

## Decreased Ras–Mitogen-Activated Protein Kinase Signaling May Cause DNA Hypomethylation in T Lymphocytes From Lupus Patients

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**Objective.** Previous studies have shown that inhibiting T cell DNA methylation causes a lupus-like disease by modifying gene expression. T cells from patients with lupus exhibit diminished levels of DNA methyltransferase (MTase) enzyme activity, hypomethylated DNA, and changes in gene expression similar to those exhibited by T cells treated with methylation inhibitors, suggesting that DNA hypomethylation may contribute to human lupus. Since it is known that DNA MTase levels are regulated by the ras–mitogen-activated protein kinase (MAPK) pathway, this study sought to determine whether decreased ras–MAPK signaling could account for the DNA hypomethylation in lupus T cells.

**Methods.** DNA MTase messenger RNA (mRNA) from lupus patients and from healthy controls was quantitated by Northern analysis, and ras–MAPK signaling was determined by immunoblotting with antibodies to the activated forms of extracellular receptor–associated kinase (ERK). Results were compared with those in T cells in which ras–MAPK signaling was inhibited with a soluble inhibitor of MAPK ERK 1 (MEK1).

**Results.** T cells from patients with active lupus had diminished DNA MTase mRNA levels and de-

creased signaling through the ras–MAPK pathway. Inhibiting signaling through the ras–MAPK pathway with the MEK1 inhibitor decreased DNA MTase mRNA and enzyme activity to the levels seen in lupus T cells, and resulted in DNA hypomethylation resembling that seen in lupus T cells.

**Conclusion.** These results suggest that a decrease in signaling through the ras–MAPK pathway may be responsible for the decreased MTase activity and DNA hypomethylation in patients with lupus.

Although the primary defect in idiopathic human lupus remains unknown, the phenomenon of drug-induced lupus suggests that drug-induced biochemical changes can lead to the development of lupus-like diseases. Thus, elucidation of the mechanisms by which drugs induce lupus may provide clues to the pathogenesis of the idiopathic disease. Our group has demonstrated that some lupus-inducing drugs, including procainamide and hydralazine, inhibit T cell DNA methylation (1). We have also shown that various agents that inhibit DNA methylation in T cells, including procainamide, hydralazine, 5-azacytidine, and ultraviolet light, induce leukocyte function–associated antigen 1 (LFA-1) overexpression (2,3), which makes T cells autoreactive (3,4). Finally, we have demonstrated that adoptive transfer of T cells made autoreactive by treatment with DNA methylation inhibitors (2,4–6) or by transfection with LFA-1 (4) is sufficient to cause a lupus-like disease in unirradiated syngeneic mice. Together, these studies indicate that one mechanism causing a lupus-like disease is the induction of T cell DNA hypomethylation, with subsequent effects on LFA-1 expression and autoreactivity.

To determine the relevance of these studies to human lupus, we investigated T cell DNA methylation in

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**Table 1.** Characteristics of the study patients\*

Disease	Number of patients	Sex, no. female/no. male	Age, mean $\pm$ SD years (range)	Treatment, no. of patients						
				NSAID	Steroids	Anti-malarial	CYC	AZA	MTX	Gold
RA	10	7/3	50 $\pm$ 11 (36–69)	6	4	4	0	0	4	1
Inactive SLE	16	13/3	35 $\pm$ 13 (18–65)	2	13	14	2	3	0	0
Active SLE	12	10/2	39 $\pm$ 14 (18–61)	2	12	8	2	1	1	0

\* NSAID = nonsteroidal antiinflammatory drug; CYC = cyclophosphamide; AZA = azathioprine; MTX = methotrexate; RA = rheumatoid arthritis; SLE = systemic lupus erythematosus.

lupus. We obtained evidence that T cells from patients with active lupus have hypomethylated DNA and an autoreactive T cell subset that overexpresses LFA-1 (7,8). These observations have been confirmed by independent groups (9,10). We have also reported that T cells from lupus patients have a significant decrease in DNA (cytosine-5-)-methyltransferase (MTase) activity, the enzyme mediating maintenance DNA methylation (11). Since treatment with procainamide, a competitive DNA MTase inhibitor, results in a similar decrease in enzyme activity (12), the reduced DNA MTase activity observed in lupus T cells may be an important step in the development of lupus. However, the basis for the reduced MTase activity in lupus is unknown.

We have demonstrated that DNA methyltransferase 1 (Dnmt1) levels increase following T cell stimulation, and our group, as well as others, has reported that Dnmt1 levels are regulated through the ras-mitogen-activated protein kinase (MAPK) signaling pathway and activator protein 1 (AP-1) sites (13–16). The studies presented in this report were undertaken to determine the role of ras-MAPK signaling in the reduced DNA MTase activity and DNA hypomethylation in lupus T cells. We provide evidence that a decrease in signaling through the ras-MAPK pathway may be responsible for the decreased MTase activity and for the hypomethylated DNA in lupus.

## PATIENTS AND METHODS

**Cells and cell culture.** Peripheral blood mononuclear cells (PBMC) were isolated from the venous blood of healthy controls, patients with rheumatoid arthritis (RA), and patients with active and inactive lupus, using density gradient centrifugation as previously described (7). The clinical parameters of these subjects are described in Table 1. Patients with RA met the American College of Rheumatology (ACR; formerly, the American Rheumatism Association) criteria for RA (17), and patients with lupus met the ACR criteria for systemic lupus erythematosus (SLE) (18). Lupus disease activity was assessed as previously described (19) or by the Systemic Lupus Activity Measure (SLAM) (20). Lupus was considered inactive if the

activity index was  $<1$  or the SLAM was  $\leq 5$ . PBMC were stimulated with 1  $\mu$ g/ml phytohemagglutinin (PHA; Burroughs Wellcome, Greenville, NC) and cultured in interleukin-2 (IL-2) for 7–10 days as previously described (7). Exceptions are noted in the text. In some experiments, the CD8<sup>+</sup> population was depleted by lysis with anti-CD8 (PharMingen, San Diego, CA) and complement (Pel-Freeze, Brown Deer, WI) using published protocols (21), and purity was checked by staining with anti-CD4-fluorescein isothiocyanate followed by flow cytometric analysis. Jurkat cells were cultured as previously described (1). Where indicated, the cells were treated with PD 98059 (New England Biolabs, Beverly, MA), a selective inhibitor of MAPK extracellular receptor-associated kinase (ERK) 1 (MEK1) (22). Proliferation was measured using tritiated thymidine (<sup>3</sup>H-TdR) incorporation as previously described (1).

**Flow cytometric cell cycle analysis.** For cell cycle analysis, peripheral blood T cells were stimulated with PHA and IL-2 as above, then the cells were stained with propidium iodide (Sigma, St. Louis, MO) as previously described (8). The stained cells were analyzed on a Coulter ELITE flow cytometer (Hialeah, FL), and the percentage of cells in G0/G1, S, and G2/M was determined by DNA content.

**RNA isolation and Northern analysis.** Total RNA was isolated from T cells using TRIzol reagents from Gibco BRL (Gaithersburg, MD). Twenty micrograms of total RNA was electrophoresed in 1% agarose, 2.2M formaldehyde gels and transferred to nylon membranes (Micron Separations, Westboro, MA). Care was taken to include RNA from at least 2 normal controls on each membrane and to calculate the expression of RNA relative to these controls, to avoid variability due to technical differences such as efficiency of random priming and hybridization. The membranes were fixed by baking for 2 hours at 80°C and hybridized with a 2.5-kb human Dnmt1 complementary DNA (cDNA) fragment (encoding the 3' catalytic domain), kindly donated by Dr. Stephen Baylin (23), or a previously described 2.0-kb  $\beta$ -actin cDNA fragment (8). The cDNA probes were labeled by random priming according to the manufacturer's suggestions (Boehringer Mannheim Biochemicals, Indianapolis, IN) with  $\alpha$ -<sup>32</sup>P-dCTP. After hybridization, the membranes were exposed to Kodak radiographic film or developed on a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). Results are calculated as the ratio of the Dnmt1 band intensity to that of  $\beta$ -actin. Where indicated, the blots were also restripped and hybridized with a cDNA encoding a fragment of histone H4 (24). For comparison between groups, the mean of this ratio was determined for

the normal controls, and the results from individual patients are expressed as a percentage of this mean value.

**Measurement of MAPK activation.** Cultured T cells were treated with 50 ng/ml phorbol myristate acetate (PMA; Sigma) with or without 50 ng/ml ionomycin (Sigma) for 15 minutes, then harvested and disrupted by sonication. Insoluble material was removed by centrifugation at 15,000g for 20 minutes, and the supernatant was recovered. Proteins were fractionated through 10–20% polyacrylamide gradient gels (Ready gels; Bio-Rad, Hercules, CA) using 25  $\mu$ g protein per lane, then electrophoretically transferred to polyvinylidene difluoride membranes (Immobilon-P; Millipore, Bedford, MA). Total MAPK and activated MAPK were quantitated using antibodies to the activated, dually phosphorylated form of human ERKs 1 and 2 (Anti-ACTIVE MAPK; Promega, Madison, WI) and previously described protocols (15). Since the monoclonal antibodies (mAb) to the dually phosphorylated forms of ERKs 1 and 2 react only with the activated form of the proteins, MAPK activation is proportional to the intensity of the band produced by this antibody. Controls included isotype-matched irrelevant antibodies, and similar blots were hybridized with an mAb to ERKs 1 and 2, obtained from Zymed (South San Francisco, CA). Band intensity was measured by densitometry as described (8). The ratio of activated to total MAPK was then determined for each subject, and results are presented as the ratio of PMA-treated to untreated preparations. Care was taken to include at least 1 control for each patient on every blot.

**Measurement of DNA MTase activity.** DNA MTase enzyme activity was measured as previously described (7), except that 2  $\mu$ g of a hemimethylated synthetic oligonucleotide was used as substrate (25). All determinations were performed in triplicate.

**Measurement of genomic deoxymethylcytosine (d<sup>m</sup>C) content.** An index of genomic d<sup>m</sup>C content was obtained by digesting 4  $\mu$ g of genomic DNA with the isoschizomers *Hpa* II and *Msp* I (both from Sigma), then fractionating the fragments by electrophoresis. *Hpa* II only cleaves unmethylated CCGG sequences, while *Msp* I cleaves CCGG sequences regardless of their methylation status. The fractionated DNA was stained with propidium iodide, and staining intensity was quantitated by densitometry between 2 kb and 3 kb. The results are expressed as the ratio of the *Hpa* II to *Msp* I digests, which increases as DNA demethylates and is more readily digested by *Hpa* II. *Sss* I assays were similarly used to obtain an index of genomic d<sup>m</sup>C content, performed as previously described by our group (16). *Sss* I catalyzes the transfer of methyl groups from S-adenosylmethionine to unmethylated dC residues in CpG pairs. Using S-adenosyl-L-[methyl-<sup>3</sup>H]methionine as the methyl donor, <sup>3</sup>H-TdR incorporation is inversely proportional to the extent of DNA methylation (16).

**Statistical analysis.** The significance of differences between groups was determined using analysis of variance (ANOVA) or Student's *t*-test. Regression analysis was performed with SySTAT software (Evanston, IL).

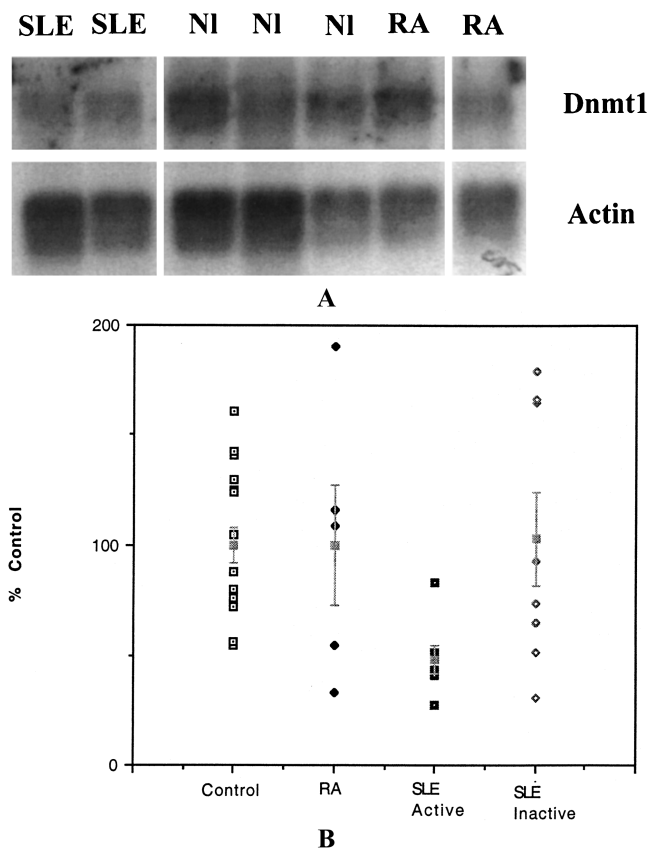
## RESULTS

**Decreased Dnmt1 messenger RNA (mRNA) levels in lupus T cells.** To determine if Dnmt1 mRNA levels are decreased in lupus T cells, PBMC were

isolated from 7 patients with active lupus, 8 patients with inactive lupus, 5 patients with RA, and normal controls. The control population consisted of 17 healthy individuals (44% women and 56% men), ages  $32 \pm 7$  years (mean  $\pm$  SD; range 22–47 years). To measure Dnmt1 expression, the PBMC were first stimulated with PHA and IL-2 as described previously (7). Stimulation is essential because little, if any, Dnmt1 mRNA is expressed in unstimulated T cells (15). RNA was isolated, and DNA MTase mRNA was measured by Northern analysis. At least 2 normal control PBMC samples were included on every gel. The filters were then stripped, rehybridized with a  $\beta$ -actin cDNA, and developed as autoradiograms.  $\beta$ -actin was chosen as a control because its expression does not change in lupus T cells (26). To confirm that  $\beta$ -actin levels are not altered in lupus T cells, the  $\beta$ -actin band intensities were compared between the patients with active lupus and the healthy controls, and expressed as the mean ratio of lupus to controls. This ratio was  $1.09 \pm 0.33$  (mean  $\pm$  SEM). Representative Northern blots are shown in Figure 1A.

All subjects demonstrated Dnmt1 transcripts of the same size. Band intensities were quantitated by densitometry, and relative Dnmt1 levels were calculated as the ratio of the intensity of the Dnmt1 band to that of the  $\beta$ -actin band for each subject. In Figure 1B, the results are expressed relative to the mean band intensity ratio of normal controls, arbitrarily defined as 100%. The results demonstrate that DNA MTase mRNA levels in patients with active lupus were  $\sim 50\%$  lower than those in healthy controls and RA patients as well as patients with inactive lupus ( $P = 0.034$  overall by ANOVA;  $P < 0.05$  for active lupus versus each of the other 3 groups by pairwise post hoc comparison). This abnormality was detectable for up to 10 days in culture. These mRNA levels are consistent with our prior activity measurements that showed an  $\sim 50\%$  decrease in DNA MTase enzyme activity in lupus patients relative to healthy controls and RA patients (7).

The decrease in Dnmt1 mRNA in the T cells from patients with active lupus could not be attributed to corticosteroids, because similar numbers of patients with active and inactive lupus were receiving this drug (Table 1). Furthermore, we have reported that corticosteroids do not affect DNA methylation (7). More subjects with inactive lupus than those with active disease were receiving antimalarial agents, suggesting that these agents were also not contributing to the decrease in Dnmt1 mRNA levels in those with active disease. Similar numbers of patients were also receiving cyclophosphamide. There were



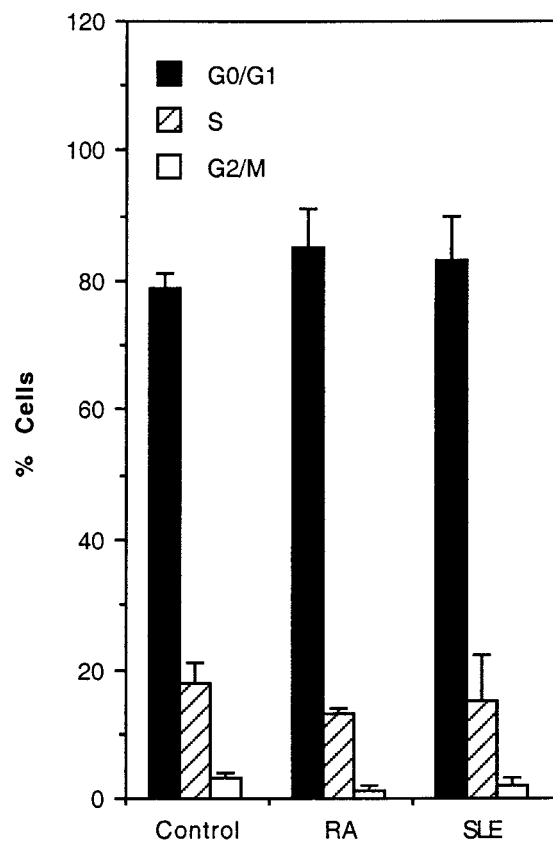
**Figure 1.** DNA (cytosine-5)-methyltransferase messenger RNA (mRNA) levels in lupus patients and controls. **A**, Peripheral blood mononuclear cells (PBMC) from 2 patients with active systemic lupus erythematosus (SLE), 2 patients with rheumatoid arthritis (RA), and 3 healthy controls (NI) were stimulated with phytohemagglutinin and interleukin-2 as described in Patients and Methods. RNA was then isolated, fractionated by agarose gel electrophoresis, transferred to nylon membranes, hybridized with a  $^{32}\text{P}$ -labeled complementary DNA (cDNA) encoding the 3' half of human DNA methyltransferase 1 (Dnmt1), then developed as an autoradiogram (upper panel). The membranes were then stripped and rehybridized with a  $^{32}\text{P}$ -labeled  $\beta$ -actin cDNA (lower panel). **B**, PBMC from 7 patients with active lupus, 8 with inactive lupus, 5 with RA, and 17 normal controls were stimulated and Dnmt1 and  $\beta$ -actin mRNA were detected as in **A**. The ratios of Dnmt1 to actin band densities were normalized to the average of the ratios from at least 2 healthy controls included on each blot. Each point represents a single subject, and some of the points overlap. Bars show the mean  $\pm$  SEM of the group.

similar numbers of women in the active and inactive lupus groups, thus arguing against a sex-specific effect. In addition, no significant differences in Dnmt1 mRNA levels were observed between healthy men and women ( $112 \pm 17\%$  versus  $92 \pm 12\%$  [mean  $\pm$  SEM] for 5 men and 7 women, respectively; compared on the same gels).

Since T cells from patients with active lupus are

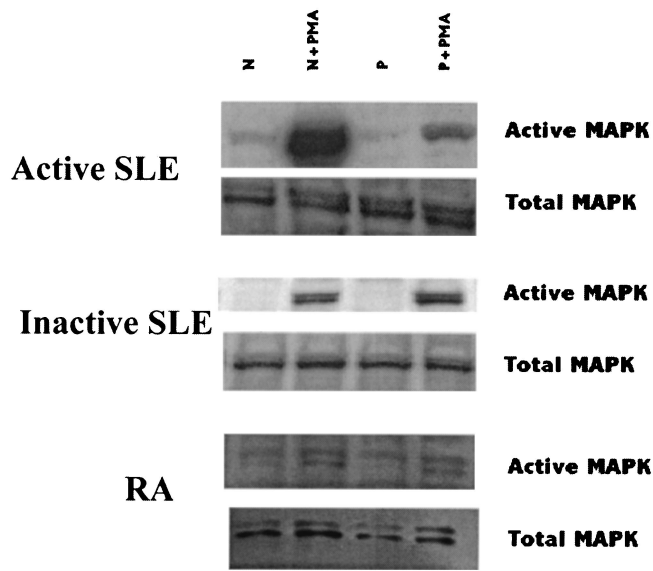
relatively anergic (for review, see ref. 27), it is possible that the decreased Dnmt1 levels reflected a lower percentage of proliferating cells. To exclude this possibility, cell cycling, as determined by propidium iodide staining, was measured in the stimulated T cells from 5 healthy controls, 5 patients with active lupus, and 4 patients with RA (Figure 2). No differences were seen, indicating that the decrease in DNA MTase was not due to differences in the kinetics of cell cycling.

We then asked if the decrease in Dnmt1 could be due to decreased ras-MAPK signaling. T cells from lupus patients with active and inactive disease and from RA patients were stimulated with PHA and IL-2 as

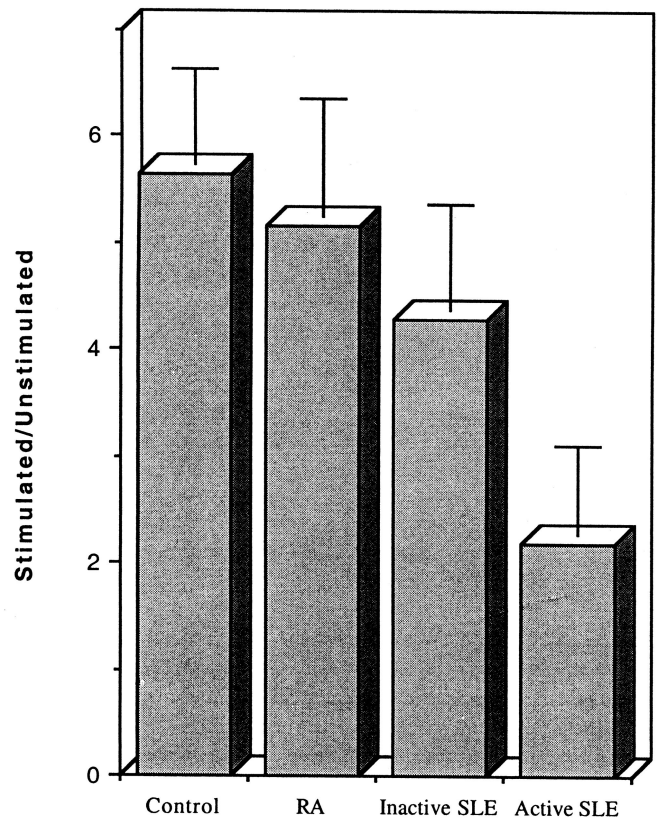


**Figure 2.** Cell cycle analysis of activated T cells from lupus patients and controls. Phytohemagglutinin- and interleukin-2-stimulated T cells from healthy controls ( $n = 5$ ), rheumatoid arthritis (RA) patients ( $n = 4$ ), or patients with active systemic lupus erythematosus (SLE;  $n = 5$ ) were analyzed by propidium iodide staining and flow cytometric analysis. Results represent the mean and SEM percentage of cells from the subjects in each group.

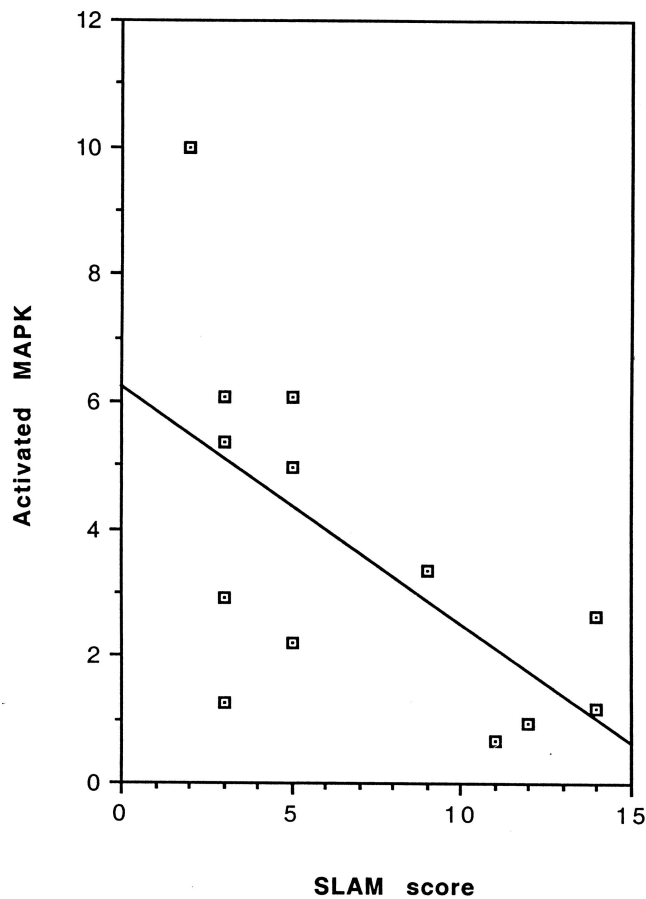
## Subjects



**A**

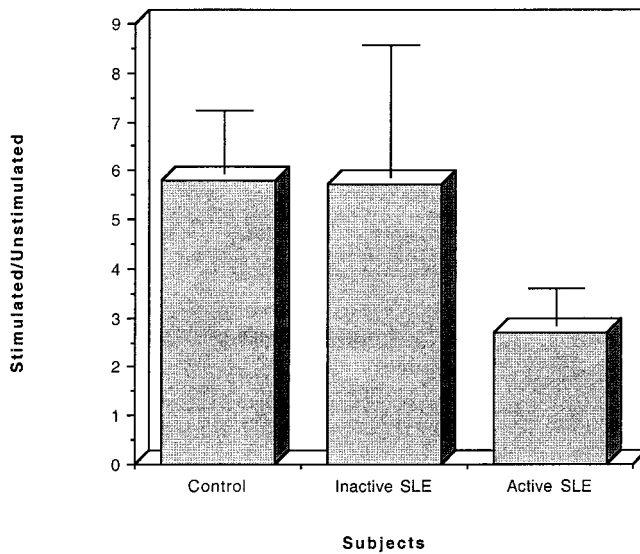


**Subjects**  
**B**



**C**

**Figure 3.** Decreased mitogen-activated protein kinase (MAPK) phosphorylation in lupus T cells. **A**, T cells from a healthy control (N) or from patients (P) with active or inactive systemic lupus erythematosus (SLE) or rheumatoid arthritis (RA) were stimulated with phytohemagglutinin and interleukin-2 as in Figure 1. Aliquots of cells were treated with 50 ng/ml phorbol myristate acetate (PMA) for 15 minutes, then treated (+PMA) and untreated cells were lysed and proteins separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis using 25  $\mu$ g protein per lane. The fractionated proteins were transferred to polyvinylidene difluoride membranes and developed as immunoblots using antibodies to the activated, dually phosphorylated form of human extracellular receptor–associated kinases 1 and 2 (Active MAPK) or to framework determinants (Total MAPK). **B**, Quantitative immunoblot analysis of activated and total MAPK was performed as described in Patients and Methods, using T cells from 18 healthy controls, 5 patients with RA, 8 patients with inactive lupus, and 5 patients with active lupus, and the ratio of activated to total MAPK was determined for unstimulated and PMA-stimulated cells from each subject. Results are presented as the ratio of PMA-stimulated to unstimulated preparations, and represent the mean and SEM of the determinations within each group. **C**, Regression analysis comparing the Systemic Lupus Activity Measure (SLAM) score of the patients with lupus with the degree of MAPK phosphorylation.



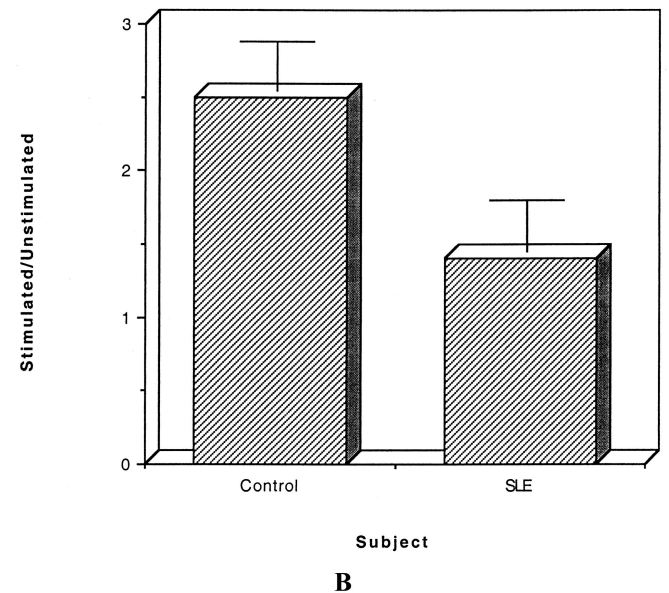
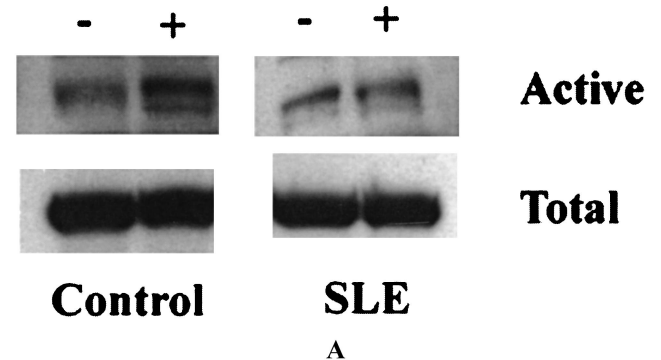
**Figure 4.** Decreased MAPK phosphorylation in CD4+ lupus T cells. PBMC were isolated from 5 healthy controls, 3 patients with inactive lupus, and 4 patients with active lupus. CD4+ T cells were depleted by treatment with anti-CD8 plus complement, and MAPK activation was studied as in Figure 3B. Cells were ~98% CD4+ at the time of analysis by cytofluorography. Bars show the mean and SEM. See Figures 1 and 3 for definitions.

before. The cells were then treated with PMA, and total MAPK was quantitated by immunoblotting. PMA was used, rather than T cell receptor crosslinking agents, because others have reported abnormalities in early signaling events in lupus T cells (28), which would confound interpretation of the results. No differences in the amount of total MAPK protein was observed between the groups (105%, 99%, and 87%, for active lupus, inactive lupus, and RA T cells, respectively, relative to normal controls). Parallel gels were reacted with an mAb that is reactive with the dually phosphorylated, activated forms of ERKs 1 and 2.

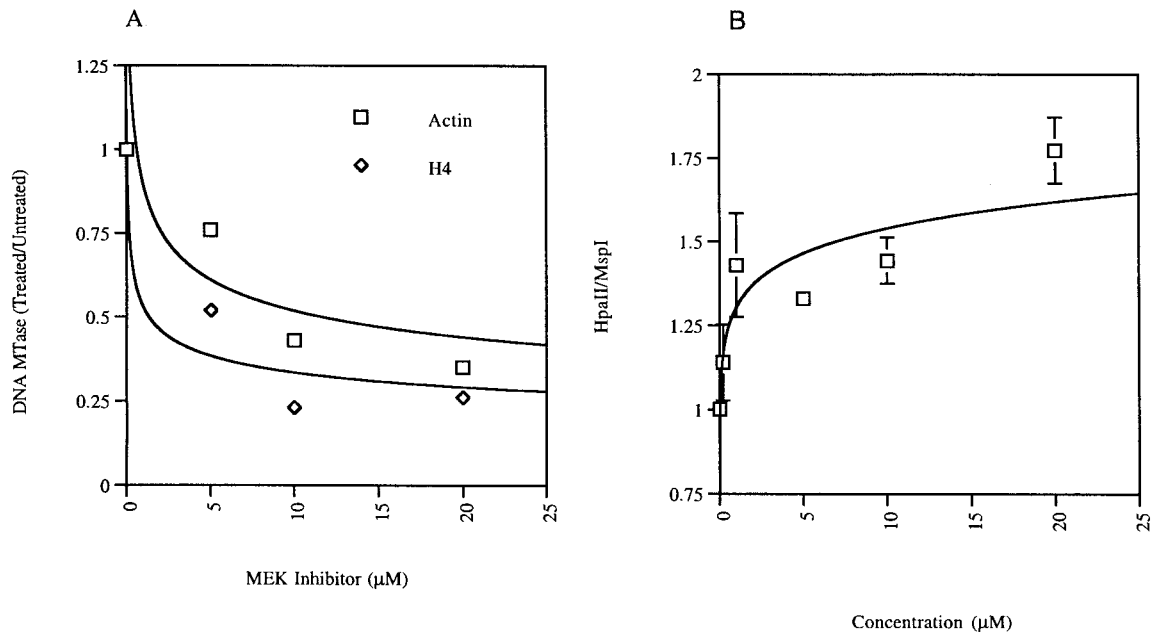
Figure 3A shows that PMA treatment caused significantly less MAPK activation in T cells from a representative lupus patient relative to that in a healthy control. The addition of ionomycin gave identical results (not shown). Kinetic studies demonstrated that both lupus patients and controls had optimal MAPK activation at 15 minutes. For reference, similar studies using T cells from patients with inactive lupus and RA are also shown (Figure 3A), and the results resembled those in the controls. Similar studies were performed on T cells from 18 healthy controls, 5 patients with RA, 8 patients with inactive lupus, and 5 patients with active lupus. The healthy controls consisted of 10 men and 8 women, age  $32 \pm 7$  years (mean  $\pm$  SD; range

21–49 years). Figure 3B compares the relative amounts of activated MAPK in untreated and PMA-treated T cells from the lupus patients and controls, adjusted for total MAPK protein content from each patient. While PMA caused an increase in MAPK phosphorylation in T cells isolated from all groups, the increase in T cells from lupus patients was the smallest, and significantly less than that in normal controls ( $P = 0.01$  by Student's *t*-test).

The effect of disease activity on MAPK activation was tested by comparing SLAM scores with MAPK



**Figure 5.** Effect of phytohemagglutinin (PHA) stimulation on MAPK signaling in control and lupus T cells. **A**, PBMC from a healthy control and a patient with active lupus were cultured alone (–) or stimulated with interleukin-2 and PHA (+), and 30 minutes later, active MAPK was measured as in Figure 3 (upper panel). Total MAPK was also measured as described in Figure 3, analyzed using a duplicate gel (lower panel). **B**, PBMC from 4 controls and 3 patients with active lupus were stimulated with PHA and analyzed as in **A**. Bars show the mean and SEM of the ratio of activated to total MAPK, as in Figure 3. See Figures 1 and 3 for other definitions.



**Figure 6.** Effect of decreased MAPK signaling on Dnmt1 mRNA and DNA methylation. **A**, Jurkat cells were treated with the indicated concentrations of PD 98059, and 90 minutes later, mRNA was isolated and analyzed for DNA (cytosine-5)-methyltransferase (MTase) and  $\beta$ -actin expression as described in Figure 1, then stripped and restudied for histone H4 expression. Results are expressed as the DNA MTase to  $\beta$ -actin ratio or the DNA MTase to H4 ratio in treated/untreated cells, and are representative of 3 experiments. **B**, Jurkat cells were treated with the indicated concentrations of the MAPK extracellular receptor-associated kinase 1 (MEK1) inhibitor, and 48 hours later, DNA was isolated, digested with *Hpa* II or *Msp* I, then fractionated by electrophoresis and stained with ethidium bromide. Results are presented as the ratio of the *Hpa* II to *Msp* I integrated intensities between 2 kb and 3 kb, and represent the mean  $\pm$  SEM of 3 independent experiments. See Figures 1 and 3 for other definitions.

activation. Figure 3C shows that as disease activity increased, the degree of MAPK phosphorylation decreased significantly (slope  $-0.47$ ,  $P = 0.004$ ). This suggests that ras-MAPK signaling is decreased in T cells from patients with active lupus, and may contribute to the decrease in DNA MTase activity. This defect was also stable in culture for up to 10 days.

Since these studies were performed on polyclonal populations containing both CD4+ and CD8+ cells, it was important to determine if the same results could be obtained using purified CD4+ T cells. CD8+ cells were removed by cytolytic depletion, and then the cells were stimulated and cultured as before. These cells were routinely 98% CD4+ by flow cytometric analysis. Figure 4 compares MAPK phosphorylation in PMA-stimulated CD4+ cells from 5 healthy controls, 3 patients with inactive lupus, and 4 patients with active lupus. The CD4+ T cells from patients with active lupus had significantly less MAPK phosphorylation relative to that in healthy controls ( $P < 0.05$ ).

To determine if the defect in MAPK signaling was reflected by differences in the basal phosphorylation

of ERKs 1 and 2 in the unstimulated T cells, the ratio of activated to total MAPK was also compared between controls and patients, for unstimulated cells only. There was no difference in this ratio between controls and patients with RA or patients with inactive lupus (mean  $\pm$  SEM  $1.00 \pm 0.33$  and  $1.51 \pm 0.33$  in RA and inactive lupus patients, respectively, relative to paired controls). In contrast, there was relatively less basal MAPK activation in the T cells from patients with active lupus ( $0.69 \pm 0.12$ ) ( $P = 0.049$  versus controls by Student's paired  $t$ -test).

It was possible that the defect in MAPK signaling was detectable only with PMA stimulation, but not with stimulation using ligands of cell surface receptors. To exclude this possibility, the effect of PHA stimulation on MAPK activation was examined. Preliminary studies demonstrated that MAPK activation was maximal 30 minutes after the addition of PHA. PBMC from 3 patients with active lupus and 4 normal controls (59–83% CD2+) were stimulated with PHA and IL-2 for 30 minutes, and MAPK activation was then measured. Figure 5A shows representative immunoblots from 1 patient and 1 control, and Figure 5B shows the average

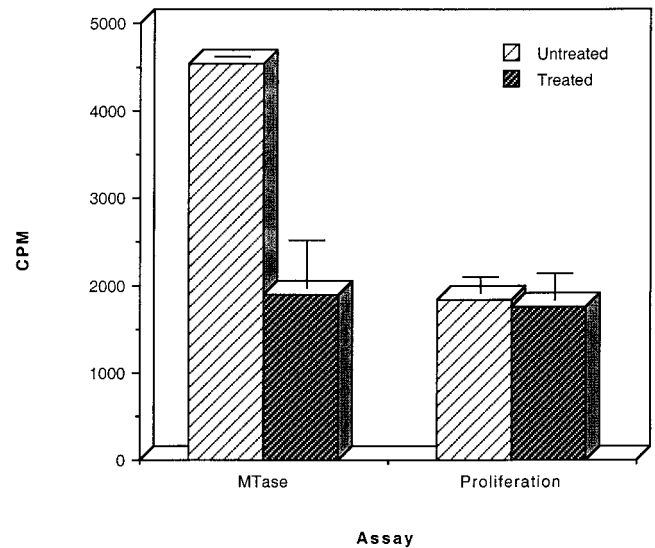
ratio of activated to total MAPK in the subjects ( $P = 0.011$ ). The results indicate that a signaling defect resembling that detected using PMA in lupus T cells is detectable with PHA stimulation as well, and is present in freshly isolated PBMC.

**Effect of decreased ras-MAPK signaling on T cell DNA methylation.** To determine the functional significance of decreased ras-MAPK signaling on T cell DNA methylation, we compared Dnmt1 mRNA expression, enzyme activity, and genomic d<sup>m</sup>C content in Jurkat T cells treated with PD 98059, a selective MEK1 inhibitor. At the concentrations used in this study, this agent does not affect the activity of at least 18 other known kinases, including those involved in the c-Jun N-terminal kinase (JNK) and p38 cascades, and a >80% inhibition of MEK1 activity is usually achieved at 50  $\mu$ M (22).

Figure 6A demonstrates that the MEK1 inhibitor decreased the levels of Jurkat mRNA by ~50% relative to those with  $\beta$ -actin and histone H4 (a cell cycle-specific gene), similar to the decrease seen in the lupus patients. Figure 6B compares genomic d<sup>m</sup>C content in untreated and MEK1 inhibitor-treated Jurkat cells. For this assay, DNA was digested with *Hpa* II and *Msp* I. *Hpa* II recognizes and cuts unmethylated, but not methylated, CCGG sequences, while *Msp* I cuts CCGG sequences regardless of their methylation status. Results are expressed as the ratio of *Hpa* II to *Msp* I digests, and the ratio increases as DNA becomes more hypomethylated and thus more readily digested by *Hpa* II. The results show that treatment with PD 98059 decreased total genomic d<sup>m</sup>C content (20  $\mu$ M versus 0 or 0.2  $\mu$ M;  $P < 0.01$ ).

To confirm that inhibiting ras-MAPK signaling will also decrease DNA MTase enzyme activity, Jurkat cells were treated with 20  $\mu$ M PD 98059. The level of DNA MTase enzyme activity per  $\mu$ g nuclear protein in the treated cells was  $62 \pm 2\%$  of controls (mean  $\pm$  SEM of 3 determinations, each performed in triplicate) ( $P < 0.01$  by univariate *t*-test of the mean), thus correlating with mRNA levels.

These results were confirmed by treating PHA-stimulated, IL-2-dependent normal T cells with 20  $\mu$ M PD 98059, then measuring DNA MTase activity (Figure 7). PD 98059 decreased enzyme activity by  $58 \pm 12\%$  (mean  $\pm$  SEM of triplicate determinations) ( $P < 0.01$ ). In contrast, no effect was seen on the proliferation of these cells, indicating that the decrease did not represent an effect on cell cycling. *Sss* I assays were used to compare d<sup>m</sup>C content in the treated and untreated cells. Jurkat cells, as well as normal T cells similarly stimulated



**Figure 7.** Effect of an MEK1 inhibitor on DNA MTase activity in normal T cells. T cells were stimulated with phytohemagglutinin and interleukin-2 as in Figure 1, then cultured alone (untreated) or with 20  $\mu$ M PD 98059 (treated) for 24 hours. DNA MTase activity was measured as described in Patients and Methods. Results represent the counts per minute (CPM) of <sup>3</sup>H-thymidine incorporated in hemimethylated synthetic oligonucleotides per  $\mu$ g protein, and are presented as the mean and SEM of 3 determinations. Proliferation was measured at the same time by culturing 20,000 untreated or treated cells with <sup>3</sup>H-thymidine for 2 hours in microtiter wells, then <sup>3</sup>H-thymidine incorporation into DNA was assessed. See Figure 6 for definitions.

with PHA and IL-2, were treated with 20  $\mu$ M PD 98059 for 24 hours, then DNA was isolated and *Sss* I-catalyzed <sup>3</sup>H-TdR incorporation was compared. PD 98059 treatment increased <sup>3</sup>H-TdR incorporation by  $10.2 \pm 1.3\%$  in normal T cells and by  $8.8 \pm 0.2\%$  in Jurkat T cells (mean  $\pm$  SEM of duplicate determinations), indicating that inhibiting ras-MAPK signaling inhibits DNA methylation in normal T cells as well as in Jurkat cells.

The observation that inhibiting ras-MAPK signaling causes decreased Dnmt1 mRNA levels and enzyme activity, as well as producing DNA hypomethylation, argues strongly that the decrease in DNA MTase activity and the DNA hypomethylation seen in lupus T cells is due to decreased signaling through the ras-MAPK pathway.

## DISCUSSION

The goal of these studies was to determine the mechanism causing a decrease in DNA MTase enzyme activity and DNA hypomethylation in lupus T cells. Since inhibiting DNA MTase was previously shown to be



sufficient to cause a lupus-like disease (5,6), early experiments sought evidence for an endogenous inhibitor. However, mixing studies gave no evidence for an inhibitor in lupus T cells (Richardson B et al: unpublished results). More recently, we reported that DNA MTase levels depend on T cell stimulation, and that the levels are regulated, in part, by the ras-MAPK signaling pathway (15,16). In the present studies, we therefore asked if regulation of Dnmt1 by this pathway might be abnormal in lupus T cells.

DNA MTase levels were compared using activated T cells. This was necessary because Dnmt1 is not expressed in unstimulated T cells (15). The results demonstrated a decrease in the amount of Dnmt1 mRNA in lupus T cells, which was almost identical in magnitude to the decrease in enzyme activity previously reported (7). Since we have previously reported a close correlation between the levels of the T cell DNA MTase enzyme and Dnmt1 mRNA levels (15), this suggests that the decrease in enzyme activity is due to a decrease in the level of the mRNA. It should be noted that T cells from patients with active lupus can be refractory to stimulation (27). To avoid this problem, which could result in an artifactual decrease in DNA MTase, the cells were stimulated with PHA and IL-2, then activation was compared by propidium iodide cell cycling studies (also done in a previous study [7]). This strategy confirmed equivalent stimulation between groups. Thus, the DNA MTase decrease is not due to inefficient stimulation of the lupus T cells. Furthermore, the decrease persisted for up to 10 days in culture, suggesting a stable biochemical abnormality.

We next asked if ras-MAPK signaling might be defective in these cells. Decreased MAPK phosphorylation following PMA stimulation was observed in T cells from patients with active lupus, implying that the decrease in DNA MTase could be due to a decrease in ras-MAPK signaling. Inhibiting MEK1 activation caused a similar 50% decrease in Jurkat Dnmt1 mRNA and a corresponding decrease in enzyme activity in Jurkat and normal T cells, and induced DNA hypomethylation resembling that seen in lupus T cells, further suggesting that impaired signaling causes the decrease in Dnmt1 enzyme activity and DNA hypomethylation characteristic of lupus. It should be noted that the MEK1 inhibitor did not completely suppress Dnmt1 expression, implying the existence of other elements providing the remaining promoter activity. Indeed, there is a CpG island, typical of housekeeping genes and containing promoter elements (29), located 5' to the MTase gene AP-1 sites

implicated in ras-MAPK signaling (30), which may also drive this expression.

Other investigators have made observations consistent with the notion of defective ras-MAPK signaling in lupus. For example, IL-2 secretion has been shown to be defective in lupus T cells (31), and IL-2 is regulated, in part, by the ras-MAPK pathway (32,33). Other investigators, using electrophoretic mobility shift assays, have demonstrated that lupus T cells contain less of the AP-1 transcription factor (34), the activation of which is also regulated by this pathway (35). How decreased signaling through the ras-MAPK pathway relates to other signaling abnormalities in lupus T cells (36,37) is unknown at this time.

The mechanism causing the decrease in signaling is unknown. The strategy of stimulating with PMA and measuring MAPK phosphorylation suggests that the defect may lie in protein kinase C, ras, raf, or MEK. Interestingly, preliminary evidence suggests that JNK pathway signaling is normal in patients with active SLE (38), raising the possibility that the defect is unique to the MAPK pathway. The precise mechanisms causing the decrease in signaling, as well as its relationship to lymphocyte subsets and disease activity, will be the subject of future studies.

Recently, additional enzymes modifying DNA methylation have been identified. These include Dnmt3a and Dnmt3b, which mediate *de novo* methylation (39), as well as a putative demethylase (40). The identification of these enzymes implies that cells have the ability to modify methylation patterns in unstimulated cells, as well as during mitosis through the effects on Dnmt1. Whether these enzymes are expressed in mature T lymphocytes is unknown, and their potential role in the DNA hypomethylation associated with lupus is yet to be determined. However, the observation that inhibiting ras-MAPK signaling decreases Dnmt1 expression to the same extent as that observed in lupus, and that this decrease correlates with DNA hypomethylation *in vitro* and *in vivo*, suggests that the defect in Dnmt1 is the primary reason for DNA hypomethylation in SLE.

In summary, these studies suggest a novel mechanism that could contribute to the development of idiopathic lupus, and perhaps to the characteristic disease flares occurring after stresses such as infections (41). In this model, T cells are stimulated to divide by exogenous antigens, but a failure of the cell to adequately up-regulate Dnmt1 results in DNA hypomethylation with subsequent T cell autoreactivity, lasting until the cells more completely remethylate the DNA, or until T cells are suppressed by other immune mechanisms.

Since inhibiting DNA MTase with lupus-inducing drugs has similar effects on T cells, and T cells treated with DNA methylation inhibitors cause a lupus-like disease in vivo, these results suggest a common mechanism for drug-induced and idiopathic lupus.

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