EVIDENCE FOR IMPAIRED T CELL DNA METHYLATION IN SYSTEMIC LUPUS ERYTHEMATOSUS AND RHEUMATOID ARTHRITIS

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Procainamide and hydralazine inhibit T cell DNA methylation and induce autoreactivity in cloned CD4+ T cells. These drugs also induce an autoimmune syndrome, suggesting a possible relationship between DNA hypomethylation, T cell autoreactivity, and certain autoimmune diseases. To test this relationship, DNA methylation was studied in T cells from patients with rheumatoid arthritis and patients with systemic lupus erythematosus, and was found to be impaired. These results support a relationship between DNA hypomethylation and some forms of autoimmune disease.

Cytosine methylation in regulatory sequences of some genes is associated with transcriptional inactivation, while hypomethylation of these sequences is associated with active transcription (1–4). DNA methyltransferase inhibitors such as 5-azacytidine inhibit methylation of newly synthesized DNA and allow expression of genes suppressed by mechanisms associated with DNA methylation (5).

In earlier experiments, cloned, antigen-specific, interleukin-2 (IL-2)–dependent CD4+ T cells were treated with 5-azacytidine, and it was found that the cells transiently lost antigen restriction and responded to autologous class II major histocompatibility complex (MHC) determinants without antigen. The altered reactivity was not due to exogenous lymphokines, was inhibited by anti-CD3 antibodies, and may be due to altered gene expression (6). Cloned CD8+ T cells treated with 5-azacytidine re-expressed CD4, but did not become autoreactive (7). Procainamide and hydralazine were subsequently found to inhibit T cell DNA methylation and induce a similar autoreactivity (8).

Some patients receiving procainamide or hydralazine develop an autoimmune syndrome characterized by arthritis, serositis, and antinuclear antibodies (9), which could be caused by autoreactive T cells. This putative association between drug-induced T cell autoreactivity and autoimmunity is suggested by similarities to the murine chronic graft-versus-host (GVH) disease model, in which CD4+ T cells responding to host class II MHC determinants produce a disease resembling systemic lupus erythematosus (SLE), but with features of other connective tissue diseases, such as rheumatoid arthritis (RA), in some strains (10,11). Taken together, these results suggested a possible relationship between T cell DNA hypomethylation, T cell autoreactivity, and some forms of autoimmune disease. Therefore, we examined T cell DNA methylation in patients with SLE and RA, diseases in which autoreactive T cells could be pathogenic (12,13).

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PATIENTS AND METHODS

Patients. Patients were recruited from the University of Michigan Hospitals and Clinics, the Ann Arbor Veterans Administration Hospital, and University of Michigan Affiliated Hospitals. RA patients fulfilled 4 or more of the American Rheumatism Association (ARA) criteria for RA (14), and SLE patients fulfilled 4 or more of the ARA criteria for SLE (15). The activity of SLE was assessed using the method of Barada et al (16), and RA activity was assessed according to the ARA criteria for clinical remission (17). To be included in the study, patients with osteoarthritis (OA) were required to have radiographic evidence of the disease, and crystal identification was required for patients with gout. The diagnosis of multiple sclerosis (MS) was based on clinical manifestations, as described (18). Healthy individuals who answered advertisements for volunteers served as controls. Informed consent was obtained in all cases.

Isolation of T cells and T cell subsets. Peripheral blood mononuclear cells were isolated from venous blood by density gradient centrifugation, and T cells were separated from non–T lymphocytes by rosetting with sheep erythrocytes (19). T cells purified by this method are usually ≥93% CD2+. Where indicated, purified T cells were cultured in RPMI 1640 medium (Gibco, Grand Island, NY) supplemented with 10% fetal calf serum (Hyclone, Logan, UT) and activated with 0.5–1.0 μg/ml of phytohemagglutinin (PHA) (Burroughs Wellcome, Greenville, NC). Activated T cells were maintained as proliferating lines by the regular addition of IL-2 (supernatant from the MLA-144 cell line) (20). Purified T cells were fractionated into CD4+ and CD8+ subsets using anti-CD8 or anti-CD4 (Ortho Pharmaceuticals, Raritan, NJ) and complement (Pel-Freez, Brown Deer, WI), as described (21).

Table 1. Methylation of T cell DNA in autoimmune disease patients and in controls*

Group (n)	Age, years	% deoxymethylcytosine content
Control (26)	33.6 ± 2.6	3.81 ± 0.09
MS (7)	36.4 ± 3.3	3.69 ± 0.06
Active SLE (8)	32.0 ± 2.9	3.15 ± 0.19†
Control (13)	43.5 ± 3.4	3.78 ± 0.10
Inactive SLE (12)	45.8 ± 3.6	3.50 ± 0.16
Control (7)	52.4 ± 3.7	3.63 ± 0.08
OA or gout (8)	57.1 ± 4.0	3.54 ± 0.12
Chronic infection (6)	59.3 ± 3.6	3.61 ± 0.18
RA (22)	57.1 ± 2.1	3.28 ± 0.11‡

* DNA deoxymethylcytosine content was measured as described in Figure 1. Values are the mean ± SEM. The first control group listed is age-matched controls for the MS and active SLE groups, the second control group is age-matched controls for the inactive SLE group, and the third control group is age-matched controls for the OA or gout group, the chronic infection group, and the RA group. MS = multiple sclerosis; SLE = systemic lupus erythematosus; OA = osteoarthritis; RA = rheumatoid arthritis.

† $P = 0.001$ versus aged-matched control group (n = 26), by analysis of variance.

‡ $P = 0.02$ versus age-matched control group (n = 7), by analysis of variance.

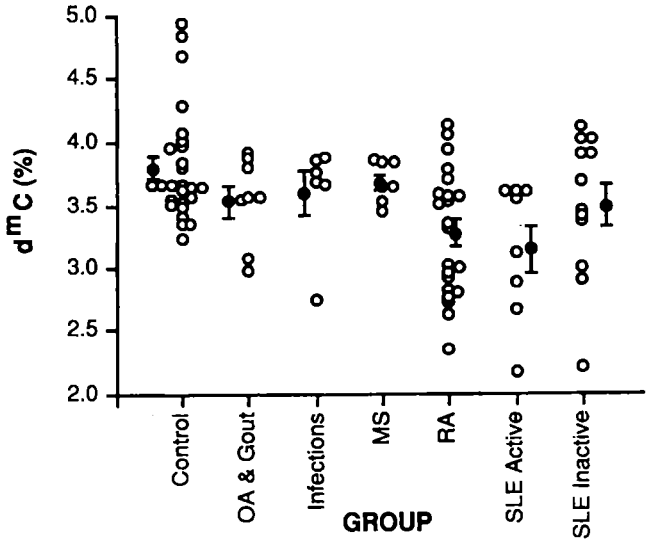

Figure 1. T cell DNA deoxymethylcytosine (d^mC) content in patients with autoimmune diseases and in controls. T cells were isolated from venous blood of subjects in the indicated study groups. DNA was isolated from the T cells, digested to nucleosides, and the dC and d^mC contents were quantitated by high performance liquid chromatography. Percent d^mC was calculated as $(d^mC)/(dC + d^mC)$. Each open circle represents a different subject. In the majority of cases, each point represents the mean of 2 or more determinations made from 1 DNA sample. The solid circles and bars represent the mean ± SEM of the points within each group. OA = osteoarthritis; MS = multiple sclerosis; RA = rheumatoid arthritis; SLE = systemic lupus erythematosus.

Measurement of DNA deoxymethylcytosine (d^mC) content. T cell nuclei were lysed with sodium dodecyl sulfate, treated with RNase and proteinase K, and DNA was extracted as previously described (8). The purified DNA was digested with DNase I, phosphodiesterase, and alkaline phosphatase (Sigma, St. Louis, MO), and the resulting nucleosides were separated by reverse-phase high performance liquid chromatography (HPLC) using a modification of a previously published technique (8). The modification included using 0.01*M* sodium acetate buffer, pH 3.5, monitoring ultraviolet-absorbing material from 220 nm to 320 nm with a Waters (Milford, MA) 990 diode array detector, and performing peak integrations at 300 nm. These changes resulted in a slight (<6%) decrease in estimates of d^mC content in a standardized T cell line.

Deoxymethylcytosine content from 9 young (<30 years) controls, 10 RA patients, and 6 SLE patients was measured using a previously described technique (8), and the data are included in Table 1 and Figure 1. It should be noted that inclusion of these data diminishes, rather than augments, differences between the disease and control groups. In most cases, 2 or more determinations were made on DNA isolated from each subject. These values were averaged, and entered into the analysis as a single value for each subject. The standard deviation of these replicate determinations was ~0.05%.

The effect of medications on T cell d^mC content was determined by treating the transformed human T cell leukemia line Jurkat with drugs as described (8), and then determining DNA d^mC content. The drugs (dexamethasone, aurothioglucose, chloroquine, D-penicillamine, and indomethacin) used in these studies were obtained from Sigma.

DNA methyltransferase activity. T cells were activated with PHA and expanded with IL-2 as described above. Nuclei were isolated with Triton X-100 as described (8) and stored at −70°C until used. DNA methyltransferase was prepared using a modification of the method of Lu and Randerath (22). Nuclei were suspended in 5 volumes of methylase buffer (50 m*M* Tris HCl, 1 m*M* dithiothreitol, 1 m*M* EDTA, 10% glycerol, 0.1 m*M* phenylmethylsulfonyl fluoride, pH 7.8) containing 0.4*M* NaCl. The mixture was stirred at 4°C for 20–30 minutes, and centrifuged at 15,000*g* for 30 minutes. DEAE-cellulose (Sigma) was prepared as described by Roe et al (23), and was added to the supernatant to make a 25% (volume/volume) suspension. The suspension was incubated at 4°C for 30 minutes and centrifuged at 12,000*g* for 30 minutes at 4°C. The protein content of the supernatant was measured using a modification of the procedure of Lowry et al (24).

The supernatant was assayed for DNA methyltransferase activity in round-bottom microtiter wells. The reaction mixture contained (in a final volume of 150 μl of methylase buffer) 25–75 μg of methyltransferase, 2 μg of RNase A (Sigma) preheated at 80°C for 10 minutes, 2.5 μg of *Micrococcus luteus* DNA (Sigma), and 5.0 μ*M* [CH_3-^{3}H]-labeled S-adenosylmethionine (SAM) (5–15 Ci/mmole; New England Nuclear, Boston, MA). The mixture was incubated at 37°C for 1 hour. Pronase (50 μg; Calbiochem, San Diego, CA) was then added, and the sample was incubated at 37°C for an additional 30 minutes. The reaction mixture was adjusted to a concentration of 0.5*M* NaOH and incubated at 60°C for 30 minutes. The DNA was precipitated with ethanol onto fiberglass strips using a PHD cell harvester (Cambridge Tech Inc., Watertown, MA). Precipitated ^{3}H-labeled DNA was measured with a 1209 Rackbeta liquid scintillation spectrometer (LKB, Turku, Finland). All determinations were performed in triplicate. Using this protocol, DNA methyltransferase activity from a reference Jurkat T cell line was 6.4 ± 0.4 disintegrations per minute/μg of protein/hour (mean ± SEM of 3 independent experiments).

SAM and S-adenosylhomocysteine determinations. Levels of SAM and S-adenosylhomocysteine were measured using a modification of the method described by Wagner et al (25). A 10% solution (100 μl) of trichloroacetic acid was added to ~5 × 10^7 washed, activated T cells, which were cultured as described above. A soluble fraction was recovered by centrifugation through a filter excluding compounds >10,000 kd (Ultrafree-MC; Millipore, Bedford, MA) at 12,000*g* for 30 minutes at 4°C. Soluble compounds present in the filtrate were subjected to HPLC analysis on a 25-cm C18 column (Ultrasphere; Beckman, San Ramon, CA). The compounds of interest were separated using the following gradient elution: mobile phase (eluent) A contained 0.1*M* NaH_2PO_4, 0.5 m*M* EDTA, 8 m*M* heptane sulfonic acid (Sigma), and 1.1% (v/v) acetonitrile, pH 3.10, adjusted with H_3PO_4; mobile phase (eluent) B contained 0.1*M* NaH_2PO_4, 5.69 m*M* heptane sulfonic acid, and 50% (v/v) acetonitrile, pH 3.10. An exponential gradient (curve 4, Waters 680 system controller) was used. The elution was initiated with 90% eluent A and 10% eluent B and ended with a mixture of 35% eluent A and 65% eluent B over a 30-minute time period. The column was equilibrated with 90% eluent A and 10% eluent B for 15 minutes prior to each injection. The column effluent was monitored with a Waters 990 diode array detector. The spectra were recorded from 220 nm to 320 nm at intervals of 1 second. Integrations were performed at 260 nm. SAM and S-adenosylhomocysteine levels were quantitated relative to purified standard (obtained from Sigma). Reference SAM preparations were further purified before use, by chromatography on sulfopropyl Sephadex (Pharmacia, Uppsala, Sweden), as described by Glazer and Peale (26).

Flow cytometric analysis. Purified T cells were stained with RD1–anti-CD4 and fluorescein isothiocyanate (FITC)–conjugated anti-CD8, FITC-conjugated anti-CD2 and RD1–anti–HLA–DR, or FITC-conjugated anti-CD2 and RD1–anti–IL-2 receptor (IL-2R) (Coulter, Hialeah, FL) and analyzed by 2-color flow cytometry, as described previously (7).

Statistical analysis. Differences between groups were analyzed using analysis of variance, and differences between means were tested, where indicated, using Student's *t*-test, with correction for multiple comparisons.

RESULTS

Hypomethylation of DNA in T cells from patients with RA and active SLE. DNA was isolated from peripheral blood T cells of 26 healthy controls (age range 21–65 years) and from 7 patients with OA, 1 with gout, 5 with chronic osteomyelitis, 1 with chronic histoplasmosis, 7 with MS, 22 with RA, 8 with active SLE, and 12 with inactive SLE. The purified DNA was hydrolyzed to nucleosides, and the percentage of methylated cytosine residues was determined by HPLC analysis. The results are shown in Figure 1. The percent d^mC values overlapped between groups, but the healthy controls had the highest values, while RA patients and SLE patients with active disease had the lowest values ($P < 0.001$). The study patients fell into 3 age ranges. Since T cell d^mC content normally decreases with age (27), the healthy controls were divided into groups that were age matched with those of the patients (Table 1). Patients with active SLE had significantly less T cell d^mC than age-matched controls ($P = 0.001$), while patients with inactive SLE did not, although a trend toward decreased values was noted.

T cell DNA from patients with RA was also significantly hypomethylated relative to age-matched controls ($P = 0.02$). In contrast to the SLE groups, no difference in T cell d^mC was noted between patients

Table 2. Non–T lymphocyte DNA methylation in autoimmune disease patients and in controls*

Group (n)	% deoxymethylcytosine content
Control (11)	3.65 ± 0.17
Active SLE (6)	3.52 ± 0.25
OA or gout (7)	3.29 ± 0.17
RA (16)	3.23 ± 0.15

* DNA deoxymethylcytosine content was measured as described in Figure 1. Values are the mean ± SEM. See Table 1 for definitions. No significant differences between groups were observed, by analysis of variance.

with active and inactive RA (mean ± SEM 3.27 ± 0.16% versus 3.28 ± 0.15%, respectively). Within the RA and SLE groups, 10 of 22 RA patients and 4 of 8 SLE patients with active disease had d^mC levels more than 1 SD outside the normal range. No clinical parameters were found to distinguish these patients. T cell d^mC content in patients with OA, gout, chronic infection, and MS was not significantly different from that in age-matched controls.

DNA methylation in SLE and RA non–T lymphocytes. To test if DNA was hypomethylated in cells other than T cells, DNA d^mC content was measured in non–T lymphocytes isolated from RA, SLE, and OA or gout patients and from controls (Table 2). In contrast to the results obtained using T cell DNA, DNA d^mC content was comparable in non–T lymphocytes from the RA and OA/gout groups, and in patients with active SLE and age-matched controls. Although a trend toward DNA hypomethylation was suggested in the older groups, the difference was not statistically significant, due to interpatient variability. DNA d^mC content was lower in non–T lymphocytes than in T lymphocytes in the OA/gout group, but this difference was also not statistically significant.

Mechanisms of DNA hypomethylation. The T cell DNA hypomethylation observed in patients with RA and in patients with active SLE could be secondary to T cell activation, differences in d^mC content between T cell subsets, or differences in T cell DNA methylation between men and women. The effect of the sex of the individual and of T cell activation on T cell d^mC content is presented in Table 3. DNA methylation was essentially identical in T cells from healthy men and women. The effect of T cell activation on DNA methylation was examined by stimulating T cells from 14 healthy donors with PHA and measuring d^mC content 3–4 days later. No change in DNA methylation was seen following PHA activation.

Table 3. DNA deoxymethylcytosine content in T cells of healthy controls, classified according to the sex of the individual and T cell stimulation by phytohemagglutinin (PHA)*

Group (n)	% deoxymethylcytosine content
All controls (26)	3.81 ± 0.09
Men (16)	3.84 ± 0.14
Women (10)	3.76 ± 0.06
PHA-stimulated cells (14)	3.85 ± 0.21

* DNA content was measured as described in Figure 1. Values are the mean ± SEM.

Table 4. Effect of phytohemagglutinin (PHA) stimulation and T cell subsets on the DNA deoxymethylcytosine (d^mC) content of T cells from healthy controls*

T cells	d^mC content: % (range)	d^mC content: Ratio (mean ± SEM)
Control:cultured control†	3.16–3.61	1.01 ± 0.05
CD4:CD8‡	3.22–4.15	0.99 ± 0.01

* DNA d^mC content was measured as described in Figure 1.
† DNA d^mC content was measured in T cells from 6 healthy controls on day 0 and 2 weeks after T cell activation by phytohemagglutinin and culturing with interleukin-2 (cultured control). Values are the range of d^mC levels in the 6 subjects and the mean ± SEM of the control cell d^mC: cultured cell d^mC ratios in the 6 subjects.
‡ T cells from 6 healthy controls were fractionated into CD4+ and CD8+ populations, and the DNA d^mC content was determined for each population. Values are the range of d^mC levels in the 6 subjects and the mean ± SEM of the CD4 d^mC:CD8 d^mC ratios in the 6 subjects.

The effect of chronic activation on T cell DNA methylation was examined by comparing d^mC content in freshly isolated T cells and in PHA-activated T cells that had been maintained as proliferating lines for 2 weeks with IL-2 (Table 4). The T cell d^mC content in 6 healthy controls ranged from 3.16% to 3.61%. After 2 weeks in culture, T cell d^mC was again measured, and the ratio of (d^mC in freshly isolated cells):(d^mC in cultured cells) was determined for each subject. The measured d^mC values were essentially identical, indicating that DNA methylation is stable in proliferating T cells for at least 2 weeks. To study whether d^mC varied between T cell subsets, T cells from 6 controls were separated into CD4+ and CD8+ subsets, and d^mC content was assayed (Table 4). The range of values obtained was 3.22–4.15%, and for each subject, the d^mC content in each subset was nearly identical.

Lack of effect of drug therapy on T cell DNA methylation. Medications could also be responsible for T cell DNA hypomethylation in patients with RA and

Table 5. Medications taken by the study subjects*

Group (n)	NSAID	HCQ	Gold salts	Pred	AZA	MTX	CYC
OA or gout (8)	7	0	0	0	0	0	0
Chronic infection (6)	0	0	0	0	0	0	0
MS (7)	0	0	0	0	0	0	0
RA (22)	21	3	8	8	1	2	0
Active SLE (8)	4	4	0	5	1	1	0
Inactive SLE (12)	6	6	0	4	0	0	0

* NSAID = nonsteroidal antiinflammatory drug; HCQ = hydroxychloroquine; Pred = prednisone; AZA = azathioprine; MTX = methotrexate; CYC = cyclophosphamide. See Table 1 for other definitions.

Table 6. DNA methyltransferase activity in T cell nuclear proteins from patients with rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE)*

Group (n)	DNA methyltransferase activity (dpm/μg/hour)
Control (5)	6.1 ± 0.74
RA (5)	4.7 ± 0.58
Active SLE (5)	2.1 ± 0.36†

* T cells were isolated from the indicated populations, activated with phytohemagglutinin, and expanded with interleukin-2. DNA methyltransferase was isolated and measured as described in Patients and Methods. Values are the dpm of ^3H incorporated into DNA per μg of protein per hour, mean ± SEM of triplicate determinations from the indicated number of subjects.
† $P = 0.001$ versus controls and versus RA patients, by analysis of variance.

active SLE. Table 5 summarizes the antirheumatic medications taken by these patients, and lists the number of patients in the other 4 disease groups who were taking similar medications. No other medications were being taken by significant numbers of patients in the RA and SLE groups. Adrenocorticosteroids and gold salts were the only agents used widely in RA and SLE patients and not in other disorders. The T cell d^mC content in RA patients receiving gold salts was (mean ± SEM) 3.43 ± 0.18%, compared with 3.19 ± 0.13% in RA patients not taking gold salts ($P > 0.05$). The T cell d^mC content in RA patients taking corticosteroids was 3.00 ± 0.14%, compared with 3.43 ± 0.13% in RA patients not taking these drugs ($P > 0.05$). No significant differences in T cell d^mC content were found between patients who were taking corticosteroids and those not taking these drugs, in either the inactive SLE group (3.61 ± 0.12% versus 3.45 ± 0.24%, respectively), or the active SLE group (3.21 ± 0.28% versus 3.05 ± 0.28%, respectively).

Since a trend toward DNA hypomethylation was found in the steroid-treated RA patients, the effect of corticosteroids on DNA methylation was examined further. The leukemic T cell line Jurkat was treated with $10^{-8}M$ to $10^{-5}M$ dexamethasone, and the DNA d^mC content was determined with the procedures previously used to demonstrate inhibition by procainamide (8). Seven to 9 determinations were made at each dexamethasone concentration. No inhibition of DNA methylation was observed at any of the concentrations tested. Similar studies using $10^{-4}M$ to $10^{-6}M$ aurothioglucose, $10^{-4}M$ to $10^{-7}M$ chloroquine, $10^{-4}M$ to $10^{-7}M$ D-penicillamine, and $10^{-5}M$ to $10^{-8}M$ indomethacin confirmed that these medications did not inhibit DNA methylation in vitro.

Effect of DNA methyltransferase activity and SAM and S-adenosylhomocysteine levels on T cell DNA methylation. It is possible that DNA hypomethylation in RA or SLE T cells could be due to diminished DNA methyltransferase activity, decreased intracellular SAM pools, or increased S-adenosylhomocysteine pools (28). Since DNA methyltransferase is expressed only in proliferating lymphocytes (29) and SAM levels are maximal in activated cells (30), T cells from patients with RA and active SLE and from normal controls were activated with PHA and expanded in media supplemented with IL-2. The nuclei were then isolated, DNA methyltransferase was partially purified, and enzymatic activity was measured. The comparison of methyltransferase activity between these groups is shown in Table 6. Controls without autoimmune disease and RA patients had approximately equivalent amounts of methyltransferase activity. The slightly higher value in the control group is due to 1 healthy individual with reproducibly greater amounts of methyltransferase activity than the other subjects (8.8 dpm/μg/hour). It should be noted that the DNA d^mC content in these RA patients, determined simultaneously with methyltransferase activity, was (mean ± SEM) 3.20 ± 0.24%. In contrast to controls and RA patients, T cell nuclear proteins from the patients with active SLE examined contained significantly less enzymatic activity ($P = 0.001$).

SAM and S-adenosylhomocysteine levels were determined in activated T cells from the RA patients and controls, to test whether changes in these intermediate products could be responsible for DNA hypomethylation in the RA group. No significant differences were found, suggesting that this possibility is unlikely.

Coexpression of CD4 and CD8 in T cells from RA and SLE patients. Since CD8+ cells treated with DNA methylation inhibitors in vitro may coexpress CD4 and

CD8 (7), we tested whether hypomethylated T cells from RA and SLE patients coexpressed CD4 and CD8, using 2-color flow cytometry. T cells from these patients were also tested for expression of HLA–DR and IL-2R because CD4+, CD8+ cells transiently appear during activation of CD4+ cells (31). Eight RA patients with diminished DNA d^mC content were examined, and a small increase in cells coexpressing CD4 and CD8 was observed compared with 70 normal controls (mean ± SEM 2.73 ± 0.70% versus 1.12 ± 0.27%; $P < 0.025$). In contrast, none of the 11 SLE patients tested (5 with active disease and 6 with inactive disease) demonstrated elevated numbers of CD4+, CD8+ cells. There was no increase in HLA–DR-bearing or IL-2R–bearing cells in the RA patients, suggesting that the CD4+, CD8+ cells were not due to T cell activation.

DISCUSSION

DNA methylation can affect gene expression, and changes in cellular DNA methylation have been implicated in differentiation (32) and carcinogenesis (33). This is the first study examining DNA methylation in idiopathic autoimmune diseases. We report that T cell DNA from ~50% of the patients with RA and active SLE contained reduced amounts of 5-methylcytosine. Comparisons were made with T cell DNA from normal subjects and patients with inactive SLE or other diseases, and no similar decrease was found. There was also no decrease of DNA methylation in the SLE non–T cell population.

It is not certain why only half the patients with RA and active SLE had hypomethylated DNA. The variability in d^mC content in patients with active SLE could reflect a transient abnormality in all SLE patients, or may identify a disease subset characterized by DNA hypomethylation. Due to the large amount (100 ml) of blood required for accurate T cell d^mC measurement, and the fact that patients with SLE were anemic (34), we were unable to perform serial d^mC measurements in the same patients. However, many of the patients with inactive SLE had previously experienced disease flares similar to the active disease group, and their DNA could have been hypomethylated at those times.

In contrast to SLE, no correlation with disease activity was found in RA patients. However, disease activity in this group was determined by clinical criteria, and many of the patients were receiving corticosteroids, gold salts, and/or nonsteroidal antiinflammatory drugs, which could prevent detection of some of the clinical manifestations of disease activity. Therefore, we cannot exclude a relationship between disease activity in RA and DNA methylation.

The decrease in DNA methylation could be confined to 1 T cell subset, rather than all T cells. In healthy donors with a wide range of total T cell methylcytosine content, CD4+ and CD8+ cells demonstrated similar d^mC values. However, accurate measurement of d^mC content in the less prevalent CD8+ population required 500 ml of blood. Again, since the RA and SLE patients were anemic, it was impractical to obtain this volume of blood from these patients.

We examined mechanisms predisposing to DNA hypomethylation. The T cell DNA hypomethylation could not be accounted for by differences in age, sex, intracellular levels of methylation precursors, T cell activation, or type of medication. Chronic T cell activation could be a factor affecting T cell DNA methylation, and remains a possibility, especially in RA. Others have proposed that DNA methyltransferase, the enzyme which replicates DNA methylation patterns, may occasionally fail to methylate a cytosine residue, and an accumulation of these errors in chronically proliferating cells could lead to DNA hypomethylation (3). To control for this, we examined patients with MS and patients with active, chronic infections and found that they did not have hypomethylated T cell DNA. Furthermore, PHA-stimulated T cells and IL-2–dependent T cell lines cultured for 2 weeks showed no changes in d^mC content. Taken together, these results provide evidence against chronic T cell stimulation as the primary cause of DNA hypomethylation. However, it remains possible that chronic T cell stimulation, as seen in RA, could slowly produce DNA hypomethylation over a period of years and not be found in these controls.

When DNA methyltransferase activity was measured in controls, RA patients, and patients with active SLE, the SLE patients were found to have approximately one-third to one-half as much enzymatic activity as the other groups. Although the study group was small, the results were statistically significant, and suggest that impaired methyltransferase activity may contribute to DNA hypomethylation in some SLE patients. This conjecture is supported by our finding that procainamide inhibits T cell DNA methyltransferase in vitro (35). Since procainamide also induces a lupus-like syndrome (9), it is possible that diminished DNA methyltransferase activity is the common link between idiopathic and procainamide-

induced lupus. The reason for diminished enzymatic activity is unknown. Possible explanations include increased levels of methyltransferase inhibitors such as methinin (36), or altered amounts or specific activity of DNA methyltransferase in these cells. Studies evaluating these mechanisms in larger numbers of patients will be informative, and are currently under way.

Environmental factors have also been shown to affect DNA methylation. For example, procainamide, hydralazine (8), and ultraviolet light (37) are inhibitors of DNA methylation. These factors are particularly interesting because of their relationship with SLE, and other environmental factors may be identified in further studies. It is possible that some of the patients studied were exposed to an environmental DNA methylation inhibitor, causing DNA hypomethylation that was then maintained by DNA methyltransferase.

Finally, others have noted abnormal DNA metabolism in lymphocytes from SLE patients. In these studies, greater amounts of low molecular weight DNA fragments were found in PHA-stimulated lymphocytes from SLE patients than from controls (38,39). It is possible that DNA hypomethylation may be related to this alteration in DNA metabolism, but the mechanism linking these 2 observations is unclear.

In summary, multiple mechanisms may contribute to DNA hypomethylation in these patients. The evidence obtained in these studies suggests that hypomethylation in SLE may be due to impaired methyltransferase activity in some patients. In RA patients, no clear cause was identified, and the hypomethylation most likely represents an accelerated loss of d^mC secondary to aging or chronic T cell stimulation.

The DNA hypomethylation observed in RA and SLE could be involved in the pathogenesis of these diseases by altering expression of certain crucial genes and thereby inducing T cell autoreactivity. The extent of hypomethylation required to alter expression of these putative genes is unknown. Cytosine methylation appears to affect protein binding to DNA (40,41), and relatively few methylcytosine residues in regulatory sequences can affect gene expression (42). Assuming that cytosine demethylation is random and that relatively few methylcytosines regulate expression of the putative gene(s) contributing to autoreactivity, the probability of demethylating the crucial residue(s) would follow a normal distribution, and progressive DNA demethylation would increase the likelihood of demethylating the crucial base(s). Also, genetic factors clearly predispose certain individuals to RA and SLE (43). It is likely that if DNA hypomethylation contributes to the pathogenesis of RA or SLE, additional genetic factors are also required. These observations could explain the overlap in DNA methylcytosine levels between some of the controls and the patients with RA and active SLE.

It is possible to estimate the number of genes potentially affected by changes in DNA methylation. In RA patients, the decrease in d^mC content relative to young healthy controls is 0.53%, and the decrease in patients with active SLE is 0.66%. Methylcytosine occurs in CG basepairs, and in vertebrate DNA, there are approximately 20,824 CG basepairs/10^6 bases (44). Assuming 3×10^9 basepairs/cell, a decrease of 0.53% corresponds to a decrease of 331,000 demethylated cytosine residues, and a decrease of 0.66% corresponds to a decrease of 412,000 residues. These numbers are sufficient to affect the expression of a large number of genes, and are comparable in magnitude with the decrease induced by 5-azacytidine, procainamide, and hydralazine (8).

The genes affected by DNA methylation are not well identified. Our group has reported that cloned CD8+ cells treated with 5-azacytidine can re-express CD4 (7), and in the present study, a small but statistically significant number of T cells from RA patients, but not SLE patients, expressed both markers. CD4+, CD8+ cells have been previously described in RA (45). It is possible that this abnormal phenotype could be due to DNA hypomethylation. However, this phenotype was expressed on a small percentage of T cells, while DNA hypomethylation appears to affect a majority of the T cells. Furthermore, none of the patients with active SLE expressed this marker. Therefore, these results suggest that if DNA methylation directly affects CD4 expression in CD8 cells, either crucial sequences regulating CD4 expression are only rarely demethylated in these patients or cells expressing both markers are eliminated or sequestered away from venous blood.

Alternatively, additional factors beyond DNA hypomethylation may be required to affect CD4 gene expression, since cloned, IL-2–dependent cell lines are exposed to conditions not found in vivo. More persuasive evidence for altered gene expression in RA and SLE patients will await identification of other T cell genes affected by DNA methylation inhibitors, some of which may be responsible for the T cell autoreactivity observed when T cells are treated with these inhibitors in vitro.

Evidence for the presence of autoreactive T cells in RA and SLE remains hypothetical, based on

the chronic GVH model (10,11). While autoreactive T cells in these diseases have been sought using the autologous mixed lymphocyte reaction, with largely negative results (46), autoreactive cells could be distributed in places other than peripheral venous blood, such as lymphatic tissue, or could be refractory to restimulation (47). Therefore, our results remain consistent with a model in which T cell DNA hypomethylation alters gene expression and generates autoreactive T cells, which then participate in the pathogenesis of RA and SLE.

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REFERENCES

1. Cedar H: DNA methylation and gene activity. Cell 53:3–4, 1988
2. Doerfler W: DNA methylation and gene activity. Annu Rev Biochem 52:93–124, 1983
3. Holliday R: The inheritance of epigenetic defects. Science 238:163–170, 1987
4. Cedar H: DNA methylation and gene expression. DNA Methylation: Biochemistry and Biological Significance. Edited by A Razin, H Cedar, AD Riggs. New York, Springer-Verlag, 1984
5. Jones PA: Gene activation by 5-azacytidine. DNA Methylation: Biochemistry and Biological Significance. Edited by A Razin, H Cedar, AD Riggs. New York, Springer-Verlag, 1984
6. Richardson B: Effect of an inhibitor of DNA methylation on T cells. Hum Immunol 17:456–470, 1986
7. Richardson B, Kahn L, Lovett EJ, Hudson J: Effect of an inhibitor of DNA methylation on T cells. I. 5-azacytidine induces T4 expression on $T8^+$ T cells. J Immunol 137:35–39, 1986
8. Cornacchia E, Golbus J, Maybaum J, Strahler J, Hanash S, Richardson B: Hydralazine and procainamide inhibit T cell DNA methylation and induce autoreactivity. J Immunol 140:2197–2200, 1988
9. Harmon CE, Portanova J: Drug-induced lupus: clinical and serological studies. Clin Rheum Dis 8:121–135, 1982
10. Rolink AG, Gleichmann E: Allosuppressor- and allohelper-T cells in acute and chronic graft-vs.-host (GVH) disease. J Exp Med 158:546–558, 1983
11. Pals ST, Radaszkiewicz T, Roozendaal L, Gleichmann E: Chronic progressive polyarthritis and other symptoms of collagen vascular disease induced by graft-vs-host reaction. J Immunol 134:1475–1482, 1985
12. Janossy G, Duke O, Poulter LW, Panayi G, Bofill M, Goldstein G: Rheumatoid arthritis: a disease of T-lymphocyte/macrophage immunoregulation. Lancet II:839–842, 1981
13. Eisenberg RA, Cohen PL: Class II major histocompatibility antigens and the etiology of systemic lupus erythematosus. Clin Immunol Immunopathol 29:1–6, 1983
14. Arnett FC, Edworthy SM, Bloch DA, McShane DJ, Fries JF, Cooper NS, Healey LA, Kaplan SR, Liang MH, Luthra HS, Medsger TA Jr, Mitchell DM, Neustadt DH, Pinals RS, Schaller JG, Sharp JT, Wilder RL, Hunder GG: The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. Arthritis Rheum 31:315–324, 1988
15. Tan EM, Cohen AS, Fries JF, Masi AT, McShane DJ, Rothfield NF, Schaller JG, Talal N, Winchester RJ: The 1982 revised criteria for the classification of systemic lupus erythematosus. Arthritis Rheum 25:1271–1277, 1982
16. Barada FA Jr, Andrews BS, Davis JS IV, Taylor RP: Antibodies to Sm in patients with systemic lupus erythematosus: correlation of Sm antibody titers with disease activity and other laboratory parameters. Arthritis Rheum 24:1236–1240, 1981
17. Pinals RS, Masi AT, Larsen RA, The Subcommittee for Criteria of Remission in Rheumatoid Arthritis of the American Rheumatism Association Diagnostic and Therapeutic Criteria Committee: Preliminary criteria for clinical remission in rheumatoid arthritis. Arthritis Rheum 24:1308–1315, 1981
18. Swanson JW: Multiple sclerosis: update in diagnosis and review of prognostic factors. Mayo Clin Proc 64:577–586, 1989
19. Golbus J, Salata M, Greenwood J, Hudson J, Richardson BC: Increased immunoglobulin response to γ-interferon by lymphocytes from patients with systemic lupus erythematosus. Clin Immunol Immunopathol 46:129–140, 1988
20. Rabin H, Hopkins RF III, Ruscetti FW, Neubauer RH, Brown RL, Kawakami TG: Spontaneous release of a factor with properties of T cell growth factor from a continuous line of primate tumor T cells. J Immunol 127:1852–1856, 1981
21. Richardson BC, Liebling MR, Hudson JL: CD4+ cells treated with DNA methylation inhibitors induce autologous B cell differentiation. Clin Immunol Immunopathol 55:368–381, 1990
22. Wang Lu L-J, Randerath K: Long term instability and molecular mechanism of 5-azacytidine-induced DNA hypomethylation in normal and neoplastic tissues in vivo. Mol Pharmacol 26:594–603, 1984
23. Roe B, Sirover M, Dudock B: Kinetics of homologous and heterologous aminoacylation with yeast phenylala-

nyl transfer ribonucleic acid synthetase. Biochemistry 21:4146–4154, 1973
24. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ: Protein measurement with the Folin phenol reagent. J Biol Chem 193:265–275, 1951
25. Wagner J, Danzin C, Mamont P: Reversed-phase ion-pair liquid chromatographic procedure for the simultaneous analysis of s-adenosylmethionine, its metabolites and the natural polyamines. J Chromatogr 227:349–368, 1982
26. Glazer RI, Peale AL: Measurement of s-adenosyl-l-methionine levels of SP-sephadex chromatography. Anal Biochem 91:516–520, 1978
27. Golbus J, Palella TD, Richardson BC: T cell DNA methylation in differentiation and aging. Eur J Immunol (in press)
28. Adams RLP, Burdon RH: The methylation machinery: the DNA methyltransferases, Molecular Biology of DNA Methylation. Edited by A Rich. New York, Springer-Verlag, 1985
29. Kaul S, Drahovsky D: Identification of proliferatively active cells by monoclonal antibodies against DNA-cytosine-5-methyltransferase. Protides Biol Fluids 32: 521–524, 1985
30. German DC, Bloch CA, Kredich NM: Measurements of s-adenosylmethionine and l-homocysteine metabolism in cultured human lymphoid cells. J Biol Chem 258: 10997–11003, 1983
31. Blue ML, Daley JF, Levine H, Schlossman SF: Coexpression of T4 and T8 on peripheral blood T cells demonstrated by two-color fluorescence flow cytometry. J Immunol 134:2281–2286, 1985
32. Jahner D, Jaenisch R: DNA methylation in early mammalian development, DNA Methylation: Biochemistry and Biological Significance. Edited by A Razin, H Cedar, AD Riggs. New York, Springer-Verlag, 1984
33. Goelz SE, Vogelstein B, Hamilton SR, Feinberg AP: Hypomethylation of DNA from benign and malignant human colon neoplasms. Science 228:187–190, 1985
34. Budman DR, Steinberg AD: Hematologic aspects of systemic lupus erythematosus. Ann Intern Med 86:220–229, 1977
35. Scheinbart LS, Johnson MA, Gross LA, Pike MC, Richardson BC: Procainamide inhibits DNA methyltransferase in cultured human T cells. Submitted for publication
36. Lyon SB, Buonocore L, Miller M: Naturally occurring methylation inhibitor: DNA hypothylation and hemoglobin synthesis in human K562 cells. Mol Cell Biol 7:1759–1763, 1987
37. Lieberman MW, Beach LR, Palmiter RD: Ultraviolet radiation-induced metallothionein-I gene activation is associated with extensive DNA demethylation. Cell 35:207–214, 1983
38. Compton LJ, Steinberg AD, Sano H: Nuclear DNA degradation in lymphocytes of patients with systemic lupus erythematosus. J Immunol 133:213–216, 1984
39. Sano H, Imokawa M, Steinberg AD, Morimoto C: Accumulation of guanine-cytosine-enriched low M.W. DNA fragments in lymphocytes of patients with systemic lupus erythematosus. J Immunol 130:187–190, 1983
40. Meehan RR, Lewis JD, McKay S, Kleiner EL, Bird AP: Identification of a mammalian protein that binds specifically to DNA containing methylated CpGs. Cell 58:499–507, 1989
41. Antequera F, Macleod D, Bird AP: Specific protection of methylated CpGs in mammalian nuclei. Cell 58:509–517, 1989
42. Bussliner M, Hurst J, Flavel RA: DNA methylation and the regulation of globin gene expression. Cell 34:197–206, 1983
43. Todd JA, Acha-Orbea H, Bell JI, Chao N, Fronek Z, Jacob CO, McDermott M, Sinha AA, Timmerman L, Steinman L, McDevitt HO: A molecular basis for MHC class II-associated autoimmunity. Science 240:1003–1009, 1988
44. Adams RLP, Burdon RH: Other effects of cytosine methylation on DNA structure, Molecular Biology of DNA Methylation. Edited by A Rich. New York, Springer-Verlag, 1985
45. Pincus SH, Clegg DO, Ward JR: Characterization of T cells bearing HLA–DR antigens in rheumatoid arthritis. Arthritis Rheum 28:8–15, 1985
46. Weksler ME, Moody CE Jr, Kozak RW: The autologous mixed-lymphocyte reaction. Adv Immunol 31:271–312, 1981
47. Silver RM, Redelman D, Zvaifler NJ: Studies of rheumatoid synovial fluid lymphocytes. Clin Immunol Immunopathol 27:15–27, 1983