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MECHANISMS OF DRUG-INDUCED LUPUS

IV. Comparison of Procainamide and Hydralazine with Analogs In Vitro and In Vivo

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Objective. **T cells treated with DNA methylation inhibitors overexpress lymphocyte function–associated antigen 1 (LFA-1), which results in autoreactivity, and the autoreactive cells cause a lupus-like disease in vivo, suggesting a mechanism by which some agents may cause drug-induced lupus. This study compared the effects of procainamide (Pca) and hydralazine (Hyd) with those of structural analogs, to determine if the degree of LFA-1 overexpression and T cell autoreactivity correlated with the ability of the agents to induce autoimmunity.**

Methods. **Cloned murine T helper 2 cells were treated with Pca, *N*-acetylprocainamide, Hyd, Phthalazine, or hydroxyurea (HU). The treated cells were then compared for LFA-1 overexpression, autoreactivity, and the ability to induce autoimmunity in vivo.**

Results. **Pca and Hyd were more potent than their analogs or HU in all 3 assays.**

Conclusion. **The results support a relationship between LFA-1 overexpression, T cell autoreactivity, and autoimmunity, and suggest a mechanism by which Pca and Hyd, but not the analogs, may cause drug-induced lupus.**

Procainamide (Pca) and hydralazine (Hyd) cause a lupus-like disease in some individuals, but the mechanism by which these drugs induce autoimmunity is uncertain. Understanding the mechanism is important, because similar mechanisms could contribute to the development of idiopathic human lupus. Our group has reported that Pca and Hyd inhibit DNA methylation and induce autoreactivity in human T lymphocytes (1,2). We have also reported that inhibiting DNA methylation with Pca or 5-azacytidine (5-azaC), in cloned as well as polyclonal CD4+ murine T cells, causes a similar autoreactivity, and that the CD4+ T cells made autoreactive by these drugs cause a lupus-like disease in nonirradiated syngeneic mice (3,4). The autoreactivity correlates with an increase in lymphocyte function–associated antigen 1 (LFA-1) expression (5), and overexpression of LFA-1 by transfection causes a similar autoreactivity in vitro and autoimmune disease in vivo (6,7), demonstrating that LFA-1 overexpression is important and probably the major mechanism responsible for these effects. These results led us to hypothesize that some agents causing drug-induced lupus do so, in part, by interacting with T cells to induce LFA-1 overexpression, which results in autoreactivity, and the autoreactive cells then interact with immune cells in the host to produce the autoimmune disease (3,4).

To further test the proposed relationship between LFA-1 overexpression, T cell autoreactivity, and autoimmunity, we compared the effects of Pca and Hyd with those of structural analogs on murine T cell LFA-1 expression and autoreactivity in vitro. We also compared cells treated with these drugs for their ability to induce autoimmunity in vivo. The effects of Pca were compared with those of *N*-acetylprocainamide (Napa), a metabolite which does not induce lupus (8,9). The effects of Hyd were compared with those of phthalazine (Phth), the parent molecule of Hyd, but lacking the hydrazine side chain. Phth has no clinical utility, so its ability to induce lupus is unknown. However, hydrazine compounds have been implicated in triggering some cases of lupus (10), and the comparison of Hyd and Phth allows analysis of the role of hydrazine in this system. Finally, the effects of hydroxyurea (HU), a DNA synthesis

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inhibitor (11), were also studied. In contrast to Pca and Hyd, HU increases DNA methylation (12).

The results showed that Pca and Hyd were more potent than their analogs and HU in increasing LFA-1 expression, and in inducing autoreactivity and autoimmunity. Together, these results lend further support to the proposed relationship of LFA-1 overexpression, T cell autoreactivity, and lupus, and suggest a mechanism by which Pca and Hyd may cause lupus in patients receiving these drugs. The results also provide an explanation as to why Napa does not induce lupus, and indicate that the hydrazine side chain of Hyd can increase the potency of the parent Phth molecule in this system.

MATERIALS AND METHODS

Mice. Six-week-old female AKR and New Zealand black × New Zealand white (NZB × NZW) mice were purchased from Jackson Laboratory (Bar Harbor, ME). These mice were housed in a pathogen-free environment maintained by the Unit for Laboratory Animal Medicine at the University of Michigan (Ann Arbor, MI).

T cell lines. The conalbumin-reactive, cloned murine T helper 2 line, D10.G4.1 (derived from AKR mice), was purchased from American Type Culture Collection (ATCC, Rockville, MD) and maintained as previously described (3,4,7). D10 cells were subcloned by limiting dilution and a nonautoreactive subclone selected for use in these studies. The cells were restimulated every 7–8 days with conalbumin (100 μg/ml; Sigma, St. Louis, MO) and with irradiated (3,000R) AKR splenocytes, as previously described (3,4,7). The cells were used in proliferation assays or for flow cytometric analysis at least 7 days after restimulation. Where indicated, the cells were treated for 6 days with Pca (Sigma), Napa (Sigma), Hyd (Sigma), Phth (Aldritch, Milwaukee, WI), or HU (Sigma), beginning 24 hours after restimulation.

T cell proliferation assays. T cell proliferative responses were measured as previously described (3,4,7). Briefly, 2×10^4 D10 cells were cultured in round-bottom 96-well microtiter plates with 2×10^4 irradiated (3,000R) thioglycolate-induced AKR peritoneal macrophages, with or without 100 μg/ml conalbumin. T cell proliferation was determined 4 days later by ^{3}H-thymidine incorporation. Results are the mean ± SEM of quadruplicate determinations, and are presented as stimulation indices (SI) in counts per minute, calculated as follows:

$$SI = \frac{\text{T cells cultured with macrophages}}{\text{T cells cultured alone} + \text{macrophages cultured alone}}$$

Flow cytometric analysis. D10 cells were reacted with anti-murine CD11a (purified from the supernatant of the M17/4.2 hybridoma cell line [13], obtained from ATCC), washed 3 times, then reacted with fluorescein isothiocyanate (FITC)–conjugated goat anti-mouse Ig (Cappel Laboratories, West Chester, PA), as previously described (3,4,7). The stained cells were analyzed using a Coulter ELITE flow cytometer.

Quantitation of T cell DNA methylation. T cell DNA was isolated and hydrolyzed to nucleosides with DNAse 1, phosphodiesterase, and alkaline phosphatase, using previously published protocols (1,2). Total genomic deoxycytosine (dC) and deoxymethylcytosine (d^mC) content in the hydrolysate was determined by reverse-phase high-performance liquid chromatography (HPLC), as previously described (1,2). Results are presented as a percentage of the total d^mC content, calculated as $\% d^mC = (d^mC/[d^mC + dC]) \times 100$.

Adoptive transfers. Female AKR mice received 5×10^6 untreated or drug-treated D10 cells via the tail vein every 2 weeks for a total of 6 injections, using 5 mice per group. The mice were killed for serologic and histologic analysis 4 weeks after the last injection.

Autoantibody enzyme-linked immunosorbent assays (ELISAs). Anti–double-stranded DNA (dsDNA) and anti–single-stranded DNA (ssDNA) ELISAs were performed on mouse sera using previously published protocols (7). Purified ssDNA was obtained from Sigma, while dsDNA consisted of cesium chloride–purified KS+−SV2CAT plasmid. Pooled sera from 6-month-old female NZB × NZW mice were used as positive controls.

Histologic analysis. Hematoxylin and eosin staining on sections of the kidney, liver, lung, brain, heart, spleen, thymus, skin, and intestine from the experimental mice was performed using published protocols (3,4,7). Immunofluorescent staining of the kidney tissues using FITC-conjugated goat anti-mouse IgG (Sigma) was performed as previously described (3,4,7).

RESULTS

Drug effects on T cell autoreactivity. Initial experiments compared the study drugs for their ability to induce autoreactivity. D10 cells were used, because they had been previously shown to become autoreactive following treatment with Pca and 5-azaC (4). The D10 cells were treated with 0–50 μ*M* Pca, Napa, Hyd, or Phth, or 10 μ*M* HU. Ten μ*M* HU is the highest concentration tolerated by cloned T cells, and is sufficient to modify expression of some genes (e.g., CD4) (14). After at least 2 cell cycles (6 days) in the presence of these drugs, the cells were washed and the proliferative response to syngeneic macrophages was measured. Figures 1A and B show that Pca, Napa, Hyd, and Phth all increased the proliferative response to macrophages without antigen. Pca was more effective than Napa, as reported previously (2), and Hyd was more potent than Phth. Treatment with HU produced no significant autoreactive response (mean SI 1.1), as observed previously (14).

Drug effects on T cell LFA-1 expression. D10 cells were then treated for 6 days with 10 μ*M* HU, 50 μ*M* Pca or Napa, or 10 μ*M* Hyd or Phth, and examined for LFA-1 expression by flow cytometry. These concentrations were chosen because they are close to the thera-

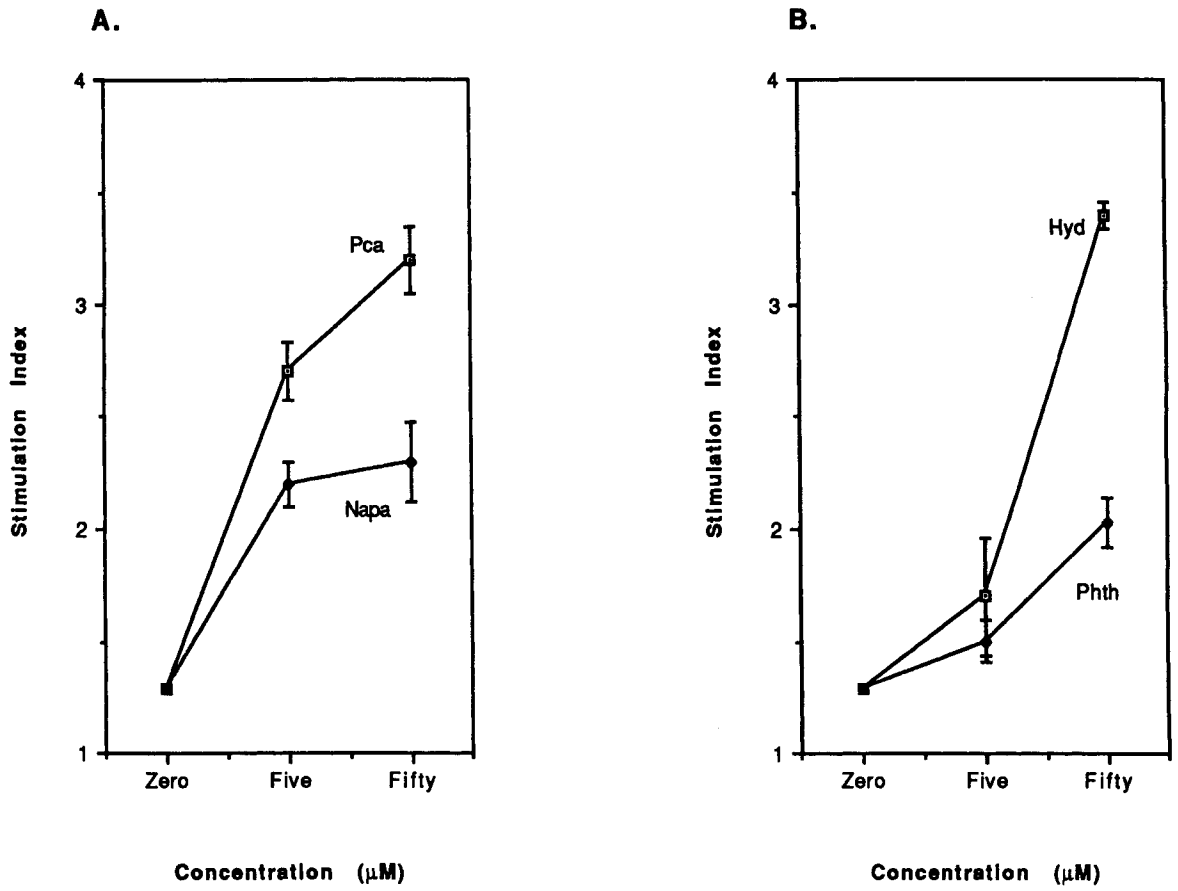

Figure 1. Autoreactivity in drug-treated D10 cells that were treated with the indicated concentrations of the test drugs, and, 6 days later, cultured with equal numbers of irradiated syngeneic peritoneal macrophages. Proliferation was measured 4 days later by ^{3}H-thymidine incorporation. **A,** Procainamide (Pca)– versus *N*-acetylprocainamide (Napa)–treated cells ($P < 0.005$). **B,** Hydralazine (Hyd)– versus phthalazine (Phth)–treated cells ($P < 0.001$). Bars show the mean ± SEM of quadruplicate determinations in 3 representative, independent experiments. For reference, T cells cultured alone gave an average background response of 2,034 ± 230 counts per minute (mean ± SEM), and the average autoreactive response for cells treated with the 50-μM concentrations for all 4 drugs was 6,270 ± 779 cpm.

peutic concentrations of Pca (10–30 μM) and Hyd (0.5–5.0 μM) (1,2), and because autoreactivity was detected at these concentrations. The results are shown in Figures 2A–F. Untreated (control) cells expressed relatively low levels of LFA-1, as did HU-treated cells. Pca induced a subset that overexpressed LFA-1, as previously described (7). This subset was smaller in Napa-treated cells. Nearly all Hyd-treated cells overexpressed LFA-1, and Phth induced LFA-1 overexpression on a smaller subset. These results correlate with the degree of autoreactivity seen. However, it should be noted that in both the autoreactivity assays and the LFA-1 expression studies, greater variability was seen in the serial repeat experiments with the Hyd- and Phth-treated cells than in the cells treated with the other 3 drugs. This was also seen in human T cells treated with these drugs (1,2), and may be due to the delayed effect that Hyd has on DNA methylation (1).

Drug effects on T cell DNA methylation. D10 cells were treated with the same concentrations of the test drugs as in Figure 2, and, 6 days later, DNA was isolated and hydrolyzed to nucleosides with DNAse, phosphodiesterase, and alkaline phosphatase, and total dC and d^mC content was measured by reverse-phase HPLC. We found that d^mC represented 3.3% of the total dC content in untreated D10 cells. Pca caused a 5.5% decrease, and Hyd a 3.6% decrease, in total d^mC content. The other drugs were relatively less potent, with

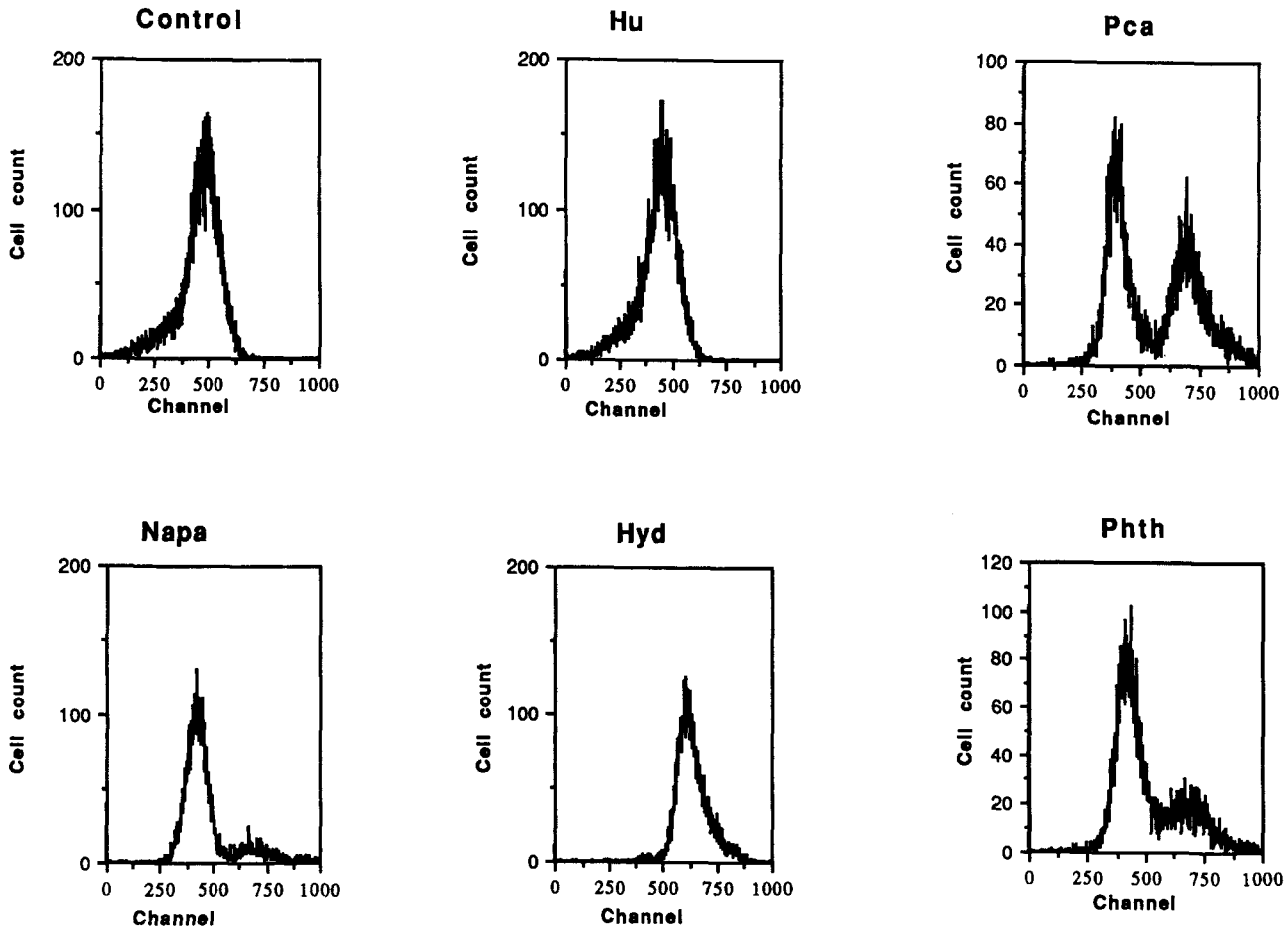

Figure 2. Lymphocyte function–associated antigen 1 expression in drug-treated D10 cells that were either cultured alone (Control), or treated with 50 μM Pca or Napa, or 10 μM Hyd, Phth, or hydroxyurea (Hu). Six days later, the cells were stained with anti-CD11a followed by fluorescein isothiocyanate–conjugated goat anti-mouse Ig, and analyzed by flow cytometry. See Figure 1 for other definitions.

Phth causing a 2.4% decrease, and Napa a 1.8% decrease. In contrast, HU increased total d^mC content by 9.7%, as reported by others (12) (mean of 5 independent experiments for each test drug, with each determination performed in duplicate; $P < 0.05$, Pca versus HU). Overall, this trend is similar to that seen previously with these drugs in human (Jurkat) T cells (2), although the decreases were quantitatively smaller in our study.

Autoantibody responses induced by drug-treated T cells. Using 5 animals for each drug-treated line, 5×10^6 D10 cells were then treated with the same concentrations of each drug as above, and injected intravenously into syngeneic recipients. Controls included untreated D10 cells. The mice received a total of 6 injections spaced 2 weeks apart, and were killed 4 weeks later. Sera were diluted 1:100 and antibodies to ssDNA and dsDNA were measured by ELISA. Figure 3A shows the anti-ssDNA antibody response in the 5 groups of mice, using NZB × NZW mice as a positive control. Overall, cells treated with Pca and Hyd gave the highest anti-ssDNA response, and HU and Napa, the lowest. The Phth-induced response was slightly less than that seen with Hyd and Pca. This distribution was highly significant overall ($P = 0.0001$ by analysis of variance [ANOVA]). An IgG-specific ELISA gave similar results (mean ± SEM optical density [OD] units Pca 0.327 ± 0.042, Napa 0.071 ± 0.018, Hyd 0.326 ± 0.129, Phth 0.194 ± 0.050, and HU 0.094 ± 0.014). Figure 3B shows the same groups analyzed for anti-dsDNA antibodies. Again, Pca and Hyd gave the greatest results, and HU, Napa, and Phth, the lowest. Untreated D10 cells gave no significant anti-ssDNA or anti-dsDNA responses (0.180 ± 0.080 and 0.080 ± 0.035 OD units, respectively). By ANOVA, the distribution was again significant

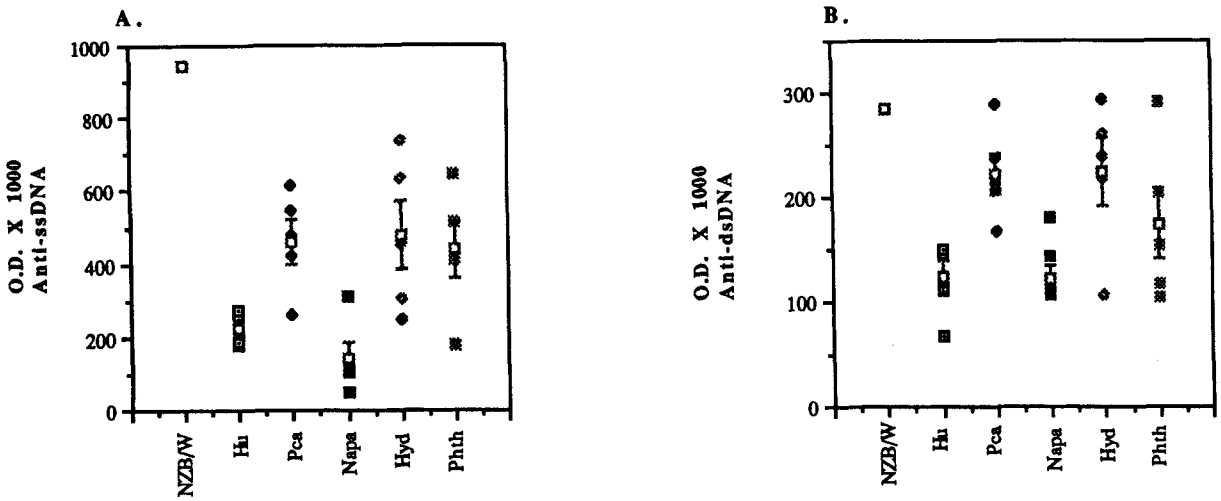

Figure 3. Anti-DNA antibodies in sera from mice receiving drug-treated D10 cells. Female AKR mice were given 6 intravenous injections of D10 cells treated with the test drugs. Four weeks after the last injection, the mice were killed. Sera were diluted 1:100 and tested for **A,** anti–single-stranded DNA (anti-ssDNA) or **B,** anti–double-stranded DNA (anti-dsDNA) antibodies by enzyme-linked immunosorbent assay. Pooled sera from New Zealand black × New Zealand white (NZB/W) mice were included as a positive control. Each point represents the mean of quadruplicate determinations on sera from a single mouse. Bars show the mean ± SEM for each group. O.D. = optical density units; Hu = hydroxyurea; see Figure 1 for other definitions.

(P = 0.0042). Using Tukey's Studentized Range and Bonferroni t-tests, the response induced by Pca, Hyd, and Phth was significantly ($P < 0.05$) greater than that by Napa and HU.

Histologic changes induced by drug-treated T cells. Similar to the results seen in the other assays, only mice receiving Pca- or Hyd-treated D10 cells developed significant proliferative glomerulonephritis, pulmonary alveolitis, or periductal lymphocytic infiltration in the liver. The histologic changes induced by Pca have been previously shown (4). Figures 4A, C, and E show the liver, lung, and renal disease induced by Hyd-treated cells and observed in 5 of 5 mice receiving these cells. Figures 4B, D, and F show representative sections from mice receiving Phth-treated cells, and appear normal. The liver, lung, and kidney in mice receiving HU- and Napa-treated cells also appeared normal (not shown). Using an arbitrary 0–4+ scale to quantitate the degree of histologic change in the kidneys, livers, and lungs, and combining the scores of all 3 organs for each mouse, mice receiving Pca-treated cells developed significantly more total tissue damage than did mice receiving Napa-treated cells (mean ± SEM 2.1 ± 0.2 versus 0.5 ± 0.2; $P < 0.001$ by Student's t-test), and mice receiving Hyd-treated cells developed greater tissue damage than did mice receiving Phth-treated cells (1.8 ± 0.3 versus 0.5 ± 0.2; $P < 0.001$). Mice receiving HU-treated cells developed minimal changes (0.2 ± 0.1).

DISCUSSION

These experiments were designed to further test the proposed relationship between T cell LFA-1 overexpression, autoreactivity, and autoimmunity, using 2 drugs known to cause drug-induced lupus and analogs known or proposed to be less potent. The effects of Pca were compared with Napa, which is known to be less potent in causing lupus (8,9), and those of Hyd were compared with Phth to test the proposed role of the hydrazine side chain in inducing autoimmunity. HU was included to control for possible effects due to DNA synthesis inhibition, and to evaluate the effect of a drug that increases DNA methylation (11,12). We have previously compared these drugs in human T cell clones, and found that Pca, Napa, Hyd, and Phth can inhibit DNA methylation and induce autoreactivity. However, Pca was ~100-fold more potent than Napa in inducing autoreactivity, while Hyd and Phth were comparable in effect (2).

The system used in the present study was one previously described by our group, in which murine CD4+ T cells are treated with DNA methylation inhib-

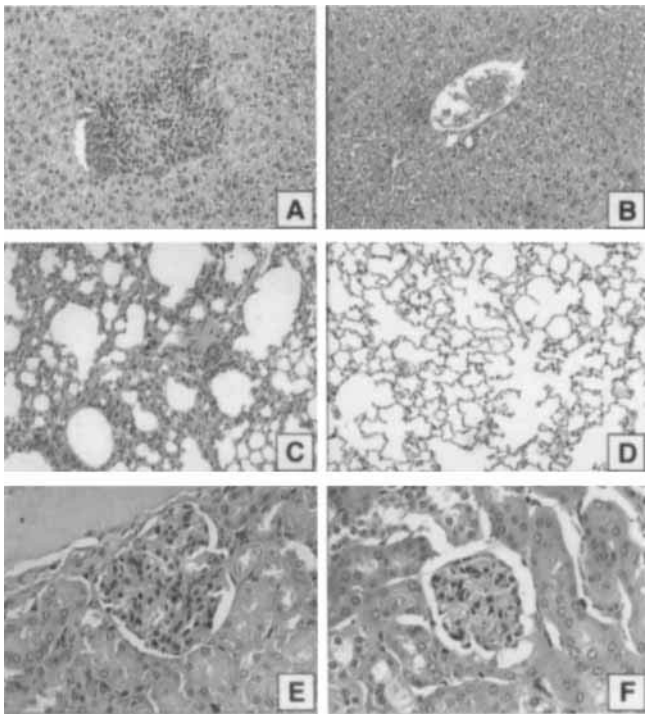

Figure 4. Histologic analysis of the liver, lung, and kidney from mice receiving drug-treated D10 cells. Liver, lung, and kidney samples from representative mice were fixed, sectioned, and stained with hematoxylin and eosin, then analyzed by light microscopy. **A,** Liver section from a mouse receiving hydralazine (Hyd)–treated cells, showing a mononuclear periductal infiltrate with destruction of the bile duct (original magnification × 60). **B,** Liver section from a mouse receiving phthalazine (Phth)–treated cells. The histologic structure appears normal (original magnification × 60). **C,** Lung section from a mouse receiving Hyd-treated cells, showing an interstitial pneumonitis with septal widening due to infiltration by inflammatory cells (original magnification × 60). **D,** Lung from a mouse receiving Phth-treated cells, with normal architecture. **E,** Glomerulus from a mouse receiving Hyd-treated cells, showing hypercellularity and increased mesangial matrix (original magnification × 120). **F,** Normal glomerulus from a mouse receiving Phth-treated cells (original magnification × 120).

itors, then given in adoptive transfer to syngeneic female recipients. Inhibiting T cell DNA methylation causes an increase in LFA-1 expression, making the cells responsive to normally subthreshold stimuli, including self class II major histocompatibility complex molecules without appropriate antigen (6,7). The recipients develop an autoimmune disease resembling murine chronic graft-versus-host disease, with an immune complex glomerulonephritis, pulmonary alveolitis, liver lesions resembling primary biliary cirrhosis, and IgG antibodies to ssDNA and dsDNA (3,4,7). Both chronic graft-versus-host disease and the D10 model appear to use a similar mechanism (7). It is recognized that humans with drug-induced lupus rarely develop many of these manifestations (15). However, the clinical manifestations seen in murine chronic graft-versus-host disease are strain-dependent (16). It is possible that differences between the present murine model and human drug-induced lupus also reflect basic genetic differences between mice and men.

The present study confirmed that Pca is more potent than Napa in inducing T cell autoreactivity. This suggests that the aromatic amine on the Pca molecule is necessary for inducing autoreactivity. Hyd was also more potent than Phth in this system. The difference between Hyd and Phth potency in this and the previous report (2) could be due to differences in the T cells used, or to species-specific differences in the response to these drugs. It is worth noting that the kinetics of DNA hypomethylation induced by Pca and Hyd are different. While Pca is a competitive inhibitor of DNA MTase (17) and causes a rapid decrease in total T cell d^mC content (1), the effects of Hyd are delayed (1). This led us to propose that a metabolite of Hyd may be responsible for the DNA hypomethylation and subsequent effects on the cell (1). The variability in the potency of Hyd and Phth thus may reflect differences in the metabolism of these drugs to active compounds. In both the human and murine systems, it is apparent that Phth is capable of inducing some degree of autoreactivity, confirming that the hydrazine side chain is not required for this effect. In addition, some of the mice receiving Phth-treated cells also developed autoantibodies. However, the hydrazine side chain does appear to increase potency in the present system.

The present studies also compared the effects of these drugs on T cell LFA-1 expression. A positive correlation was observed between LFA-1 overexpression and T cell autoreactivity. This is not unexpected, since inducing LFA-1 overexpression by transfection also induces autoreactivity (6,7).

The 5% decrease in d^mC content induced by Pca was less than the 14% previously reported using Jurkat cells (1,2). The reason for this is unclear, but may reflect a species-specific difference in the sensitivity of T cells to DNA methylation inhibitors, since Pca also caused an identical 5% decrease in murine EL-4 cells (3). Others have noted species and even strain-specific differences in overall levels of DNA methylation (18–20), supporting this interpretation. The decrease corresponds to the demethylation of ~110,000 cytosine residues (21). Since relatively few methylated cystosines in regulatory sequences can suppress gene expression (22), a change of this magnitude could be significant.

The ability of D10 cells treated with these drugs to induce autoimmunity followed the same trends that were seen with LFA-1 overexpression and autoreactivity. Again, Pca and Hyd induced the highest autoantibody titers and the greatest amount of tissue damage. The use of 6 adoptive transfers minimizes interexperimental variability, so the results observed probably represent a valid approximation of the relative potency of these drugs. The correlation of the degree of autoreactivity with disease severity argues that the autoreactivity observed in vitro also occurs in vivo, resulting in autoimmunity. We have previously pointed out the similarities between the present system and chronic graft-versus-host disease, another model in which T cells responding to Ia molecules in vivo produce a similar lupus-like disease (16,23). The similarities between these systems argue that similar mechanisms are causing the autoimmunity. Alternatively, others have proposed that hypomethylated DNA may contribute to a lupus-like disease by directly activating B cells (19). We have reported that D10 cells made autoreactive by LFA-1 transfection cause a similar disease without DNA hypomethylation (7), thus arguing against this interpretation. Furthermore, heat-killed hypomethylated cells do not induce autoimmunity, further arguing against this as the primary mechanism. However, the hypomethylated DNA could potentially contribute to disease severity by augmenting autoantibody responses.

Together, these results support the proposed relationship between LFA-1 overexpression, T cell autoreactivity, and autoimmunity. They also suggest a reason why Napa does not induce autoimmunity, and indicate that the hydrazine side chain on Hyd can increase potency of the parent molecule in this system. Together with the findings of earlier work, these results indicate a mechanism by which Pca and Hyd could cause autoimmunity in humans, and which could contribute to the development of idiopathic human lupus. We have already reported that T cells from patients with idiopathic lupus have hypomethylated DNA, as well as an autoreactive subset that overexpresses LFA-1 (5,25). Similar studies in patients receiving Pca and Hyd would help determine whether this mechanism also contributes to drug-induced lupus. It should be noted that drug-induced lupus caused by Pca and Hyd have certain key differences, including the specificity of the autoantibodies made and in the incidence of arthritis and serositis (15), although there is considerable overlap in both clinical and serologic manifestations (26). The reasons for this are uncertain, but could reflect effects of other metabolites of these drugs, or differences in the interactions of these drugs with DNA, modifying antigenicity as proposed by others (27–29). Nonetheless, this system indicates a mechanism by which these drugs can be shown to cause a lupus-like autoimmune disease. Other agents or events increasing LFA-1 expression and inducing autoreactivity by inhibiting DNA methylation or other mechanisms could cause a similar lupus-like disease.

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