METHODS FOR PROTECTING AGAINST CHEMOTHERAPY-INDUCED HAIR LOSS

The invention relates to methods for protecting against chemotherapeutic agent induced alopecia. In one aspect, the invention discloses a method for protecting against chemotherapeutic agent induced alopecia by administering to a mammalian subject therapeutically effective amounts of: cytokine release inducing biologic response modifier, therapeutically effective amounts of compound(s) selected from the group consisting of recombinant cytokine IL-1, endotoxin, Lipid A, and monophosphoryl Lipid A, and antioxidants. According to the teaching of the invention, the cytokine release inducing biologic response modifier, compound(s) selected from the group consisting of recombinant cytokine IL-1, endotoxin, Lipid A, monophosphoryl Lipid A and non-toxic antioxidant can be administered parenterally or can be applied topically. In addition the invention relates to use of cytokine release inducing biologic response modifiers, compound(s) selected from the group consisting of recombinant cytokine IL-1, endotoxin, Lipid A, monophosphoryl Lipid A, and antioxidant in the manufacture of medicament packs for use in protecting against chemotherapeutic agent induced alopecia.
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METHODS FOR PROTECTING AGAINST
CHEMOTHERAPY-INDUCED HAIR LOSS

Field of the Invention

This invention relates generally to methods for protecting against chemotherapy-induced hair loss. More particularly, the invention relates methods for protecting against chemotherapy induced hair loss by administering to a mammalian subject therapeutically effective amounts of biologic response modifier, recombinant cytokine interleukin-1 (rIL-1), endotoxin, Lipid A, monophosphoryl Lipid A, antioxidant(s), or combinations thereof, either parenterally or topically. In addition the invention relates to use of biologic response modifier, recombinant cytokine IL-1, endotoxin, Lipid A, monophosphoryl Lipid A, and antioxidant(s) in the manufacture of medicament packs for use in the treatment of chemotherapy-induced hair loss.

Background of the Invention

Various types of chemotherapeutic agents have been used successfully in treating cancer. However, a frequent and distressing side effect of many of these agents is partial or complete hair loss, i.e., alopecia. Alopecia not only can be a source of embarrassment and inconvenience to a patient, but in some cases can result in depression and has even caused patients to refuse potentially curative chemotherapy.

Unfortunately, although this complication of chemotherapy has been observed for decades, little progress has been made in its prevention or treatment. Although some success has been reported using certain chemical agents, such success has been limited. Moreover, many compounds which show promise in this
respect may cause undesirable side effects. Prevention of alopecia has also been attempted by temporarily reducing blood flow to the scalp. Results in this connection have been inconsistent.

It is an object of the present invention to provide an improved method for protecting against chemotherapeutic agent induced alopecia.

Another object of the invention is to provide a method for protecting against chemotherapeutic agent induced alopecia without the administration of potentially harmful chemical agents.

Another object of the invention is to provide medicament packs suitable for treating chemotherapeutic agent induced alopecia which will not require administration of potentially harmful chemical agents.

Other objects of the invention will become apparent to those skilled in the art from the following description.

SUMMARY OF THE INVENTION

Very generally, the invention discloses methods for protecting against chemotherapeutic agent induced alopecia. In one aspect, the invention discloses a method for protecting against chemotherapeutic agent induced alopecia by administering to a mammalian subject therapeutically effective amounts of biologic response modifier that induces release of cytokine(s), especially IL-1. In another aspect, the invention discloses a method for protecting against chemotherapeutic agent induced alopecia by administering to a mammalian subject therapeutically effective amounts of recombinant cytokine IL-1, endotoxin, Lipid A, monophosphoryl Lipid A, alone or in combination. In still another aspect, the invention discloses a method for
protecting against chemotherapeutic agent induced alopecia which comprises administering to a mammalian subject therapeutically effective amounts of biologic response modifier that induces release of cytokine(s), in combination with therapeutically effect amounts of compound(s) selected from the group consisting of recombinant cytokine IL-1, endotoxin, Lipid A, and monophosphoryl Lipid A. In yet another aspect, the invention discloses a method for protecting against alopecia caused in whole or in part by non cycle-specific chemotherapeutic agents. According to this method, non-toxic antioxidant is administered to a mammalian subject in combination with therapeutically effective amounts of agents selected from the group consisting of biologic response modifier that induces release of cytokine(s), recombinant cytokine IL-1, endotoxin, Lipid A, and monophosphoryl Lipid A. According to the teaching of the invention, the disclosed combinations of biologic response modifier, recombinant IL-1, endotoxin, Lipid A, monophosphoryl Lipid A, and antioxidant(s) can be administered parenterally or topically. In addition the invention relates to use of biologic response modifier, recombinant cytokine IL-1, endotoxin, Lipid A, monophosphoryl Lipid A, and antioxidant(s) in the manufacture of medicament packs for use in protecting against chemotherapy-induced hair loss.

Detailed Description of the Invention

Unlike most animals, in humans, hair growth is asynchronous, that is, with constant shedding and regrowth throughout life. A typical human scalp contains approximately 100,000 hairs, each hair comprising a hair bulb, containing the germinative cells, the hair root, and the hair shaft or fiber. As the germinative cells in the hair bulb proliferate,
the hair is pushed from the root up through the scalp surface.

In a typical human scalp, approximately 85% of the hair follicles are actively growing. Factors affecting hair growth include age, race, sex, hormonal production, and the particular topographical location of the hair. Other factors include the overall nutrition and body temperature of the individual.

Alopecia may occur in different degrees, depending upon the particular drug administered, the route of administration, the dosage level, and the dosage schedule. Typical hair loss will occur approximately two to four weeks after the initial dose of chemotherapy. High dose intermittent intravenous therapy commonly causes sudden and almost complete hair loss. Re-growth of hair may occur despite continued treatment. However, this may take as long as three to six months following chemotherapy.

Despite the known clinical impact of chemotherapy-induced alopecia, there has been no effective method of preventing this complication. In the course of studying ways to induce leukemic cells to mature, it was surprisingly discovered that cytokine release inducing biologic response modifier, either alone or in combination with rIL-1, endotoxin, Lipid A, monophosphoryl Lipid A, and/or antioxidant(s) can protect against chemotherapeutic agent induced alopecia.

It was known that circulatory monocytes (cells which function as part of the immune defense system) when isolated from the blood and stimulated, secrete maturation factor(s) which are capable of inducing the leukemic cell to mature. It was also known that CTI-BRM is capable of activating peripheral blood mononuclear cells in vitro causing the release
of a number cytokines including interleukin-1 and tumor necrosis factor. See Example 8.

Therefore in the initial studies that led to the discovery of the present invention, leukemia-bearing rats were injected with an agent (CTI-BRM) that would stimulate the rats' own monocytes to produce maturation factors, resulting in maturation of the leukemic cells and therefore a remission or cure. (CTI-BRM is a bacterial product and a powerful activator of monocyte/macrophages.) Following the CTI-BRM injections, a significant number of rats (40%) so treated became free of leukemia. In contrast, all untreated control rats died of leukemia.

It was reasoned that if a chemotherapeutic drug was used first to reduce the number of leukemic cells, then the CTI-BRM will have a better chance of forcing all the remaining cells to mature resulting in higher percentage cure. To test this hypothesis, one group of leukemia-bearing rats was given the chemotherapeutic agent Cytosine Arabinoside (ARA-C) alone, and another group ARA-C plus CTI-BRM. Only 10% of the rats give ARA-C alone were cured of their leukemia whereas 80% of those give ARA-C/CTI-BRM were cured. In observing the animals it was discovered that rats treated with ARA-C alone lost all their hair becoming totally nude by the 10th day after treatment, whereas those treated with ARA-C/CTI-BRM were completely protected from hair loss. The same experiment was repeated in normal rats (without leukemia) and similar results were obtained (see Example 1, Table 1).

This startling observation suggested that CTI-BRM was protecting the hair root from the toxic action of chemotherapy, and suggested that CTI-BRM could offer protection from chemotherapy-induced hair loss in the cancer patient.
Knowing that CTI-BRM could protect against ARA-C induced alopecia, studies were designed to determine whether CTI-BRM could protect against alopecia caused by other chemotherapeutic agents. Such agents are broadly divided into two groups: cycle-specific drugs that damage the cell primarily during cell division, and non cycle-specific drugs that can damage resting cells. ARA-C is a cycle-specific drug; cytoxan (CTX), an alkylation agent is a non cycle specific drug. (See "Definitions" section of this specification for further discussion of these drugs.)

As the results in Examples 1 and 2 indicate, CTI-BRM protects against hair loss from cycle-specific drugs ARA-C and doxorubicin (DX), but unfortunately not against the non cycle-specific drug cytoxan (CTX). See Example 2.

Cytoxan is an alkylation agent which exerts its cell damage by producing toxic O₂ radicals. These radicals which cause oxidant damage, and cell death. Therefore experiments were designed to determine if antioxidant(s) would protect against cytoxan-induced hair loss. The results of these experiments are illustrated in Examples 3-5. In Examples 3, subjects were given cytoxan along with the antioxidant N-acetylcysteine (NAC). As the results in Table 3 illustrate, cytoxan-induced alopecia can be effectively prevented by N-acetylcysteine, whether administered parenterally, or topically in liposomes. This latter finding was most surprising, and important since systemic antioxidants such as NAC may attenuate the chemotherapeutic efficacy of agents such as CTX. Topical treatment could circumvent these problems, thus providing an ideal approach for protection against alopecia from agents such as cytoxan without interfering with their chemotherapeutic efficacy.
Although the mechanism of protection from chemotherapy-induced alopecia by CTI-BRM remains uncertain, it is believed it could be mediated by a single cytokine or could represent the summation effect of a number of cytokines. To investigate this further, recombinant cytokine IL-1 and TNF were tested for their ability to provide protection against chemotherapeutic agent induced alopecia. As the results in Example 7 indicate, IL-1, and to a lesser degree TNF, provide protection from cycle-specific chemotherapeutic agent induced hair loss. The same is true of endotoxin, which is a sub-component of CTI-BRM, and Lipid A, which is a sub-component of endotoxin, and monophosphoryl Lipid A, which is a non toxic form of Lipid A. See Example 9.

In practicing the methods of the present invention, CTI-BRM is preferred as a cytokine release inducing biologic response modifier. Other biologic response modifiers can be used if they induce cytokine release, which can be determined by the method of Example 8.

It is clear from the above studies that cytokine release inducing biologic response modifier, used alone or in combination with the other compounds of the present invention, is useful for providing protection against chemotherapy-induced alopecia.

Definitions

In the present specification and claims, reference will be made to phrases and terms of art which are expressly defined for use herein as follows:

As used herein, "recombinant" means produced by means of genetic engineering, as opposed to merely being isolated from nature. Recombinantly produced molecules are indicated by "r", e.g., "rIL-1". Recombinant molecules derived from human genes are
identified as "Hu", e.g., "rHuIL-1". Recombinant molecules derived from murine genes are identified as "Mu", e.g., rMuTNF. Molecules are further identified by Greek letters, alpha α, beta β, etc.

As used herein, "IL-1" means interleukin 1; "rHuIL-1" means recombinant human type interleukin 1.

As used herein, "TNF" means tumor necrosis factor; "rMuTNFα" means recombinant murine type tumor necrosis factor alpha.

As used herein, "IFNα" means interferon gamma.
As used herein, "GM-CSF" means Granulocyte/Macrophage Colony Stimulating Factor.

Commonly used chemotherapeutic agents can be broadly divided into two groups: (1) cycle-specific drugs that damage the cell primarily during cell division, and (2) non cycle-specific drugs that can damage resting cells that are not undergoing cell division. Cycle-specific chemotherapeutic agents include Cytosine Arabinoside (ARA-C) (also known as Cytarabine), Doxorubicin (DX) (also know as Adriamycin, or ADX), Methotrexate, 5-Fluorouracil (5-FU), 6-Mercaptopurine (6-MP), Thioguanine (TG), etc. Non cycle-specific chemotherapeutic agents include alkylating agents such as Nitrogen mustard, Melphalan, Cisplatin, Nitrosourea, DTIC, Procarbazine, Cyclophosphamide (also known as Cytoxan or CTX), etc. Other chemotherapeutic agents, that do not fit into these two broad categories include Bleomycin, Etoposide, Teneposide, Actinomycin D, etc.

As used herein, "ARA-C" means the cycle-specific chemotherapeutic agent Cytosine Arabinoside.
As used herein, "DX" means the cycle-specific chemotherapeutic agent Doxorubicin.
As used herein, "CTX" means the non cycle-specific chemotherapeutic agent cyclophosphamide, which is also known by the trade name Cytoxan.
As used herein, "NAC" means the antioxidant, N-acetylcysteine (a sulfhydryl compound).

As used herein, "non-toxic" means within a level of toxicity which is tolerable by the mammalian subject receiving the compound (e.g., antioxidant) or the biologic response modifier according to the methods of the invention.

As used herein, "substantially non-pathogenic in humans" means not or rarely associated with disease in humans of normal health. Since most microorganisms are capable of causing opportunistic infections under the right circumstances, such as in persons whose immune system has been compromised, this definition excludes only those organism which typically cause non-opportunistic infections.

As used herein, "tolerable level of endotoxin, cell walls, and cell membrane fragments" means that any such fractions, if present, have a low enough level of biologic activity to maintain a non-toxic characteristic as defined herein.

As used herein, "natural membrane vesicles" means membrane vesicles prepared from membranes which are derived from living or dead natural cells.


ImuVert® is the registered (United States Patent and Trademark Office) trademark of Cell Technology, Inc., Boulder, Colorado, USA, and indicates the source of a biologic response modifier comprised of a natural membrane vesicle - ribosome preparation derived from the bacterium Serratia marcescens by a series of lytic and separation techniques. ImuVert® is the most preferred of the

As used herein, "cytokine release inducing biologic response modifier" means a biologic response modifier that can induce or stimulate release of cytokines from human cells (e.g., peripheral blood mononuclear cells) in vitro or in vivo. An in vitro method for determining whether a biologic response modifier can induce or stimulate peripheral blood mononuclear cells to release cytokines, e.g., IL-1, is given in Example 8.

As used herein, "protecting against chemotherapeutic agent induced alopecia in a mammalian subject" means no detectable alopecia or less severe alopecia as a result of treatment according to the methods of the present invention. In this regard, "treatment" means administration of therapeutically effective amounts of biologic response modifier, recombinant IL-1, endotoxin, Lipid A, monophosphoryl lipid A, and antioxidant, etc, wherein therapeutically effect amounts are those non-toxic amounts that result in no alopecia or a lessening of chemotherapeutic agent induced hair loss. Additional variants of therapeutically effective amounts of biologic response modifiers, recombinant cytokine IL-1, endotoxin, Lipid A, monophosphoryl lipid A, and antioxidants, alone or in combination, may be readily determinable by the treating physician through observation, and from the information provided herein and elsewhere (e.g., toxicity tests, Clinical Trial data), without undue experimentation. Such are intended to be encompassed within the scope of term "therapeutically effective amounts" as used herein and in the appended claims.
This application claims priority from U.S.S.N. 500,577, filed March 28, 1990 in the United States Patent and Trademark Office. The contents of the priority application are expressly incorporated by reference herein.

EXAMPLES

Example 1

This example demonstrates that parenteral administration of CTI-BRM provides protection from cytosine arabinoside (ARA-C) (a cycle-specific drug) induced alopecia in leukemic and normal subjects.

Subjects with leukemia: Young (6-8 day old) rats treated with cytosine arabinoside (ARA-C) for rat myelogenous leukemia regularly developed severe alopecia. When these mammalian subjects were given a therapeutically effective amounts of cytokine release inducing biologic response modifier such subjects were virtually completely protected from alopecia.

In one study, a total of 84 eight day old rats were randomly divided into four groups. Each rat received 10^5 MIA C51 leukemia cells intraperitoneally (IP). Six hours after cell transfer, the rats received the following once-daily IP injections for 7 days: Group I (21 rats), control group, 0.1 ml buffer; Group II (23 rats), ARA-C 20 mg/kg; Group III (20 rats), CTI-BRM 25 μg; and Group IV (20 rats), ARA-C 20 mg/kg plus CTI-BRM 25 μg.

Prior to treatment, all animals had a near full coat of hair. On day six of treatment, all rats in Group II (ARA-C alone) had alopecia with hair loss starting over the head and rapidly progressing to include the entire body. By day nine, sixteen of the twenty rats had total hair loss, another four animals had lost over 50% of their hair, and three animals had mild alopecia. In sharp contrast, alopecia was totally absent in fifteen of the twenty rats in Group
IV, and was mild in the remaining five rats. The results in the foregoing experiments are shown below in Table 1. Rats in Group III were indistinguishable from the control Group I.

### Table 1

Occurrence of Alopecia, Rats with Chloroleukemia Treated with ARA-C

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<tr>
<td>I (Controls)</td>
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<tr>
<td>II (ARA-C)</td>
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<tr>
<td>III (CTI-BRM)</td>
<td>20</td>
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<td>IV (ARA-C + CTI-BRM)</td>
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Hair loss was graded as described in Hussein, et al., Science 249:1564-1566 (1990). 0, No detectable alopecia; 1+, Mild alopecia defined as less than 50% hair loss; 2+, Moderately severe alopecia with more than 50% hair loss; 3+, Total or virtually total absence of hair.

Normal Subjects: In another set of experiments, doses of BRM were compared with ARA-C induced alopecia in normal rats. In Experiment 1, 38 8-day old rats were randomly divided into two groups of 19 rats each. Group I received ARA-C (25 mg/kg per day IP) for 7 days, and Group II received ARA-C in the same dose plus CTI-BRM (25 μg/day SC) for 7 days. Data were recorded on day 9 of the experiment (2 days after ARA-C treatment was stopped). Experiment 2 was carried out as in Experiment 1, except that there were 12 rats per group and the CTI-BRM dose was 10 μg/day.
Protection from ARA-C induced alopecia was observed both at the 25- and at the 10-μg dose of CTI-BRM. See Table 2.

Table 2
Occurrence of Alopecia, Normal Rats Treated with ARA-C
Effect of CTI-BRM
Dose Comparison

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<td>0</td>
<td>8</td>
<td>11</td>
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<td>II (ARA-C) + CTI-BRM (25 μg)</td>
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<td>0</td>
<td>0</td>
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<td>7</td>
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<tr>
<td>II (ARA-C) + CTI-BRM (10 μg)</td>
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<td>2</td>
<td>0</td>
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</table>

Hair loss was graded as described in Hussein, et al., *Science* 249:1564-1566 (1990). 0, No detectable alopecia; 1+, Mild alopecia defined as less than 50% hair loss; 2+, Moderately severe alopecia with more than 50% hair loss; 3+, Total or virtually total absence of hair.
Example 2

This example demonstrates that parenteral administration of CTI-BRM provides protection from alopecia induced by doxorubicin (DX) (a cycle-specific drug), but not cytoxan (CTX) (a non cycle-specific drug).

Two other chemotherapeutic agents known to produce alopecia in humans, doxorubicin (DX) (a cycle-specific chemotherapeutic agent) and cytoxan (CTX) (a non cycle-specific chemotherapeutic agent) were also examined. In one set of experiments, twenty 8-day-old rats were treated with DX (2 mg per kilogram of body weight per day intraperitoneally (IP) with or without CTI-BRM 25 μg subcutaneously (SC)) for 7 days. Group I control, buffer; Group II, DX 2 mg/kg; Group III, CTI-BRM 25 μg; Group IV, DX 2 mg/kg + CTI-BRM 25 μg.

All rats receiving DX alone (Group II) had complete alopecia over the head and proximal part of the neck. None of the animals given CTI-BRM (Group IV) had alopecia.

In another set of experiments animals were treated with a single dose of CTX (25 μg/kg IP) without or with CTI-BRM (25 μg/day IP for 7 days). Group I, control, buffer; Group II, CTX 25 μg/kg; Group III, CTI-BRM 25 μg; Group IV, CTX 25 μg/kg + CTI-BRM 25 μg.

Four of ten rats treated with CTX alone (Group II) had total body alopecia indistinguishable from that produced by ARA-C; four others had moderately severe alopecia (loss of more than 50% of body hair); and the remaining two rats had mild alopecia. A similar pattern was noted in the animals treated with CTX + CTI-BRM (Group IV); no protection against alopecia was observed with CTI-BRM in these experiments.
The apparent lack of protection from CTX-induced alopecia strongly suggests that this agent causes alopecia by a mechanism distinct from that caused by ARA-C or DX. It is believed that CTI-BRM protection of the hair follicles from ARA-C and DX occurs at some point in the S phase of the cell cycle, since both of these chemotherapeutic agents are cell cycle-specific and exert their action during DNA synthesis.

Example 3

This example demonstrates that cytoxan-induced alopecia can be effectively prevented by N-acetylcysteine, administered parenterally or applied topically in liposomes.

It has been postulated that alkylating agents such as cytoxan (CTX) exert their cell damage by producing toxic O_2 radicals which attack the cell's vital structure leading to its death (oxidant damage). Therefore, experiments were run to see whether an antioxidant (N-acetylcysteine, also known as NAC) would protect against cytoxan-induced hair loss.

In the first experiment, 8-day-old rats were randomized into two groups of 9 rats each. All rats received each CTX 30 mg/kg intraperitoneally (IP) in a single dose. Group I rats received in addition injections of 2 mg NAC in 0.1 ml PBS subcutaneously (SC) in the flank area 3 x daily for 2 days. Group II received PBS injection and served as control. The degree of alopecia was scored on day 10. All rats in Group II developed alopecia with 6 of 9 animals losing over 50% of their body hair. In contrast, 6 of 9 rats in Group I (treated with NAC) had no detectable alopecia (see Exp. 1 in Table 3). It was further noted that some of the rats in Group I which were not totally protected from alopecia had excellent
protection around the site of NAC injection indicating a local effect.

In the second experiment (Table 3, Exp. 2) 18 rats were divided into 2 groups of 9 rats each. All rats were given CTX 30 mg/kg IP in a single dose. Group I received in addition NAC 2 mg injections which were attempted intradermally in the scalp 3 times daily for 2 days. Group II received PBS injections and served as control. Alopecia was scored on day 10. All 9 rats in Group II had moderately severe to severe alopecia (Table 3, Exp. 2). In contrast, 5 of 9 rats in Group I were virtually completely protected from alopecia and the remaining 4 had moderately severe alopecia. In these latter rats, good protection could nevertheless be observed at the site of NAC injections, indicating topical effect.

Accordingly, a third experiment was performed as follows: 12 8-day old rats received each CTX 30 mg/kg IP as a single injection and randomized into two groups of 6 rats each. Immediately after CTX injection, rats in Group I were each placed erect tail first in a tube containing 1 ml of 30 mg/kg NAC-liposome suspension and kept in for 12 hours. They were then taken out for 12 hours, then resubmerged for another 12-hr-period. The suspension was changed every 4 hrs. Control rats were similarly treated using liposome suspension without NAC. All 6 rats in the control group had total body alopecia. In contrast, all rats in the NAC-treated group were virtually completely protected over the caudal half of their body (Table 3, Exp.3).

In a separate experiment involving 20 8-day old rats, 10 rats in each of two groups, NAC given IP did not protect from ARA-C induced alopecia. (Data not shown.)
As the results in Table 3 indicate, mammalian subjects given Cytoxan (CTX) along with the antioxidant N-acetylcysteine (NAC) were protected from hair loss.

Table 3

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<tr>
<th>Group</th>
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<td><strong>Experiment 1</strong></td>
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</tr>
<tr>
<td>I CTX + NAC</td>
<td>6</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>II CTX alone</td>
<td>0</td>
<td>3</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td><strong>Experiment 2</strong></td>
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</tr>
<tr>
<td>I CTX + NAC</td>
<td>5</td>
<td>1</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>II CTX alone</td>
<td>0</td>
<td>0</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td><strong>Experiment 3</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I CTX + Topical NAC</td>
<td>6</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>II CTX alone</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>6</td>
</tr>
</tbody>
</table>

Hair loss was graded as described in Hussein, et al., Science 249:1564-1566 (1990). 0, No detectable alopecia; 1+, Mild alopecia defined as less than 50% hair loss; 2+, Moderately severe alopecia with more than 50% hair loss; 3+, Total or virtually total absence of hair.

Example 4

This example demonstrates parenteral administration of BLM-NAC provides protection from alopecia caused by the combination of cytoxan (CTX)
non cycle-specific drug) and cytosine arabinoside (ARA-C) (a cycle-specific drug).

Twenty 8-day old rats were randomized into two groups of 10 rats each and were each given CTX 50 mg/kg in a single injection IP plus ARA-C 50 mg/kg IP daily for 5 days. Group I received beginning on the first day and continuing for 5 days CTI-BRM 10 μg plus NAC 4 mg SC. Group II received PBS injections. The degree of alopecia was scored on day 10 of experiment. All rats in Group II developed total body alopecia. In contrast, 3 rats in Group I developed 2+ alopecia, and 7 of 10 rats had only 1+ alopecia. See Table 4.

Table 4

<table>
<thead>
<tr>
<th>Group</th>
<th>Alopecia</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>0</td>
</tr>
<tr>
<td>ARA-C + CTX BRM + NAC</td>
<td>0</td>
</tr>
<tr>
<td>II</td>
<td>0</td>
</tr>
<tr>
<td>ARA-C + NAC</td>
<td></td>
</tr>
</tbody>
</table>

Hair loss was graded as described in Hussein, et al., *Science* 249:1564-1566 (1990). 0, No detectable alopecia; 1+, Mild alopecia defined as less than 50% hair loss; 2+, Moderately severe alopecia with more than 50% hair loss; 3+, Total or virtually total absence of hair.
Example 5

This example demonstrates that topical administration of BRM-NAC (in liposomes) provides protection from cytoxan (CTX) induced alopecia.

Fifty eight-day old rats were randomized into 5 groups of 10 rats each. All rats received CTX 50 mg/kg IP as a single injection and ARA-C 50 mg/kg IP daily for 4 days. Group I received no additional treatment and served as control. All the remaining four groups received topical treatments in 0.25 ml suspension applied over the entire back from the shoulders to the tail daily for four days as follows: Group II, Liposomes (liposome control); Group III, Liposome/CTI-BRM; Group IV, Liposome-NAC; Group V, Liposome/CTI-BRM/NAC. After each application, rats were individually kept separate for 3 hrs.

All rats in Groups I and II developed total body alopecia. Slight but significant protection was observed in all rats of Group III and IV localized to the caudal part of the back. In contrast, all 9 rats in Group V had excellent protection manifested by thicker hair growth over most of the back and in 3 rats covered the entire body. (Data not shown.)

Example 6

This example discloses how to prepare drug-in-liposome suspensions. In preferred form, drug-in-liposome suspensions are prepared according to Huang, C.H., Biochem. 8:344-351 (1969), and for use herein were prepared as follows: soybean L-α-lecithins were suspended in phosphate buffered saline (PBS) at a concentration of 0.6 gm/ml with NAC, 100 mg/ml and CTI-BRM 1 mg/ml. The mixture was sonicated at 0° - 4° for 30 min, or two 15 min periods, separated by a 10 min cooling interval, with the standard tip of the sonic dismembrator (Fischer Scientific, Pittsburg,
PA) at a setting of 60 W. When necessary, suspensions were filtered with 0.45 \( \mu \)m filter.

**Example 7**

This example demonstrates that recombinant cytokine IL-1, provides protection from cytosine arabinoside (ARA-C) induced alopecia. CTI-BRM is a powerful immunomodulator: it is rapidly phagocytosed by monocytes/macrophages which then show increased phagocytic, bactericidal and tumoricidal activity. Patients injected subcutaneously with CTI-BRM show significant rises in granulocyte counts 24 hours later. Co-culture of CTI-BRM with human peripheral blood mononuclear cells results in elevation of NK activity, increased T-cell mediated cytotoxicity, and augmented lymphocyte and monocyte antibody mediated cytotoxicity (ADCC). The enhancement of these cellular effector functions is believed to be the result of a cascade of cytokine release which occurs after CTI-BRM stimulation. Supernatants from CTI-BRM stimulated human peripheral blood mononuclear cells contain IL-1, interferons alpha and gamma, TNF and GM-CSF. Both human and rat CTI-BRM stimulated monocytes produce substantial quantities of a myeloid differentiation factor, as measured by the ability to induce differentiation of the rat C51 chloroleukemia in vitro and in vivo. See, for example, United States Patent 4,971,801 and published PCT/US87/01397.

Accordingly, experiments were run to determine if protection of some chemotherapy induced alopecia is mediated by cytokines such as IL-1 and TNF. Thirty four 7-day old Sprague Dawley rats were randomized into 2 groups of 17 rats each. All rats received ARA-C 50 \( \mu \)g/kg/day for 7 days. The following additional treatment was given in once daily
intraperitoneal injections of 0.2 ml for 7 days. Group I (17 rats), control, buffer; Group II (17 rats) - rHuIL-1β 0.5 μg.

Fifteen of 17 rats in Group I developed complete and total body alopecia and 2 rats lost more than 75% of their hair. In contrast, 10 of the 17 rats in Group II had no detectable hair loss, 6 had less than 25% hair loss and only 1 rat had complete hair loss. In a similar set of experiments (using rMu-TNFα 0.5 μg IP or 1 μg IP), TNF gave some protection from ARA-C induced alopecia. These results indicate that protection from ARA-C-induced alopecia is mediated by cytokines IL-1 and, to a lesser extent, TNF. They also indicate that recombinant IL-1 can be used to prevent chemotherapeutic agent induced alopecia in mammalian subjects.

**Example 8**

This example discloses a method for determining the ability of a biologic response modifier to induce cytokine release.

The biologic response modifier (BRM) is evaluated for the ability to induce the expression of the cytokines interleukin 1 beta (IL-1β), tumor necrosis factor alpha (TNFα), interleukin 6 (IL-6), granulocyte macrophage-colony stimulating factor (GM-CSF), and the inflammation mediator prostaglandin E₂ (PGE₂) following the exposure of the human peripheral blood mononuclear cells (PBMCs) to the BRM (i.e., the BRM being evaluated, hereinafter referred to as the "BRM sample") in vitro. PBMCs are isolated by the application of heparinized human blood to leukoprep gradients (Becton-Dickinson, Lincoln Park, NJ) and centrifuging for 20 minutes at 1,800 x g. Mononuclear cells are removed from the gradient by aspiration and washed twice by resuspension in phosphate buffered
saline (PBS) followed by centrifugation at 200 x g for 10 minutes. Cell pellets are resuspended in X-Vivo 10 medium (Whittaker Biologicals) containing 2 mM glutamine (Sigma) at a density of 2 x 10^6 cells per milliliter and 1.3 x 10^6 cells are added to each well of a 24 well plate (Costar, Cambridge, MA). "BRM sample" is diluted in X-vivo medium and added to the wells to make a final concentration of 2 micrograms per milliliter. "BRM sample" containing cell suspensions are incubated for 24 hr. at 37°C in 5% CO₂. Cell supernatants are recovered following centrifugation, divided equally into 5 separate aliquots, and stored frozen at -70°C. Supernatants experience one freeze-thaw prior to each cytokine assay. All reagents must be below the limits of detection for endotoxin as determined by Limulus amebocyte lysate assay LAL.

Enzyme-linked immunosorbent assay (ELISA) is used to measure TNFα, IL-1β (Cistron), IL-6, and GM-CSF (Genzyme). PGE₂ is quantified by radioimmunoassay (Advanced Magnetics). Human IL-1β, TNFα, IL-6, GM-CSF, and PGE₂ serve as standards in each of the respective assays. The results of each assay are reported as picograms of component per milliliter of cell supernatant.

**Example 9**
This example demonstrates that endotoxin, Lipid A, and MPL (monophosphoryl Lipid A) provide protection from cycle-specific (ARA-C) induced alopecia.

The preferred biologic response modifier of the present invention, ImuVert®, contains endotoxin, which in turn contains Lipid A. MPLA is a non-toxic analogue of Lipid A. See United States Patent 4,971,801.
In one experiment, the effect of Lipid A on the occurrence of alopecia in rats treated with ARA-C was investigated. Lipid A was given as follows: Day one, 2.5 μg; day two, 5 μg; day three through seven, 10 μg. Experimental results are shown in Table 5.

<table>
<thead>
<tr>
<th></th>
<th>0</th>
<th>3+</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Control</strong></td>
<td></td>
<td>10</td>
</tr>
<tr>
<td><strong>Lipid A S.C.</strong></td>
<td>6</td>
<td></td>
</tr>
</tbody>
</table>

Hair loss was graded as described in Hussein, et al., *Science* 249:1564–1566 (1990). 0, No detectable alopecia; 1+, Mild alopecia defined as less than 50% hair loss; 2+, Moderately severe alopecia with more than 50% hair loss; 3+, Total or virtually total absence of hair.

In another experiment, the effect of Lipid A and endotoxin (lipopolysaccharide, LPS) were examined. Lipid A and LPS were given as follows: Day one, 2.5 μg; day two 5 μg; day three through seven, 10 μg. Lipid A from Sigma. *Salmonella minnesota* Re 595. Lipopolysaccharide (LPS) from Sigma. Experimental results are shown in Table 6.
Table 6
Occurrence of Alopecia in Rats Treated with ARA-C
Effect of Lipid A or LPS

<table>
<thead>
<tr>
<th>Alopecia</th>
<th>0</th>
<th>1+</th>
<th>2+</th>
<th>2.5+</th>
<th>3+</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lipid A I.P.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lipid A S.C.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lipid A Topical</td>
<td></td>
<td></td>
<td>3</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Lipopolysaccharide</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(S. typhosa) I.P.</td>
<td>4</td>
<td></td>
<td>2</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Data from two separate experiments. Hair loss was graded as described in Hussein, et al., Science 249:1564-1566 (1990). 0, No detectable alopecia; 1+ Mild alopecia defined as less than 50 percent hair loss; 2+ Moderately severe alopecia with more than 50% hair loss; 3+ Total absence of hair.

In still another experiment, the effect of MPLA was examined. (MPL, Monophosphoryl Lipid A from S. typhimurium) MPLA I.P was given as follows: Day one, 2.5 μg; day two 5 μg; day three through seven, 10 μg. MPLA S.C. given as follows: Day one through seven, 5 μg. Experimental results are shown in Table 7.
Table 7
Occurrence of Alopecia in Rats Treated with ARA-C

Effect of MPL

<table>
<thead>
<tr>
<th>Alopecia</th>
<th>0</th>
<th>1+</th>
<th>2+</th>
<th>3+</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
<td>6</td>
</tr>
<tr>
<td>MPL* I.P.</td>
<td>4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MPL S.C.</td>
<td>4**</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* MPL = Monophosphoryl Lipid A from *S. typhimurium*.
RIBI. ** = 4 rats, complete hair protection at site of injection, approximately 2 cm square. Hair loss was graded as described in Hussein, *et al.*, *Science* 249:1564-1566 (1990). 0, No detectable alopecia; 1+ Mild alopecia defined as less than 50 percent hair loss; 2+ Moderately severe alopecia with more than 50% hair loss; 3+ Total absence of hair.

Summary

It may be seen, therefore, that the method of the invention provides methods for protecting against chemotherapeutic-agent induced alopecia. Various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description. Such modifications are intended to fall within the scope of the appended claims.
WHAT IS CLAIMED IS:

1. A method for protecting against chemotherapeutic agent induced alopecia in a mammalian subject, comprising, administering to a mammalian subject therapeutically effective amounts of cytokine release inducing biologic response modifier.

2. A method for protecting against chemotherapeutic agent induced alopecia in a mammalian subject, comprising, administering to a mammalian subject therapeutically effective amounts of compound(s) selected from the group consisting of recombinant cytokine IL-1, endotoxin, Lipid A, and monophosphoryl Lipid A.

3. A method for protecting against chemotherapeutic agent induced alopecia in a mammalian subject, comprising, administering to a mammalian subject therapeutically effective amounts of cytokine release inducing biologic response modifier, in combination with therapeutically effective amounts of compound(s) selected from the group consisting of recombinant cytokine IL-1, endotoxin, Lipid A, and monophosphoryl Lipid A.

4. The method of any of Claims 1 or 3 wherein said biologic response modifier is comprised of two major particle populations, one such population being of lesser size particles comprised of ribosomes and the other such population being comprised of natural membrane vesicles, in a suspending buffer, said membrane vesicles and ribosomes being endogenous to a selected microorganism which is substantially non-pathogenic in humans, said biologic response modifier being substantially free of intact cells, and having tolerable levels of endotoxin, cell walls, and cell membrane fragments.
5. The method of any of Claims 1-3 wherein said therapeutically effective amounts of said biologic response modifier, and said compound(s) selected from the group consisting of recombinant cytokine IL-1, endotoxin, Lipid A, and monophosphoryl Lipid A are administered parenterally or topically.

6. The method of Claim 1 or Claim 3 wherein said biological response modifier is derived from the microorganism Serratia marcescens.

7. The method of any of Claims 1-3 wherein said biological response modifier is administered at intervals from 2 to 7 days in an amount between about 0.5 mg - 5 mg, and said compounds(?) are selected from the group consisting of recombinant cytokine IL-1, endotoxin, Lipid A, and monophosphoryl Lipid A, are administered at intervals from 2 to 7 days in therapeutically effective amounts.

8. A method according to any of Claims 1-3 wherein said biological response modifier and/or said compound(s) selected from the group consisting of recombinant cytokine IL-1, endotoxin, Lipid A, and monophosphoryl Lipid A, are administered in combination with antioxidant that is non-toxic to mammalian subjects.

9. A method according to any of Claims 1-3 wherein said biological response modifier and/or said compound(s) selected from the group consisting of recombinant cytokine IL-1, endotoxin, Lipid A, and monophosphoryl Lipid A, are administered in combination with the antioxidant, N-acetyl cysteine (NAC).

10. The method of Claim 1 wherein said biological response modifier is administered in combination with compound(s) selected from the group consisting of recombinant cytokine IL-1, endotoxin,
Lipid A, monophosphoryl Lipid A, and non-toxic antioxidants.

11. A method for protecting against alopecia induced by cycle-specific chemotherapeutic agents, comprising, administering to a mammalian subject therapeutically effective amounts of cytokine release inducing biological response modifier.

12. A method for protecting against alopecia induced by cycle-specific chemotherapeutic agents, comprising, administering to a mammalian subject therapeutically effective amounts of compound(s) selected from the group consisting of recombinant cytokine IL-1, endotoxin, Lipid A, and monophosphoryl Lipid A.

13. A method for protecting against alopecia induced by cycle-specific chemotherapeutic agents, comprising, administering to a mammalian subject therapeutically effective amounts of cytokine release inducing biologic response modifier, in combination with therapeutically effective amounts of compound(s) selected from the group consisting of recombinant cytokine IL-1, endotoxin, Lipid A, and monophosphoryl Lipid A.

14. The method of any of Claims 11 or 13 wherein said biologic response modifier is comprised of two major particle populations, one such population being of lesser size particles comprised of ribosomes and the other such population being comprised of natural membrane vesicles, in a suspending buffer, said membrane vesicles and ribosomes being endogenous to a selected microorganism which is substantially non-pathogenic in humans, said biologic response modifier being substantially free of intact cells, and having tolerable levels of endotoxin, cell walls, and cell membrane fragments.
15. The method of Claim 11 or Claim 13 wherein said biological response modifier is derived from the microorganism Serratia marcescens.

16. The method of any of Claims 11-13 wherein said cycle-specific chemotherapeutic agents are selected from the group consisting of Cytosine Arabinoside (ARA-C), doxorubicin (DX), methotrexate, 5-Fluorouracil (5-FU), 6-Mercaptopurine (6-MP), and Thioguanine (TG).

17. A method for protecting against alopecia induced by non cycle-specific alkylating agent type chemotherapeutic agents, comprising, administering to a mammalian subject therapeutically effective amounts of cytokine release inducing biological response modifier in combination with therapeutically effective amounts of a non-toxic antioxidant.

18. A method for protecting against alopecia induced by non cycle-specific alkylating agent type chemotherapeutic agents, comprising, administering to a mammalian subject therapeutically effective amounts of compound(s) selected from the group consisting of recombinant cytokine IL-1, endotoxin, Lipid A, and monophosphoryl Lipid A, in combination with therapeutically effective amounts of non-toxic antioxidant.

19. A method for protecting against alopecia induced by non cycle-specific alkylating agent type chemotherapeutic agents, comprising, administering to a mammalian subject therapeutically effective amounts of cytokine release inducing biologic response modifier in combination with therapeutically effective amounts of compound(s) selected from the group consisting of recombinant cytokine IL-1, endotoxin, Lipid A, and monophosphoryl
Lipid A, in combination with therapeutically effective amounts of non-toxic antioxidant.

20. The method of any of Claims 11 or 13 wherein said biologic response modifier is comprised of two major particle populations, one such population being of lesser size particles comprised of ribosomes and the other such population being comprised of natural membrane vesicles, in a suspending buffer, said membrane vesicles and ribosomes being endogenous to a selected microorganism which is substantially non-pathogenic in humans, said biologic response modifier being substantially free of intact cells, and having tolerable levels of endotoxin, cell walls, and cell membrane fragments.

21. The method of Claim 11 or Claim 13 wherein said biological response modifier is derived from the microorganism Serratia marcescens.

22. The method of any of Claims 17-19 wherein said non cycle-specific chemotherapeutic agents are selected from the group consisting of cyclophosphamide (CTX), Nitrogen mustard, Melphalan, Cisplatin, Nitrosourea, DTIC, and Procarbazine.

23. The method of any of Claims 17-19 wherein said antioxidant is selected from the group consisting of N-acetyl cysteine (NAC) and other sulfhydryl compounds.

24. Use of cytokine release inducing biological response modifier, either alone or in combination with compound(s) selected from the group consisting of recombinant cytokine IL-1, endotoxin, Lipid A, monophosphoryl Lipid A, and antioxidants in the manufacture of medicament packs for use in protecting against chemotherapeutic agent-induced alopecia.

25. A biologic response modifier of Claim 24 wherein said biologic response modifier is
comprised of two major particle populations, one such population being of lesser size particles comprised of ribosomes and the other such population being comprised of natural membrane vesicles, in a suspending buffer, said membrane vesicles and ribosomes being endogenous to a selected microorganism which is substantially non-pathogenic in humans, said biologic response modifier being substantially free of intact cells, and having tolerable levels of endotoxin, cell walls, and cell membrane fragments.

26. A biologic response modifier of any of Claims 24-25 wherein said biologic response modifier is derived from the microorganism Serratia marcescens.

27. A method for protecting against alopecia in a mammalian subject who has been subjected to a combination of at least two chemotherapeutic agents, wherein at least one agent is selected from the group consisting of cytosine arabinoside (ARA-C), doxorubicin (DX), methotrexate, 5-Fluorouracil (5-FU), 6-Mercaptopurine (6-MP), and Thioguanine (TG) and the other is selected from the group consisting of cyclophosphamide (CTX), Nitrogen mustard, Melphalan, Cisplatin, Nitrosourea, DTIC, and Procarbazine, said method comprising, administering to a mammalian subject therapeutically effective amounts of cytokine release inducing biologic response modifier, in combination with therapeutically effective amounts of non-toxic antioxidant.

28. A method for protecting against alopecia in a mammalian subject who has been subjected to a combination of at least two chemotherapeutic agents, wherein at least one agent is selected from the group consisting of cytosine arabinoside (ARA-C), doxorubicin (DX), methotrexate, 5-Fluorouracil (5-FU), 6-Mercaptopurine (6-MP), and Thioguanine (TG) and the other is selected from the group consisting of
cyclophosphamide (CTX), Nitrogen mustard, Melphalan, Cisplatin, Nitrosourea, DTIC, and Procarbazine, said method comprising, administering to a mammalian subject therapeutically effective amounts of cytokine release inducing biologic response modifier, in combination with therapeutically effective amounts of compound(s) selected from the group consisting of recombinant IL-1, endotoxin, Lipid A, and monophosphoryl Lipid A, in combination with non-toxic antioxidant.

29. A method according to any of Claims 27-28 wherein said non-toxic antioxidant is N-acetylcysteine.

30. A method according to any of Claims 27-28 wherein said combinations are administered parenterally or topically.
INTERNATIONAL SEARCH REPORT

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) *

According to International Patent Classification (IPC) or to both National Classification and IPC

INT. CL.5 A61K 9/127, 45/05
U.S.CL. 424/85.2, 450; 428/402.2; 514/880,885,889,908.

II. FIELDS SEARCHED

Classification System Classification Symbols

U.S. CL. 424/85.2, 450; 428/402.2; 514/880, 885, 889, 908.

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

III. 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>X,P</td>
<td>US, A, 4,971,801 URBAN 20 NOVEMBER 1990 SEE ABSTRACT; PATENT CLAIMS; COL. 11, LINE 75-COL. 12, LINE 39; COL. 16, LINE 27-COL. 17, LINE 18; COL. 18 LINES 47-58; COL. 19, LINES 33-52; AND COL. 20, LINES 8-28.</td>
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<td>X,Y</td>
<td>US, A, 4,985,241 ZIMMERMAN ET AL. 15 JANUARY 1991 SEE COL. 4, LINES 45-66; COL. 5, LINES 19-60; COL. 6, LINES 26-57; COL. 7, LINES 3-29; AND COL. 8, LINES 51-COL. 9, LINE 39</td>
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<td>A</td>
<td>US, A, 4,670,185 FUJIVARE ET AL. 02 JUNE 1987</td>
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<td>JP, A, 58-49311 EISAI KK 23 MARCH 1983</td>
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* Special categories of cited documents: 10

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubt 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

"Y" document of particular relevance; the claimed invention 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

IV. CERTIFICATION

Date of the Actual Completion of the International Search 20 JUNE 1990

Date of Mailing of this International Search Report 26 JUL 1991

International Searching Authority ISA/US

Signature of Authorized Officer

RICHARD D. LOVERING