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Modulation of Interleukin Production in Anthrax Lethal Toxin-treated Macrophages by Melatonin and Dehydroepiandrosterone

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Anthrax lethal toxin, which consists of two separate protein, protective antigen (83 KDa) and lethal factor (85 KDa) is responsible for major symptoms and death from systemic infection of *Bacillus anthracis*. High concentrations of this toxin are cytolytic to macrophages, whereas sublytic concentrations of lethal toxin induce these cells to produce interleukin 1β (IL-1β). It is proposed that melatonin and dehydroepiandrosterone (DHEA) may play an important role in modifying immune dysfunction. In this study, we investigated whether or not melatonin and DHEA could prevent IL-1β production that is induced by anthrax lethal toxin in mouse peritoneal macrophages. Treatment of melatonin or DHEA alone, as well as together, prevented the production of IL-1β caused by anthrax lethal toxin. We found that melatonin at a concentration of 10^{-6}-10^{-7} M inhibits IL-1β production induced by anthrax lethal toxin. As expect, treatment of DHEA at a concentration 10^{-6}-10^{-7} M also suppressed production of IL-1β by lethal toxin stimulated macrophages. The results of these studies suggest that melatonin and DHEA, immunomodulators, may have an important role in reducing the increase of cytokine production in anthrax lethal toxin-treated macrophages.

Keywords: DHEA, Lethal toxin, IL-1b, Melatonin

Introduction

The involvement of melatonin and dehydroepiandrosterone (DHEA), in many aspects of the immune function and immune responsiveness has been intensively investigated. The hormone melatonin, N-acetyl-5-methoxytryptamine, is synthesized by the number cells and tissues, but in mammals, the pineal gland is the main source of this indolamine (Reiter, 1991; Brzezinski, 1997). The pineal gland translates the photoperiodic message into a chemical signal, melatonin, which serves as a messenger to every organ in the body (Reiter, 1993). Melatonin has also been implicated in the modulation of the immune system. In many studies, melatonin prevented stress-induced immunodepression, restored immune-function after a hemorrhagic shock, and provided protection against gram-negative septic shock (Morrey *et al.*, 1994; Mohan *et al.*, 1995; Wichmann *et al.*, 1996). Additionally, melatonin is an effective scavenger of hydroxyl free radicals (Poeggeler *et al.*, 1993). Although melatonin receptors are present on a variety of cells, melatonin being a lipid soluble can readily pass membranes without aid of carrier protein. This property implies that melatonin could have various roles in the cell.

Dehydroepiandrosterone (DHEA), an androgenic 17-ketosteroid derived from pregnenolone, is known to serve as an intermediary in sex steroid hormones (Miller, 1988). Dehydroepiandrosterone sulfate (DHEAS), the principle adrenal gland steroid that is the inactive sulfated form of DHEA, is converted into DHEA through the activation of the enzyme DHEAS sulfatase that facilitates the conversion of hydrophilic DHEAS to the hydrophobic species DHEA (Hennebold and Daynes, 1994). The major physiological functions of DHEA have not been fully identified although DHEA has been shown to have specific positive effects on the immune response. Recent reports suggested that DHEA has significant immunomodulatory activity in older mice, with DHEA supplementation overcoming their cytokine dysregulation and immune dysfunction (Watts *et al.*, 1996). It was also found to prevent the production of the endotoxin-

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induced tumor necrosis factor (Danenberg *et al.*, 1992). Correlations also exist between DHEA levels and human immunodefiency virus (HIV)-infected patients (Christeff *et al.*, 1992). It was reported that a DHEA loss correlated with a progression towards human acquired immune deficiency syndrome (Lowisniewski *et al.*, 1993).

Anthrax is a zoonotic infection caused by *Bacillus anthracis*. The disease occurs in three forms: cutaneous, respiratory, gastrointestinal (Cieslak and Eitzen, 1999; Duesberry and Woude, 1999). *Bacillus anthracis* is a large, square-ended, nonmotile, aerobic, gram-positive rod, with a centrally located spore. When *Bacillus anthracis* finds favorable milieu for growth, the organism produces an antiphagocytic capsule and at least three proteins, which appear to play a major role in virulence. These proteins are known as protective antigen (PA), lethal factor (LF), and edema factor (EF). Following the A-B model of toxicity, PA serves as a necessary carrier molecule for LF and EF and permits penetration into cells. Lethal toxin (LeTx) results from the combination of LF+PA. Edema toxin (EdTx) results from EF+PA (Hanna 1998; Pile *et al.*, 1998). Lethal toxin is the central effector of shock and death from systemic anthrax (Hanna 1998; Leppla 1995). Thus animal injected with purified LeTx succumb in a manner that closely mimics the natural systemic infection (Hanna *et al.*, 1993). Lethal toxin is selectively cytotoxic for macrophages (Friedlander, 1986; Hanna *et al.*, 1993). Higher levels of lethal toxin are cytolytic to macrophages. Low levels of lethal toxin induce the macrophage production of shock-inducing cytokines, such as TNF-α and IL-1β (Hanna *et al.*, 1994). These observations imply that the release of inflammation mediators might be part of the mechanism of shock and sudden death in anthrax (Hanna, 1998).

Although melatonin and dehydroepiandrosterone have been used in clinical and experimental settings as an immunomodulator agent in many immune dysfunction diseases (Watts *et al.*, 1996; Bubenik *et al.*, 1998), there is no information available on the effect of these immunomodulators on anthrax toxin-induced IL-1β production. Therefore, it was the aim of this study to determine whether or not melatonin and dehydroepiandrosterone can exert their cytokine-suppressing effects in anthrax toxin-treated macrophages. Our data indicate that pretreatment of melatonin and dehydroepiandrosterone on macrophages had significant effect on anthrax lethal toxin-induced IL-1β production.

Materials and methods

Materials Melatonin, RPMI 1640, and fetal calf serum were purchased from Sigma Chemical Co. (St. Louis, USA). Dehydroepiandrosterone was purchase from Fluka (Buchs, Switzerland). DEAE-Sepharose CL-4B and Mono Q column were from Pharmacia (Uppsala, Sweden). Hydroxyapatite was obtained from Calbiochem (La Jolla, U.S.A.). All reagents were of analytical grade.

Animals Macrophages were obtained from an ICR mouse weighing in the range of 27-30 g.

Preparation and treatment of peritoneal macrophages Elicited peritoneal macrophages were harvested as described previously (Shin *et al.*, 1999). Mice were injected i.p. with 3 ml of 2.5% thioglycolate, and peritoneal cells were harvested 3 days later. The cells were plated on 96-well plastic plates at 1×10^6 cells/ml and incubated in a RPMI 1640 medium containing 10% fetal calf serum, 2.6 mg/ml $NaHCO_3$, and antibiotics (20 μg/ml penicillin-streptomycin) for 3 hr at 37°C in a 5% CO_2 incubator. Nonadherent cells were removed by rinsing the plates three times with RPMI 1640. Adherent cell viability was more than 95% as determined by trypan blue (0.1%) dye-exclusion assay. Cells were treated various concentrations of lethal toxin (PA+LF) and the production of IL-1β was assayed. The level of IL-1β was determined by ELISA, as described below.

Preparation of anthrax lethal toxin Lethal toxin (PA and LF) was purified from the Sterne strain of *Bacillus anthracis* according to the method previously described (Leppla, 1988). The culture was grown in a defined RM toxin-production medium. Hydroxyapatite (5 g/L) was added to the mixture was gently agitated at 4°C for 1 hr. The toxin was eluted with 660 mM potassium phosphate buffers (pH 7.0) and was diluted against 10 mM Tris containing 2 mM EDTA (pH 8.0). The dialyzed crude toxin was applied to a column of hyroxyapatite and the column was developed with a gradient of 0-500 mM potassium phosphate buffer (pH 7.0). The components were eluted in the order PA>LF>EF. The toxin containing fractions were pooled and diluted with a 10 mM Tris buffer (pH 8.0). The dialyzed toxin was reapplied on DEAE-Sepharose CL-4B column. The column was subsequently washed with a 10 mM Tris buffer (pH 8.0) containing a linear gradient of increasing NaCl (0-250 mM) for PA, and a buffer containing a linear gradient of increasing NaCl (0-400 mM) for LF. Final purification of PA and LF was achieved by a FPLC Mono Q anion exchange column with a 0.02 M Tris buffer (pH 8.0) and linear 0-400 mM NaCl gradient elution. PA was eluted at 130-140 mM NaCl, and LF eluted at 250-270 mM NaCl. The purity of PA and LF was determined by a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis. Neither PA nor LF alone was cytotoxic to macrophages.

Detection of IL-1β IL-1β concentrations in cells were determined by ELISA kits that are specific against mouse IL-1β. Cells (1×10^6 cells/ml) were cultured in triplicate on 96-well flat-bottom culture plates. Cells were then stimulated with various concentrations of lethal toxin for induction of IL-1β with 8 hr incubation at 37°C in 5% CO_2 incubator. After incubation, cells were collected and stored at 70°C until assay. The values represent the sum of extracellular plus intracellular IL-1β pools (Hanna *et al.*, 1993). The mouse IL-1β (Cat. No. 80-3384-01) ELISA kits were purchased from Genzyme (Cambridge, USA).

Treatment of cell to melatonin and DHEA Macrophages were preincubated with various concentrations of melatonin or DHEA that was dissolved in ethanol 10 min before addition of the lethal toxin. The final concentration of ethanol in cell suspension was

0.25%. Control cells were exposed to the same amount of ethanol that had no effect on control parameters in the test system.

Trypan blue test Cell viability was measured as the viability of live cells to exclude trypan blue vital dye at a 0.1% final concentration. Living cells were counted in a hematocytometer.

Data analysis Data are reported as a mean of triplicate cultures±S.E. Each experiment is representative of 3-5 independent experiments. Comparisons among groups were done by means of ANOVA. Tukeys method was used for multiple comparisons. A $p<0.05$ was considered as statistically significant.

Results

The effect of lethal toxin on the production of IL-1β in macrophages We tested for IL-1β production after addition of various concentrations of LeTx in cultured macrophages. Production of IL-1β as measured by the ELSIA method, was observed at LF concentrations in the range of 10^{-3}-10^{-7} mg per ml in the presence of 0.1 μg/ml of PA (Fig. 1). Maximum stimulation of IL-1β production by LeTx was observed at a LF concentration of 10^{-6} mg/ml. Figure 2 demonstrates time dependence for IL-1β production induced by toxin. The release of IL-1β was very fast; a significant amount was detected within 2 hr after addition of LeTx (PA 0.1 μg, LF 10^{-6} μg per ml), then the amount increased markedly and the maximum was observed at 8 hr. Thereafter, the amount of IL-1β decreased gradually. Therefore, 8-hr-time-point was chosen to assess the amount of IL-1β produced by the LeTx (PA 0.1 μg, LF 10^{-6} μg per ml) treatment for subsequent experiments.

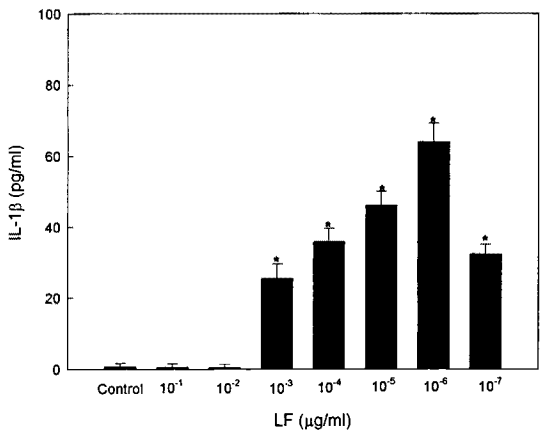

Fig. 1. Effect of various concentrations of LeTx on the production of IL-1β. Cells were treated with LeTx (PA at 0.1 μg/ml + LF, as indicated). Cultures were collected 8 hr after incubation with toxin and assayed for IL-1β content. The control consisted of cells incubated in the absence of LeTx. Values are the mean of 3-5 experiments run in triplicates and bars represent ±S.E. Asterisks indicate statistically significant differences ($P<0.05$; ANOVA) corresponding to control.

The suppression effect of melatonin on IL-1β production Recently, it has been suggested that melatonin regulate the production of Th1 and Th2 cell cytokines in mice (Inserra *et al.*, 1998). Suppression of interleukin production by melatonin has also been noted in retrovirus-infected mouse (Zhang *et al.*, 1999). Therefore, we tested whether or not melatonin affects the production of LeTx-induced IL-1β. To address this point melatonin was added to the culture medium prior to exposure of lethal toxin. The addition of 10^{-6} M melatonin in the culture medium significantly ($P<0.05$) lowered the LeTx-induced IL-1β production (Fig. 3) compared to the control cells (LeTx treatment only). After 8 hr incubation in the presence of LeTx (PA 0.1 μg, LF 10^{-6} μg per ml), the level of IL-1β in cell was 64.3 ± 2.4 pg/ml. However, an addition of 10^{-6} M melalatonin decreased the IL-1β level to 48.1 ± 1.8 pg/ml.

The suppression effect of DHEA on IL-1β production Recent progress in immunological research has demonstrated that DHEA affects the production of interleukin such as IL-2, IL-5, IL-6, and IL-10 (Araghi-Niknam *et al.*, 1997; Inserra *et al.*, 1998). Bearing in mind the possibility that DHEA has an immune regulatory role in interleukin other than IL-2, IL-5, IL-6, and IL-10, we examined whether DHEA affects the production of IL-1β by anthrax lethal toxin treated macrophages. Production of IL-1β was significantly higher in LeTx-treated cells than in untreated cells (Fig. 2). Figure 4 shows the effect of DHEA on LeTx-induced IL-1β production. DHEA, at concentrations of 10^{-7}-10^{-6} M, inhibited IL-1β production by LeTx. The production of the IL-1β in 10^{-7} M DHEA-treated cells was reduced by 40% as compared to its respective control (cells were given LeTx but no DHEA).

Additionally, we studied the synergistic/additive effects of

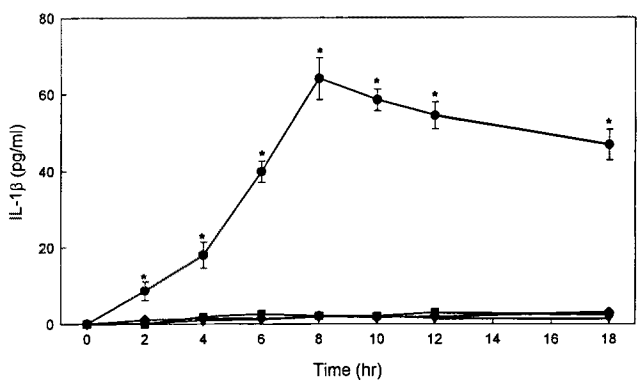

Fig. 2. Time course for the production of IL-1β. Cells were challenged with LeTx (●; PA, 0.1 μg/ml+LF, 10^{-6} μg/ml), PA alone (▼; 0.1 μg/ml), LF alone (■; 10^{-6} μg/ml), and control (◆). At the indicated times after toxin treatment, the levels of IL-1β were determined by ELISA. The control consisted of cells incubated in the absence of LeTx. Values are the mean of 3-5 experiments run in triplicates and bars represent ±S.E. Asterisks indicate statistically significant differences ($P<0.05$; ANOVA) corresponding to control.

Table 1. Combination effect of melatonin and DHEA on IL-1β production by LeTx-stimulated peritoneal macrophages from mice

Treatment	IL-1β (pg/ml)
LeTx	64.3±2.4
LeTx+melatonin	48.3±1.8*
LeTx+DHEA	40.1±3.8*
LeTx+ melatonin+DHEA	41.6±4.9*

Macrophages were preincubated with melatonin (10^{-6} M), DHEA (10^{-7} M) or melatonin+DHEA for 10 min. Anthrax lethal toxin (PA at 0.1 μg/ml + LF at 10^{-6} μg/ml) was then added, and cells were incubated at 37°C for 8 hr as described in Materials and Methods. After incubation, the levels of IL-1β were assayed by ELISA. Data are the mean of 3-5 experiments run in triplicates and represent ±S.E. Asterisks indicate statistically significant differences (P<0.05; ANOVA) corresponding to LeTx alone.

DHEA and melatonin on the inhibition of LeTx-induced IL-1β production. As shown in table 1, their combined use produced no significant effect.

Discussion

Lethal toxin (LeTx) is responsible for the massive death and shock associated with systemic anthrax. The systemic shock caused by LeTx somewhat resembles lipopolysaccharide (LPS)-mediated septic shock, with the characteristic of sudden death observed in the terminal phase of anthrax. In general, during the course of microbial infections, bacterial cell wall products such as endotoxin (LPS) are released and LPS stimulates macrophages and other cells to produce a variety of cytokines, including TNF-α and IL-1β (Beutler *et al.*, 1986). It is well known that low levels of TNF-α or IL-1β coordinate the hosts immune response globally, however at high levels these cytokines mediate damaging inflammatory cascades, shock, and death (Tracey and Cerami, 1993). Based on these observations, it appears that release of large amount of TNF-α, IL-1β, and other potent stored mediators of inflammation from macrophages to the circulation may contribute to the sudden death seen in anthrax.

In this study, we demonstrate a stimulatory effect of LeTx on IL-1β production by mouse peritoneal macrophages. Treatment of cultured macrophages with high levels (>10^{-3} μg/ml) of LeTx showed no induction of IL-1β expression. However, when concentrations of LF dropped to a low dose (10^{-3}-10^{-7} μg/ml), macrophage production of IL-1β was stimulated. In the presence of 10^{-6} μg/ml of PA, small amounts of LF (10^{-5}-10^{-6} μg/ml) resulted in IL-1β level of 50-65 pg/ml. No induction of IL-1β by high levels of LF (>10^{-3} M) is most likely due to the fact that a higher dose of LF is cytolytic, causing cell death in cultured macrophages. The induction pattern of IL-1β by LeTx (in our study) is consistent with previous observations (Hanna *et al.*, 1993).

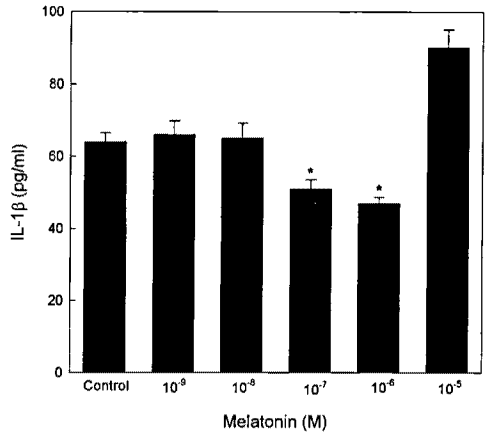

Fig. 3. Effect of melatonin on IL-1β production by LeTx-stimulated macrophages from mice. Macrophages were preincubated with melatonin for 10 min at indicated concentrations. Anthrax lethal toxin (PA at 0.1 μg/ml+LF at 10^{-6} mg/ml) was then added, and cells were incubated at 37°C for 8 hr as described in Materials and Methods. After incubation, the levels of IL-1β were assayed by ELISA. Data are the mean of 3-5 experiments run in triplicates and bars represent ±S.E. Asterisks indicate statistically significant differences (P<0.05; ANOVA) corresponding to LeTx alone.

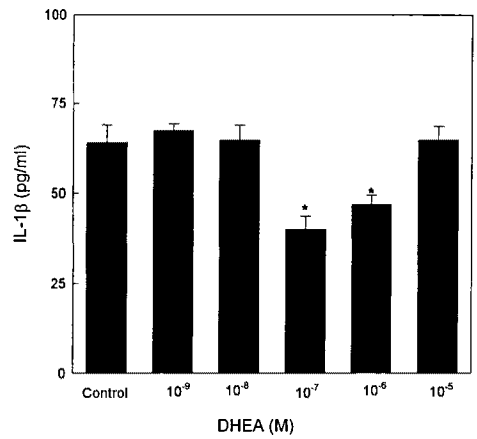

Fig. 4. Effect of DHEA on IL-1β production by LeTx-stimulated macrophages from mice. Macrophages were preincubated with DHEA for 10 min at indicated concentrations. The anthrax lethal toxin (PA at 0.1 μg/ml+LF at 10^{-6} μg/ml) was then added, and cells were incubated at 37°C for 8 hr as described in Materials and Methods. After incubation, the levels of IL-1β were assayed the by ELISA. Data are the mean of 3-5 experiments run in triplicates and bars represent ±S.E. Asterisks indicate statistically significant differences (P<0.05; ANOVA) corresponding to LeTx alone.

Our further studies demonstrated that the LeTx-induced production of IL-1β was prevented by melatonin and DHEA, indicating that these hormones have a protective effect on anthrax infection. A large body of evidence exists supporting the involvement of the pineal hormone melatonin in the modulation of the cytokine network (Regelson and Pierpaoli

1987; Maestroni, 1993; Brzezinski, 1997). Exogenous melatonin administration has been shown to decrease the production of Th2 cytokines, IL-6 and IL-10 in old mouse (Inserra *et al.*, 1998). Furthermore, it has been observed that melatonin was found to regulate the IL-4 secretion on ovalbumin-specific T cells (Shaji *et al.*, 1998). It has also been reported that administration of melatonin increases the immune function after trauma-hemorrhage by the regulation of macrophage interleukin production (Wichmann *et al.*, 1996). Although it has been suggested that low levels of anthrax lethal toxin lead to a marked change of interleukin production (Hanna *et al.*, 1993), the therapeutical potential of melatonin for the treatment of cytokine dysregulation associated with anthrax lethal toxin has not been studied. In this study, mouse peritoneal macrophages were used to test the effect of melatonin on anthrax lethal toxin. These cells were known to be able to produce melatonin (Finocchiaro *et al.*, 1991). Our results indicated that pretreatment of melatonin (concentrations ranging from 10^{-6}-10^{-7} M) inhibits IL-1β production in LeTx-treated macrophages. Similar concentration (10^{-7} M) of melatonin was reported to exert prevention effect of TNF-α production on endotoxin-treated mononuclear cells (Sacco *et al.*, 1998). The results presented here clearly show that melatonin treatment before LeTx addition on macrophages significantly prevented the LeTx-induced IL-1b production.

We also observed that DHEA treatment prevented the LeTx-induced IL-1β production. It has been suggested that DHEA acts as a cytokine buffer, regulating concentrations of cytokines by reducing high levels of one cytokine or by increasing the deficient secretion of another (Mclachlan *et al.*, 1996). For example, aged animals display dysregulated IL-6 production that can be corrected by DHEA treatment (Daynes *et al.*, 1993). Moreover, recent observations suggested that preincubation of peripheral blood mononuclear cells with DHEA reduced the IL-4 production by concanvalin A-stimulated peripheral blood mononuclear cells (Tabata *et al.*, 1997). Dehydroepiandrosterone sulfate (DHEAS) is an end organ metabolized by sulfatase activity into DHEA. It has been determined that macrophages possess this necessary enzyme for the activation of DHEA (Hennebold and Daynes, 1994). Many studies showed that DHEA concentrations within 10^{-4} to 10^{-8} M range were effective in resulting cytokines production *in vivo* and *in vitro* (Mclachlan *et al.*, 1996; Tabata *et al.*, 1997). From our studies, pretreatment of 10^{-6} to 10^{-7} M concentrations range of DHEA inhibits IL-1β production in LeTx-treated macrophages. These data, in combination with previous research, show the positive effect of on DHEA on the immunologic function. In addition, these inhibition effects on lethal toxin-induced IL-β production was also achieved when melatonin or DHEA was added to the culture media at the same time as the lethal toxin (data not shown).

We propose that modifying the transcription of the IL-1β gene is one of possible molecular mechanisms by which melatonin and DHEA can prevent the anthrax lethal toxin induced cytokine production. It has been suggested that melatonin's antioxidant properties may prevent the production of cytokines, since free radicals can affect signal transduction pathways leading to cytokine synthesis (Sacco *et al.*, 1998). In fact, melatonin has been shown to inhibit the transcription factor, nuclear factor-kB (NF-kB), as do other antioxidants (Chuang *et al.*, 1996). In addition, DHEA was shown to have antioxidant properties (Araghi-Niknam *et al.*, 1996). However, in the present study, we do not know the clear mechanism of action of melatonin or DHEA. Further studies are needed to explore the mechanisms by which melatonin and DHEA affect the production of IL-1β following LeTx treatment.

The main significance of the findings reported here is that situation in which melatonin or DHEA may decrease the vulnerability of the macrophages to anthrax lethal toxin. These protective effects are mediated by the reduction of cytokine production. These findings point the way toward a new therapeutic approach for those suffering anthrax toxin toxicity.

References

Araghi-Niknam, M., Chung, S., Nelson-White, T., Eskelson, C. and Watson, R. R. (1996) Antioxidant activity of dioscorea and dehydroepiandrosterone in older humans. *Life Science* **59**, 147-157.

Araghi-Niknam, M., Liang, B., Zhang, Z., Ardestani, S. K. and Watson, R. R. (1997) Modulation of immune dysfunction during murine leukaemia retrovirus infection of old mice by dehydroepiandrosterone sulphate. *Immunol.* **90**, 344-349.

Beutler, B., Krochin, N., Milsark, I. W., Luedke, C. and Cerami, A. (1986) Control of cachectin synthesis: mechanism of endotoxin resistance. *Science* **232**, 977-980.

Bubenik, G. A., Blask, D. E., Brown, G. M., Maestroni, G. J. M., Pang, S. F., Reiter, R. J., Viswanathan, M. and Zisapel, N. (1998) Prospects of the clinical utilization of melatonin. *Biol. Signals Recept.* **7**, 195-21.

Brzezinski, A. (1997) Melatonin in humans. *New Eng. J. Medicine* **336**, 186-195.

Christeff, N., Gharakhanian, S., Thobie, N., Rozenbaum, W. and Nuenz, E. A. (1992) Evidence for changes in adrenal and testicular steroids during HIV infection. *J. Acq. Immune. Defic. Syndr.* **5**, 841-849.

Chuang, J. I., Mohan, N., Meltz, M. L. and Reiter, R. J. (1996) Effect of melatonin on NF-kB DNA binding activity in the rat spleen. *Cell Biol. Int.* **20**, 686-692.

Cieslak, T. J. and Eitzen, E. M. (1999) Clinical and epidemiologic principles of anthrax. *Emer. Infect. Dis.* **5**, 552-555.

Danenberg, H. D., Alpert, G., Lusting, S. and Ben-Nathan, D. (1992) Dehydroepiandrosterone protects mice from endotoxin toxicity and reduces tumor necrosis factor production. *Antimicro. Agents Chemother.* **36**, 2275-2279.

Daynes, R. A., Araneo, B. A., Ershler, W. B., Maloney, C., Li, G. Z. and Ryu, S. Y. (1993) Altered regulation of IL-6 production with normal aging. *J. Immunol.* **150**, 5219-5226.

Duesbery, N. S. and Woude, G. F. (1999) Anthrax toxins. *Cell*

Mol. Life Sci. **55**, 1599-1609.

Finocchiaro, L. M. E., Nahmod, V. E. and Launay, J. M. (1991) Melatonin biosynthesis and metabolism in peripheral blood mononuclear leukocytes. *Biochem. J.* **280**, 727-731.

Friedlander, A. M. (1986) Macrophages are sensitive to anthrax lethal toxin through an acid-dependent process. *J. Biol. Chem.* **261**, 7123-7126.

Hanna, P. C., Acosta, D. and Collier, R. J. (1993) On the role of macrophages in anthrax. *Proc. Natl. Acad. Sci.* **90**, 10198-10201.

Hanna, P. C., Kruskal, B. A., Ezekowitz, R. A. B., Bloom, B. R. and Collier, R. J. (1994) Role of macrophage oxidative burst in the action of anthrax lethal toxin. *Mol. Med.* **1**, 7-18.

Hanna, P. C. (1998) Anthrax pathogenesis and host response. Current Topic Microbiol. *Immunol.* **225**, 13-35.

Hennebol, J. D. and Daynes, R. A. (1994) Regulation of the macrophage dehydroepiandrosterone sulfate metabolism by inflammatory cytokines. *Endocrinol* **135**, 67-75.

Inserra, P., Zhang, Z., Ardestani, S. K., Araghi-Niknam, M., Liang, B., Jiang, S., Shaw, D., Molior, M., Elliott, K. and Watson, R. R. (1998) Modulation of cytokine production by dehydroepiandrosterone plus melatonin supplementation of old mice. *Proc. Soc. Biol. Med.* **218**, 76-82.

Leppal, S. H. (1988) Production and purification of anthrax toxin. *Methods Enzymol.* **165**, 103-116.

Leppla, S. H. (1995) Anthrax toxins; in *Bacterial toxins and virulence factors in disease,* Moss, J., Iglewski, B., Vaughan, M. and Tu, A. T. (eds.), pp. 543-572, New York, Marcel Deckker.

Lowisniewski, T., Hilton, C. W., Morse, E. V. and Svec, F. (1993) The relationship to serum DHEA-S and cortisol levels to measure of immune function in human immunodeficiency virus-related illiness. *Am. J. Med. Sci.* **305**, 79-86.

Maestroni, G. J. M. (1993) The immunoneuroendocrine role of melatonin. *J. Pineal Res.* **14**, 1-10.

Mclachlan, J. A., Serkin, C. D. and Bakouche, O. (1996) Dehydroepiandrosterone modulation of lipopolysaccharide-stimulated monocyte cytotoxicity. *J. Immunol.* **156**, 328-335.

Miller, W. L. (1988) Molecular biology of steroid hormone synthesis. *Endocr. Rev.* **9**, 295-318.

Mohan, N., Sadeghi, K., Reiter, R. and Meltz, M. (1995) The neurohormone melatonin inhibits cytokine, mitogen, and ionizing radiation induced by NF-kappa B. *Biochem. Mol. Biol. Int.* **37**, 1063-1070.

Morrey, K. M., McLachlan, J. A., Serkin, C. D. and Bakouche, O. (1994) Activation of human monocytes by the pineal hormone melatonin. *J. Immunol.* **153**, 2671-2680.

Pile, J. C., Malone, J. D., Eitzen, E. M. and Friedlander, A. M. (1998) Anthrax as a potential biological warefare agent. *Arch. Intern. Med.* **158**, 429-434.

Poeggeler, B., Reiter, R. J., Tan, D. X., Chen, L. D. and Manchester, L. C. (1993) Melatonin, hydroxyl radical-mediated oxidative damage and aging: A hypothesis. *J. Pineal Res.* **14**, 151-168.

Regelson, W. and Pierpaoli, W. (1987) Melatonin: a rediscovered antitumor hormone. *Cancer Invest.* **5**, 379-385.

Reiter, R. J. (1991) Pineal melatonin: Cell biology of its synthesis and of its physiological interactions. *Endocrine Rev.* **12**, 151-180.

Reiter, R. J. (1993) The melatonin rhythm: Both a clock and a calendar. *Experientia.* **49**, 654-664.

Sacco, S., Aquilini, L., Ghezzi, P., Pinza, M. and Guglielmotti, A. (1998) Mechanism of the inhibitory effect of melatonin on tumor necrosis factor production *in vivo* and *in vitro*. *Eur. J. Pharmacol.* **343**, 249-255.

Shaji, A. V., Kulkarni, S. K. and Agrewala, J. N. (1998) Regulation of secretion of IL-4 and IgG1 isotype by melatonin-stimulated ovalbumin-specific T cells. *Clin. Exp. Immunol.* **111**, 181-185.

Shin, S., Kim, Y. B. and Hur, G. H. (1999) Involvement of phospholipase A_2 activation in anthrax lethal toxin-induced cytotoxicity. *Cell Biol. Toxicol.* **15**, 19-29.

Tabata, N., Tagami, H. and Terui, T. (1997) Dehydroepiandrosterone may be one of the regulators of cytokine production in atopic dermatitis. *Arch. Dermatol. Res.* **289**, 410-414.

Tracey, K. J. and Cerami, A. (1993) Tumor necrosis factor, and other cytokines, and diseases. *Ann. Rev. Cell Biol.* **9**, 317-343.

Watts, R. R., Hulls, A., Araghi-Niknam, M. and Chung, S. (1996) Dehydroepiandrosterone and diseases of aging. *Drugs Aging*, **9**, 272-291.

Wichmann, M. W., Zellweger, R., Demaso, C. E., Ayala, A. and Chaudry, I. H. (1996) Melatonin administration attenuates depressed immune functions after trauma-hemorrhage. *J. Sur. Res.* **63**, 256-262.

Zhang, Z., Araghi-Niknam, M., Liang, B., Inserra, P., Ardestani, S. K., Jiang, S., Chow, S. and Watson, R. R. (1999) Prevention of immune dysfunction and vitamin E loss by dehydroepiandrosterone and melatonin supplementation during murine retrovirus infection. *Immunol.* **96**, 291-297.