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Disruption of sphingolipid homeostasis by myriocin, a mycotoxin, reduces thymic and splenic T-lymphocyte populations

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Abstract

Myriocin is a naturally occurring fungal metabolite possessing potent immunosuppressive properties. The biochemical mechanism of action of this compound is inhibition of serine palmitoyltransferase (SPT), the key rate limiting enzyme in sphingolipid biosynthesis, intermediates of which are important mediators of immune signaling. Previous studies have shown that myriocin strongly suppressed immune function with T-lymphocyte functions being most sensitive. To further our understanding of the mechanisms of this effect, we investigated the impact of subacute treatment with myriocin on lymphocyte populations in the thymus and spleen of male BALB/c mice following intraperitoneal injection of myriocin at 0, 0.1, 0.3, and 1.0 mg/kg daily for 5 consecutive days. Cellular analysis of the thymus demonstrated that total cellularity was dose-dependently reduced and the reduction was significant in mice treated with 1.0 mg/kg myriocin. Phenotyping showed that $CD4^+$ and $CD4^+/CD8^+$ double positive lymphocyte populations were sensitive to myriocin. No change in total cellularity of the spleen was noted but there was a significant reduction in the $CD4^+$ lymphocyte population in mice treated with 1.0 mg/kg myriocin. There was a strong positive correlation between total $CD4^+$ lymphocytes in the thymus and those in the spleen. Analysis of sphingolipid levels showed a dose-dependent reduction of sphinganine in the thymus, which were positively correlated with all reductions in lymphocyte populations. These results suggest that the immunosuppressive properties of myriocin may be due to diminished T-lymphocyte populations likely related to inhibition of SPT and disruption of sphingolipid homeostasis.

Keywords: Myriocin; T-lymphocytes; Immunosuppression; Sphinganine; Serine palmitoyltransferase; Mycotoxin

1. Introduction

Myriocin (ISP-1, thermozymocidin, Fig. 1) is a natural product isolated from the *Isaria* (*Cordyceps*) *sinclairii* (*Paecilomyces cicadae*, ATCC 24400). *Isaria* sp. is categorized in a so called "vegetable wasps and plant worms" group of entomopathogenic fungi,

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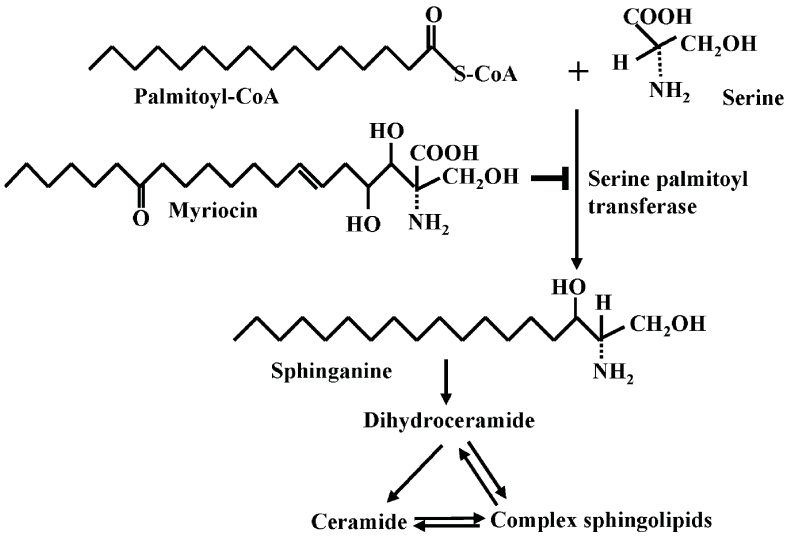

Fig. 1. Schematic showing inhibition of SPT by myriocin in the sphingolipid biosynthetic pathway. Structures of myriocin and sphinganine, along with precursors of sphinganine are indicated.

which have been long utilized in traditional Chinese medicine (Im, 2003). Myriocin was initially isolated as an antibiotic and immunosuppressant for human use but severe gastrointestinal toxicity in laboratory animals has limited its therapeutic potential (Fujita et al., 1995). It has been suggested that myriocin could be used as a therapeutic agent to treat fumonisin-related diseases based on its ability to block fumonisin B_1-induced accumulation of free sphingoid bases (Riley et al., 1999). Alternative routes of exposure to this natural product exist since *I. sinclairii* has potential industrial applications in the form of a natural pigment (Cho et al., 2002). In addition, myriocin forms the molecular basis upon which a variety of potent immunosuppressive analogues are being produced. For example, FTY720 is a novel myriocin-based immunosuppressive drug that is currently undergoing clinical trials as an anti-rejection medication following transplant surgeries (Im, 2003; Napoli, 2000). Therefore, research to further our understanding of the effects of this class of chemicals on the immune system is warranted.

Myriocin is known to be a specific inhibitor of serine palmitoyltransferase (SPT, Fig. 1), the rate-limiting enzyme in the sphingolipid biosynthetic pathway (Miyake et al., 1995). It is capable of inhibiting SPT due to its structural similarities to sphinganine, the natural product of SPT. Disruption of sphingolipid biosynthesis at SPT results in decreased free sphingoid bases sphinganine and sphingosine, cellular ceramide, sphingosine-1-phosphate (S1P) and eventually decreased complex sphingolipids, all important components of cellular signaling or lipid membranes (Miyake et al., 1995). Ultimately, such changes can lead to cell death but more subtly can interfere with cell signaling leading to altered immune regulation. It is known that many bioactive intermediates of sphingolipid metabolism including sphingosine, sphinganine (dihydrosphingosine), ceramide and sphingosine-1-phosphate modulate cellular signaling and regulate cell growth, differentiation, apoptosis, and proliferation (Merrill et al., 1997, 2001). Sphingolipids are also an integral component of membrane rafts and thus are vital to receptor-mediated signaling. Therefore, sphingolipids are important components, both structurally and as signaling molecules, in the development and regulation of immune cells and responses (Baumruker and Prieschl, 2002).

Previous studies have shown that myriocin potently inhibits proliferation of lymphocytes in mouse allogeneic mixed lymphocyte reaction (MLR), T-cell-dependent antibody production, and the generation of allo-reactive cytotoxic T-lymphocytes (CTL) in the mouse with a potency of 10–100-fold greater than that of cyclosporine A, a clinically prescribed immunosuppressant (Fujita et al., 1994). The growth

inhibition of myriocin on mouse cytotoxic T cell line, CTLL-2, was due to its induction of apoptosis, and the apoptotic effect of myriocin on these cells could be reversed by addition of sphingosine to the cultures (Nakamura et al., 1996). Myriocin also potently inhibits interleukin-2 (IL-2)-induced T cell proliferation but not IL-2 production from alloantigen-stimulated T cells after intraperitoneal or oral administration in BALB/c mice (Fujita et al., 1994). More recently, the immunosuppressive effects of myriocin and its structural analogues have been suggested to involve increased lymphocyte homing to lymph nodes resulting in a decreased circulating pool of lymphocytes (Chiba et al., 1998; Pinschewer et al., 2000).

The current study was conducted to investigate the influence of subacute myriocin treatment on lymphocyte populations in primary (thymus) and secondary (spleen) lymphoid organs. We hypothesized that sphingoid base concentrations in lymphoid tissues decreased by myriocin will be related to altered differentiation of lymphoid cells. We report that myriocin treatment significantly decreased T-lymphocyte populations in the thymus and the spleen with $CD4^+$ lymphocytes being the cells most affected. The decrease in lymphocytes correlated with a decrease in free sphinganine content in the thymus suggesting that sphingolipid metabolism may play an important role in selection and/or differentiation in the thymus. Overall, these results suggest that myriocin may have adverse effects on human and animal immune systems possibly via disruption of sphingolipid homeostasis.

2. Materials and methods

2.1. Animals and treatment

Male BALB/c mice, 22 g body weight and 7–8 weeks old, were obtained from Harlan Laboratories (Indianapolis, IN). The animals were acclimated in the University of Georgia Animal Resources facility for 1 week at 23 °C and 50% relative humidity, with a 12 h light: 12 h dark cycle. Mice were housed in groups of 5 in polycarbonate cages lined with wood chip bedding (Betachip, Northeastern Products Corporation, Warrensberg, NY, USA) which was changed every third day. Rodent chow, Harlan Teklad 22/5 (Harlan Teklad, Madison, WI, USA) and water were supplied ad libitum. Protocols for animal use followed the Public Health Service Policy on Humane Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee.

Myriocin (2*S*,3*R*,4*R*,6*E*-2-amino-3,4-dihydroxy-2-hydroxymethyl-14-oxo-6-eicosenoic acid, Fig. 1) was purchased from Biomol Research Laboratories Inc. (Plymouth Meeting, PA, USA). Four groups of five animals each were treated with five daily intraperitoneal injections of phosphate buffered saline (PBS, vehicle), or 0.1, 0.3, or 1.0 mg/kg of myriocin as a suspension in PBS. Twenty-four hours following the final injection, mice were euthanized with fluothane and the thymus, spleen, liver and kidney were removed aseptically. Organs were weighed and then single cell suspensions were made from the thymus and spleen using a Stomacher laboratory blender (STOM 80, Seward, Norfolk, UK) as previously described (Johnson and Sharma, 2001). Cells were counted using a hemocytometer and the final cell concentration was adjusted to 1×10^6 cells/ml.

2.2. Flow cytometric phenotyping of thymic and splenic lymphocyte populations

Three-color flow cytometry was used to determine the prevalence of specific lymphocyte populations in the thymus and spleen as previously described (Johnson and Sharma, 2001). Monoclonal antibodies were conjugated to fluoresceine isothiocyanate (FITC, emission at 525 nm), *R*-phycoerythrin (*R*-PE, emission at 575 nm) or TRI-COLOR (PE-Cy5 tandem transfer dye, emission at 650 nm). Antibodies specific for cell specific receptors included hamster anti-mouse CD3-FITC (pan T-lymphocyte), rat anti-mouse CD45/B220-*R*-PE (pan B-lymphocyte), rat anti-mouse CD4-*R*-PE (T-helper lymphocyte) and rat anti-mouse CD8-TRI-COLOR (T-cytotoxic/suppressor lymphocyte). Mouse anti-mouse CD32/16 antibodies were used to block non-specific binding to Fcγ II/III receptors and were added to all antibody staining combinations. Rat IgG and hamster IgG isotype control antibodies were used to determine specificity of binding. All antibodies were purchased from Caltag Laboratories (Burlingame, CA).

The following tubes were setup for each mouse (A) thymocytes, CD32/16; (B) thymocytes, CD3, CD4

and CD8; (C) splenocytes, CD32/16; (D) splenocytes, CD3 and CD45/B220; (E) splenocytes, CD3, CD4, and CD8. Antibodies were added to respective tubes containing 100 000 cells and vortexed gently followed by incubation at 4 °C for 30 min. Cells were washed three times in PAB (PBS with 1% bovine serum albumin and 0.1% NaN_3) and then resuspended in 0.5% formalin in PBS. Fixed cells were maintained at 4 °C in the dark until acquisition (<24 h). Cells were acquired (20 000 events) using an EPICS XL-MCL flow cytometer (Coulter Cytometry, Hialeah, FL) equipped with a 488 nm argon ion laser and Lysis II acquisition software. Analysis was performed using WinMidi flow analysis package.

2.3. Sphingolipid analysis

The concentrations of free sphinganine and sphingosine in the thymus and spleen were determined by high performance liquid chromatography (HPLC) utilizing a modification of the extraction method described earlier (Merrill et al., 1988). Samples from a separate set of animals using identical treatments of myriocin were employed. Details of the base extraction procedure are the same as previously described (He et al., 2001). Sphingoid bases were quantitated based on the recovery of a C_{20}-sphinganine standard (D-erythro-C_{20}-dihydro-sphingosine, Matreya Inc. Pleasant Gap, PA, USA). The HPLC apparatus and derivatization procedure were similar to the ones described before (He et al., 2001) except the fluorescence detector used in this study was Luminescence Spectrometer LS30 (Perkin-Elmer, Norwalk, CT, USA).

2.4. Statistical analysis

All statistical analyses were performed using the SAS statistical software package (SAS Institute, Cary, NC). Treatment effects were analyzed using one way analysis of variance (ANOVA) followed by post-hoc Student–Newman–Keuls analysis. A value of $P < 0.05$ was considered significant unless indicated otherwise.

3. Results

No overt clinical toxicity or behavioral changes were observed during the 5-day treatment period and no significant changes in body weight were observed between treatment groups. In addition, relative organ weights remained unchanged by myriocin treatment although there was a decreasing trend (statistically insignificant) in relative thymus weights (Table 1).

Cellular analysis of the thymus revealed that subacute treatment with myriocin resulted in a dose-dependent decrease in thymic cellularity which was statistically significant in the 1.0 mg/kg group compared to control mice (Table 2). Phenotypic dissection of the cell populations in the thymus showed that there was a dose-dependent increase in the relative percentage of $CD4^+$ and $CD8^+$ single positive lymphocytes (Fig. 2a and b) whereas the relative number of $CD4^+/CD8^+$ double positive thymocytes (Fig. 2c) was dose-dependently decreased. In contrast, the absolute number of all three major lymphocyte subsets, $CD4^+$, $CD8^+$, and $CD4^+/CD8^+$ double positive were decreased although the decrease was not significant for the $CD8^+$ lymphocyte population

Table 1
The effects of myriocin treatment on body weight (BW) and relative organ weights

Myriocin (mg/kg)	Initial BW[a] (g)	Final BW (g)	Organ/BW ratio (g/100 g)			
			Thymus	Spleen	Liver	Kidney
Vehicle[b]	21.0 ± 0.2	21.4 ± 0.3	0.18 ± 0.01	0.36 ± 0.01	4.86 ± 0.10	1.47 ± 0.05
0.1	20.7 ± 0.4	21.2 ± 0.4	0.19 ± 0.02	0.36 ± 0.02	4.63 ± 0.18	1.48 ± 0.04
0.3	21.6 ± 0.3	22.5 ± 0.1	0.17 ± 0.01	0.36 ± 0.01	4.89 ± 0.10	1.47 ± 0.04
1.0	20.3 ± 0.4	21.5 ± 0.3	0.16 ± 0.01	0.39 ± 0.02	4.87 ± 0.13	1.41 ± 0.02

[a] Body weight.
[b] Phosphate buffered saline.

Table 2
The effects of myriocin treatment on thymic and splenic cellularity

Myriocin (mg/kg)	Total thymic cellularity ($\times 10^{-6}$)	Total splenic cellularity ($\times 10^{-6}$)	Spleen T-lymphocytes[a] ($\times 10^{-6}$)	Spleen B-lymphocytes[b] ($\times 10^{-6}$)
Vehicle[c]	26.0 ± 1.5	32.9 ± 2.5	14.9 ± 1.6	15.0 ± 1.5
0.1	17.7 ± 3.0	25.4 ± 4.6	12.2 ± 1.9	11.0 ± 2.3
0.3	16.9 ± 2.7	27.9 ± 3.3	11.9 ± 0.9	13.0 ± 2.4
1.0	9.0 ± 4.0*	32.4 ± 2.7	11.4 ± 0.9	17.0 ± 1.6

[a] T-lymphocytes were identified as $CD3^+$ splenocytes.
[b] B-lymphocytes identified as $CD45/B220^+$ splenocytes.
[c] Phosphate buffered saline.
* Significantly different from vehicle control at $P < 0.05$; Newman–Keuls test.

(Fig. 2d–f). There was a dose-dependent decrease in $CD4^+$ (Fig. 2d) and $CD4^+/CD8^+$ double positive (Fig. 2f) populations that paralleled the observed decrease in total cellularity in the thymus.

No change in the absolute number of splenocytes was observed under these treatment conditions (Table 2). Total T- and B-lymphocyte numbers also remained unchanged in the spleen although there was a decreasing trend for T-lymphocytes in myriocin-treated mice. Further dissection of the T-lymphocyte population in the spleen demonstrated that there was a dose-related decrease in both $CD4^+$ and $CD8^+$ lymphocytes (Fig. 3) and the decrease in $CD4^+$ lymphocytes was significant in the 1.0 mg/kg myriocin group compared to control mice when examined as relative (Fig. 3a) and absolute counts (Fig. 3c). Importantly, there was a strong positive correlation ($R^2 = 0.9652$) between total $CD4^+$

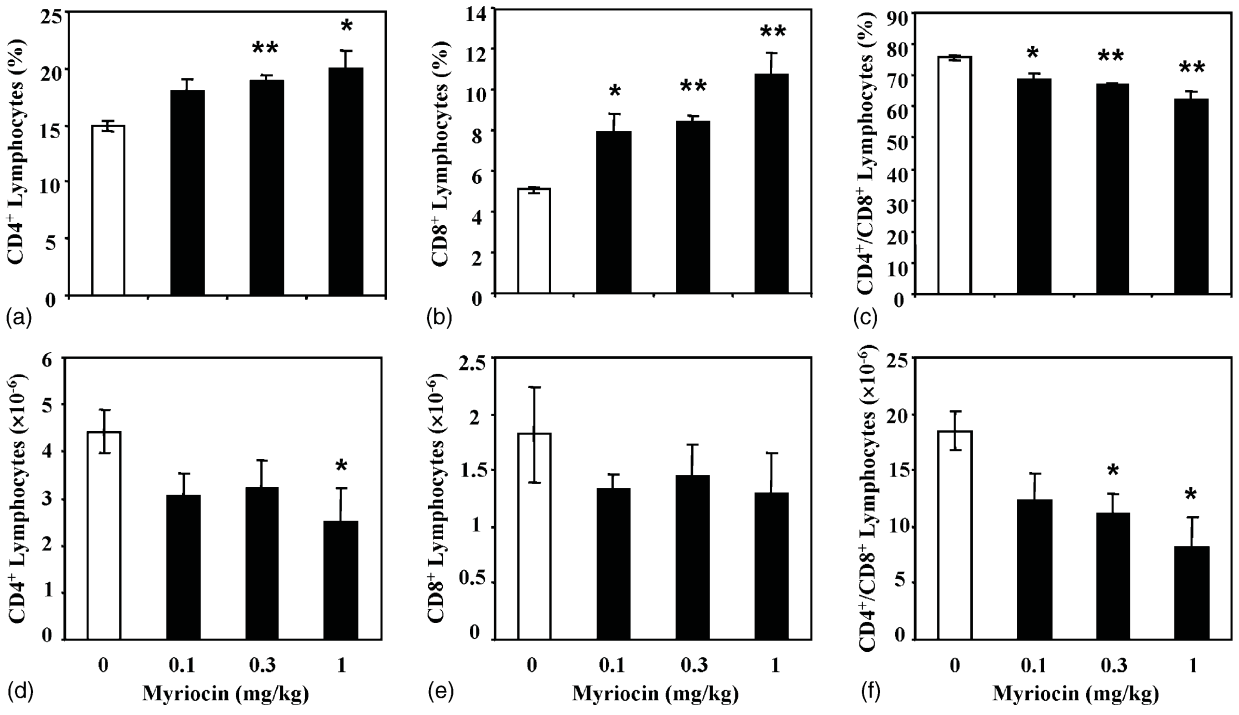

Fig. 2. Decreased lymphocyte populations in the thymus following treatment with myriocin for 5 days. Flow cytometric phenotyping was used to determine the relative and absolute number of $CD4^+$ (a and d), $CD8^+$ (b and e) and $CD4^+/CD8^+$ double positive (c and f) lymphocytes in the thymus. Mean ± S.E.M. ($n = 5$). Significantly different from control group at $^*P < 0.05$, $^{**}P < 0.01$.

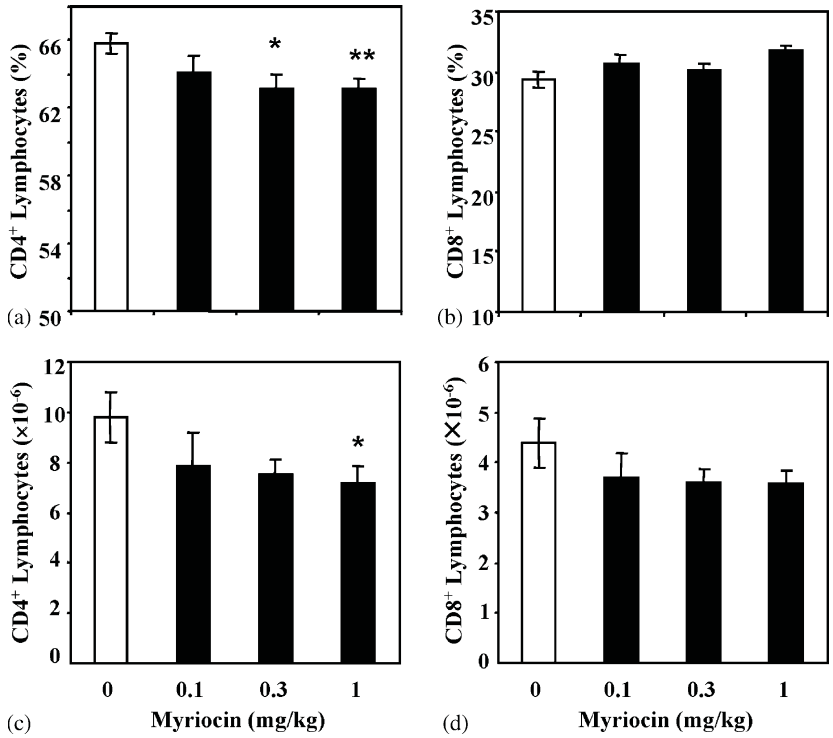

Fig. 3. Myriocin treatment decreased the $CD4^+$ lymphocyte population in the spleen. Mice were treated with myriocin for 5 consecutive days following which flow cytometric phenotyping was performed to determine the relative (upper panels) and absolute number (lower panels) of $CD4^+$ (a and c) and $CD8^+$ (b and d) lymphocytes in the spleen. Mean ± S.E.M. ($n = 5$). Significantly different from control group at $^*P < 0.05$, $^{**}P < 0.01$.

lymphocytes in the thymus and spleen indicating the possibility of decreased emergence of T-lymphocytes from the thymus.

Treatment of mice with myriocin resulted in a dose-dependent decrease in free sphinganine content in the thymus (Fig. 4a) suggesting a direct impact on sphingolipid homeostasis. The decrease in sphinganine corresponded with a dose dependent decrease in SPT in the liver of treated mice (He et al., 2004; Osuchowski et al., 2004). A similar trend was observed for thymic free sphingosine levels (Fig. 4b). No changes in free sphinganine (Fig. 4c) or sphingosine (Fig. 4d) were observed in the spleen. Decreased sphinganine in the thymus was highly associated with the observed decreases in lymphocyte populations. This is supported by the strong positive correlations between the levels of sphinganine in the thymus and total thymocytes, $CD4^+$ thymocytes, $CD8^+$ thymocytes, $CD4^+/CD8^+$ thymocytes, $CD4^+$ splenocytes, and $CD8^+$ splenocytes (Table 3).

Table 3
Correlation between free sphinganine levels in the thymus and lymphocyte populations in the thymus and spleen

Lymphocyte population	Correlation (R^2) with thymus sphinganine levels
Total thymocytes	0.9401
$CD4^+$ thymocytes	0.8881
$CD8^+$ thymocytes	0.8399
$CD4^+/CD8^+$ thymocytes	0.9508
$CD4^+$ splenocytes	0.9664
$CD8^+$ splenocytes	0.9528

4. Discussion

Present data indicated that in vivo treatment with myriocin resulted in marked alterations in lymphocyte populations in the thymus and spleen, which were related to disruption of sphingolipid metabolic pathways, a known property of this mycotoxin. We show that subacute treatment with myriocin resulted

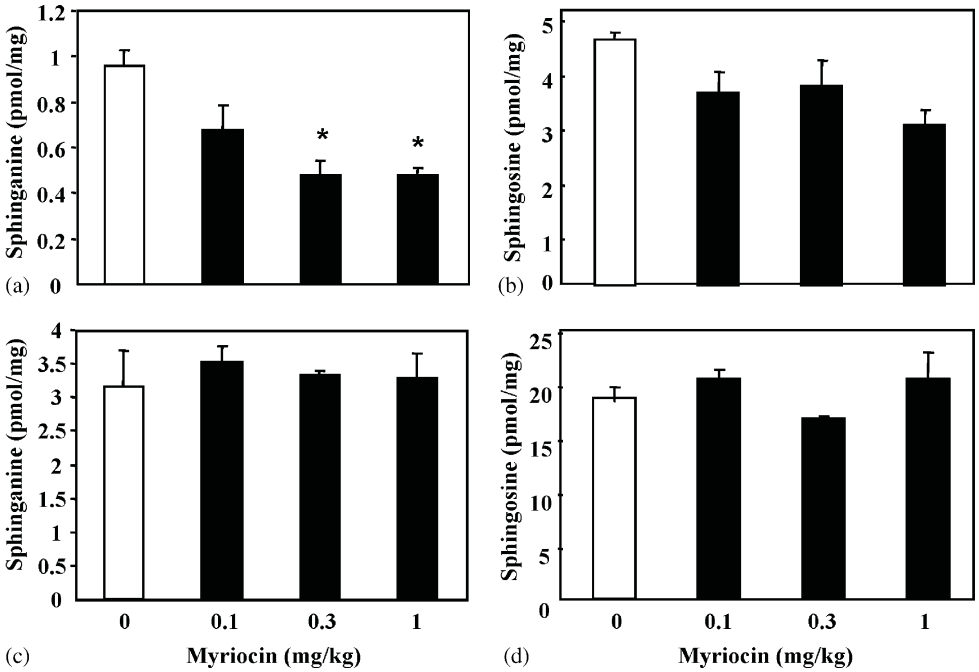

Fig. 4. Free sphingolipid concentrations are reduced in the thymus following treatment with myriocin for 5 consecutive days. Free sphingoid base levels as thymus sphinganine (a), thymus sphingosine (b), spleen sphinganine (c), and spleen sphingosine (d) were determine in base-treated lipid extracts using HPLC. Concentration in whole tissue as mean ± S.E.M. ($n = 4$). *Significantly different from control group at $P < 0.05$.

in a shift in the ratio between single and double positive thymocytes with the relative number of $CD4^+$ and $CD8^+$ single positive cells increasing in the thymus. Due to the decrease in total cellularity in the thymus, total cell numbers in each thymocyte population were decreased. A parallel decrease in $CD4^+$ splenocytes was observed despite unaltered organ cellularity. Total $CD8^+$ lymphocytes in the spleen also tended to decrease but statistical significance was not reached. These changes in lymphoid populations were highly correlated with decreased sphinganine levels in the thymus. These data suggest that decreased T-lymphocyte populations may play a major role in the potent immunosuppressive activity of myriocin, a natural compound and mycotoxin.

Sphingolipids and bioactive intermediates of sphingolipid metabolism play numerous and important roles in the immune regulation and homeostasis. Myriocin targets serine palmitoyltransferase, the rate-limiting enzyme in the sphingolipid biosynthetic pathway (Miyake et al., 1995) and inhibition of this enzyme results in decreased free sphingoid bases, cellular ceramide, sphingosine-1-phosphate and eventually decreased complex sphingolipids (Merrill et al., 1997, 2001). Sphingolipids are also components of membrane rafts and thus are important in receptor mediated signaling. Therefore, sphingolipids are important components, both structurally and as signaling molecules, in the development and regulation of immune cells and responses (Baumruker and Prieschl, 2002). Previous studies have shown that disruption of sphingolipid metabolism using myriocin resulted in marked suppression of immune responses, myriocin being up to 100 times more potent than cyclosporin A (Fujita et al., 1994). Importantly, Fujita et al. (1994) showed potent reduction in T-lymphocyte-dependent antibody production at doses, route and frequency of treatment very similar to those used in the present study. Intraperitoneal administration of myriocin for 4 consecutive days at 0.1, 0.3, 1, and 3 mg/kg resulted in potent suppression of the antibody response to sheep red blood cells of 7, 36, 79, and 93%, respectively (Fujita et al., 1994). This group demonstrated even greater potency of myriocin for inhibition of the

CTL response (40.3, 82.5, 91.9, 98.4% inhibition at 0.03, 0.1, 0.3, and 1.0 mg/kg myriocin, respectively). Therefore, the present data indicates that alterations in lymphocyte populations may play an important role in the earlier observed immunosuppression.

Previous studies showed that FTY720, derived by the chemical modification of myriocin structure, possesses potent immunosuppressive properties that have been related to alterations in peripheral lymphocyte populations (Chiba et al., 1998; Kiuchi et al., 2000; Pinschewer et al., 2000). Treatment of mice with FTY720 resulted in decreased circulating mature lymphocytes following sequestration in lymph node depots (Chiba et al., 1998). This finding suggests that FTY720 alters lymphocyte homing activity and this may possibly be through upregulation of homing receptors including CD62L, CD49s, and CD11a (Chiba et al., 1998). Increased peripheral lymphocyte homing is observed in the absence of defects in T-lymphocyte effector function, expansion and memory development (Pinschewer et al., 2000). These properties make FTY720 a promising therapeutic candidate following transplants and for autoimmune disease and effectiveness have been demonstrated in animal models (Pinschewer et al., 2000; Yang et al., 2003). The effect of myriocin on lymphocyte homing was not investigated in the present study and thus a similar mechanism of action is possible.

The present results suggest that altered lymphocyte traffic may play an important role in myriocin-induced immunosuppression. We observed a decrease in $CD4^+$ lymphocytes in the spleen that correlated with decreased thymic lymphocyte populations. Interestingly, the decrease in peripheral spleen T-lymphocyte numbers was inversely correlated with an increase in the relative percentage of $CD4^+$ and $CD8^+$ single positive thymocytes. This observation suggests the possibility that myriocin may interfere with emigration of new lymphocytes from the thymus resulting in decreased peripheral populations. A similar mechanism was proposed for FTY720 which has been shown to decrease the release of thymic lymphocytes through down regulation of sphingosine-1-phosphate-1 (S1P-1) receptor which is critical for lymphocyte emigration from primary lymphoid organs (Matloubin et al., 2004). There is no evidence that myriocin impacts S1P-1 receptor levels but inhibition of SPT reduces S1P levels. Therefore, a decrease in the ligand for S1P-1 receptor may achieve a similar effect as down regulation of the receptor itself. Analysis of T-cell receptor excision circle (TREC) containing peripheral lymphocytes (Ye and Kirschner, 2002) would provide insight into this hypothesis.

It is also possible that the increase in relative numbers of $CD4^+$ and $CD8^+$ positive lymphocytes is secondary to a decrease in the $CD4^+/CD8^+$ double positive population as observed in the present study. This may be a direct toxic effect of disruption of sphingolipid homeostasis. We previously demonstrated that fumonisin B_1-dependent sphingolipid disruption markedly decreased thymic lymphocyte populations in female BALB/c mice, the $CD4^+/CD8^+$ populations being the most sensitive (Johnson and Sharma, 2001). Increased apoptosis in the thymus may be responsible for the observed effects since the $CD4^+/CD8^+$ positive population of lymphocytes is sensitive to many apoptotic stimuli. Nakamura et al. (1996) showed that myriocin induced apoptosis in the cytotoxic T-cell line CTLL-2 but not in F7 cells derived from a pro-B cell line, suggesting that the death inducing effect of this compound may be unique to specific types of T-lymphocytes. Thus, activation of T-lymphocyte cell death could be a mechanism for myriocin's effects on thymic and splenic lymphocyte populations although this cannot be proven based on data from this study.

In conclusion, the results of the present study demonstrate that subacute treatment of mice with myriocin decreased the T-lymphocyte populations in the thymus and the spleen. Specifically, the $CD4^+$ T-lymphocyte population was the most sensitive to the effects of myriocin although similar trends were observed for $CD8^+$ lymphocytes. The decrease in spleen $CD4^+$ T-lymphocytes was correlated with changes in $CD4^+$ thymocytes, possibly due to $CD4^+$ lymphocyte death or decreased emigration of mature naïve T-lymphocyte from the thymus. All changes observed in lymphocyte populations were highly correlated with a dose-dependent decrease in sphinganine in the thymus. These data suggest that myriocin is detrimental to central and peripheral T-lymphocyte populations, effects associated with disruption of sphingolipid homeostasis. In addition, such a decrease in T-lymphocytes may be a major mechanism of immunosuppression caused by myriocin.

Acknowledgements

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