METHODS OF TREATING HYPERTENSION AND OF IMPROVING IMPAIRED RENAL FUNCTION

A method of treating hypertension and related symptoms thereof is described. A suspension of small, unilamellar vesicles composed primarily of phospholipids similar in nature to those of egg phosphatidylcholine is administered parenterally to a subject in need of such treatment repeatedly and over an extended period of time of at least several days, until a significant drop in blood pressure is observed. A method of improving renal function is also disclosed. In the treatment method, a suspension of liposomes is administered parenterally to a subject in need of such improvement repeatedly and over an extended period of time of at least several days, until a significant improvement in renal function is observed, as evidenced by an improvement in glomerular filtration rate characterized by a drop in plasma creatinine concentration of at least 10 percent.
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METHODS OF TREATING HYPERTENSION AND
OF IMPROVING IMPAIRED RENAL FUNCTION

1. Field of the Invention

The present invention relates to a method for treating hypertension and to a method for improving impaired renal function, and in particular, to treatment by administration of phosphatidylcholine liposomes.

2. References


CRC Press, Boca Raton, FL.

Harrison, T.R., editor-in-chief, Harrison’s Principles of Internal Medicine, Twelfth Ed.,


Kasten, P.H., Tissue Culture: Methods and Applications, Kruse, P., Patterson, M.,
Levida, M. Handbook of Nutrition in the Aged (R.R. Watson ed.), CRC Press, pp 89-


3. Background of the Invention

Hypertension, which refers to elevated arterial pressure, is a widespread health problem in developed countries. Diagnosis of hypertension depends on measurement of blood pressure, which is typically reported as a ratio of systolic pressure (arterial pressure during contraction of the heart muscle) to diastolic pressure (residual arterial pressure during relaxation of the heart muscle), reported in units of mmHg. A normal diastolic blood pressure is between about 60-85 mmHg. Typically, adult hypertension is defined as a resting blood pressure greater than 140 mm Hg (systolic)/90 mm Hg (diastolic). Diastolic pressures above about 85-90 mmHg are
generally diagnostic of hypertension. By some estimates, the arterial blood pressure of fifteen percent of American adults is in a hypertension range that requires medical treatment.

A number of factors have been implicated in the development of hypertension. These include heredity and a number of environmental factors such as salt intake, obesity, occupation, family size, and crowding. Additional factors which may modify the course of hypertension include age, race, sex, stress, diet, smoking, serum cholesterol, and glucose intolerance.

The majority of individuals suffering from hypertension have no obvious cause for their elevated blood pressure levels; such individuals are described as suffering from essential hypertension. In about ten percent of hypertensive cases, a definite cause can be identified, including various disorders of the kidney, adrenal glands and coartation of the aorta.

The effects of hypertension are numerous, with the most severe being premature death, commonly caused by heart disease related to hypertension. Hypertension imposes an increased work load on the heart; related effects on the heart include angina pectoris, increased myocardial mass or hypertrophy (enlarged heart), and, late in the disease, evidence of ischemia or infarction.

Neurologic effects of hypertension are commonly divided into retinal and central nervous system changes. With respect to retinal impact, increasing severity of hypertension is associated with focal spasm as well as hemorrhages, exudates and papilledema, which often produce scotomata, blurred vision and even blindness. Central nervous system dysfunction may cause occipital headaches, dizziness, lightheadedness, vertigo, tinnitus and dimmed vision.

Drug therapy is a common approach to treatment of hypertension. In general, antihypertensive drugs belong to one of five classes of compounds: diuretics, antiadrenergic agents, vasodilators, calcium entry blockers, and angiotensin-converting enzyme (ACE) inhibitors (e.g., Harrison, Katzung). Each of the types of drugs, though generally effective in reducing hypertension, has side effects, such as potassium depletion, hyperglycemia, hypokalemia, depression, carbohydrate intolerance, tachycardia, and/or allergic skin rashes, and in more severe cases, vomiting, fever, diarrhea, angina, and cardiac failure.

One possible complication of untreated hypertension, among many, includes damage to the kidney. The kidney is the organ responsible for filtering blood and excreting waste products and excess water in the form of urine. Kidney or renal failure can be acute (of sudden onset) or chronic (developing gradually). Typically, renal failure refers to a reduction in the ability of the kidneys to i) filter waste products from the blood and excrete them in the urine, ii) to control the body’s water and salt balance, and to regulate blood pressure.
Acute renal failure (ARF) refers to a deterioration in renal function sufficient to result in the accumulation of nitrogenous wastes in the body. In general, patients suffering from acute renal injury can regain kidney function if the condition is diagnosed early and treated promptly. Chronic renal failure, however, involves irreversible kidney damage, typically progressive and permanent destruction of nephrons. Early diagnosis and treatment of acute renal failure can in some cases arrest or prevent progression to a chronic condition.

Approximately five percent of all hospitalized patients develop acute renal failure; in some clinical settings, such as intensive care units, ARF can occur in up to 20 percent of the patients. Acute renal failure may be due to prerenal, renal or postrenal causes. About 40 to 80 percent of all cases of acute renal failure are caused by decreased renal perfusion (prerenal azotemia), often resulting from varied conditions or events such as gastrointestinal hemorrhage, burns, diarrhea, severe congestive heart failure, pancreatitis, or sepsis. Postrenal causes, such as bladder neck constriction, account for about 10 percent of all cases of ARF while renal causes include disorders such as thrombosis, emboli, and vasculitis. Additionally, two types of common pharmacologic agents, non-steroidal anti-inflammatory agents (NSAID's) and angiotensin converting enzyme inhibitors may cause ARF in predisposed patients. Cross sectional studies have also found that aging is typically accompanied by a decline in renal function (Harrison).

ARF is typically detected by determination of glomerular filtration rate (GFR) or blood urea nitrogen or serum creatinine levels. GFR is the rate of ultrafiltration of plasma across the walls of the glomerular capillaries and measurement of total GFR of both kidneys provides a sensitive index of overall renal excretory function. Normal renal excretory function is indicated by a GFR of about 125 mL/min (180 L/day), although when renal excretory capacity is impaired, total GFR declines. Often, measurements of urea and creatinine concentrations are used to assess the glomerular filtration rate. Both substances are produced at a relatively constant rate by the liver and muscles; an increase in their respective serum concentrations occurs as GFR declines due to the fact that both compounds undergo complete glomerular filtration and are not reabsorbed by the renal tubules. Creatinine provides a more reliable index of GFR than urea because urea can back diffuse more completely from tubule lumen to peritubular blood than creatinine. Thus, blood urea nitrogen levels are typically higher than serum creatinine levels; a normal ratio of blood urea nitrogen to serum creatinine is about 10.

Chemical analysis of both urine and serum samples are useful indicators of ARF. For example, the range of urine osmolalities that can be achieved by an individual with normal-
functioning kidneys (40 to 1200 mosmol/kg) is much larger than the range achievable in diseased kidneys (250-350 mosmol/kg). Typically, acute renal failure is characterized by urine osmolalities of below about 400 mosmol/kg, urine sodium concentrations above about 40 mmol/L, a ratio of urine-to-plasma creatinine levels below 20, and a fractional excretion of filtered sodium, defined as the ratio of urine sodium concentration/serum sodium concentration to urine creatinine concentration/serum creatinine concentration multiplied by 100, of about 2 (Harrison).

As mentioned above, the early effective treatment of ARF can arrest the progression of the condition to chronic renal failure, where permanent kidney damage occurs.

4. Summary of the Invention

In one aspect, the present invention is based on the discovery that treatment of human subjects with intravenously administered liposomes of the type described herein results in a significant lowering of blood pressure without apparent side effects. The treatment also leads to a reversal of myocardial hypertrophy, a common side effect of elevated arterial pressure.

In one aspect, the invention includes a method for treating hypertension in a subject having elevated blood pressure. The method includes intravenously administering to the subject a suspension of small unilamellar liposomes composed primarily of phosphatidylcholine phospholipids having phase transition temperatures in the range between about -10 and 37°C. Administration is repeated over a period of at least several days and in an amount effective to produce a significant reduction in both systolic and diastolic blood pressure of at least 10 percent.

Preferably, the liposomes in the suspension have sizes ranging predominantly between 0.02 and 0.08 microns and are composed of egg phosphatidylcholine.

In one embodiment of this aspect of the invention, the liposomal suspension is administered 1-3 times per week, at a dose of between about 0.05 and 1 µg lipid/kg body weight. Administration of the liposomes is continued until a drop in blood pressure of at least about 10% is observed.

Another aspect of the present invention is based on the discovery that treatment of subjects with intravenously administered liposomes of the type described herein results in a significant improvement in renal function in a subject with diminished or impaired renal function. The effect of liposomal treatment on kidney function, where the liposomes are acting as the therapeutic agent for improving impaired renal function, is evidenced by measurable
changes in kidney serum creatinine levels, urine maximal osmolality and sodium concentration, as well as medullary Na-K ATPase activity.

The invention includes a method of improving renal function in a subject in need of such treatment, i.e., a subject having diminished or failing renal function as evidenced by a subnormal rate of glomerular filtration of less than 180 liters per day. In the method of this aspect of the invention, a suspension of liposomes composed primarily of phosphatidylcholine (PC) phospholipids is administered in an amount of liposomes effective to produce, over an extended period of several dosings, a substantial improvement in overall renal function of the treated subject. Such substantial improvement is evidenced by an improvement in glomerular filtration rate characterized by a reduction in serum creatinine concentration of at least 10 percent. The PC phospholipids composing the liposomes have a phase-transition temperature between about -10 to 37 °C, preferably a transition temperature of less than about 5°C, as exemplified by egg phosphatidylcholine (egg PC) which has a transition temperature of -5°C.

In one embodiment, the liposome suspension is for use in preventing chronic renal failure in a patient suffering from acute renal failure, where such acute renal failure is evidenced by a ratio of urine-to-plasma creatinine concentration below 20. The liposomal composition is repeatedly administered to the patient over an extended period and in an amount effective to improve the ratio of urine-to-plasma creatinine concentration above 20.

In an alternative embodiment, the liposome suspension of the invention is administered to a middle aged subject suffering from age-related diminution of renal function. The liposomes are administered intravenously in an amount effective to produce, over an extended period of several dosings, a substantial improvement in overall renal capacity as determined by an increase of at least 10 percent in urine maximal osmolality.

Preferably, the liposome suspension is administered 1-3 times per week, at a dose of between about 0.05 and 1 g lipid/kg body weight.

The features and details of the invention will become more apparent and appreciated by one skilled in the art to which this invention pertains from the following detailed description of the invention.

**Brief Description of the Drawings**

Fig. 1 illustrates the blood circulation time of intravenously administered PC SUV's in rat, measured as a function of plasma PC concentration over time;
Fig. 2 illustrates the blood circulation time of intravenously administered PC SUV's in rat, measured as a function of percent injected dose over time;

Fig. 3 is a graph of systolic (open triangles) and diastolic (open squares) blood pressure in one human volunteer as a function time after liposome treatment in accordance with the invention;

Fig. 4 is a plot similar to Fig. 3 for a second human volunteer;

Fig. 5 is a bar graph showing uptake of $^{46}$Ca$^{2+}$ in myocyte cells that are 4 days old, 12 days old, and 12 days old treated with PC SUV liposomes;

Fig. 6 is a bar graph showing the reduction in plasma creatinine concentration as a function of intravenous liposome treatment (solid bars: treated subjects; striped bars: untreated) for three age groups of male rats;

Fig. 7 is a bar graph showing the elevation of urine maximal osmolality as a function of intravenous liposome treatment (solid bars: treated subjects; striped bars: untreated) for three age groups of male rats;

Fig. 8 shows the reduction in sodium excretion as a function of intravenous liposome treatment (solid bars: treated subjects; striped bars: untreated) for three age groups of male rats; and

Fig. 9 illustrates enhanced renal medullary Na-K-ATPase activity as a function of intravenous liposome treatment (solid bars: treated subjects; striped bars: untreated) for three age groups of male rats.

Detailed Description of the Invention

Definitions:

The following terms, as used herein, have the meanings indicated:

Elevated blood pressure (e.g., hypertension) is defined as a resting blood pressure greater than 140 mm Hg (systolic)/85-90 mm Hg (diastolic) in an adult human subject.

Impaired renal function or diminished renal function, typically caused by damage to the nephrons (the glomeruli and tubules which make up the functioning units of the kidney) refers to a diminished capacity of the kidneys to regulate blood and electrolytes, to eliminate waste products, and to control the body’s acid-base balance.
Renal failure refers to a reduction in the ability of the kidneys to i) filter waste products from the blood and excrete them in the urine, ii) to control the body's water and salt balance, and to regulate blood pressure.

Acute renal failure (ARF) refers to a deterioration in renal function sufficient to result in the accumulation of nitrogenous wastes in the body. Usually, patients suffering from acute renal injury can regain kidney function if the condition is diagnosed early and treated. Chronic renal failure (CRF) involves irreversible damage to the kidneys, typically progressive and permanent destruction of nephrons.

Glomerular filtration rate or GFR is the rate of ultrafiltration of plasma across the walls of the glomerular capillaries.

Therapeutic agent, as used herein in reference to the liposomes of the present invention, refers to an agent used to treat a specific condition, wherein administering of such an agent has a beneficial effect in treating the condition.

I. Preparation of Liposomes

The present invention involves, in one aspect, administering a suspension of liposomes parenterally to an individual (a veterinary animal or human) to reduce hypertension. The invention also includes, in another aspect, administering to a subject (an animal or a human) a suspension of liposomes to improve impaired renal function. The subject in need of such treatment is one suffering from acute renal failure, or diminished renal function, or age-related diminution of renal function. The treatment of acute renal failure by the method of the invention can arrest progression of the condition to chronic renal failure.

In a preferred embodiment of the invention, described and used in the examples below, the liposomes are composed predominantly (more than 50 mole percent, preferably more than 80-90 mole percent) of phosphatidylcholine (PC) having a phase transition temperature less than about 37°C, preferably between about -10 to 24°C, e.g., below about 5°C. The preparation of liposomes composed primarily of phosphatidylcholine has been previously described (Barenholz, 1989).

The liposome composition used in the method of the present invention is composed primarily of PC phospholipids. PC phospholipids include those phospholipids having a choline moiety and where the fatty acid chain portion of the phospholipid may vary in length and degree of unsaturation.

One preferred vesicle composition includes egg PC, which has a transition temperature of -5°C, and contains predominantly 1-palmitoyl, 2-oleyl PC and 1-palmitoyl,2-linoleyl PC.
The liposomes may be composed entirely of the egg PC, or may contain other lipid components which (i) are not immunogenic, (ii) do not contribute a significant portion, i.e., more than 25-50 mole percent, of lipids with high phase transition temperature. Additional components may include negatively charged lipids, such as phosphatidylglycerol (PG) or phosphatidylserine (PS).

Of course, the mole percentage of these lipids should be relatively low with respect to PC. The liposomes may also include cholesterol or other sterols, in an amount preferably less than about 40 mole percent.

Lipid protective agents, such as α-tocopherol, α-tocopherol acetate, or α-tocopherol succinate, may also be included in the lipids forming the liposomes, to protect the lipid components against free radical damage (Levida). Typically such agents are included at a mole percentage between about 0.5% and 2%. It is advantageous to add α-tocopherol to the liposomes to maintain a balance between vitamin E and lipids in the liposomes.

A. Unsized Liposomes

A variety of methods for producing liposomes are available, and these have been extensively reviewed (Szoka 1980). In general these methods produce liposomes with heterogeneous sizes from about 0.02 to 10 microns or greater. As will be discussed below, liposomes which are relatively small and well defined in size are preferred for use in the present invention, hence a second processing step for reducing the size and size heterogeneity of liposomal suspensions will usually be required.

In one preferred method for forming the initial liposome suspension, the vesicle-forming lipids are taken up in a suitable organic solvent system, and dried in vacuo or under an inert gas to form a lipid film in a vessel. An aqueous suspension medium, such as a sterile saline solution, is added to the film, and the vessel is agitated until the lipids have hydrated to completion, typically within 1-2 hours. The amount of aqueous medium added is such as to produce a final liposome suspension containing preferably between about 10 and 30 g lipid per 100 ml media.

The lipids hydrate to form multilamellar vesicles (MLVs) whose sizes range between about 0.5 microns to about 10 microns or larger. In general, the size distribution of MLVs can be shifted toward slightly smaller sizes by hydrating the lipids under more vigorous shaking conditions. Example 1 describes the preparation of egg PC MLVs, prior to treating the MLVs with ultrasonic irradiation to reduce the liposome sizes.

The aqueous medium used in forming the liposomes may contain water-soluble agents which enhance the stability of the liposomes upon storage. A preferred stabilizing agent is an
iron-specific trihydroxamine chelating agent, such as desferrioxamine. The use of this compound in reducing lipid peroxidation and free radical damage in drug-containing liposomes has been reported in co-owned U.S. Patent No. 4,797,285. Briefly, it was shown that the combination of a lipophilic free-radical quencher, such as α-tocopherol, and the water-soluble chelator gave substantially better protection against lipid peroxidation damage than did either protective agents alone. The chelator is included in the aqueous medium in molar excess of the amount of free iron in the medium. Typically, a chelator concentration of between about 10-200 micromolar is sufficient.

B. Sizing Liposomes

The suspension of liposomes prepared as above is preferably treated to produce a desired liposome size and size homogeneity for use in the treatment methods of the invention.

The liposome suspension may be sized to achieve a selective size distribution of vesicles in a size range less than about 1 micron and preferably less than about 0.2-0.3 microns. Liposomes in this size range can be readily sterilized by filtration through a depth filter. Smaller vesicles also show less tendency to aggregate on storage, thus reducing potentially serious vascular blockage problems when the composition is administered parenterally. Finally, liposomes which have been sized down to the submicron range show more uniform biodistribution and drug clearance characteristics.

Preferred liposomes are small unilamellar vesicles (SUVs), i.e., single-bilayer liposomes having sizes between about 0.02 to 0.08 microns. SUVs have relatively long blood circulation halflives, when administered intravenously. This is illustrated in Figs. 1 and 2, which show plots of liposome retention in the bloodstream, measured up to 1,000 minutes after IV injection, and expressed either as PC measured in plasma (Fig. 1), or as percent injected dose (Fig. 2). As seen, significant amounts of liposomes remained in the bloodstream even at 1,000 minutes.

Several techniques are available for reducing the sizes and size heterogeneity of liposomes, in a manner suitable for the present invention. Ultrasonic irradiation of a liposome suspension either by bath or probe sonication produces a progressive size reduction down to SUVs. A sonicating procedure used to produce SUVs is described in Example 1. Homogenization is another method which relies on shearing energy to fragment large liposomes into smaller ones. In a typical homogenization procedure, MLVs are recirculated through a standard emulsion homogenizer until selected liposome sizes, typically less than 0.1 microns, are observed.
Extrusion of liposomes through a small-pore polycarbonate membrane is an effective method of reducing liposome size down to a relatively well-defined size distribution. An average range is between about 0.03 and 1 micron, depending on the pore size of the membrane, such as described in Example 2. Typically, the suspension is cycled through the membrane several times until the desired liposome size distribution is achieved. The liposomes may be extruded through successively smaller-pore membranes, to achieve a gradual reduction in liposome size.

The size-processed liposome suspension may be readily sterilized by passage through a sterilizing membrane having a particle discrimination size of about 0.2 microns, such as a conventional 0.22 micron depth membrane filter. If desired, the liposome suspension can be lyophilized for storage and reconstituted shortly before use.

II. Utility

A. Treatment Method For Elevated Blood Pressure

In the treatment method of one aspect of the invention, namely treatment of hypertension, a liposomal suspension of the type described above in which the liposomes are acting as the therapeutic agent is administered parenterally over an extended period of time of at least several days until a significant reduction in blood pressure is produced. The liposomal suspension is typically administered at a dosing frequency no greater than once per day, although dosing frequency may vary depending on the severity of the hypertensive condition, the age and overall general health of the subject, and other pre-existing conditions.

Additionally, and at any point in the treatment, the liposomal suspension may be administered in combination with other known antihypertensive agents to produce the desired reduction and or maintenance of blood pressure levels.

The liposomes may be conveniently administered as a series of dosages, given over a period of at least several days, and preferably maintained by continued doses at one to several month intervals over the lifetime of the treated individual. The amount of liposomes administered at each dose is between about 0.01 and 10 g lipid per kg of body weight, and preferably between about 0.05-1.0 g lipid per kg of body weight, although the dose may be substantially less. Long term dosages are typically delivered at a rate of between about 0.01-1 g lipid per kg body weight per day. In a preferred embodiment, the liposome suspension is administered 1-3 times per week, at a dose of between about 0.05 and 1 g lipid/kg body weight.
A typical dose for an 80 kg individual is between about 40 and 80 grams lipid, corresponding to between 200 and 400 ml of an up to 20 g lipid/100 ml suspension. Administration may be by iv (intravenous) bolus injection, but is preferably done by iv drip over a period of at least about 1 hour, to minimize tissue and blood trauma at the site of administration. The liposomes may be suspended in sterile saline or in a nutritional or drug-containing medium, such as a glucose/salt medium, to combine liposome treatment with other parenteral therapy.

Before the first dose is given, a minimum of two blood pressure measurements may be taken to provide a baseline for comparison over the course of liposome treatment. The liposome treatment is continued until a significant drop in both systolic and diastolic blood pressure of at least 5 percent is produced, and preferably, of at least about 10% or more.

Following the first liposome administration, blood pressure is measured, and a second liposome dose is given, typically between 2 and 7 days subsequent the first dose. Blood pressure is monitored in the conventional fashion in the upper arm with a sphygmomanometer. Values for both systolic and diastolic pressure are obtained and recorded. Further liposomal doses may likewise be given at 2-7 day intervals until either the blood pressure measurements begin to plateau or blood pressure measurements within the "normal" range are achieved. Thereafter, the subject may be maintained within the target blood pressure range by periodic maintenance liposome treatments, e.g., every 1-2 months.

B. Results: Effect on Blood Pressure

In support of the invention, a suspension of SUV liposomes was administered to two human volunteers. As described in Example 3, blood pressure was measured over the treatment period of 16 days, during which time seven liposomal treatments were administered. The results are shown in Table I and in Figs. 3 and 4. As seen in Table I, blood pressure levels declined by approximately 20% from the Day 1 baseline value in both of the human subjects after the seventh treatment.

**TABLE I**

<table>
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<tr>
<th>Subject</th>
<th>1st Treatment Day 1</th>
<th>2nd Day 3</th>
<th>3rd Day 7</th>
<th>4th Day 9</th>
<th>5th Day 11</th>
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<tr>
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The blood pressure data for volunteers A and B is plotted in Figs. 3 and 4, respectively. The figures show that a reduction in both systolic pressure (open triangles) and diastolic pressure (open squares) occurs following treatment with the liposomal suspension, in accordance with the method of the invention.

C. Results: Effect on Cardiac Hypertrophy

As discussed above, one side effect of hypertension is cardiac hypertrophy or an enlargement of the heart or heart tissue due to an increase in size of its constituent cells. Experiments were performed to show that a reduction in cardiac hypertrophy is observed in hypertrophied cells treated according to the method of the invention.

As described in Example 4, the effect of egg PC liposome treatment on myocardial hypertrophy was determined by administering liposomes to laboratory rats ranging in age from 1.5 to 2 years. The animals were treated by iv (femoral vein) administration of SUVs every three days for six days with 0.5-1 g lipid per animal.

Three days after the final injection, the animals were sacrificed, and the heart mass, expressed as heart mass (wet mass) to total body mass of the animals, was determined for both the treated and the control animals, and the results shown in Table II below. The data in the table represent the average values for each group (treated versus untreated). As seen, the treatment method resulted in a marked reduction in average heart mass of about 20%.

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D. Results: Calcium Uptake

Experiments were carried out to investigate the effect of liposome treatment on hypertrophic myocyte cells, and in particular, on the levels of cellular calcium uptake. Calcium overload is known to be one of the more prominent features of the hypertrophic heart and is considered to be a critical factor in causing irreversible ischemic damage in hyper-
tension. It was discovered that increased levels of calcium incorporated in older, hypertrophied cells were reversed upon treatment with egg PC SUVs, as reported in the study described in Example 5.

In this study (Example 5), cellular calcium uptake in hypertrophied and in non-hypertrophied cells was measured after incubation of the cells in $^{45}$Ca$^{2+}$ for up to 2 hours at 37°C. The results are shown in Fig. 5, and, as seen, the older, hypertrophic cells (12 days old) exhibited a 3-fold increase in the level of calcium uptake as compared to the younger, non-hypertrophic cells (4 days old). However, after a 72 hour treatment with egg PC SUVs, a greater than 50 percent decrease in cellular calcium uptake was detected in the older cells, as seen in the bar of Fig. 5 labeled "Day 12 + SUV".

E. Treatment Method for Acute Renal Failure

According to another aspect of the present invention, a liposomal suspension as has been described is administered parenterally over an extended period of time of at least several days until a significant improvement in impaired renal function is achieved. Impaired renal function may be characterized by a number of different indicators. Generally, such renal impairment is evidenced by a subnormal rate of glomerular filtration of less than about 180 liters per day. Alternatively, diminished renal function such as that characterized by acute renal failure is characterized by a ratio of urine-to-plasma creatinine concentration below about 20.

Diminished renal function is also characterized by a drop in urine concentrating capacity as evidenced by urine maximal osmolality values.

In one aspect, the method is used for treatment of renal failure, and more particularly, to improve renal function by preventing progression of ARF to chronic renal failure and for use in improving age-related diminution of renal function in a middle-aged subject. Generally, adults from about 40-60 years of age are considered to be middle-aged.

Improvement in renal function is commonly indicated by plasma creatinine levels; commencement of the recovery phase of ARF occurs when the glomerular filtration rate increases so that blood urea nitrogen and serum creatinine concentrations no longer continue to rise. However, significant recovery is typically indicated by reduction in plasma creatinine levels or change in urinary osmolality.

The liposomal suspension is typically administered at a dosing frequency no greater than once per day, although dosing frequency may vary depending on the severity of the renal disorder, the age and overall general health of the subject, and other pre-existing conditions.
Additionally, and at any point in the treatment, the liposomal suspension may be administered in combination with other known agents or methods of treatment to produce the desired improvement in renal function and glomerular filtration rates and or maintenance of serum creatinine levels.

The liposomes may be conveniently administered as a series of dosages, given over a period of at least several days, and preferably maintained by continued doses at one to several month intervals over the lifetime of the treated individual. The amount of liposomes administered at each dose is between about 0.01 and 1.0 g lipid per kg of body weight, and preferably between about 0.05-1.0 g lipid per kg of body weight, although the dose may be substantially less. Long term dosages are typically delivered at a rate of between about 0.001-1 g lipid per kg body weight per day. In a preferred embodiment, the liposome suspension is administered 1-3 times per week, at a dose of between about 0.05 and 1 g lipid/kg body weight.

As described above, a typical dose for an 80 kg individual would be between about 40 and 80 grams lipid, corresponding to between 200 and 400 ml of an up to 20 g lipid/100 ml. Administration may be by iv (intravenous) injection, but is preferably done by iv drip over a period of at least about 1 hour, to minimize tissue and blood trauma at the site of administration. The liposomes may be suspended in sterile saline or in a nutritional or drug-containing medium, such as a glucose/salt medium, to combine liposome treatment with other parenteral therapy.

Typically, before the first dose is given, both urine and plasma samples are withdrawn and biochemical analyses performed, such as urine maximal osmolality, urine sodium concentration, and both urine and plasma concentrations of urea and creatinine, to provide a baseline for comparison over the course of liposome treatment. The liposome treatment is continued until renal function is improved as evidenced by a change in creatinine levels (in urine or in plasma) or urine concentrations of pertinent indicators (e.g., urine maximal osmolality, urine sodium concentration, urine urea concentration, and the like).

Following the first liposome administration, plasma creatinine, and optionally other biochemical markers of renal function, are measured, and a second liposome dose is given typically between 2 and 7 days subsequent to the first dose. Further doses may likewise be given at 2-7 day intervals until either the plasma creatinine measurements no longer decrease or desired plasma creatinine concentrations are achieved. Thereafter, the subject may be maintained within the target plasma creatinine range by periodic maintenance liposome treatments, e.g., every 1-2 months.
F. Restoring Diminished Renal Function

According to a related aspect of the invention, the method is useful in improving renal function in a subject who has age-related diminution of renal function and decreased GFR. In this method, the liposomal suspension is administered parenterally over an extended period of time of at least several days to a subject suffering from renal failure until a major improvement in renal function is achieved.

The liposome treatment for age-related decline in renal function was applied to laboratory animals, as described in Examples 6-8. In one series of tests, three groups of Sabra male rats, aged 5 months (young), 12 months (middle age), and 22 month (old) were treated with a suspension of egg PC SUVs. The treatment consisted of 6 intravenous administrations of 1 gram SUV per 1 kg body weight over a three week period. Two months following the final SUV treatment, the function of the kidney was assessed by evaluating plasma creatinine levels, urine maximal osmolality and sodium concentration, and medullary Na-K-ATPase activity as described in Examples 6-8.

As seen in Fig. 6, a decline in plasma creatinine levels after PC SUV treatment for all three groups of animals was observed, with the most prominent lowering of plasma creatinine observed in the middle aged group (approximately 15 percent reduction in plasma creatinine). A decrease in plasma creatinine levels correlates with increased glomerular filtration rate and accordingly, enhanced renal excretory function. The animal ages given in Figs. 6-9 refer to the ages at the start of the treatment. The actual measurements were thus made about 2.5 months after the indicated ages.

An improvement in the ability to concentrate urine following liposomal treatment was also observed, as shown in Fig. 7. The limited ability to concentrate urine is a common indication of impaired renal function; significant improvement in urine maximal osmolality was seen in both the young and the middle-aged groups. The middle aged subjects showed an improvement of nearly 30% in urine concentrating capacity.

Changes were also observed in the levels of sodium excretion and in renal medullary Na-K-ATPase activity upon liposome treatment, as seen in Figs. 8 and 9, respectively. Impaired renal function is often accompanied by a suppression of fractional sodium reabsorption thus leading to an increase in fractional salt excretion. Fig. 8 shows that all three groups of treated subjects experienced an improvement in overall renal function as evidenced by reduced sodium excretion.
The above noted biochemical changes indicate improved renal function upon liposomal treatment for all three groups of treated subjects with the most significant improvement resulting for the middle-aged group.

From the foregoing, it will be appreciated how various objects and features of the invention are met.

Referring to one aspect of the present invention, experiments demonstrate that the liposomal treatment method is effective to significantly reduce blood pressure after a relatively short treatment period, e.g., two weeks. The treatment method employs only natural phospholipids and is relatively safe.

It was also shown that the treatment is effective to reduce myocardial hypertrophy, one of the pathologies associated with elevated blood pressure.

Referring now to another aspect of the present invention, administration of PC-liposomes to a subject is effective in significantly lowering plasma creatinine concentrations and in improving urine osmolality, sodium excretion and renal medullary Na-K ATPase activity. These improvements are observed after only a relatively short treatment period, e.g., three weeks.

The following examples illustrate methods of preparing liposome suspensions for use in the various treatment methods of the invention. The examples are intended to illustrate, but in no way limit, the scope of the invention.

MATERIALS

Egg phosphatidylcholine (egg PC) was prepared according to known methods (Shinitisky). High purity egg PC may also be purchased from Avanti Polar Lipids (Alabaster, AL) or Lipoid KG (Ludwigshafen, Germany). The egg PC was determined to be greater than 99% pure, based on thin layer chromatography (TLC) analysis. The egg PC fatty acid composition was similar to the reported composition (Hertz). The main PCs of the preparation included 1-palmitoyl,2-oleyl PC and 1-palmitoyl,2-linoleyl PC. Thin-layer chromatography plates, 0.25 silica gel HR and 0.024 silica gel, were obtained from Merck (Darmstadt, Germany) and Analtech (Newark, Del.), respectively.

Example 1

Preparation of Small Unilamellar Vesicles: Sonication

Egg PC dissolved in chloroform was placed in a 100 ml vessel and dried to a thin film under nitrogen. Sterile saline was added to the lipid film to a final concentration of about 100
mg/ml, and the lipid film was hydrated with swirling. The resulting multilamellar vesicle (MLV) suspension was then bath sonicated for 1 hour using a Heat System Sonicator, Model 375W, at a power setting of 40-50% full value. The temperature of the suspension was maintained at about 4°C under nitrogen during sonication. The sonicated suspension was separated from large vesicles by ultracentrifugation at 100,000 g for 1 hour (Barenholz, 1977). The suspension of SUVs, having a concentration of about 100 mg/ml, was filter sterilized.

Example 2
Preparation of Small Unilamellar Vesicles: Extrusion

Homogeneous small unilamellar vesicles of egg PC with an average diameter of 39 ± 8 nm, in 0.15 M NaCl were prepared by extrusion using serial filtration through polycarbonate filters in a GH 76-400 pressure cell (Nucleopore) (Amselem et al, 1993). Liposomal size was determined using a Coulter model N4 sub-micron particle analyzer equipped with a size distribution processor analyzer (Barenholz et al, 1993). The final extrusion step was through a 0.05 micrometer pore polycarbonate filter. Egg PC SUV’s were sterilized by filtration through sterile 0.22 micrometer Millipore filters.

Example 3
Effect of Egg PC SUV Treatment on Blood Pressure

To test the effect of egg PC liposome treatment on blood pressure, two volunteers were treated with egg PC SUV’s made according to the procedures in Examples 1 or 2. The first human subject, herein designated as Volunteer A, was a 63 year old human male with a total body mass of 72 kg. The second subject, designated as Volunteer B, was a 29 year old human male with a body mass of 84 kg. Over a period of 16 days, seven liposome treatments, designated as Treatment 1, Treatment 2, etc., were administered intravenously to each subject.

The dose of Treatments 1, 2 and 3 was 100 mg egg PC SUV per kg body weight. For Treatments 4, 5 and 6, a dose of 200 mg egg PC SUV/kg body weight was administered and Treatment 7 was dosed at 300 mg egg PC SUV per kg body weight.

The first liposome treatment, Treatment 1, was administered on day 1, and Treatments 2-7 were administered on days 3, 7, 9, 11, 13, and 16, respectively.

Blood pressure and pulse were monitored over the course of the study. Blood pressure measurements were taken in the artery of the upper arm and an initial measurement was taken in each subject prior to administration of the first liposome treatment, providing a baseline blood pressure for comparison with subsequent measurements.
The results of the study are shown in Figs. 3 and 4 and in Table I. The blood pressure data corresponding to a given treatment represent an average of multiple blood pressure measurements taken over the 48 hour period following that treatment. As seen, Volunteer A (Fig. 3) exhibited a nearly 20% drop in both systolic and diastolic blood pressure; similarly, Volunteer B (Fig. 4) exhibited a nearly 20% drop in systolic pressure and a 25% drop in diastolic pressure.

Example 4

Effect of Egg PC SUV Treatment on Myocardial Hypertrophy

Liposomes were administered to laboratory rats ranging from 1.5 to 2 years of age. The treated group contained 5 animals; the untreated group contained 6 animals. The animals were injected through the femoral vein with either sterile saline (untreated group) or with the SUV suspension of Example 1. The treated animals were injected every three days for six days with 0.5-1 g lipid per animal. The untreated animals received a similar volume of sterile saline over the same period. Three days after the final injection, the animals were sacrificed. The weight of each animal was determined. The heart was then removed, washed with cold saline and weighed. The ratios of heart mass (wet mass) to total body mass of the rats is given in Table II. The data in the table represent the average values for each group (treated versus untreated).

Example 5

Effect of Liposome Treatment on Cellular Calcium Uptake in Hypertrophic Myocytes

A. Preparation of Cell Cultures

Myocyte cultures were prepared from the ventricular portion of one day old rat hearts (Sabra, Hebrew University-Hadassah Medical School). Fibroblasts were depleted by differential attachment for 60 minutes based on published procedures (Kasten, Yechiel). Myocytes were plated to reach a final density of approximately 10^6 cells/35mm dish. Cultures were grown in Ham's F-10 media with the following supplements: sodium penicillin 200 U/l, streptomycin 200 mg/L, 20% fetal calf serum, and 0.1 mM 5-bromo-2-deoxyuridine. The combination of differential attachment, confluent plating of myocytes and the use of 5-bromo-2-deoxyuridine ensured less than 10% contamination with fibroblasts. Cultures were grown in 35 or 60 mm X 15 mm Falcon culture dishes at 37°C in 5% CO_2 for 14 days. The medium was changed every 48 h.
B. Characterization of Cells

The viability of 14 day cells was determined by trypan blue exclusion of trypsinized cells resuspended in 0.04% trypan blue for 15 min. Cells were then examined microscopically for uptake of the dye; trypan blue exclusion was greater than 95% in all samples of trypsinized cells. DNA was determined using calf thymus DNA as a standard (Cesarone). Determinations of DNA content and trypan blue exclusion in day 4 and day 14 cultures indicated that proper plating of cultures, i.e., those well dispersed before seeding and having greater than 90% confluency post-plating, ensured fluctuations of less than 10% in number. An additional indication of cell viability in older cultures was the full recovery of the beating rate (which had decreased by 70%) after treatment of cells at day 12 with egg PC liposomes.

Hypertrophy of cells was confirmed by measuring protein levels (Lowry) and protein synthesis based on [3H]leucine incorporation in day 4 and day 14 cultures. Cells were incubated with 1 μCi of [3H]leucine (342 mCi/mmol) in leucine-free Ham's F-10 media supplemented with 1 μm leucine and 5% fetal calf serum for 24 h at 37°C. The cells were lysed in 0.1% SDS and protein was precipitated with ice-cold trichloroacetic acid (TCA) on Whatman GF/B glass filters. Filters were washed 3 times with 10% TCA and dried thoroughly before counting in 40% lumax in toluene. Cell size was quantitated by measuring the average surface area of cell monolayers labelled for 24 h with ceramide-lissamine-rhodamine in F-10 medium without phenol red and serum (Monti). Myocyte plasma membranes under these conditions were totally labelled after 24 h and the cell area was assessed using an ACAS 570 Interactive Laser Cytometer and Meridian computer image analysis program. An area of the culture dish was irradiated at room temperature (approximately 24°C) using a 514 nm line argon laser operating at 100 mW power. For optimization of the spectral fit, a dichroic filter of 575 nm was used.

C. Effect of Liposomes

Cells (4 day old and 12 day old, hypertrophic) were incubated with 1 μCi of 45Ca2+ (2.32 mCi/mL) for up to 2 h at 37°C in incubation media. Incorporation of labelled Ca2+ was determined by the removal of the incubation media and the rapid addition of ice-cold media followed by four consecutive rapid washes in cold media. Non-specific binding was measured by the addition of 1 mM EGTA in the rapid washes and also by incubating the cells for 1 h at 4°C to allow binding of Ca2+ without allowing appreciable uptake. The level of calcium uptake was determined by subtracting the cell-associated 45Ca2+ obtained by incubation at 4°C.
Calcium uptake results, shown below in Table III and in Fig. 5 above, represent the average of two different experiments, each performed in triplicate. As seen, a three-fold increase in the uptake of calcium was detected in the older (day 12) cells than in the younger cells (day 4). The higher incorporation of \(^{40}\text{Ca}^2+\) in the older, hypertrophic cultures correlates with the characteristic calcium overload of the hypertrophic heart. Sterilized egg PC SUVs were added to the older cultures to give a final lipid concentration of 1.0 mM and the liposome treatment was extended for 72 h. The uptake of calcium was determined to be reduced by over 50% for the liposome-treated cells.

<table>
<thead>
<tr>
<th>Calcium Uptake, cpm/2n/10^6 cells</th>
<th>Day 4</th>
<th>Day 12</th>
<th>Day 12, PC SUV treated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>12,500</td>
<td>42,000</td>
<td>17,000</td>
</tr>
</tbody>
</table>

**Example 6**

*Effect of Egg PC SUV Treatment on Plasma Levels of Creatinine*

The serum creatinine levels in three groups of Sabra male rats aged 5 months (young), 12 months (middle age), and 22 months (old) following liposome treatment were examined to provide a measure of kidney function. Egg PC SUV's were prepared as described in Examples 1 and 2. The young group consisted of 5 treated rats and 6 controls, the middle aged group consisted of 12 treated rats and 12 controls, and the old group consisted of 10 treated rats and 14 controls. The rats were injected through the femoral vein with either sterile saline (controls) or with a suspension of egg PC SUV's. The animals were treated with 6 i.v. administrations of 1 gram SUV lipid per 1 kg subject body weight over a 3 week period. After two months, the function of the kidney was assessed by examining plasma creatinine levels. Blood samples from the three groups of animals were centrifuged at low speed to remove blood cells and the resulting serum fractions were assayed for creatinine using standard procedures. The plasma creatinine data are shown in Table IV below and in Fig. 6. Plasma creatinine is expressed in units of \(\mu\text{mol creatinine per liter of plasma}\). All data represent the average of each age group.
Table IV

<table>
<thead>
<tr>
<th>Age (months)</th>
<th>Plasma Creatinine Concentration (μmols/L)</th>
<th>Plasma Creatinine Concentration (μmols/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TREATED</td>
<td>UNTREATED</td>
</tr>
<tr>
<td>5</td>
<td>65.5</td>
<td>71.3</td>
</tr>
<tr>
<td>12</td>
<td>60.1</td>
<td>67.7</td>
</tr>
<tr>
<td>22</td>
<td>61.7</td>
<td>62.8</td>
</tr>
</tbody>
</table>

The plasma creatinine levels are reduced in all three age groups upon treatment with PC SUVs, with the most significant reduction observed in the 12 month old rats.

Example 7

Effect of Egg PC SUV Treatment on Urine Maximal Osmolality and Sodium Excretion

The effect of administration of egg PC SUV's on kidney function was assessed by examining urine content. Urine samples from the three groups of rats in Example 6 were assayed for sodium content and maximal osmolality. The results are shown in Table V and in Figs. 7 and 8, where the urine maximal osmolality is expressed in units of mosm per liter and sodium excretion is expressed in units of microequivalents per day.

Table V

<table>
<thead>
<tr>
<th>Age (months)</th>
<th>SUV Treatment</th>
<th>Urine Maximal Osmolality, mosm/L</th>
<th>Urine Sodium Content, μEq/day</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>+</td>
<td>4120</td>
<td>1308</td>
</tr>
<tr>
<td>5</td>
<td>-</td>
<td>3269</td>
<td>1461</td>
</tr>
<tr>
<td>12</td>
<td>+</td>
<td>3754</td>
<td>1215</td>
</tr>
<tr>
<td>12</td>
<td>-</td>
<td>2908</td>
<td>1686</td>
</tr>
<tr>
<td>22</td>
<td>+</td>
<td>2306</td>
<td>1331</td>
</tr>
<tr>
<td>22</td>
<td>-</td>
<td>2242</td>
<td>1437</td>
</tr>
</tbody>
</table>

The data show an improvement in the ability to concentrate urine for all three SUV-treated groups as evidenced by increased urine maximal osmolality. Significant increases were seen in both the young and middle age subjects. A decrease in sodium excretion was observed.
for the SUV treated groups, with a very significant decrease in sodium excretion observed for the 12 month old rats.

Example 8

Effect of Egg PC SUV Treatment on Renal Medullary Na-K ATPase Activity

The effect of administration of egg PC SUV's on kidney function was assessed by examining Na-K ATPase activity in the rats from Example 6. The Na-K-ATPase activity in the red outer medulla was colorimetrically defined as the difference between the inorganic phosphate liberated in both the presence and absence of ouabain, corrected for nonenzymatic breakdown of ATP. The reaction was initiated by addition of ATP and terminated by addition of an ammonium molybdate solution. The results are summarized in Table VI and shown in Fig. 9, where Na-K-ATPase activity is reported in units of ($\mu$mol P$_i$/mg protein)/hour. The results demonstrate that medullary Na-K-ATPase activity is elevated by PC SUV treatment for all three groups of rats, most significantly for the 12 month old rats.

<table>
<thead>
<tr>
<th>Age (months)</th>
<th>Na-K-ATPase Activity ($\mu$mol P$_i$/mg protein)/hour</th>
<th>Na-K-ATPase Activity ($\mu$mol P$_i$/mg protein)/hour</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TREATED</td>
<td>UNTREATED</td>
</tr>
<tr>
<td>5</td>
<td>96.7</td>
<td>90.1</td>
</tr>
<tr>
<td>12</td>
<td>91.2</td>
<td>68.6</td>
</tr>
<tr>
<td>22</td>
<td>62.8</td>
<td>53.6</td>
</tr>
</tbody>
</table>

While various embodiments of the invention have been described herein, it will be apparent that various modifications can be made without departing from the intended scope of the invention.
IT IS CLAIMED:

1. A method for treating hypertension in a subject having elevated blood pressure, comprising:

   intravenously administering to the subject a suspension of small unilamellar liposomes composed primarily of phosphatidylcholine phospholipids having phase transition temperatures in the range between about -10 and 37°C, and

   repeating said administering over a period of at least several days and in an amount effective to produce a reduction in both systolic and diastolic blood pressure of at least 10 percent from said elevated blood pressure.

2. The method of claim 1, wherein the liposomes in the suspension have sizes ranging between 0.02 and 0.08 microns.

3. The method of claim 1, wherein the liposomes in the suspension are composed of egg phosphatidylcholine.

4. The method of claim 1, wherein said liposome suspension is administered 1-3 times per week, at a dose of between about 0.05 and 1 g lipid/kg body weight.

5. The method of claim 1, wherein the phase transition temperature is less than about 5°C.

6. The method of claim 1, wherein said administering is continued until a drop in blood pressure of at least about 10% is observed.

7. A method for improving impaired renal function in a subject in need of such treatment, as evidenced by a subnormal rate of glomerular filtration of less than 180 liters per day, comprising:

   intravenously administering to the subject, a suspension of small unilamellar liposomes composed of greater than 80 mole percent phosphatidylcholine phospholipids having phase transition temperatures between -10° and 37°C, said liposomes acting as the therapeutic agent for said improving, and
repeating said administering over a period of at least several days and in an amount of liposomes effective to produce an improvement in glomerular filtration rate in said subject characterized by a reduction in serum creatinine concentration of at least 10 percent.

8. The method of claim 7, wherein the liposomes in the suspension have sizes between 0.02 and 0.08 microns.

9. The method of claim 7, wherein the liposomes in the suspension are composed of egg phosphatidylcholine.

10. The method of claim 7, wherein said phospholipids have phase transition temperatures less than 5°C.

11. The method of claim 7, wherein said liposomal suspension is administered 1-3 times per week, at a dose of between about 0.05 and 1 g lipid/kg body weight.

12. The method of claim 7, for use in preventing chronic renal failure in a subject suffering from acute renal failure, said acute renal failure evidenced by a ratio of urine-to-plasma creatinine concentration below 20, wherein said repeating is further effective, over said period, to improve the ratio of urine-to-plasma creatinine concentration above 20.

13. The method of claim 7, for use in improving age-related diminution of renal function in a middle-aged subject, wherein said repeating is further effective, over said period, to produce an improvement in urine concentrating capacity characterized by an increase of at least 10 percent in urine maximal osmolality.
Fig. 1

Plasma phosphatidylcholine (cpm/μL)

Fig. 2

% Injected Dose

TIME (minutes)
Fig. 5
**INTERNATIONAL SEARCH REPORT**

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(6) : A61K 9/127
US CL : 424/450
According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/450

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

NONE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

NONE

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

<table>
<thead>
<tr>
<th>Category*</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
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<td><strong>Y</strong></td>
<td>Perspectives In Biology and Medicine, Volume 27, No. 3, issued Spring 1984, Williams et al, &quot;Intravenously Administered Lecithin Liposomes: A Synthetic Antiatherogenic Lipid Particle&quot;, pages 417-431, see entire document.</td>
<td>1-13</td>
</tr>
</tbody>
</table>

☐ Further documents are listed in the continuation of Box C.  ☐ See patent family annex.

* Special categories of cited documents:
  "A" document defining the general state of the art which is not considered to be of particular relevance
  "E" earlier document published on or after the international filing date
  "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
  "O" document referring to an oral disclosure, use, exhibition or other means
  "P" document published prior to the international filing date but later than the priority date claimed
  "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
  "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
  "Y" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
  "Z" document member of the same patent family

Date of the actual completion of the international search

13 JULY 1995

Date of mailing of the international search report

21 AUG 1995

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