Defective signal-transduction pathways in T-cells from autoimmune MRL-*lpr/lpr* mice are associated with increased polyamine concentrations

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We previously reported that difluoromethylornithine (DFMO), an inhibitor of polyamine biosynthesis, exerted significant beneficial effects on the lifespan and disease expression of MRL-*lpr/lpr* mice, which spontaneously develop a lupus-like syndrome. Polyamine levels in splenic T-cells of MRL-*lpr/lpr* mice were significantly higher than those of Balb/c mice. In the present investigation, we examined the role of endogenous polyamines in transmembrane Ca^{2+} influx, generation of InsP_3 and tyrosine phosphorylation of the p56^{lek} protein in concanavalin A-stimulated splenic T-cells. Cytosolic free calcium concentrations ($[\text{Ca}^{2+}]_i$) in concanavalin A-stimulated T-cells of MRL-*lpr/lpr* and Balb/c mice were 250 ± 25 and 450 ± 42 nM respectively. Treatment of MRL-*lpr/lpr* mice with DFMO increased $[\text{Ca}^{2+}]_i$ to 360 ± 30 nM ($P < 0.05$). InsP_3 levels of concanavalin A-stimulated MRL-*lpr/lpr* splenic T-cells were only 20 % higher than those of unstimulated controls, whereas those of Balb/c T-cells

were 90 % higher. DFMO treatment increased InsP_3 levels in concanavalin A-treated MRL-*lpr/lpr* T-cells to 67 %. Western-blot analysis showed a 7-fold higher level of p56^{lek} phosphorylation of MRL-*lpr/lpr* splenic T-cells than that of Balb/c mice. DFMO treatment reduced tyrosine phosphorylation of p56^{lek} of MRL-*lpr/lpr* mice significantly ($P < 0.001$). Two-colour flow-cytometric analysis revealed no significant difference in the $\text{CD4}^+/\text{CD8}^+$ ratio in splenic T-cells of MRL-*lpr/lpr* mice after DFMO treatment. Polyamine levels in splenocytes were significantly reduced by DFMO treatment. These data show that DFMO treatment could alter signal-transduction pathways of splenic T-cells of MRL-*lpr/lpr* mice. Increased levels of polyamines in T-cells of untreated *lpr* mice contribute to defective signal-transduction pathways and the pathogenesis of lupus-like symptoms.

INTRODUCTION

Polyamines (putrescine, spermidine and spermine) are small organic cations that are essential for cell growth and differentiation [1,2]. Ornithine decarboxylase (ODC) is a key enzyme in their biosynthesis [3,4]. ODC activity is induced by mitogens, growth factors and other trophic signals, maintaining tight control of intracellular polyamine levels. Delicate regulation of ODC includes transcriptional, translational and post-translational mechanisms [5]. Increased levels of polyamines participate in the suppression of their own synthesis by inhibiting the translation of ODC mRNA and by facilitating the degradation of ODC protein [6–8]. Derailment of one or more of these control mechanisms is found in tumour cells as well as in certain autoimmune disorders [9–11].

Puri et al. [12] first reported that patients with systemic lupus erythematosus, an autoimmune disorder, have increased levels of polyamines in their serum compared with normal controls. In animal models of lupus, MRL-*lpr/lpr* and NZB/W mice, we found a 3-fold higher level of intracellular polyamines in their splenocytes than in normal Balb/c mice [11–14]. Treatment of these mice with an ODC inhibitor, difluoromethylornithine (DFMO), prolonged their lifespan, ameliorated renal disease and prevented anti-DNA antibody production. DFMO treatment also altered the pattern of thymic differentiation as judged by

changes in the expression of differentiation markers in thymic T-cells of MRL-*lpr/lpr* mice [14]. Other studies also support a role for polyamines in the pathogenesis of immunological disorders. Flescher et al. [9] reported that mononuclear cells of patients with rheumatoid arthritis have intracellular polyamine concentrations 2–20-fold higher than in normal controls. Colombatto et al. [10] showed that polyamine levels are significantly higher in peripheral blood mononuclear cells of AIDS patients than in normal controls. Despite these intriguing findings, the role of polyamines in autoimmunity is not yet understood.

Our finding [11,15] of a beneficial effect of DFMO on MRL-*lpr/lpr* mice suggests that polyamines may have an important role in the pathogenic mechanisms of murine lupus. DFMO prevented the production of pathogenic anti-DNA antibodies in this strain without seriously compromising total immunoglobulin production [15], thereby suggesting that the action of this drug might be selectively targeted at the T-cell compartment. Involvement of T-cells in the development of a lupus-like disease in MRL-*lpr/lpr* mice has been well documented by earlier studies using thymectomy [16] as well as by recent studies using chimaeric/transgenic mice [17]. We therefore questioned whether interference with the polyamine-biosynthetic pathway in MRL-*lpr/lpr* mice would induce alterations in T-cell signalling in response to mitogens.

Cellular responses to cell-surface stimuli by antigens or mito-

Abbreviations used: ODC, ornithine decarboxylase; DFMO, difluoromethylornithine; Con A, concanavalin A; $[\text{Ca}^{2+}]_i$, cytosolic free Ca^{2+} concentration; FITC, fluorescein isothiocyanate; PE, phycoerythrin.

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gens are translated into biochemical events by transmembrane signalling pathways leading to gene transcription, DNA replication, cell proliferation and cell differentiation [18–20]. Association of the extracellular signal such as the antigen with the T-cell receptor and the co-receptor CD4 activates tyrosine kinases. Lck, a member of the Src family of tyrosine kinases, is particularly important in signal transduction in T-cells although it may not be the sole tyrosine kinase activated by T-cell receptor [21–23]. Pleiotropic responses, including the activation of phospholipase C and polyphosphoinositide phosphodiesterase, follows the activation of p56^{lck} [24]. The hydrolysis of PtdInsP₂ results in the production of two second messengers, InsP₃ and 1,2-diacylglycerol [25]. InsP₃ is responsible for the increase in intracellular Ca²⁺, and diacylglycerol activates protein kinase C [26,27]. We hypothesized that an altered regulation of these critical signalling processes could occur in MRL-*lpr/lpr* mice as a result of abnormal concentrations of polyamines in their lymphoid cells.

We report here that splenic T-cells from MRL-*lpr/lpr* mice exhibit defective PtdInsP₂ hydrolysis, transmembrane Ca²⁺ influx and elevated tyrosine phosphorylation of p56^{lck} protein compared with that of normal Balb/c mice. DFMO treatment resulted in increased InsP₃ generation, improved Ca²⁺ influx and normalized levels of p56^{lck} tyrosine phosphorylation.

EXPERIMENTAL

Mice

Female Balb/c and MRL-*lpr/lpr* mice were purchased at 4 weeks of age from The Jackson Laboratory, Bar Harbour, ME, U.S.A. Balb/c mice were used as a control because this strain is not predisposed to develop a lupus-like disease at any stage of life. DFMO was a gift from the Marion Merrell Dow Research Institute, Cincinnati, OH, U.S.A. All animals were housed in the same area of a certified vivarium of the UMDNJ–Robert Wood Johnson Medical School. They were on a normal 12 h light/12 h dark cycle and were given food and drinking fluid (water or DFMO solution) *ad libitum*. A group of 20 MRL-*lpr/lpr* mice were administered 1% DFMO in tap water as the only source of drinking fluid from 4 weeks of age. DFMO consumption was 160 mg/100 g body weight per day. There was no significant loss of body weight in mice as a result of DFMO treatment. All mice were killed at 20 weeks of age, the median lifespan of MRL-*lpr/lpr* mice.

Reagents and chemicals

Indo 1 acetoxymethyl ester (indo 1/AM) was obtained from Molecular Probes (Junction City, OR, U.S.A.) in aliquots of 50 µg and stored at –70 °C. It was dissolved in anhydrous DMSO at a concentration of 0.5 mM and stored at 4 °C in the dark. Ionomycin was purchased from Calbiochem (San Diego, CA, U.S.A.) and concanavalin A (Con A) was from Sigma Chemical Co. (St. Louis, MO, U.S.A.). [³H]Inositol and inositol phosphate standards were obtained from Dupont–NEN Research Products (Boston, MA, U.S.A.). Monoclonal anti-phosphotyrosine antibody was obtained from Upstate Biotechnology (Lake Placid, NY, U.S.A.). Rabbit antiserum to p56^{lck} (serum 195.7) was a gift from Professor Roger Perlmutter (University of Washington, Seattle, WA, U.S.A.). An enhanced chemiluminescent Western-blot detection system was obtained from Amersham Corp. (Arlington Heights, IL, U.S.A.). All other reagents were from Sigma Chemical Co.

Preparation and purification of T-cells

Twenty-week old untreated and DFMO-treated MRL-*lpr/lpr* as well as Balb/c mice were killed by cervical dislocation, spleens were removed and single cell suspensions made by gentle teasing in Hanks balanced-salt solution containing 1.2 mM Ca²⁺. The cells were filtered through a nylon mesh to remove debris and possible tissue contamination.

Purified T-cells were obtained from splenocytes by selectively depleting B-cells using J11D antibody (Accurate Chemical and Scientific Corporation, Westbury, NY, U.S.A.). To each 100 × 10⁶ cells, 100 µl of J11D antibody was added and mixed well. After 1–2 min, the cell suspension was treated with 9 vol. of the low toxic rabbit complement (Accurate Chemical and Scientific Corporation) and diluted in cytotoxicity medium (Cedarlane, Toronto, Ont., Canada). The mixture was incubated for 1 h at 37 °C, centrifuged at 1000 *g* for 5 min, washed with cytotoxicity medium, and then with RPMI. Viable cells were counted with a haemocytometer using the Trypan Blue-exclusion method. They were found to be more than 98% pure by flow cytometry using a fluorescein isothiocyanate (FITC)-conjugated anti-CD3 antibody (Pharmingen, San Diego, CA, U.S.A.). These cells were 95% viable for 8 h at 22 °C, as determined by the Trypan Blue-exclusion method.

Measurement of cytosolic free Ca²⁺ concentration, [Ca²⁺]_i

Transmembrane Ca²⁺ influx was measured using a Coulter Epics 753 flow cytometer equipped with a Coherent model Innova 90 argon ion laser as described previously [27]. Purified T-cells were resuspended in serum-free RPMI medium supplemented with 0.1% crystalline BSA at a concentration of 1.5 × 10⁶ cells/ml. The cells were loaded with indo 1/AM at concentrations of 0.5 M and incubated at 37 °C for 40 min. The dye-loaded cells were stimulated with Con A at 25 µg/ml and the ratio of indo 1/AM fluorescence at 500 nm to that at 400 nm ($R = F_{500}/F_{400}$) was recorded for a period of 10 min. Indo 1/AM has a fluorescence maximum at 500 nm, which shifts to 400 nm when the dye is bound to Ca²⁺. A decrease in indo 1/AM fluorescence ratio *R* is therefore a measure of increased accumulation of [Ca²⁺]_i by either transmembrane Ca²⁺ influx or release from intracellular stores. Two-parameter histograms of time versus *R* were recorded. The ratios were then converted into [Ca²⁺]_i by using a calibration method described by Chused et al. [28] and used in our recent publication [27]. Using Multi-parameter Data Acquisition and Display System (MDADS; Coulter Electronics) list mode-analysis programs, we further analysed the histograms to generate graphs showing relative cell number against [Ca²⁺]_i.

In order to confirm the [Ca²⁺]_i measurements performed with the flow cytometer, we also used a spectrofluorimetric technique, as described by Rabinowitch et al. [29]. A Perkin–Elmer luminescence spectrometer LS 50, thermostatically controlled at 37 °C, was used for this purpose. Our measurements of [Ca²⁺]_i using this method were in good agreement (within 15%) with those calculated by the flow-cytometric technique. The data were further analysed to generate curves representing the time courses of mean [Ca²⁺]_i after Con A treatment.

Measurement of [³H]inositol phosphates

Inositol phosphates were separated and quantified as described by Berridge et al. [30]. Briefly, freshly isolated T-cells from Balb/c as well as untreated and DFMO-treated MRL-*lpr/lpr* mice were incubated in RPMI medium (10⁷ cells/ml) containing

40 $\mu\text{Ci}/\text{ml}$ [^3H]inositol for 3 h at 37 °C. After extensive washing, cells were resuspended in RPMI medium containing 10% fetal bovine serum at 0.5×10^7 – 1.0×10^7 cells/ml. LiCl was added to a final concentration of 10 mM, and cells were incubated for 20 min at 37 °C. The cells were transferred to Microfuge tubes, incubated with 25 $\mu\text{g}/\text{ml}$ Con A for 10 min at 37 °C and centrifuged. After aspiration of the medium, 0.75 ml of chloroform/methanol (1:2, v/v) was added to the cell pellet. The aqueous and organic phases were separated by the addition of 250 μl of water and 250 μl of chloroform. The upper aqueous phase was diluted to 2.5 ml. Free inositol and the inositol phosphates were separated from these extracts using a Dowex 1 (X8; formate form) resin (Pharmacia, Piscataway, NJ, U.S.A.) anion-exchange column. The eluent used was 5 mM sodium borate and 60 mM sodium formate (inositol and glycerophosphoinositol), 0.1 M formic acid and 0.2 mM ammonium formate (InsP_1), 0.1 M formic acid and 0.4 mM ammonium formate (InsP_2) and 0.1 M formic acid and 1 mM ammonium formate (InsP_3).

The resolved inositol phosphates were quantified by liquid-scintillation spectroscopy. After the collection of each peak, the column was washed with 3–4 column vol. of the same buffer before elution of the next peak; during these washes, eluted radioactivity returned to background levels. The identities of InsP_1 , InsP_2 and InsP_3 were confirmed with radiolabelled standards. This method unequivocally separates InsP_1 , InsP_2 and InsP_3 from one another, but does not resolve isomers of the inositol phosphates [30].

We also incubated a set of Balb/c T-cells with putrescine for 1 h in RPMI medium and then measured inositol phosphates in order to examine the effect of exogenous polyamines on T-cells. Previous studies showed that exogenous putrescine is readily internalized within 30 min of its addition to cell culture [31].

Western blots

Con A-stimulated (25 $\mu\text{g}/\text{ml}$ for 10 min) T-cells from untreated and DFMO-treated MRL-*lpr/lpr* as well as Balb/c mice were lysed in Nonidet P40 lysis buffer (containing 1% Nonidet P40, 20 mM Tris/HCl, pH 8.0, 150 mM NaCl and 1 mM PMSF) at a concentration of 10^7 cells/ml at 4 °C. Total protein content was quantified using a kit from BRL (Gaithersburg, MD, U.S.A.), and 20 μg of protein was loaded in each lane of an SDS/10% polyacrylamide gel. Electrophoresis was conducted at 200 mV for 3 h, and the proteins were transferred to an Immobilon membrane for 1 h at 100 mV. The blots were incubated overnight with Carnation non-fat dry milk [1.5 g/50 ml of 0.1% Tween/Tris-buffered saline (TTBS)], with 0.003% NaN_3 to block non-specific binding. After 18 h, the blot was incubated with a monoclonal anti-phosphotyrosine antibody (1:2000 dilution in TTBS) or p56^{lck} antibody (1:500 dilution in TTBS) for 2 h. The membranes were subsequently treated with 1:5000 dilution of horseradish peroxidase-conjugated goat anti-mouse IgG (in the case of anti-phosphotyrosine antibody) and peroxidase-conjugated goat anti-rabbit IgG (in the case of p56^{lck} antibody). Immunoreactivity was determined with an enhanced chemiluminescence detection kit and the signal intensity was quantified with a scanning densitometer (Hoefer Scientific Instruments, San Francisco, CA, U.S.A.).

Two-colour fluorescence staining for phenotype distribution

To determine the effect of DFMO on the distribution of CD4 and CD8 subpopulations of T-cells in mouse splenocytes, we conducted fluorescence-activated cell-sorting analysis after two-colour fluorescent staining for CD4 and CD8. Purified thymic T-

cells were treated with L3T4 (CD4)/PE and Lyt2 (CD8)/FITC at a concentration of 4 $\mu\text{l}/10^6$ cells and incubated at 4 °C for 30 min. Negative controls consisted of cells incubated with purified IgG_{2a}/FITC and IgG_{2a}/PE. Flow-cytometer analysis was performed on a Becton-Dickinson FACstar plus instrument. Cells were gated from debris using forward-angle light scatter and 90° light scatter (log). The cells were surface stained for CD4 and CD8 and were gated on each subpopulation. Fluorescence data were collected with logarithmic amplification. For each sample, data from 10000–20000 light-scatter-gated viable cells were collected.

Polyamine assay

Measurements of polyamine concentration were conducted on a Hewlett-Packard HP 1090 liquid chromatograph equipped with an HP 1060 A programmable fluorescence detector. Some 10^7 cells were washed three times with PBS, and pelleted. The pellet was treated with 2 ml of 8% sulphosalicylic acid and sonicated for 15 s in ice. The solution was incubated in ice for 1 h with intermittent vortexing and centrifuged at 900 g for 5 min to remove the precipitated protein. Intracellular polyamine levels were measured after pre-column derivatization with dansyl chloride [31]. 1,6-Diaminohexane was used as the internal standard.

Measurement of ODC activity

ODC activity was measured as described previously [7,11]. Briefly, 10^7 spleen cells were sonicated in ice for 15 s, and centrifuged at 5000 g to remove the precipitated proteins. The supernatant was used for the polyamine assay. The reaction mixture consisted of 250 μl of the cell supernatant, 25 μl of freshly prepared dithiothreitol (62 mg/10 ml of water), 50 μl of ornithine solution (0.5 μCi) containing [^{14}C]ornithine and unlabelled ornithine at a ratio of 1:50, and 25 μl of freshly prepared pyridoxal 5'-phosphate (24.7 mg/100 ml of water) in 15 ml polypropylene tubes fitted with rubber stoppers and centre wells (Kontes Co., Vineland, NJ, U.S.A.). Whatman GF/A filter papers were folded and placed inside the centre wells. The contents of the tubes were incubated for 90 min at 37 °C, and the reaction was stopped by chilling the mixture in an ice/water bath and injecting 0.5 ml of citric acid into the incubation mixture. Hyamine hydroxide (0.2 ml) was then injected into the centre well containing the filter paper. The tubes were further incubated for 30 min at 37 °C. The liberated $^{14}\text{CO}_2$ was trapped in the filter paper and quantified by liquid-scintillation spectroscopy. ODC activity is expressed as pmol of $\text{CO}_2/10^6$ cells.

Statistical significance

All experiments were conducted in triplicate and repeated two to four times, and results are means $\pm$ S.E.M. Statistical significance (taken to be at $P < 0.05$) was calculated using Student's *t* test.

RESULTS

Measurement of $[\text{Ca}^{2+}]_i$

Figure 1 shows representative histograms of transmembrane Ca^{2+} mobilization against time for purified T-cells of Balb/c (a), and untreated (b) and DFMO-treated (c) MRL-*lpr/lpr* mice. We calculated mean indo 1/AM fluorescence ratios by projecting slices of the two-parameter histograms on a computer screen using software available with the Coulter instrument. Mean ratios were converted into $[\text{Ca}^{2+}]_i$ by an *in situ* calibration

Figure 1 Two-parameter histogram of Con A-induced Ca^{2+} mobilization in T-cells from Balb/c (a), untreated MRL-*lpr/lpr* (b) and DFMO-treated MRL-*lpr/lpr* (c) mice

The break in the histogram indicates the time of addition of Con A (25 $\mu\text{g}/\text{ml}$) by stopped-flow techniques. $[\text{Ca}^{2+}]_i$ was calculated as described previously [27,28].

method [27,28]. All cells were stimulated with Con A at a concentration of 25 $\mu\text{g}/\text{ml}$. Balb/c T-cells responded to Con A-induced Ca^{2+} influx with a peak value occurring at 2–3 min after Con A addition by the stopped-flow technique. T-cells from untreated MRL-*lpr/lpr* mice showed significant inhibition of Ca^{2+} mobilization compared with those from Balb/c mice. There was also increased heterogeneity in the response of the cells to Con A compared with that of Balb/c T-cells: 30% exhibited normal transmembrane Ca^{2+} mobilization, whereas 70% were refractory to Con A-stimulated Ca^{2+} influx. T-cells from DFMO-treated mice exhibited significantly increased Ca^{2+} influx and the percentage of the responder population increased to 75%.

Mean $[\text{Ca}^{2+}]_i$ in normal Balb/c T-cells increased from a resting level of 100 ± 20 nM to 450 ± 42 nM in the presence of Con A. Mean $[\text{Ca}^{2+}]_i$ in untreated MRL-*lpr/lpr* T-cells increased only up to 250 ± 25 nM, whereas that in DFMO-treated mice showed a significant increase to 360 ± 30 nM. There was a significant difference in mean $[\text{Ca}^{2+}]_i$ of T-cells from untreated and DFMO-treated mice ($P < 0.001$; $n = 4-5$).

We also analysed these histograms using Coulter MDADS list mode-analysis programs in order to generate graphs showing relative cell number against $[\text{Ca}^{2+}]_i$ (Figure 2). The predominant population of T-cells from untreated MRL-*lpr/lpr* mice remained with low $[\text{Ca}^{2+}]_i$, whereas that from Balb/c mice had a broader distribution, as judged by their $[\text{Ca}^{2+}]_i$ after Con A treatment. With DFMO treatment, there was a major redistribution of T-

Figure 2 Relative splenic T-cell populations of Balb/c (—), untreated MRL-*lpr/lpr* (---) and DFMO-treated MRL-*lpr/lpr* (···) mice at maximum Ca^{2+} influx after Con A stimulation

These plots were generated by creating histogram files of data in Figure 1 using EPICS CYTOLOGIC software.

cells of MRL-*lpr/lpr* mice, indicating a shift toward increased accumulation of intracellular Ca^{2+} in the presence of Con A.

Measurement of $[\text{Ca}^{2+}]_i$ by a spectrofluorimetric technique gave similar results. The time courses of $[\text{Ca}^{2+}]_i$ changes calculated from these experiments for the three groups of splenic T-cells are shown in Figure 3. The peak $[\text{Ca}^{2+}]_i$ values (Balb/c, 435 ± 25 nM; untreated MRL-*lpr/lpr*, 300 ± 30 nM; and DFMO-treated MRL-*lpr/lpr*, 370 ± 25 nM) were obtained 2–3 min after the addition of Con A. $[\text{Ca}^{2+}]_i$ remained high up to 8 min of Con A treatment. Control experiments showed that DFMO treatment had no significant effect on $[\text{Ca}^{2+}]_i$ (450 ± 45 nM) of Balb/c

Figure 3 Time courses of Ca^{2+} influx in splenic T-cells from Balb/c (—), untreated MRL-*lpr/lpr* (---), and DFMO-treated MRL-*lpr/lpr* (···) mice after Con A treatment

The arrow indicates time of Con A addition. Data shown are means of three independent spectrofluorimetric measurements.

Figure 4 Anion-exchange chromatographic separation of inositol phosphates in splenic T-cells from Balb/c (○), untreated MRL-*lpr/lpr* (●) and DFMO-treated MRL-*lpr/lpr* (▲) mice on mitogenic stimulation

Inositol phosphates were separated using increasing concentrations of formate buffers as described in the Experimental section. The separated phosphates were quantified by liquid-scintillation spectroscopy.

T-cells stimulated with Con A. As reported earlier [11], long-term DFMO treatment was toxic to Balb/c mice.

In order to confirm the source of the increased intracellular Ca^{2+} in our system, we measured the fluorescence ratio R when cells were stimulated with Con A in the presence of a Ca^{2+} chelator, EGTA (2 mM). There was no significant increase in $[\text{Ca}^{2+}]_i$ when Con A treatment was conducted in the absence of Ca^{2+} or when Ca^{2+} was chelated by EGTA: basal $[\text{Ca}^{2+}]_i$ was 100 ± 20 nM and that measured in cells after Con A treatment in the absence of Ca^{2+} was 110 ± 25 nM. In contrast, release of Ca^{2+} from intracellular sources was normal when these cells were treated with ionomycin [27]. These results confirmed that the increase in $[\text{Ca}^{2+}]_i$ found in cells treated with Con A was due to influx of Ca^{2+} from extracellular sources.

Quantification of inositol phosphates

We next examined inositol phosphate hydrolysis in T-cells from Balb/c mice, as well as that from untreated and DFMO-treated MRL-*lpr/lpr* mice. Splenic T-cells were treated with 25 $\mu\text{g}/\text{ml}$ Con A in the presence of $[\text{^3H}]$ inositol. Water-soluble lipids from both control and Con A-stimulated cells consisted of three distinct radioactive peaks, corresponding to InsP_1 , InsP_2 and InsP_3 when resolved on an anion-exchange column (Figure 4). Table 1 presents c.p.m. values obtained for InsP_3 in the presence and absence of Con A for T-cells from all three groups of mice. For Balb/c mice, a 90% increase in InsP_3 production occurred as the result of Con A stimulation. In contrast, T-cells from MRL-*lpr/lpr* mice showed only a 20% increase in InsP_3 production after stimulation with Con A, suggesting a significantly lower level of PtdInsP_2 hydrolysis in T-cells from Balb/c mice. DFMO treatment significantly increased the level of InsP_3 production in T-cells of MRL-*lpr/lpr* mice to 67% ($P < 0.001$; $n = 4$) on stimulation with Con A. The generation of total inositol phosphates in Con A-stimulated splenocytes of Balb/c as well as untreated and DFMO-treated MRL-*lpr/lpr* T-cells showed a similar correlation. These results indicate that the deficient Ca^{2+} influx in MRL-*lpr/lpr* T-cells might be related to

Table 1 InsP_3 generation in Con A-stimulated splenocytes from 20-week-old MRL-*lpr/lpr* and normal Balb/c mice

When present, the concentration of Con A was 25 $\mu\text{g}/\text{ml}$. Results are means $\pm$ S.E.M.

Strain	Presence of Con A	InsP_3 (c.p.m.)
Balb/c	—	1000 ± 75
	+	1900 ± 50
Untreated MRL- <i>lpr/lpr</i>	—	350 ± 25
	+	425 ± 35
DFMO-treated MRL- <i>lpr/lpr</i>	—	600 ± 40
	+	1000 ± 60

Figure 5 Tyrosine phosphorylation of p56^{lck}

Total proteins were extracted from Con A-stimulated T-cells of Balb/c, untreated MRL-*lpr/lpr* and DFMO-treated MRL-*lpr/lpr* mice, subjected to SDS/PAGE and transferred to Immobilon membrane filters. In (a) the membrane was incubated with a monoclonal mouse anti-phosphotyrosine antibody followed by peroxidase-conjugated sheep anti-mouse IgG antibody. In (b) the membrane was incubated with rabbit anti- p56^{lck} antibody, followed by peroxidase-conjugated goat anti-rabbit IgG antibody. Immunoreactivity was detected using an enhanced chemiluminescence detection system. In (a) lane 1, Balb/c; lane 2, untreated MRL-*lpr/lpr*; lane 3, DFMO-treated MRL-*lpr/lpr*. In (b) lane 1, molecular-size markers; lane 2, Balb/c; lane 3, untreated MRL-*lpr/lpr*; lane 4, DFMO-treated MRL-*lpr/lpr*.

other events in the signal-transduction pathways, including the production of InsP_3 .

We further confirmed that elevated levels of polyamines had a significant effect on inositol phosphate hydrolysis by measuring InsP_3 in Balb/c T-cells treated with 1 mM putrescine. As reported previously [31], there was a facile transport of putrescine within the first 30 min of treatment. In Balb/c T-cells, the basal level of putrescine was negligible (see Table 3 below), whereas that measured after 30 min of putrescine treatment was 250 pmol/ 10^6 cells. In the absence of Con A treatment, $[\text{^3H}]\text{InsP}_3$ in cells not exposed to putrescine (control cells) was 1200 ± 35 c.p.m., whereas that in putrescine-treated cells was 800 ± 45 c.p.m. After Con A (25 $\mu\text{g}/\text{ml}$) treatment, $[\text{^3H}]\text{InsP}_3$ production increased to 2100 ± 100 and 1500 ± 75 c.p.m. respectively in control and putrescine-treated cells. This result confirms that accumulation of intracellular polyamines inhibits phosphate hydrolysis, although this inhibition is not as marked as in MRL-*lpr/lpr* splenocytes.

Quantification of tyrosine phosphorylation by Western-blot analysis

In the next set of experiments, we studied tyrosine phosphorylation of proteins from T-cells of all three groups of mice. T-cells were stimulated with 25 $\mu\text{g}/\text{ml}$ Con A for 10 min and proteins were extracted as described in the Experimental section. Figure 5(a) shows results of Western-blot analysis using a

Table 2 Effect of DFMO treatment on splenic T-cell subpopulations of 20-week-old MRL-*lpr/lpr* miceResults are means $\pm$ S.E.M.

Treatment	Percentage of total splenocytes			
	CD4 ⁺ CD8 ⁻	CD4 ⁻ CD8 ⁺	CD4 ⁻ CD8 ⁻	CD4 ⁺ CD8 ⁺
None	18 $\pm$ 5	3 $\pm$ 1	77 $\pm$ 4	2 $\pm$ 0.5
DFMO (1%)	16 $\pm$ 4	3 $\pm$ 1	80 $\pm$ 7.5	1 $\pm$ 0

monoclonal anti-phosphotyrosine antibody. Two major bands corresponding to 63 and 56 kDa were phosphorylated at the tyrosine residues. The 63 kDa band was present in samples prepared from all three sources. The 56 kDa band was barely visible in samples from splenic T-cells of Balb/c mice, whereas this band was very intense in T-cells from MRL-*lpr/lpr* mice. DFMO treatment significantly reduced the intensity of the 56 kDa band. Quantification of the intensity of this band, using a scanning densitometer, gave proportions of 1:7:2 for normal Balb/c, untreated MRL-*lpr/lpr* and DFMO-treated MRL-*lpr/lpr* mice respectively.

We conducted further Western-blot analysis using an anti-serum to p56^{lck} protein (Figure 5b). T-cells from Balb/c as well as untreated and DFMO-treated MRL-*lpr/lpr* mice had comparable p56^{lck} protein levels. This result, in conjunction with that presented in Figure 5(a), shows that p56^{lck} is hyperphosphorylated in T-cells of untreated MRL-*lpr/lpr* mice compared with normal Balb/c mice. *In vivo* treatment of MRL-*lpr/lpr* mice with DFMO produced a significant reduction in the phosphorylation of the p56^{lck} protein (Figure 5a, lane 3).

Effect of DFMO treatment on CD4 and CD8 T-cell subpopulations

To examine whether the altered signal-transduction pathways found in splenic T-cells of DFMO-treated MRL-*lpr/lpr* mice is due to a preferential loss of the CD4⁺ or CD8⁺ subpopulations of T-cells, we determined the concentrations of these cells using two-colour flow cytometry. There was no significant difference between T-cell subpopulations of DFMO-treated and untreated mice (Table 2). These data suggest that DFMO-induced changes in signal-transduction pathways do not represent selective depletion of CD4 or CD8 subpopulations in splenic T-cells of MRL-*lpr/lpr* mice.

Measurement of ODC activity and polyamine concentrations

In order to quantify the effect of DFMO on polyamine biosynthesis, we measured ODC activity and polyamine levels in splenocytes of Balb/c and untreated and DFMO-treated MRL-*lpr/lpr* mice. Basal ODC activity in MRL-*lpr/lpr* splenocytes was 4-fold higher than in T-cells of Balb/c mice, and DFMO treatment reduced this activity to a level lower than that in Balb/c mice (Table 3). Putrescine was not detectable in splenocytes of Balb/c and DFMO-treated MRL-*lpr/lpr* mice under the conditions of our assay, whereas its concentration was 78 $\pm$ 25 pmol/10⁶ cells in untreated MRL-*lpr/lpr* mice. Spermidine and spermine levels were 5- and 3-fold higher in the splenocytes of untreated MRL-*lpr/lpr* mice than in those of Balb/c mice. There was a significant reduction in spermidine concentration on DFMO treatment, whereas spermine levels were not significantly affected. These results are consistent with

Table 3 Effect of DFMO treatment on ODC activity and polyamine concentrations in splenocytes of 20-week-old MRL-*lpr/lpr* miceResults are means $\pm$ S.E.M. ND, Not detectable.

Strain	ODC activity (pmol of CO ₂ /h per mg of protein)	Polyamine concentration (pmol/10 ⁶ cells)		
		Putrescine	Spermidine	Spermine
Balb/c	14 $\pm$ 3	ND	190 $\pm$ 45	290 $\pm$ 35
Untreated MRL- <i>lpr/lpr</i>	50 $\pm$ 11	78 $\pm$ 25	1060 $\pm$ 40	840 $\pm$ 80
DFMO-treated MRL- <i>lpr/lpr</i>	6.6 $\pm$ 2.5	ND	620 $\pm$ 80	870 $\pm$ 140

the known mode of action of DFMO as a mechanism-based specific inhibitor of ODC [1].

DISCUSSION

The results presented in this paper show deficiencies in transmembrane Ca²⁺ influx and InsP₃ generation in Con A-treated splenic T-cells of MRL-*lpr/lpr* mice compared with normal Balb/c mice. These deficiencies were largely corrected by DFMO treatment, indicating an important role for polyamines in modulating the signal-transduction pathways in autoimmune T-cells. The mean [Ca²⁺]_i in T-cells from Balb/c mice increased from 100 to 450 nM after Con A stimulation, whereas in MRL-*lpr/lpr* T-cells it increased to 250 nM only. DFMO treatment *in vivo* significantly increased the ability of T-cells to take up Ca²⁺, leading to a level of 360 nM. Con A-stimulated changes in InsP₃ closely paralleled those of [Ca²⁺]_i. The addition of Con A to T-cells from MRL-*lpr/lpr* mice led to only a 20% increase in the generation of InsP₃, whereas a 90% increase was observed for T-cells from Balb/c mice. Splenocytes from mice treated with DFMO showed an increase in InsP₃ generation to 65–70%. Deficiencies in Ca²⁺ influx and phosphoinositide metabolism appear to be partially compensated for by hyperphosphorylation of the p56^{lck} protein. Administration of DFMO suppressed p56^{lck} phosphorylation to a level comparable with that of Balb/c T-cells. DFMO treatment down-regulated ODC activity and polyamine levels in splenocytes of MRL-*lpr/lpr* mice, suggesting that the normalization of signal-transduction pathways in DFMO-treated mice is linked to polyamine depletion.

Flow-cytometric analysis of CD4 and CD8 subpopulations of T-cells from the spleen of untreated and DFMO-treated MRL-*lpr/lpr* mice shows that the ratio of these cell populations is not significantly affected by DFMO treatment. Thus the normalization of signal-transduction pathways in splenic T-cells by polyamine depletion is not a result of preferential elimination of a subset of T-cells.

Inadequate signal transduction during thymocyte ontogeny and activation of mature T-cells has been attributed to functional T-cell defects in murine lupus. Scholz et al. [32] reported that hyporesponsiveness of *lpr* T-cells to mitogens is linked to a reduction in phosphoinositide hydrolysis. Budd et al. [25] observed that the functional deficiencies observed in the double-negative T-cells of MRL-*lpr/lpr* mice could be correlated with the defects in Ca²⁺ influx and InsP₃ production in response to mitogenic stimulation. Samelson et al. [33] reported a constitutive

tyrosine phosphorylation on p21 in *lpr* and *gld* T-cells, even in the absence of mitogenic stimulation, suggesting abnormalities in the signal-transduction pathway involving tyrosine kinases.

A link between Ca^{2+} influx, phospholipid hydrolysis and polyamines has been reported by us and other investigators [27,34–37]. We found that polyamines inhibited Ca^{2+} influx in Balb/c splenic T-cells in a cell-specific manner [27], the CD4 subset being more susceptible to the inhibitory effect of polyamines than the CD8 subset. Feuerstein et al. [34] demonstrated that polyamine inhibition by DFMO altered Ca^{2+} signalling in platelet-derived-growth-factor-stimulated A172 brain tumour cells in culture. Polyamine-mediated inhibition of the phospholipid-sensitive Ca^{2+} -dependent protein kinase C has also been reported [35]. It is possible that the effect of polyamines might be exerted through their oxidation products [36]. Furthermore, polyamines may react with biological membranes and induce a variety of effects including the promotion of membrane fusion and inhibition of the association of protein kinases with the membranes [37]. For example, Smith and Wells [38] have previously shown that polyamines affect protein tyrosine kinase activities. Our recent studies show that polyamines modulate epidermal-growth-factor-stimulated epidermal growth factor receptor tyrosine kinase activity in A431 human epidermoid carcinoma cells [39].

Our Western-blot analysis using a monoclonal anti-phosphotyrosine antibody showed that Con A-stimulated splenocytes of MRL-*lpr/lpr* mice are hyperphosphorylated on p56^{lck} . Splenocytes from Balb/c and DFMO-treated MRL-*lpr/lpr* mice exhibited comparable levels of phosphorylation on p56^{lck} . These data are in agreement with the results of Oetken et al. [40] who reported that DFMO treatment of LSTRA cells suppressed the amount of tyrosine phosphorylation in several cellular substrates, including p56^{lck} . They also reported that polyamines inhibited phosphotyrosine phosphatases. Thus it is possible that polyamines may modulate the rate of dephosphorylation of p56^{lck} by CD45 and/or other membrane-bound phosphatases.

Several studies [41–43] show that p56^{lck} -related phosphorylation events enhance T-cell-receptor-mediated functions and are critical to the co-receptor role of CD4. p56^{lck} has two tyrosine residues, Tyr-394 and Tyr-505, of which the former is an autophosphorylation site [21,44]. Tyr-505 is constitutively phosphorylated *in vivo*. Dephosphorylation at this site and consequent kinase activation are regulated by the tyrosine phosphatase, CD45 [24]. Reduced p56^{lck} activation and increased phosphorylation on Tyr-505 are displayed by T-cells lacking CD45. Analysis of transgenic mice overexpressing activated p56^{lck} [45] and mice that are deficient in p56^{lck} [46] demonstrate that this kinase is required for normal thymocyte maturation. Thus it is likely that increased polyamine concentrations in MRL-*lpr/lpr* mice suppress dephosphorylation of this site and consequently lead to decreased production of inositol phosphates. This effect on signal transduction appears to be specific for CD4⁺ T-cells, as our previous findings [27] indicate that polyamines inhibit Ca^{2+} influx in CD4⁺ T-cells but not in CD8⁺ T-cells. Our studies [14] also indicate that DFMO treatment normalized the defect in the thymic maturation process in terms of the proportion of cells expressing CD4 and CD8 differentiation markers.

In spite of the hyperactivation of B-cells, MRL-*lpr/lpr* T-cells are refractory to mitogenic stimulation and produce very little interleukin 2 [47]. Altered thymic development and differentiation, and consequent accumulation of immature CD4⁺ CD8[−] double-negative T-cells, are characteristics of murine lupus. We found aberrant regulation of ODC in MRL-*lpr/lpr* mice [48]. It is likely that autoimmunity-related T-cell defects arise from the combined effect of the *lpr* gene and abnormalities in background

genes such as ODC, contributing to the expression of the disease. The *lpr* modification appears to be a mutation/modification in the *fas* gene which is involved in apoptosis [49,50].

In summary, our results show that phosphoinositide hydrolysis and transmembrane Ca^{2+} influx are suppressed in Con A-stimulated MRL-*lpr/lpr* T-cells. We also found hyperphosphorylation on p56^{lck} , which was normalized by DFMO treatment. Intracellular concentrations of spermidine and spermine are 5- and 3-fold higher respectively in splenocytes of MRL-*lpr/lpr* mice than in those of Balb/c mice. Whereas putrescine is undetectable in unstimulated Balb/c splenocytes, 78 ± 25 pmol/ 10^6 cells was found in unstimulated splenocytes of MRL-*lpr/lpr* mice. Taken together, these data suggest that the defective signal-transduction pathways of MRL-*lpr/lpr* T-cells are linked to increased levels of polyamines, and suggest an important role for polyamines in T-cell signalling and function.

This work was supported, in part, by NIH grants AR 39020 and CA42439, by the Foundation of UMDNJ, Arthritis Foundation NJ Chapter, the W. H. Conzen Endowment of the Schering-Plough Foundation and by the Merck Foundation. We thank Carol A. Faaland for technical help in conducting Western blots. We thank Professor Roger Perlmutter and the Marion Merrell Dow Research Institute (Cincinnati, OH, U.S.A.) for the supply of p56^{lck} antibody and DFMO respectively for our studies.

REFERENCES

- Pegg, A. E. (1986) *Biochem. J.* **234**, 249–260
- Tabor, C. W. and Tabor, H. (1984) *Annu. Rev. Biochem.* **53**, 749–790
- Canellakis, E. S., Viceps-Madore, D., Kyriakidis, D. A. and Heller, J. S. (1979) *Curr. Top. Cell. Regul.* **15**, 155–202
- Russel, D. A. (1985) *Drug Metab. Rev.* **16**, 1–88
- Heby, O. and Persson, L. (1990) *Trends Biochem. Sci.* **15**, 153–158
- Abrahamsen, M. S., Li, R.-S., Dietrich-Goetz, W. and Morris, D. R. (1992) *J. Biol. Chem.* **267**, 18866–18873
- Gohda, L., Sidney, D., Macrae, M. and Coffino, P. (1992) *Mol. Cell. Biol.* **12**, 2178–2185
- Höltta, E. and Pohjanpelto, P. (1986) *J. Biol. Chem.* **261**, 9502–9508
- Flescher, E., Bowlin, T. L., Ballester, A., Hok, R. and Talal, N. (1989) *J. Clin. Invest.* **83**, 1356–1362
- Colombatto, S., De Agostini, M., Corsi, O. and Sinicco, A. (1989) *Biol. Chem. Hoppe-Seyler* **370**, 745–748
- Gunnia, U. B., Amenta, P. S., Seibold, J. R. and Thomas, T. J. (1991) *Kidney Int.* **39**, 882–890
- Puri, H., Campbell, R. A., Puri, A. et al. (1978) *Adv. Polyamine Res.* **2**, 359–367
- Thomas, T. J. and Messner, R. P. (1991) *J. Rheumatol.* **18**, 215–221
- Thomas, T. J., Gunnia, U. B. and Thomas, T. (1992) *Autoimmunity* **13**, 275–283
- Thomas, T. J. and Messner, R. P. (1989) *Clin. Exp. Immunol.* **78**, 239–244
- Steinberg, A. D., Roths, J. B., Murphy, E. D., Steinberg, R. T. and Faveche, E. S. (1980) *J. Immunol.* **125**, 871–877
- Zhou, T., Bluethmann, H., Eldridge, J., Berry, K. and Mountz, J. (1993) *J. Immunol.* **150**, 3651–3667
- Perlmutter, R. M. (1989) *Science* **245**, 344
- Kaczmarek, L. and Kaminska, B. (1989) *Exp. Cell Res.* **183**, 24–35
- von Boehmer, H. (1990) *Annu. Rev. Immunol.* **8**, 531–567
- Abraham, N. and Vielle, A. (1991) *Cancer Invest.* **9**, 455–463
- Carpenter, G. (1992) *FASEB J.* **6**, 3283–3290
- Collins, T. L., Uniyal, S., Shin, J., Strominger, J. L., Mittler, R. S. and Burakoff, S. (1992) *J. Immunol.* **148**, 2159–2162
- Mustelin, T., Coggeshall, K. M. and Altman, A. (1989) *Proc. Natl. Acad. Sci. U.S.A.* **86**, 6302–6306
- Budd, R. C., Winslow, G., Inokuchi, S. and Imboden, J. B. (1990) *J. Immunol.* **145**, 2862–2872
- Kuno, M. and Garner, P. (1987) *Nature (London)* **326**, 301–304
- Thomas, T., Gunnia, U. B., Yurkow, E., Seibold, J. R. and Thomas, T. J. (1993) *Biochem. J.* **291**, 375–381
- Chused, T. M., Wilson, A., Greenblat, D. et al. (1987) *Cytometry* **8**, 396–404
- Rabinovitch, P. S., June, C. H., Grossmann, A. and Ledbetter, J. A. (1986) *J. Immunol.* **137**, 952–961
- Berridge, M. J., Downes, C. P. and Hanley, M. R. (1982) *Biochem. J.* **206**, 587–595
- Singh, A. B., Thomas, T. J., Thomas, T., Singh, M. and Mann, R. A. (1992) *Cancer Res.* **52**, 1840–1847
- Scholz, W., Isakov, N., Mally, M. I., Theofilopoulos, A. N. and Altman, A. (1988) *J. Biol. Chem.* **263**, 3626–3631

-
- 33 Samelson, L. E., Davidson, W. F., Morse, H. C. and Klausner, R. D. (1986) *Nature* (London) **324**, 674–676
- 34 Feuerstein, B. G., Szollosi, J., Basu, H. S. and Marton, L. J. (1992) *Cancer Res.* **52**, 6782–6789
- 35 Mezzetti, G., Monti, M. G. and Moruzzi, M. S. (1984) *Life Sci.* **42**, 2293–2298
- 36 Flescher, E., Ledbetter, J. A., Shievan, G. L., Alboukrek, D. and Talal, N. (1992) *Arthritis Rheum.* **35**, S130
- 37 Smith, C. D. and Snyderman, R. (1988) *Biochem. J.* **256**, 125–130
- 38 Smith, C. D. and Wells, W. W. (1984) *Arch. Biochem. Biophys.* **235**, 529–537
- 39 Faaland, C. A., Laskin, J. D. and Thomas, T. J. (1995) *Cell Growth Differ.* **6**, 115–121
- 40 Oetken, C., Pressa-Morikawa, T., Autero, M., Andersson, L. C. and Mustelin, T. (1992) *Exp. Cell Res.* **202**, 370–375
- 41 Turner, J. M., Brodsky, M. H., Irving, B. A., Levin, S. D., Perlmutter, R. M. and Littman, D. R. (1990) *Cell* **60**, 755–765
- 42 Hsi, E. D., Siegel, J. N., Minami, Y., Luong, E. T., Klausner, R. D. and Samelson, L. E. (1989) *J. Biol. Chem.* **264**, 10836–10842
- 43 March, J. D., Lewis, D. B., Wilson, C. B., Gearn, M. E., Krebs, E. G. and Perlmutter, R. M. (1987) *EMBO J.* **6**, 2727–2734
- 44 Samelson, L. E. and Klausner, R. D. (1992) *J. Biol. Chem.* **267**, 24913–24915
- 45 Mustelin, T., Coggeshall, K. M., Isakov, N. and Altman, A. (1990) *Science* **247**, 1584–1587
- 46 Molina, T. J., Kishihara, K. and Siderovski, D. P. (1992) *Nature* (London) **357**, 161–164
- 47 Altman, A., Theofilopoulos, A. N., Weiner, R., Katz, D. H. and Dixon, F. J. (1981) *J. Exp. Med.* **154**, 791–808
- 48 Hsu, H. C., Seibold, J. R. and Thomas, T. J. (1994) *Autoimmunity*, **19**, 253–264
- 49 Watanabe-Fukunaga, R., Brannan, C. I., Copeland, N. G., Jenkins, N. A. and Nagata, S. (1992) *Nature* (London) **356**, 314–317
- 50 Wu, J., Zhou, T., He, J. and Mountz, J. D. (1993) *J. Exp. Med.* **178**, 461–468
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Received 5 January 1995/15 May 1995; accepted 17 May 1995