase synthesis and accelerate reductase degradation (Mo and Elson, 2004).

Since cancer cells are high in cholesterol content, they are more susceptible to infection by cholesterol-hijacking *Chlamydia*, which explains the engorged inclusions within the cells.

Tocotrienols are known to induce apoptosis of cancer cells. Contrarian to this, *Chlamydia* has been shown to inhibit the apoptosis of infected cells. *Chlamydia*-infected, tocotrienol-treated cancer cells in this example did not appear to become apoptotic. Although tocotrienol did not cause infected host cells to undergo apoptosis, it nonetheless disrupted the development of full-blown inclusions by the infecting *Chlamydia*. However, apoptosis with tocotrienol is expected with continual usage (Example 3), whereby the chlamydial EBs are eliminated with time (Example 1), as it has been numerously shown that control of *Chlamydia* is a process of controlling its progression (all earlier examples). This study shows tocotrienol will arrest opportunistic chlamydial infections in cancer patients.

Example 9

Chlamydial infection modulated activation of T-lymphocytes and was linked to a decrease in the antigen-presenting dendritic cell population in the human body. Therefore, the dendritic cells are prevented from activating the T-lymphocytes, and the immunity of the individual against *Chlamydia* and other opportunistic pathogens is decreased. This is

further supported by the implication of *Chlamydia* in autoimmune disease. Tocotrienol is known to boost the immune system to ward off viruses and bacterial infections, and has even been shown to assist T-lymphocytes in slowing down the progression of AIDS and increasing specific immune markers. Thus, tocotrienol also aids the immune system through the activation of dendritic cells and T-lymphocytes to fight off chlamydial infections.

This study shows tocotrienol will ward off pathogenic infections including *Chlamydia* via activation of antigen-presenting dendritic cells and T-lymphocytes.

This invention also applies to activation of antigen-presenting dendritic cells and T-lymphocytes against tumors and cancers.

Example 10

Tocotrienol decreases high blood pressure in hypertensive rats. Primary pulmonary hypertensive (PPH) cells have enhanced expression of caveolae, which contributes to the elevated $[Ca^{2+}]$ associated with hypertension, and when treated with cholesterol-reducing agents such as statin, caveolae expression in these cells was modified and vasoconstriction was reduced. Since PPH cells have increased caveolae expression in cell membranes and *Chlamydia* entry into host cells involves lipid rafts, hypertensive patients are more susceptible to opportunistic chlamydial infections. Tocotrienol, like other cholesterol-reducing agents, decreases caveolae expression in cell membranes, downregulates the $[Ca^{2+}]$ in PPH cells, and protects against chlamydial infections.

This invention provides the application that tocotrienol fights off opportunistic chlamydial infection of hypertensive patients, and thereby reduces their blood pressure.

Example 11

Metabolic syndrome is a cluster of cardiovascular risk factors, including elevated waist circumference, elevated triglycerides, reduced high-density lipoprotein cholesterol, elevated blood pressure, and elevated fasting glucose associated with obesity, and elevates one's risk of developing diabetes. *Chlamydia* is not a causative agent of diabetes, but seroactive chlamydial infections in diabetic patients are more frequent than in non-diabetic

patients, meaning that diabetic patients may be more susceptible to infection by *Chlamydia*. Tocotrienol reduces the cardiovascular risk factor associated with metabolic syndrome, obesity, and diabetes, and therefore may reduce opportunistic chlamydial infections due to these factors.

This invention provides the application that tocotrienol reduces cardiovascular risk factors associated with metabolic syndrome, and thereby reduces the risk of diabetes and concomitant or subsequent infection by *Chlamydia*.

Example 12

The mode of application of tocotrienols is important because *Chlamydia* is involved in many public health-related diseases. A non-limiting summary of these diseases is described in the background section. The mode of application of tocotrienol may be in form of aerosol sprays (to reach respiratory tract airways), oral ingestion via softgels, tablets or capsules (to reach vascular-organistic systems), topical creams, douches, and lotions (to reach genital sites), and topical liquid drops (to reach ocular sites).

Dosages: In one embodiment, the invention is drawn to a method comprising administering delta-T3 in a range from 10 to 1000 mg per day. In a preferred embodiment, the invention is drawn to a method comprising administering delta-T3 in a range from 20 to 500 mg per day. In a more preferred embodiment, the invention is drawn to a method comprising administering delta-T3 in a range from 50 to 150 mg per day. Treatment would be continuous with the delta-T3 being administered daily at the above-mentioned dosages (for as short as one month, preferably six months, and most preferably one year), or until infection and inflammation due to *Chlamydia* is cleared (e.g., no inclusion-forming units can be found in the patient's whole blood). However, for *prevention* of chlamydial infection the dosage can be on the lower end (e.g., 1-100 mg/day), and the dosage duration can be indefinite, and begun before the subject has any evidence of infection by *Chlamydia*.

To reach respiratory tract infections, tocotrienol may be used in form of aerosol spray, at a dosage of 1-4 sprays per day at the above-mentioned dosages. For genital tract infections, tocotrienol may be administered in form of topical creams, lotions, or douches with 1-4 applications per day at the above-mentioned dosages. Vascular-organistic systems

inflamed due to chlamydial infection can be treated with oral ingestion of tocotrienol with 1-4 softgels, tablets, or capsules per at the above-mentioned dosages. To treat ocular infections, including conjunctivitis, tocotrienol may be administered in form of liquid eye drops with 1-4 applications per day at the above-mentioned dosages.

Claims

1. The use of a tocotrienol Vitamin E tocochromanol in the manufacture of a medicament for the treatment of an *Chlamydia* infection , wherein said medicament is to be administered daily for at least one month to a mammal in need of treatment; wherein the amount of the tocotrienol is a dose between 10 mg and 1000 mg per day, and wherein the tocotrienol treats the *Chlamydia* infection.

2. The use of claim 1, wherein a geranyl geraniol is to be further administered.

3. The use of claim 1, where the tocotrienol is selected from the group consisting of a natural tocotrienol and a synthetic tocotrienol.

4. The use of claim 3, where the tocotrienol is an isomer of a tocotrienol

5. The use of claim 4, where the isomer of tocotrienol is selected from the group consisting of alpha tocotrienol, beta-tocotrienol, gamma-tocotrienol, delta, desmethyl-tocotrienol, and didesmethyl-tocotrienol.

6. The use of claim 1, where the *Chlamydia* is selected from the group consisting of *Chlamydia trachomatis, Chlamydia suis, Chlamydia muridarum, Chlamydiophila pneumoniae, Chlamydiophila psittaci, Chlamydiophila pecorum, Chlamydiophila abortis, Chlamydiophila felis,* and *Chlamydiophila caviae.*

7. The use of claim 1, where mode of application of the medicament is selected from the group consisting of aerosol spray, oral ingestion, creams, douches, lotions, and eye drops.

8. The use of claim 1, wherein said medicament is to be administered at a dose of between 20 mg and 500 mg per day.

9. The use of claim 1, wherein said medicament is to be administered at a dose of between 50 mg and 150 mg per day.

10. The use of claim 5, where the isomer of tocotrienol is delta-tocotrienol.

11. Use of tocotrienol for preparation of a medicament for the treatment of an infection by *Chlamydia,* wherein said tocotrienol is administered in combination with at least one agent selected from the group consisting of a statin, a bioflavonoid, a polyphenolic, a polymethoxylated flavone, a plant sterol, a oryzanol, a policosanol, a B vitamin, Coenzyme Q10 (CoQ10), an omega 3 fatty acid, a lecithin, garlic, a gugul lipid, an insoluble fiber, a soluble fiber, a soy protein, a chitosan, a red yeast rice, and a mineral.

12. The use of claim 11, wherein the bioflavonoid is citrus bioflavonoid.

13. Use of a tocotrienol for the preparation of a medicament for the treatment of a *Chlamydia* infection, wherein said tocotrienol is administered daily for at least one month to a mammal or avian to treat *Chlamydia*-associated disease selected from the group consisting of cardiovascular disease, hypertension, atherosclerosis, COX-I- and COX-II-induced inflammation, a sexually transmitted disease, a genital tract infection, arthritis, prediabetes, metabolic syndrome, diabetes, polycystic ovarian syndrome (PCOS), a respiratory tract infection, pneumonia, an ocular infection, a neurological disease, Alzheimer's disease, and multiple sclerosis.

USE OF VITAMIN E TOCOTRIENOLS FOR THE INHIBITION OF INTRACELLULARLY OBLIGATE PATHOGEN CHLAMYDIA

Abstract

This invention reveals the beneficial use of vitamin E tocotrienols for inhibition of chlamydial infections. Chlamydial infection levels in mouse macrophages treated with tocotrienol were decreased >50%, with concomitant aberrant pathogen development. The number of large and small inclusions in tocotrienol-versus-control cells was decreased 3-fold and 2-fold, respectively. When treated with delta tocotrienol, *Chlamydia* in human lymphocytes was inhibited by at least 2.6-fold in 1.5 days. Dietary delta tocotrienol inhibited *Chlamydia* infection and persistence in hypercholesterolemic patients with a corresponding drop in LDL. These studies demonstrate that tocotrienol lowers cholesterol, thus preventing or diminishing the cholesterol hijacking by *Chlamydia* obligatory for its infectivity and replication. Therefore, hypolipidemic agents used to treat cardiovascular diseases, metabolic syndrome, and diabetes are used as monotherapies, or in combination with tocotrienol to treat *Chlamydia*.

Figure 1.