Title: COMPOSITIONS AND METHODS FOR REGULATING IL-6 PRODUCTION IN VIVO

Abstract

Abnormally elevated levels of IL-6 are associated with a number of pathologic disorders. Compositions comprised of DHEA congeners are useful for the treatment of an individual to reduce an abnormally elevated IL-6 level.
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COMPOSITIONS AND METHODS FOR REGULATING IL-6 PRODUCTION IN VIVO

Technical field
The invention relates to methods and compositions used for the regulation of cytokines in vivo, more specifically, to the reduction of abnormally elevated levels of interleukin 6 by compositions comprised of DHEA congeners.

Background
Interleukin 6 ("IL-6") is a pleiotropic cytokine that is produced by a variety of cells and acts on a wide range of tissues, exerting growth-inducing, growth-inhibitory and differentiation-inducing effects, depending on the nature of the target cells. It is believed that IL-6 regulates immune responses, acute phase reactions and hematopoiesis, and that it may play a central role in host defense mechanisms.

Due to its pleiotropic effects, IL-6 was previously identified as β₂-interferon (IFN-β₂), B-cell stimulatory factor 2 (BSF-2), 26 kDa protein, hybridoma/plasmacytoma growth factor (HPGF or IL-HP1), hepatocyte stimulating factor (HSF) and monocyte granulocyte inducer type 2 (MGI-2). However, molecular cloning studies have shown the common identity of the polypeptide chains of all of these factors.

IL-6 is believed to be involved in induction of B-cell differentiation, induction of acute phase proteins in liver cells, growth promotion of myeloma/plasmacytoma/hybridoma cells, induction of IL-2
and IL-2 receptor expression, proliferation and differentiation in T cells, inhibition of cell growth of certain myeloid leukemic cell lines and induction of their differentiation to macrophages, enhancement of IL-3 induced multi-potential colony cell formation in hematopoietic stem cells and induction of the maturation of megakaryocytes as a thrombopoietic factor, induction of mesangial cell growth, induction of neural differentiation of PC12 cells and induction of keratinocyte growth.

Disregulation or overproduction of IL-6 is thought to be linked to a variety of pathologic states, including polyclonal B-cell abnormalities and autoantibody production (for example, cardiac myxoma, rheumatoid arthritis, Castleman's disease, AIDS, alcoholic liver cirrhosis, systemic lupus erythematosus (SLE)); proliferative diseases (mesangial proliferative glomerulonephritis, psoriasis); acute infectious neural diseases (viral meningitis, bacterial meningitis, tuberculosis meningitis, bacterial meningitis, herpes simplex meningitis); and malignancies (plasmacytoma and myeloma, lymphoma and leukemia, and renal cell carcinoma). Abnormally high levels of IL-6 in serum and in urine has also been observed in individuals after surgical trauma, for example, in kidney transplantation where it has been observed during acute rejection episodes. (For reviews on IL-6, see Hirano, T., et al., Immunology Today 11: (1990); Wolvinkamp, M.C.J. and R.L. Marquet, Immunology Letters 24:1-10 (1990); and Van Snick, J., Annu. Rev. Immunol. 8:253-278 (1990).)

Therefore, it is desirable to reduce and regulate abnormal levels of IL-6.
Summary of the Invention

Applicant's invention is directed towards methods and compositions that are useful for the reduction ("down-regulation") in individuals of elevated levels of IL-6, including those associated with pathologic conditions. Applicant's have discovered, surprisingly, that dehydroepiandrosterone ("DHEA") congeners, including DHEA and DHEA-S, are effective agents in restoring elevated IL-6 levels induced by trauma, or related to aging or autoimmune disease, to levels found in normal mature individuals.

Accordingly, one embodiment of the invention is a method for treating an individual to reduce an abnormally elevated IL-6 level, comprising administering to the individual a suitable composition comprised of a pharmacologic dose of a DHEA congener.

Another embodiment of the invention is a composition for treating an individual to reduce an abnormally elevated IL-6 level, comprised of a pharmacologic dose of a DHEA congener in a pharmaceutically acceptable excipient.

Still another embodiment of the invention is a method of preparing a composition for treating an individual to reduce an abnormally elevated IL-6 level, comprising mixing a pharmacologic dose of a DHEA congener with a pharmaceutically acceptable excipient.

Yet another embodiment of the invention is a method for treating an individual to reduce an abnormally elevated IL-6 level wherein the elevated IL-6 level is associated with a B-cell disorder, comprising administering to the individual a suitable composition comprised of a pharmacologic dose of a DHEA congener.

Another embodiment of the invention is a method for treating an individual to reduce an abnormally
elevated IL-6 level and to restore cellular responsiveness to a growth factor, comprising administering to the individual a suitable composition comprised of a pharmacologic dose of a DHEA congen.

Relevant Art


Brief Description of the Drawings

Figure 1 is a bar graph depicting the levels of IL-6 found in the cell supernatants of activated lymphoid cells isolated from mucosal (deep cervical, periaorticaic, parathymic, Peyer’s Patch) and nonmucosal (spleen, inguinal, brachial, axillary) draining lymphoid organs of mature adult, aged, and aged donors maintained on chronic DHEA-S supplementation.

Figure 2 is a bar graph depicting the levels of IL-6 in found in the plasma of mature adult and untreated
aged mice, as compared to that in DHEA and DHEA-S treated aged mice.

Figure 3 are bar graphs depicting the levels of serum IL-6 found in mature adult, untreated aged, and aged mice treated by chronic DHEA-S supplementation. Figure 3A is with C3H mice; Figure 3B is with Balb/c mice.

Figure 4 are bar graphs depicting the concentrations of serum amyloid protein found in mature adult, untreated aged, and aged mice treated by chronic DHEA-S supplementation. Figure 4A is with C3H mice; Figure 4B is with Balb/c mice.

Figure 5 are bar graphs showing the concentration of serum IgG isotypes found in mature adult, untreated aged, and aged mice treated by chronic DHEA-S supplementation.

Figure 6 is a bar graph showing serum IL-6 levels in adult mice before bacterial infection, after bacterial infection, and after bacterial infection with prior treatment with DHEA.

Figure 7 is a graph showing the kinetics of serum IL-6 levels in mice infected in control, thermally-injured, and thermally-injured mice treated with DHEA, and all infected with Listeria.

Figure 8 is a bar graph showing serum IL-6 levels in MRL/lpr mice at 10 weeks (untreated), at 14 weeks (untreated), and at 14 weeks (treated by chronic DHEA-S supplementation).

Figure 9 are graphs showing the effect of PDGF-BB on the production of IL-2, IL-4, and γ-IFN by activated T cells in the presence or absence of pretreatment with IL-6.

Figure 10 are graphs showing the effect of PDGF-BB on the production/activity of IL-2, IL-4, and γ-
IFN, in T cells from young (untreated), aged (untreated) and aged mice (treated with DHEA-S).

Detailed Description of the Invention

As used herein, the term "individual" refers to a vertebrate and preferably to a member of a species which exhibits DHEA-S sulfatase activity, and includes but is not limited to domestic animals, sports animals, and primates, including humans.

The term "effective amount" refers to an amount of congener sufficient to restore normal mature levels of IL-6 in the individual to which the DHEA-congener is administered, and/or to alleviate one or more symptoms of a pathologic condition associated with the elevated IL-6 level.

As used herein, the term "immunodeficient individual" means an individual whose response to immune stimulation to a foreign antigen is significantly less than that of the average of normal individuals of the same species. Methods of determining "immunodeficiency" are known in the art, and include, for example, an examination of lymphokine production by activated T cells; the ability of the individual to demonstrate contact hypersensitivity; the ability of the individual to raise a humoral response to antigen challenge, or the resistance of the individual to infection by microorganisms.

"Treatment" refers to the administration of a composition to an individual which yields a reduction in an abnormally elevated level of IL-6, and includes prophylaxis and/or therapy.

An "antigen" refers to a molecule containing one or more epitopes that will stimulate a host's immune system to make a secretory, humoral and/or cellular
antigen-specific response. The term is also used interchangeably with "immunogen".

An "immunological response" to a composition or vaccine comprised of an antigen is the development in the host of a cellular and/or antibody-mediated immune response to the composition or vaccine of interest. Usually, such a response consists of the subject producing antibodies, B cells, helper T cells, suppressor T cells, and/or cytotoxic T cells directed specifically to an antigen or antigens included in the composition or vaccine of interest.

As used herein, the term "prohormone" pertains to water soluble precursors of DHEA, e.g. DHEA derivatives from which DHEA may be synthesized in vivo, for example, DHEA-S (and other precursors known in the art).

As used herein, a "pharmacologic dose" is one that, when administered to an individual, gives a desired physiological effect, e.g., causes inter alia a reduction of an abnormally elevated level of IL-6 in the treated individual.

As used herein, the term "abnormally elevated level of IL-6" refers to a level of IL-6 in the individual that is associated with a detrimental response to trauma (for example, immunosuppression), a detrimental response to an infection, a level associated with the presence of auto-antibodies in autoimmune states, or a constitutive level of circulating IL-6 associated with, for example, aging.

The term "reducing an abnormally elevated level of IL-6" refers to the down-regulation of IL-6 to that equivalent to levels found in normal mature individuals of the same species and/or to a level associated with a lessening of at least one pathologic symptom of a disease associated with an elevated IL-6 level.
As used herein, "DHEA congener" refers to a compound having the general formula (I)

where \( R \) is hydrogen atom or a halogen atom (including chlorine, bromine, or fluorine); \( R_1 \) is a hydrogen atom, or an \( \text{SO}_2 \text{OM} \) group or a \( \text{PO}_2 \text{OM} \) group wherein \( M \) is a hydrogen or sodium or potassium atom, a sulfatide group

\[
\text{SO}_2\text{O-CH}_2\cdot\text{CH}\cdot\text{CH}_2\cdot\text{O-CO.R}_3
\]

\[
\quad\text{O-CO.R}_2
\]

or a phosphatide group

\[
\text{O}
\]

\[
\text{P-O-CH}_2\cdot\text{CH}_2\cdot\text{O-CO.R}_3
\]

\[
\quad\text{O-CO.R}_2
\]

wherein each of \( R_2 \) and \( R_3 \), which may be the same or different, is a straight or branched chain alkyl radical of 1 to 14 carbon atoms; or a glucuronide group
wherein the broken line in formula I represents an optional double bond, and the hydrogen atom at position five is present in the $\alpha$- or $\beta$- configuration (or the composition comprises a mixture of both configurations).


In one embodiment the invention provides methods for reducing abnormally elevated levels of IL-6 in an individual by treating the individual with a composition comprised of a pharmacologic dose of a DHEA congener. The exact amount necessary will vary from subject to subject, depending on the species, age, and general condition of the subject, the severity of the condition being treated, the mode of administration, etc. Thus, it is not possible to specify an exact effective amount. However, the appropriate effective amount may be determined by one of ordinary skill in the art using only routine experimentation. Methods of determining IL-6 levels, including, for example, immunoassays, are known
in the art, and some are exemplified infra. In addition, methods of detecting alleviation of pathologic symptomology, including, inter alia, decreases in immunodeficiency by determining immunological response to a foreign antigen, decreases in autoimmune effects by lessening of the presence of autoantibodies or physiological manifestations resulting from the autoimmune disease are also known in the art.

The individuals to be treated are those in which elevated levels associated with pathologic conditions exist, for example, individuals with polyclonal B-cell abnormalities or autoantibody production (for example, cardiac myxoma, rheumatoid arthritis, Castleman’s disease, AIDS, Alcoholic liver cirrhosis, systemic lupus erythematosus (SLE)); individuals with proliferative diseases (e.g., mesangial proliferative glomerulonephritis, psoriasis); individuals with acute infectious neural diseases (e.g., viral meningitis, bacterial meningitis, tuberculosis meningitis, bacterial meningitis, herpes simplex meningitis); and individuals with malignancies (e.g., plasmacytoma and myeloma, lymphoma and leukemia, and renal cell carcinoma). Others individuals to be treated include those in which the elevated IL-6 levels result from trauma and/or stress, and result in immunodeficiency, for example, burn victims, surgery patients, and animals with shipping fever. Still other individuals to be treated include aged individuals with constitutive levels of circulating IL-6. In addition, individuals to be treated include those with abnormally high levels of IL-6 associated with allograft transplantation, in which symptomology of graft rejection may also be monitored by means known to those of skill in the art. Included within individuals to be treated are those who have abnormally elevated IL-6, and are non-
responsive to growth factors, (as shown infra). Growth factors are known in the art, and include, inter alia, PDGF, TGF-β, and insulin (disorders associated with non-responsiveness to these growth factors are inhibition of wound healing, osteoporosis, and diabetes, respectively.)

If desired, DHEA may be administered to individuals through precursor substances which are then metabolized to DHEA or its metabolites. For example, the sulfonated form of DHEA, DHEA-S, can be administered provided that the administration is to an individual that can metabolize the prohormone to DHEA by tissue-associated DHEA-sulfatases. The presence or absence of DHEA-sulfatase in specific tissues probably allows for compartmentalization of DEAE at specific anatomical sites.

Depending upon the nature of the DHEA congener, and the time over which the desired regulatory effect on IL-6 level is to be obtained, the administration is by the mode most suitable to obtain that effect, taking into account the ease and/or efficiency of the mode chosen. Thus, it is contemplated that administration is by routes known to those of skill in the art, including, inter alia, oral, intravenous (I.V.), subcutaneous, topical, nasal and rectal routes.

Generally, the person in charge of the administration of the compositions comprised of DHEA congeners will choose the appropriate form of the steroid based upon compartmentalization effects and metabolic products resulting therefrom. For example, if the indication for administration is prophylaxis or chronic therapeutic treatment, a DHEA-congener that is a prohormone, for example, DHEA-S, is preferred to escape the side effects associated with of the administration of chronic high levels of DHEA. In this case the level of DHEA-S is relatively low, and may be in the range of
about 5 to about 100 mg per day, preferably may be in the range of about 10 to about 80 mg per day, and even more preferably may be in the range of about 15 to about 60 mg per day.

Alternatively, if the indication for treatment is acute trauma or acute infection, it may be preferable to treat with one or more high dose ("bolus") administrations of DHEA. A bolus administration may be in the range of about 1 to about 20 mg per kg of body weight, more usually may be in the range of about 2 to about 10 mg per kg of body weight, and preferably may be in the range of about 3 to about 8 mg per kg of body weight.

Under some conditions it may be desirable to use two or more methods of administration and/or two or more species of DHEA congeners, either simultaneously or sequentially, to obtain the desired systemic administration of the active ingredient.

It is also contemplated to be within the scope of the invention that the administration of a DHEA congener may be in sequence with or simultaneous with other agents that are antagonists of IL-6 in vivo, and/or are associated with a lessening of symptoms of a disorder associated with an elevated level of IL-6 in the individual, e.g., growth factors.

Other embodiments of the invention are compositions for lowering an abnormally elevated level of IL-6 in an individual. Some of the compositions are comprised of a DHEA congener (except DHEA or DHEA-S) in a pharmacologically acceptable dose. Other compositions are comprised of a DHEA congener (including DHEA or DHEA-S) and at least one other agent that is effective in lessening or preventing the pathologic response associated with the elevated IL-6 level, both the congener and the other agent being present in
pharmacologically acceptable doses. Included within these agents are antagonists of IL-6. Antagonists of IL-6 activity include agents that prevent IL-6 reactivity with normal receptors contributory to the pathologic response associated with elevated IL-6 activity, or that inhibit IL-6 synthesis. Thus, antagonists of IL-6 activity may include, for example: analogs of IL-6 that bind to the normal IL-6 receptor, but that lack or have significantly lowered IL-6 activity with respect to the pathologic response; other agents (e.g., inter alia, steroids, immune regulators, growth factors) that bind to a receptor other than an IL-6 receptor, but that alleviate the pathogenic response associated with an elevated level of IL-6; anti-IL6 antibodies; fragments or analogs of IL-6 receptors; and the like. Included within antagonists of IL-6 activity are DHEA congeners; thus some compositions are comprised of at least two different species of DHEA congeners. Compositions comprised of at least two different species of DHEA congeners may also be comprised of an IL-6 antagonist. Also contemplated within the invention are compositions comprised of at least one DHEA congener and a synergist that potentiates either the reduction of the abnormally elevated level of IL-6 and/or causes an alleviation of a pathologic condition associated with the abnormally elevated IL-6 level. Examples of the latter include, inter alia, growth factors (e.g., PDGF and TGF-β), medicaments, and the like.

The compositions described above are also usually comprised of a pharmaceutically acceptable excipient. Pharmaceutically acceptable excipients are dependent, in part, on the mode of administration, and are known in the art. See, for example, Remington’s Pharmaceutical Sciences, 17th Edition (1985, Mack Publishing Company, Easton, Penn.). Examples of suitable
pharmaceutical carriers include, inter alia, liquid carriers, such as normal saline and other non-toxic salts at or near physiological concentrations, glucose solutions, oils, and solid carriers not used for humans, such as talc or sucrose, also feed for farm animals.

The compositions comprised of a DHEA congener according to the invention may be formulated for enteral, parenteral or topical administration, or for implantation, or for nasal inhalation or spray, or for transdermal application.

Suitable formulations for oral administration include, for example, hard or soft gelatin capsules, dragees, pills, tablets, including coated tablets, elixirs, suspensions, syrups or inhalations and controlled release forms thereof.

Suitable formulations for topical administration include creams, gels, jellies, mucilages, pastes and ointments. The compounds may also be formulated for transdermal administration, for example, in the form of transdermal patches.

Suitable injectable solutions include intravenous, subcutaneous and intramuscular injectable solutions.

Another embodiment of the invention is the use of compositions comprised of one or more species of DHEA congeners in the manufacture of a medicament for use in the treatment of an individual with an abnormally elevated IL-6 level. Further the invention provides for the use of compositions comprised of one or more species of DHEA congeners and at least one IL-6 antagonist in the manufacture of a medicament for use in the treatment of an abnormally elevated IL-6 level and/or pathologic conditions associated therewith.

Described below are examples of the present invention which are provided only for illustrative
purposes, and not to limit the scope of the present invention. In light of the present disclosure, numerous embodiments within the scope of the claims will be apparent to those of ordinary skill in the art.

Examples

Example 1
Restoration by administration of DHEA-S of normal control over IL-6 lymphokine-producing potential by responsive lymphoid cells of aged mice

This study demonstrated that the capacity of activated cells obtained from a variety of mucosal and non-mucosal lymphoid organs of aged mice to produce IL-6 probably is related to the age-associated decrease in endogenous DHEA production.

C57BL/6 strain of mice were bred and housed in the University of Utah Vivarium from breeding stock originally purchased from the National Cancer Institute. Within this experiment mature adult mice were 22 weeks of age and aged mice were 117 weeks of age. In addition, a group of 100-week old C57BL/6 mice were given 100 μg/ml DHEA-S supplementation in their drinking water for a period of 17 weeks prior to the isolation of their lymphoid organs for analysis of IL-6 production following anti-CD3ε stimulation. Single cell suspensions were prepared from the indicated lymphoid organ from groups of 3-4 mice, washed twice in sterile balanced salt solution and resuspended at a density of 1 x 10^7 cells/ml/well in a 24-well Cluster culture plate (Costar, Cambridge, MA) with serum-free culture medium consisting of RPMI 1640 supplemented with 1% Nutridoma-NS (Boehringer-Mannheim), antibiotics, 200 mM L-glutamine and 5 x 10^{-5} M 2-mercaptoethanol. Anti-CD3ε (1.5 μg/ml) antibody was used
as a polyclonal T cell activator and the cultures were then incubated for 24 hours at 37°C, 10% CO₂ in a humidified incubator. Culture supernatants were harvested, clarified by centrifugation and stored at 4°C until the IL-6 assays were performed.

IL-6 bioactivity was assessed according to the method of Van Snick et al. (J. Exp. Med. 165:641 (1987)). The IL-6-dependent indicator cell line, B9, was subcultured every 3 days in a predetermined concentration of recombinant IL-6. Test supernatants were serially diluted into culture wells, with 1 x 10⁵ washed B9 cells, in 10% FCS supplemented RPMI 1640 media. During the final 2 hours of a 72-hour incubation, 5 µg of MTT was added to each culture. The contents of each culture well were then solubilized and the absorbance read on the Vmax microplate spectrophotometer (Molecular Devices, Menlo Park, CA). IL-6 in culture supernatants is reported as pg/ml based on the response of B9 cells to a dose response curve of the human recombinant IL-6 standard.

Presented in Figure 1 is the result of a representative experiment depicting the levels of IL-6 found in the cell supernatants of activated lymphoid cells isolated from mucosal (deep cervical, periaortica, parathymic, Peyer’s Patch) and nonmucosal (spleen, inguinal, brachial, axillary) draining lymphoid organs of mature adult, aged and aged donors maintained on chronic DHEA-S supplementation. A pronounced increase in the production of IL-6 was observed in most lymphoid organs from aged donors following anti-CD3ε activation of their T cells in vitro. The most dramatic age-associated changes in IL-6 production occurred within most of the mucosal-draining lymphoid organs and the spleen. DHEA-S supplementation of the old animals was able to facilitate a change in this age-associated phenotype, to levels of
IL-6 production quite consistent with that seen in mature adult lymphoid-cell donors.

The results of this thorough anatomical analysis strongly suggest that DHEA-S replacement therapy not only supports a more controlled production of IL-6 by activated lymphoid cells from aged mice (Daynes and Araneo, in press), but also "corrects" the changes in the production of IL-6 whose levels are markedly elevated as a consequence of age. Furthermore, the use of DHEA-S for supplementation (thus allowing tissue-specific end organ metabolism of this hormone to occur) appeared to restore normal compartmentalization of IL-6 producing potential. We believe that this compartmentalization of cell function may provide the host with the capacity to generate the most effective types of responses to stimulation in either mucosal or nonmucosal tissues.

Example 2

The acute treatment of aged mice with either DHEA or DHEA-S re-establishes normal plasma IL-6 levels

As an important stimulant of the acute phase response, IL-6 plays a critical role in a host's natural defense mechanisms. However, the constitutive presence of IL-6 in the blood indicates that this lymphokine is abnormally regulated. This can occur either through increased rates of biosynthesis or through decreased rates of utilization and catabolism. The constitutive presence of IL-6 in the blood is not normal. IL-6 has been reported in the plasma of HIV infected (Honda et al., J. Immunol. 145:4059 (1990), thermally injured (Poz et al. Clin. Exp. Immunol. 82:579 (1990)), tumor bearing (Hirano et al. Immunol. Today 11:443 (1990)), and individuals with autoimmune diseases (Hirano et al. Immunol. Today 11:443 (1990)) and may be responsible for
some of the clinical manifestations that associate with these conditions. Our studies show that "Normal" aging associates with the constitutive presence of IL-6 in the serum/plasma, and that the abnormally elevated level is reversed by DHEA congeners.

This study demonstrates that the acute treatment of aged mice by subcutaneous injection of 100 µg of either the active metabolite, DHEA, or the precursor form, DHEA-S, reduced the blood levels of IL-6.

Untreated Balb/c mice, 24 and 120 weeks of age, were selected at random. Equal numbers of the aged Balb/c littermates were administered 100 µg of DHEA or 100 µg DHEA-S by subcutaneous injection in 0.1 ml propylene glycol vehicle. Twenty-four hours later, blood was obtained from each of the treated and untreated mice and the individual sera were evaluated for the concentration of IL-6.

Rat anti-murine IL-6 antibodies were either prepared from culture supernatants of appropriate B-cell hybridomas adapted to growth under serum-free conditions or obtained from Pharmingen (San Diego, CA). These reagents were used for quantitation of murine IL-6 by capture ELISA according to the manufacturer's suggestions and using a modification of the protocol of Schumacher (J. Immunol. 141:1576 (1988)). Briefly, 100 µl of 2 µg/ml of an appropriate capture antibody in .05M Tris-HCl (pH 9.6) was adsorbed to the wells of a 96-well microtest plate, washed and clogged with PBS/1% BSA.

Test supernatants and two-fold serial dilutions of the appropriate reference cytokine (100 µl/well) were dispensed and after sufficient incubation and washing, 100 µl of biotinylated-detection antibody, 1 µg/mL, was dispensed into each well. The ELISA was developed using avidin-HRP and ABTS-substrate. O.D. readings were
performed at 405 nM using a Vmax 96-well microtest plate spectrophotometer (Molecular Devices, Menlo Park, CA). The lower limit of detection for most of this cytokine was 15-30 pg/ml.

The results are shown in Figure 2. While less than 60 pg/mL of IL-6 was detected in the plasma of mature adult Balb/c mice, greater than 600 pg/mL of IL-6 was measured in the untreated aged Balb/c mice. The analysis of serum IL-6 in the serum of both DHEA and DHEA-S-treated aged Balb/c mice show a striking similarity to that of the mature adults. This finding indicates that both DHEA and DHEA-S have the capacity to control the dynamics of abnormal IL-6 production and/or utilization in aged animals as evidenced by reductions in the serum levels of this lymphokine of aged mice.

Example 3

Mice supplemented chronically with DHEA-S show normal levels of serum IL-6 serum amyloid P (SAP) and serum immunoglobulins

IL-6 is a potent stimulant of acute phase proteins by hepatocytes. Furthermore, increasing evidence also implicates a role for IL-6 in the elevations of serum immunoglobulins and autoantibody production associating with certain pathologic conditions. As a consequence of normal aging, the acute phase proteins are elevated in the plasma. This has been reported to parallel a dysregulation of natural defense mechanisms. Additionally, the serum of elderly donors possess elevated levels of all types of serum immunoglobulins. Each of these clinical findings could be caused by the age-associated changes in the regulation of IL-6 synthesis that we have found to exist in the elderly. This study quantitatively evaluated the serum
of mature adult, untreated aged, and aged mice treated by chronic DHEA-S supplementation for the amount of IL-6 present. Serum samples were also quantitatively analyzed to content of serum amyloid P (as an indicator of a subacute, continuous or recurrent acute phase response) and immunoglobulin content and immunoglobulin subclasses.

Both C3H/HeN MTV- and Balb/c strains of mice were used in these experiments. Mature adult female C3H mice were 22 weeks of age and female Balb/c mice were 24 weeks of age at the time of blood collection. One set of aged female C3H and Balb/c mice were 93 weeks of age, while a second set of Balb/c mice was 120 weeks of age. Littermates of all C3H and Balb/c mice had been maintained on 100 μg/ml DHEA-S in drinking water for nine weeks (in the 93 week old subjects) or 52 weeks (in the 120 week old Balb/c mice). The n values for each experimental value were shown. Serum IL-6 was quantified by capture ELISA using reagents from PharMingen (San Diego, CA), according to a modification of the procedure by Schumacher (J. Immunol. 141:1576 (1988)). Serum amyloid P was quantified using radial immunodiffusion according to a previously published report (Gahrning, Daynes et al., Proc. Natl. Acad. Sci. 81:1198-1202 (1984). The amount of immunoglobulin subclasses in serum was evaluated by capture ELISA with horseradish peroxidase labeled antibodies using standard isotyping reagents (Southern Biologicals, Birmingham, AL) employing a minor modification of the manufacturers suggested protocol.

The results of this study provide strong support for the concept that a dysregulated production of IL-6 is responsible for the elevated levels of acute phase reactants and elevated immunoglobulin levels found in the elderly. Normal aged mice very clearly demonstrate constitutive levels of IL-6 and elevated
amounts of serum amyloid P in their plasma (Figures 3 and 4). In addition, aging animals also demonstrate an elevation in quantity of all immunoglobulin isotypes which is significant (Figure 5). Following nine weeks of treatment with DHEA-S supplementation in the drinking water, all three of these clinical findings show a reversal in phenotype similar to that of the untreated mature adult. These results suggest that DHEA-S/DHEA is involved in the physiologically normal regulation of endogenous IL-6 biosynthesis. In addition these data imply that as a result of chronic DHEA-S administration, some of the clinical features which are commonly observed in the elderly, which may be involved in compromising natural defense mechanisms and autoantibody presence, may be successfully managed.

Example 4

DHEA treatment of normal and thermally injured mice promotes resistance to elevated serum IL-6 induced by infection with Listeria monocytogenes

One consequence of bacterial or viral infection is the stimulated production and appearance of IL-6 in the plasma. In victims of thermal injury, the results of infection can lead to an elevation in plasma IL-6 which may remain for protracted periods of time. In fact, sustained blood levels of IL-6 in trauma patients correlate with increased mortality (Lephant et al., J. Clin. Endocrinol. Metab. 64:842 (1986). The studies listed below established that DHEA administration enables thermally-injured mice to regulate plasma IL-6 levels following infection with Listeria monocytogenes.

Male and female C3H/HeN MTV- were bred and housed in the University of Utah Vivarium from breeding stock originally purchased from the National Cancer
Institute. Pools of 4-6 mice, ranging in age from 6-8 weeks, were used in any single experiment and were age and sex-matched.

A virulent strain of *L. monocytogenes* was obtained as a gift from Dr. Keith Bishop (University of Utah). The bacteria were grown in trypticase-soy broth (BBL Microbiology Systems, Cockeysville, MD) and stored at 10^9 colony-forming units/ml (CFU) in saline at -70°C until used.

Scald burns were given in a standardized manner following guidelines for the use of animals in research set forth in the Guide for the Care and Use of Laboratory Animals (DHEW Publication No. NIH 78-23, revised 1985). After induction of general anesthesia with chloral hydrate, truncal hair was removed using clippers and a depilatory. Two days later, the animals were re-anesthetized with methoxyflurane for induction of deep anesthesia, and were given scald burns to 20% of total body surface area (TBSA). Thermal injuries were inflicted with hot water maintained at a constant 70°C temperature by immersion of an exposed segment of dorsal skin for 6 seconds through an insulated template. Full-thickness injury using this protocol has been documented by histologic examination. The size of the thermal injury was calculated using eschar measurements and Meeh’s formula for total body surface area: \( A = K W^{2/3} \), where \( A \) is an area in square centimeters, \( K = 8.95 \), and \( W \) is body weight in grams. Burn-injured mice were given intraperitoneal injections for fluid resuscitation of normal sterile saline: 2 ml in the first 24 hours, followed by 1 ml on days 1 and 2, in addition to standard mouse chow and water ad libitum throughout the study period. All animals were maintained in a controlled environment under warming lamps, for several hours post injury, to help maintain body temperature.
The experiment illustrated by the data in Figure 6 was designed to demonstrate that infection of normal adult mice with our stock of *L. monocytogenes* was capable of eliciting significant IL-6 response in vivo. In addition, it was designed to determine whether DHEA could prevent the elevations in IL-6 that accompany such infections. Groups of mature adult C3H/HeN mice were quantitatively evaluated, prior to any manipulation, for levels of IL-6 in their plasma. The mice were then subdivided into an untreated and a DHEA-treated group. 100 µg of DHEA in propylene glycol was given subcutaneously to the DHEA treatment group. The animals from both the treatment and control groups were then infected with 2 x 10^6 live *L. monocytogenes* organisms twenty-four hours after infection blood samples were obtained from individual mice. The mean level of serum IL-6 in the plasma was found to be approximately 3000 pg/ml as a result of infection in the untreated group of mice. However, the group of mice receiving 100 µg DHEA by subcutaneous injection 24 hours prior to infection had levels of IL-6 that were nearly identical to the levels observed in mice prior to infection.

C3H/HeN strain mice are inherently resistant to the lethal effects of infection by a small dose of *Listeria monocytogenes*. When infection is combined with thermal injury, however, this strain of animals demonstrates a markedly increased susceptibility to this pathogen (data not shown). Since an infection with this organism is followed by an acute phase response that is preceded by marked elevation in plasma IL-6, we hypothesized that thermal injury of otherwise normal C3H/HeN mice may promote a dysregulation of the normal homeostatic control over IL-6 production. Normal and thermally-injured mice were prepared as described previously (Merril et al., *Am. J. Surg.* **156**:623 (1987)).
Half of the thermally-injured animals received a single 100 μg injection of DHEA subcutaneously in propylene glycol within 1 hour after thermal injury. Three days later, all mice were infected with 2 x 10^5 viable *Listeria monocytogenes* organisms.

The kinetic evaluation of the serum IL-6 levels induced by infection with *Listeria* in control, thermally-injured, and thermally-injured mice treated with DHEA, revealed similar responses in the control and DHEA-treated thermal-injury groups (Figure 7). In contrast, the levels of serum IL-6 detected in the thermally-injured mice infected with *Listeria monocytogenes*, were markedly elevated and significantly prolonged.

**Example 5**
Abnormally elevated levels of serum IL-6 in a murine model of Autoimmune disease can be effectively controlled by DHEA-S

IL-6 has proven to be a multifunctional cytokine playing a central role in hematoepoiesis, the acute phase response, and immunoglobulin production (Van Snick, *Ann. Rev. Immunol.* 8:253 (1990)). This lymphokine has also been implicated in contributing to the pathology of certain autoimmune diseases (Hirano et al., *Immunol. Today* 11:443 (1990)). The MRL/lpr is an inbred strain of mouse of which every member develops autoimmune disorders early in life (about 14 weeks of age) that is characterized by prominent lymphoid hyperplasia, arteritis, myocardial infarcts, arthritis and premature death (Thiofilopoulos and Dixon, *Adv. Immunol.* 37:269 (1985)). The clinical feature considered to be the major cause of early death in MRL/lpr mice in a subacute proliferative form of glomerulonephritis (Thiofilopoulos and Dixon, *Adv. Immunol.* 37:269 (1985)). Glomerular
lesions contain monocytes with proliferation of both the endothelium and mesangium (Thicofilopoulos and Dixon, *Adv. Immunol.* 37:269 (1985)). The role of IL-6 in the development of glomerulonephritis is currently under investigation and is thought to be manifest by the capacity of IL-6 to serve as an autocrine growth factor for glomerular mesangial cells (Horii et al., *J. Immunol.* 143:3949 (1989)). This is supported by the finding in vivo that IL-6 transgenic mice are prone to mesangial proliferative glomerulonephritis (Hirano et al., *Immunol. Today* 11:443 (1990)). The MRL/lpr strain is believed to represent a valid animal model in which to correlate IL-6 levels with clinical disease. This strain of mice provided us with a model to determine whether chronic DHEA-S supplementation prior to onset of clinical changes could abrogate development of the elevated levels of serum IL-6 found in these animals. At age 10 weeks MRL/lpr appear disease free. However, by 14 weeks, a marked lymphadenopathy is evident and histological examination of the kidney demonstrates frank glomerulonephritis accompanied by mesangial proliferation.

Ten MRL/lpr mice, age 11 weeks, were divided into two groups. Half of these mice were started on DHEA-S supplementation (100 μL/mL of drinking water), while the other half received no treatment. When these mice reached 14 weeks, all treated and untreated mice were bled. In addition, a phenotypically normal group of 5 10-week-old MRL/lpr mice were bled to access plasma IL-6 levels. Levels of serum IL-6 were quantified by a standard capture ELISA (Schumacher, et al., *J. Immunol.* 141:1576, 1988).

The analysis of serum from young MRL/lpr (10-week-old) mice revealed minimal levels of IL-6 in the sera prior to onset of disease symptoms (Figure 8). At
14 weeks of the age the mice that were not DHEA-S-treated displayed characteristic signs of autoimmune syndromes and simultaneously expressed very high levels of serum IL-6. The 14-week-old MRL/lpr that received chronic DHEA supplementation from 11 weeks onward showed levels of serum IL-6 that were within the normal range of the 10-week-old group that was symptom-free at the time of serum collection.

This finding indicates that DHEA-S supplementation probably could serve as an effective mode of therapy to be used prophylactically to control the elevations in serum IL-6 levels associated with certain types of autoimmune conditions.

Example 6
IL-6 influences cellular responsiveness to PDGF; DHEA-S administration to aged animals re-establishes PDGF responsiveness

Platelet-derived growth factor (PDGF) is a small family of potent cytokines produced and released by a variety of cell types following their stimulation (Hart, et al., J. Invest. Dermatol. 94:53s, 1990). PDGF also exists in the granules of blood platelets. The major source of PDGF released during an inflammatory episode is derived from the platelet. The most predominant isoforms of PDGF in human platelets is PDGF-BB and PDGF-AB (Bowen-Pope, et al., J. Biol. Chem. 264:2502, 1989). In defined tissue microenvironments, an example being a tumor, PDGF-AA may be produced. All three isoforms of PDGF have been studied for their capacity to induce chemotaxis and proliferation responses of various target cell types (Hart, et al., J. Invest. Dermatol. 94:53s, 1990). These molecules also can contribute to the processes of angiogenesis (Hart, et

Wound healing, angiogenesis and other repair processes which are dependent upon growth factors are moderately or even severely compromised in the aged or the severely traumatized individual. Since these clinical conditions are paralleled by an age-associated dysregulation in IL-6 production, evidenced by sustained plasma levels of this lymphokine, we questioned whether a relationship existed between IL-6 presence and growth factor responsiveness.

C57BL/6 donor mice were sacrificed and their lymph node cells were fractionated into an IL-6 pretreatment and sham groups. Treatment was comprised of a short period of incubation with 10 ng/ml human recombinant IL-6 (although 1 ng/mL is effective) for 1 hour at 37°C. Following this pretreatment the cells were washed in balanced salt solution, reconstituted to 1 x 10^7 cells/ml in serum-free RPMI 1640 (1% Nutridoma-SR, Boehringer-Mannheim, Inc.). 1 x 10^7 cells were dispensed into 24-well macrowells with 1.5 μg anti-CD3ε plus or minus a specified concentration of human, recombinant (hr) PDGF-BB (Boehringer-Mannheim, Inc.). The dose range of PDGF-BB employed was 0.2 ng/mL, or 2.0 ng/mL. After a 24-hour incubation at 37°C, cell-free supernatants were harvested and the cytokines IL-2, IL-4 and γIFN were quantified. IL-2 values in units/ml were established by bioassay and IL-4 and γIFN in culture supernatants were measured in pg/ml by capture ELISA as previously reported (Daynes, et al., J. Exp. Med. 174:1323, 1991).

The data presented in Figure 9 reiterate the effect of PDGF-BB on murine T cells at the time of activation (Daynes, et al., J. Exp. Med. 174:1323, 1991). It is evident that production of IL-2 by activated lymphoid cells is augmented while the capacity of
activated T cells to produce IL-4 and γIFN is reduced. These effects are identical to those published by this laboratory, and demonstrate the PDGF has a significant influence on T-cell function. IL-6 pretreatment in this in vitro model completely abrogated the effect of PDGF-BB on T-cell production of lymphokines in response to stimulation. These results suggest that the target cells in microenvironments where inflammatory cytokines are present, specifically cells which display receptors for both the cytokine and the growth factor, may lose responsiveness to the growth factor when IL-6 is present.

Because the presence of IL-6 inhibits T-cell responsiveness to PDGF, we next wished to determine whether the T lymphocytes from donor animals whose physical condition associates with a dysregulation in IL-6 are able to respond to growth factors. More importantly, we questioned whether acute administration of DHEA could reverse any deficiency in growth factor responsiveness.

Lymphoid cells were prepared from mature adult C3H/HeN (13 week of age) donor mice and 2 groups of aged adult C3HeN at 100 weeks of age, one group receiving 100 μg DHEA-S by subcutaneous injection 24 hours before the experiment was initiated. Aged C3H/HeN strain mice which are known to have a dysregulation in IL-6 production were cultured as described with anti-CD3ε plus or minus PDGF-BB. After twenty-four hours culture, supernatants were evaluated for the indicated cytokine levels. Figure 10 illustrates that T cells from normal adult animals are sensitive to the effects of PDGF while normal T cells from aged donors are insensitive to the effects of this growth factor. The results of this experiment also illustrate the dramatic reinstatement of normal lymphokine and PDGF responsiveness by T cell isolated
from donor aged mice that had received a single injection of 100 μg of DHEA-S twenty-four hours prior to sacrifice.

Example 7

DHEA-S supplementation of all mice decreases serum autoantibody activity

The effect of chronic DHEA-S supplementation on autoantibody activity in aged animals was determined by comparing autoantibody activity in the sera of 3 groups of C3H/HeN mice: (1) untreated normal adults (12 to 14 weeks of age, n=8); (2) untreated aged animals (greater than 22 months of age, n=8); and (3) aged animals (greater than 22 months of age, n=8) that for 8 to 9 weeks prior to the taking of serum had been provided with supplements of DHEA-S (100 μg/ml) in their drinking water.

Autoantibody activity in sera was detected by immunohistology using a multiorgan frozen section specimen containing brain, lung, liver, kidney, and thymus from a 1 month old C3H/HeN donor. Frozen sections were cut in a cryostat at -20°C until use. After washing 3X in cold saline, various dilutions of serum ranging from 1:100 to 1:6000 were placed on the sample, kept at room temperature for 2 hours and washed with cold saline. Autoantibody activity in sera was determined by reaction with an optimally diluted peroxidase labeled goat anti-mouse immunoglobulin reagent for 1 hour at room temperature followed by appropriate color development. Autoantibody presence was then determined by microscope using the procedure described by Kato and Hirokawa in Aging: Immunology and Infectious Disease 1:177, 1988.

The results in Table 1 established that the serum of untreated 22 month old C3H/HeN donors contains significant amounts of autoantibody reactive with the
multiple organ specimen of normal murine tissue. In contrast, aged (22 month) animals provided with DHEA-S supplementation for 8-10 weeks prior to serum collection demonstrated a far lower antibody titer, approaching that seen in the mature adult control donors.

Table 1

<table>
<thead>
<tr>
<th>Age</th>
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<tr>
<td>4 month</td>
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<tr>
<td>Mean Autoantibody titer</td>
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Commercial Utility

The compositions and methods of the invention may be used for the treatment of individuals to reduce abnormally elevated levels of IL-6. Further, the compositions of the invention comprised of one or more species of DHEA congeners are useful in the manufacture of a medicament for use in the treatment of an individual with an abnormally elevated IL-6 level. In addition, the compositions comprised of one or more species of DHEA congeners and at least one IL-6 antagonist are also useful in the manufacture of a medicament for use in the treatment of an abnormally elevated IL-6 level.
CLAIMS

1. A method for treating an individual to reduce an abnormally elevated IL-6 level, comprising administering to the individual a suitable composition comprised of a pharmacologic dose of a DHEA congener.

2. The method of claim 1, further comprising determining whether the individual has an abnormally elevated IL-6 level.

3. The method of claim 1, wherein the elevated level of IL-6 is associated with trauma.

4. The method of claim 3, wherein the trauma is caused by burn, and is associated with immunodeficiency.

5. The method of claim 1, wherein the elevated level of IL-6 is constitutive.

6. The method of claim 5, wherein the individual is aged.

7. The method of claim 2, wherein the elevated level is associated with an autoimmune disease.

8. A composition for treating an individual to reduce an abnormally elevated IL-6 level, comprised of a pharmacologic dose of a DHEA congener in a pharmaceutically acceptable excipient.

9. The composition of claim 8, wherein the elevated level of IL-6 is associated with trauma.
10. The composition of claim 8, wherein the trauma is caused by burn, and is associated with immunodeficiency.

11. The composition of claim 8, wherein the elevated level of IL-6 is constitutive.

12. The composition of claim 8, wherein the individual is aged.

13. The composition of claim 8, wherein the elevated level is associated with an autoimmune disease.

14. A method of preparing a composition for treating an individual to reduce an abnormally elevated IL-6 level, comprising mixing a pharmacologic dose of a DHEA congener with a pharmaceutically acceptable excipient.

15. The method of claim 13, wherein the composition is for use in the method of claim 2.

16. The method of claim 13, wherein the composition is for use in the method of claim 3.

17. The method of claim 13, wherein the composition is for use in the method of claim 4.

18. The method of claim 13, wherein the composition is for use in the method of claim 5.

19. The method of claim 13, wherein the composition is for use in the method of claim 6.
20. A method for treating an individual to reduce an abnormally elevated IL-6 level wherein the elevated IL-6 level is associated with a B-cell disorder, comprising administering to the individual a suitable composition comprised of a pharmacologic dose of a DHEA congener.

21. The method of claim 20, wherein the B cell disorder is a malignancy.

22. The method of claim 21, wherein the disorder is myeloma.

23. The method of claim 21, wherein the B-cell disorder is B-cell lymphoma.

24. A method for treating an individual to reduce an abnormally elevated IL-6 level and to restore cellular responsiveness to a growth factor, comprising administering to the individual a suitable composition comprised of a pharmacologic dose of a DHEA congener.

25. The method of claim 24, wherein the growth factor is PDGF.

26. The method of claim 25, wherein the restoration of cellular responsiveness removes an inhibition of wound healing.

27. The method of claim 24, wherein the growth factor is TGF-β.

28. The method of claim 26, wherein the restoration of cellular responsiveness reduces osteoporosis.
29. A method according to claim 6, wherein treatment with a DHEA congener reduces autoantibody activity.

30. A method according to claim 6, wherein treatment with a DHEA congener reduces immunoglobulin levels.

31. A method according to claim 6, wherein treatment with a DHEA congener reduces a metabolite associated with acute phase response.

32. The method of claim 32, wherein the metabolite is serum amyloid P.
FIG. 2

SERUM IL-6 (pg/ml)

MATURE ADULT  AGED  AGED + ACUTE DHEA  AGED + ACUTE DHEAS

n = 19  n = 12  n = 6  n = 6
FIG. 5D

SERUM Ig ISOTYPE (µg/ml)

6/13

MATURE ADULT  AGED  DHEAS-TREATED AGED

FIG. 5E

SERUM Ig ISOTYPE (µg/ml)

MATURE ADULT  AGED  DHEAS-TREATED AGED

FIG. 5F

SERUM Ig ISOTYPE (µg/ml)

MATURE ADULT  AGED  DHEAS-TREATED AGED

SUBSTITUTE SHEET
FIG. 6
FIG. 7
FIG. 8
### INTERNATIONAL SEARCH REPORT

**A. CLASSIFICATION OF SUBJECT MATTER**
- IPC(5): A01N 45/00; A61K 31/56
- US CL: 514/171

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)
- US: 514/171

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

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

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

<table>
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<tr>
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<td>US, A, 5,077,284 (Loria et al.) 31 December 1991, see entire document.</td>
<td>1-4, 8-19</td>
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<tr>
<td>X</td>
<td>US, A, 4,628,052 (Peat) 09 December 1986, see entire document.</td>
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<td>&quot;Modulation of growth, differentiation and carcinogenesis by dehydroepiandrosterone&quot;, see abstract.</td>
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- **Further documents are listed in the continuation of Box C.**

- **See patent family annex.**

- **Date of the actual completion of the international search:** 29 July 1993
- **Date of mailing of the international search report:** 16 AUG 1993

- **Name and mailing address of the ISA/US Commissioner of Patents and Trademarks**
  - Box PCT
  - Washington, D.C. 20231
  - Facsimile No. NOT APPLICABLE

- **Authorized officer:** KAREN COCHRANE CARLSON, PH.D.
- **Telephone No.:** (703) 308-0196

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