N-ACETYLPROCAINAMIDE IS A LESS POTENT INDUCER OF T CELL AUTOREACTIVITY THAN PROCAINAMIDE

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We have reported that an inhibitor of DNA methylation, 5-azacytidine, makes cloned, antigen-specific CD4+ T cells autoreactive, and that procainamide and hydralazine mimic this effect. Those results suggested that procainamide and hydralazine may induce autoimmunity by inhibiting DNA methylation and causing T cell autoreactivity. We report now that N-acetylprocainamide, a procainamide derivative that does not induce lupus, is also a DNA methylation inhibitor, but it is 100 times less potent than procainamide in inducing T cell autoreactivity.

DNA methylation inhibitors induce expression of genes normally suppressed by mechanisms associated with cytosine methylation (1) and, in certain systems, can change cellular phenotype and alter differentiation (2,3). We have previously reported that 5-azacytidine (5-azaC), a DNA methylation inhibitor, made 4 cloned antigen-reactive T cells respond to autologous class II major histocompatibility complex (MHC) determinants without specific antigen, presumably by inducing expression of suppressed genes (4). This response was termed autoreactivity.

In those studies, we demonstrated that the 5-azaC–treated T cells responded to autologous but not allogeneic macrophages, and that monoclonal antibodies to class II but not class I MHC determinants would inhibit activation (4). Those results suggested the possibility that similar inhibition of T cell DNA methylation in vivo could lead to an autoimmune disease.

To determine if inhibitors of DNA methylation could produce an autoimmune disease, we tested whether 2 drugs known to induce a lupus-like syndrome inhibit DNA methylation and induce T cell autoreactivity. We found that hydralazine (Hyd) and procainamide (Pca) mimicked the effects of 5-azaC on T cells by inducing T cell autoreactivity in these cloned lines and decreasing deoxymethylcytidine (d^mC) content in T cell DNA, thus supporting the hypothesis that DNA methylation inhibitors may induce autoimmune disease (5).

To further test this hypothesis, we compared Pca and Hyd with structural analogs, using the assays previously developed. N-acetylprocainamide (Napa) is a procainamide derivative that rarely induces lupus (6–8), and hydralazine is a phthalazine (Phth) derivative containing a hydrazine side chain (1-hydrazinophthalazine) (9,10). Previously, investigators speculated that the hydrazine side chain of hydralazine may be responsible for the lupus-like syndrome (9); therefore, we compared Pca with Napa and Hyd with Phth, for the ability to induce T cell autoreactivity and inhibit DNA methylation.

MATERIALS AND METHODS

T cell cultures. CD4+, cloned, tetanus toxoid (TT)–reactive, interleukin-2 (IL-2)–dependent T cell lines were generated and maintained as previously described (4,11).

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Briefly, T cells were activated with TT in vitro, expanded with IL-2, cloned once by limiting dilution at 0.2 cells/well, and maintained by weekly rechallenge with fresh irradiated (2,000R) autologous antigen-presenting cells (APC) plus TT, and twice weekly addition of IL-2. The human leukemic Jurkat T cell line was cultured in RPMI 1640 containing 10% fetal calf serum (Hyclone, Logan, UT) and supplemented with 100 units/ml penicillin and 100 μg/ml streptomycin. T cells were treated with Pca, Hyd, hydroxyurea (Hu), 5-azaC (all from Sigma, St. Louis, MO), Napa, or Phth (both from Aldrich Chemicals, Milwaukee, WI) for at least 4 days before testing.

Proliferation assays. IL-2–dependent T cells were washed free of IL-2 and cultured 16 hours in RPMI supplemented with 20% human type AB serum without IL-2. Then, 2×10^4 cells were cultured with 10^5 irradiated (2,000R) autologous peripheral blood mononuclear cells in 200 μl RPMI:20% AB serum using round-bottom 96-well plates (Nunc plates; Gibco, Grand Island, NY). TT (Wyeth, Marietta, PA) was used at 1:25–1:100 dilutions, and maximum responses were usually observed at 1:50. Proliferation was measured on day 3 by tritiated thymidine (^{3}H-TdR) uptake, as previously described (4,11).

High performance liquid chromatography (HPLC) quantitation of d^{m}C. Jurkat cells were treated with $10^{-7}M$ to $10^{-4}M$ Pca, Napa, Hyd, Phth, Hu, or $10^{-6}M$ 5-azaC. DNA was isolated 4–7 days later, using the method of Bell et al (12). In addition, the DNA was treated with 100 μg/ml RNase (Sigma) at 37°C for 1–3 hours before precipitating with ethanol. The DNA was quantitatively hydrolyzed with DNase I, phosphodiesterase, and alkaline phosphatase as described (13), and the resultant nucleosides were separated by reverse-phase chromatography using a C_{18} column (particle size 5μ; Regis, Morton Grove, IL) with a mobile phase of 1% methanol in 0.01M acetate buffer (pH 5.3), at a flow rate of 1 ml/minute.

The column was washed between samples with 30% methanol in the same buffer, and absorbance was monitored at 280 nm using a Model 440 detector (Waters, Milford, MA). Data were collected using a Waters Data Module. Fluorodeoxyuridine was added to each sample as an internal standard, and deoxycytidine (dC) and d^{m}C content was determined using peak area, relative to standard curves for dC and d^{m}C. The d^{m}C peak was identified by retention time and ultraviolet spectral analysis. Deoxymethylcytidine content was expressed as a percentage of total dC:

$$\frac{d^mC}{d^mC + dC} \times 100$$

Statistical analysis. Results were compared using Student's t-test.

RESULTS

Induction of T cell autoreactivity by Pca, Hyd, Napa, and Phth. A cloned, CD4-bearing, TT-reactive T cell line (TT36C) was used to test whether Pca, Napa, Hyd, or Phth induces autoreactivity similar to 5-azaC. The reactivity of this cell line is shown in Table 1. TT36C required APC and TT for activation and did not respond to APC alone. The T cell responses to APC alone were not significantly greater than the sum of ^{3}H-TdR incorporation by T cells and APC cultured separately. Peak responses differed between experiments, possibly due to differences in cell number or viability, but in all cases optimal proliferation was usually found at TT dilutions of 1:50.

Table 1. Response of TT36C T cell line to antigen-presenting cells (APC) plus tetanus toxoid (TT)

Experiment	TT36C	TT36C + APC	TT36C + APC + TT*
1	610 ± 169	2,097 ± 1,052	19,818 ± 401
2	76 ± 18	2,009 ± 1,043	30,756 ± 4,556
3	102 ± 55	114 ± 25	6,616 ± 361

* Cells were cultured as described in Materials and Methods. Proliferation was measured on day 3 by tritiated thymidine (^{3}H-TdR) incorporation. Values are the mean ± SEM cpm of quadruplicate determinations, and maximal reactivities are shown. ^{3}H-TdR uptake by peripheral blood mononuclear cells cultured alone was 400 ± 126 cpm (mean ± SEM of 3 experiments; range 27–561 cpm).

To compare the relative potencies of Pca with Napa and of Hyd with Phth in this assay system, TT36C was treated with a broad range of concentrations of these 4 drugs. Each treated line was cultured alone or challenged with autologous APCs with and then without an optimal amount of TT.

Figure 1 illustrates the results of these experiments. The maximum antigen-induced proliferative response frequently varied among the treated lines; therefore, to compare results between the treated cell lines, the data in Figure 1 are expressed as the ratio of the T cell response to APC alone divided by the maximum antigen-induced response, expressed as a percentage, and the data are plotted against the concentration of drug used to treat the cells. Both Pca and Napa induced autoreactivity; however, approximately 100 times as much Napa was required to induce a comparable amount of autoreactivity as Pca, indicating that it is 100 times less potent in this assay.

In other experiments testing autoreactivity induced by Pca and Napa, only 100 μM Napa consistently induced significant autoreactivity, while Pca induced autoreactivity between $10^{-6}M$ and $10^{-4}M$. In contrast, in the experiment shown in Figure 1, both Hyd and Phth induced autoreactivity over the range of concentrations tested, and Phth induced a greater amount of autoreactivity at all concentrations tested.

In other experiments, no significant differences were observed in the amount of autoreactivity induced

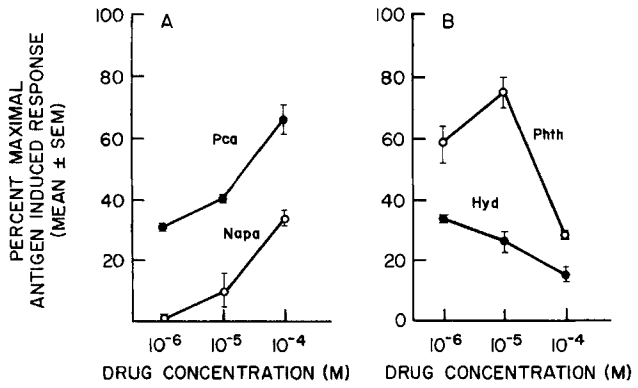

Figure 1. Comparison of antigen reactivity and drug-induced autoreactivity. T cells were treated with the indicated concentrations of **A**, procainamide (Pca) or N-acetylprocainamide (Napa) or **B**, hydralazine (Hyd) or phthalazine (Phth). Four days later, the cells were cultured as described in Materials and Methods. Proliferation was measured by tritiated thymidine (^{3}H-TdR) uptake on day 3. Results are expressed as the ratio of the T cell response to peripheral blood mononuclear cells (PBMC) divided by the maximum response to PBMC plus tetanus toxoid for each drug concentration tested. Values are the mean ± SEM of quadruplicate determinations. ^{3}H-TdR incorporation of the T cells cultured alone was 411 ± 138 cpm, and the antigen-induced response was 7,656 ± 1,040 cpm (mean ± SEM of 12 determinations).

by $10^{-6}M$ and $10^{-5}M$ Hyd; however, the decreased autoreactivity observed at $10^{-4}M$ Hyd and $10^{-4}M$ Phth was reproducible. Moreover, $10^{-4}M$ is close to the maximum concentration of Hyd and Phth at which the cloned cells survive, and in some experiments this concentration was toxic, suggesting that the decreased autoreactivity observed may be due to a toxic effect on the cells. No such toxic effects were observed for Pca and Napa in the concentration range tested.

Inhibition of DNA methylation by Pca, Napa, Hyd, and Phth. To test whether Pca, Napa, Hyd, or Phth inhibit DNA methylation, the human leukemic Jurkat T cell line was treated with various concentrations of these drugs. Similar concentrations of Hu served as a negative control, and 1 μM 5-azaC was a positive control.

The cells were harvested 4–7 days later, washed, and the DNA isolated. Previous studies had demonstrated that 5-azaC gave maximal inhibition of DNA methylation on day 4, while Pca and Hyd inhibited maximally after 7 days of continuous culture with the drugs (5). The purified DNA was digested with DNase I, phosphodiesterase, and alkaline phosphatase, and the resultant nucleosides were separated and quantitated by HPLC analysis. Figure 2 shows the decrease in d^mC content induced by Pca, Napa, Hyd, Phth, and 5-azaC at the lowest concentration giving significant ($P < 0.05$) inhibition, expressed as the percentage of d^mC content, compared with untreated controls. For reference, d^mC represents 3.98 ± 0.2% of total dC in Jurkat DNA (mean ± SEM of 12 determinations).

Pca, Napa, Hyd, Phth, and 5-azaC significantly decreased d^mC content, compared with untreated controls, while Hu failed to inhibit DNA methylation at similar concentrations. At $10^{-6}M$, Napa also decreased d^mC content (mean ± SEM of 3 determinations 74 ± 7%; $P < 0.1$), as did $10^{-6}M$ Pca (mean ± SEM of 3 determinations 56 ± 15%; $P < 0.1$). The decreases in d^mC content induced by Pca, Napa, Hyd, and Phth were not significantly different at comparable concentrations, nor were they significantly different from that induced by 1 μM 5-azaC. All 4 drugs, however, gave significant methylation inhibition compared with untreated controls, and it is noteworthy that Napa gave some inhibition at $10^{-6}M$, a concentration that failed to induce autoreactivity.

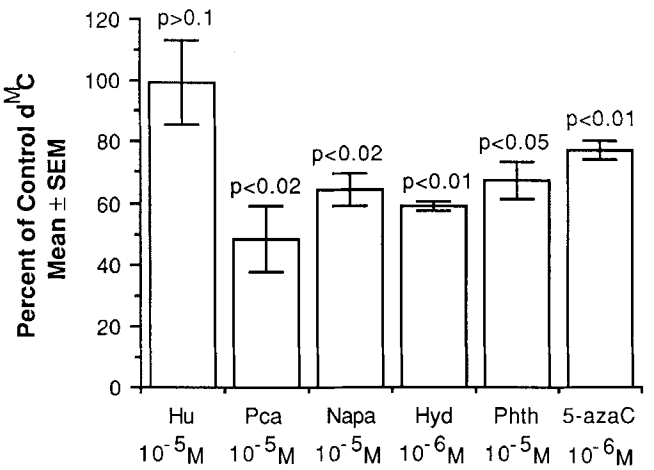

Figure 2. Inhibition of DNA methylation. Jurkat cells were treated with $10^{-7}M$ to $10^{-4}M$ hydroxyurea (Hu), Pca, Napa, Hyd, Phth, or $10^{-6}M$ 5-azacytidine (5-azaC). Seven days later the cells were recovered, and the DNA was isolated and digested as described in Materials and Methods. The 5-azaC–treated cells were harvested after 4 days of treatment. Nucleosides were separated by high performance liquid chromatography, and deoxymethylcytidine (d^mC) and deoxycytidine were quantitated. The d^mC content was calculated as described in Materials and Methods. Values are the mean ± SEM, relative to untreated Jurkat cells, of 2 separate experiments for Hyd and Phth, and 3 separate experiments for Pca, Napa, 5-azaC, and Hu. The results represent the lowest concentration of the drugs inducing significant ($P < 0.05$) inhibition. The d^mC content of untreated Jurkat cells was 3.98 ± 0.20% (mean ± SEM of 12 determinations). See Figure 1 for other definitions.

DISCUSSION

The experiments presented compare 2 drugs, known to induce a lupus-like syndrome, with analogs, using assays that test induction of autoreactivity and inhibition of DNA methylation in vitro. The data demonstrate that Pca, Napa, Hyd, and Phth each decreased d^mC in DNA, thus resembling the effects of 5-azaC, a recognized inhibitor of DNA methylation (1,14). Moreover, all 4 drugs induced autoreactivity in cloned, antigen-specific T cell lines, again similarly to 5-azaC. As was found with 5-azaC, at least a portion of the treated cells retained specificity for TT (4). These effects occurred at concentrations that are achieved in vivo by Pca and Hyd. Therapeutic levels of hydralazine are 0.5–5.0 μM (15), and Pca levels achieved in vivo are in excess of 10 μM (6,16). These results suggest that the lupus caused by Pca and Hyd may result, in part, from induction of autoreactive T cells in vivo.

To test this hypothesis, we compared relative potencies of Pca and Napa and of Hyd and Phth in the autoreactivity assay. Napa is a Pca derivative that is an effective antiarrhythmic agent like Pca, but it rarely induces the lupus-like syndrome (6–8). Hyd is a Phth derivative containing a hydrazine side chain (9,10). Hydrazine has been implicated in drug-induced lupus (17), and additionally is known to covalently react with thymidine and deoxycytidine (3), leading to the speculation that the hydrazine side chain of Hyd may be responsible for the lupus-like syndrome (9).

Phth was found to be a potent inducer of T cell autoreactivity and a DNA methylation inhibitor like Hyd, suggesting that if these properties are involved in drug-induced lupus, the hydrazine side chain is not required. More interestingly, both Pca and Napa were found to induce autoreactivity; however, Napa was 100 times less potent in inducing autoreactivity than Pca, suggesting that Napa may fail to induce lupus because insufficient concentrations are achieved in vivo to cause T cell autoreactivity. For this reason, Pca may be more "lupogenic" than Napa.

When Pca and Napa were compared for the ability to inhibit DNA methylation, however, both drugs induced significant hypomethylation at 1 μM, a concentration at which Napa did not induce autoreactivity. This suggests that inhibition of DNA methylation may not always lead to T cell autoreactivity. Other investigators have observed a dissociation between the biologic effects and degree of hypomethylation when various methylation inhibitors were compared, and this may well be the case with Pca and Napa (3).

Alternatively, Jurkat, the leukemic T cell line used to measure DNA methylation, and TT36C, the IL-2–dependent line used to measure autoreactivity, may differ in their sensitivity to Pca and Napa. Ideally, autoreactivity and DNA methylation should be measured in the same cell line; however, the MHC allele recognized by Jurkat cells is unknown, and insufficient numbers of T cells are obtainable from conventional cloning techniques to perform methylation studies, which require 30–50 $\times$ 10^6 cells per determination.

The mechanism by which these drugs inhibit DNA methylation is unknown. 5-azaC is incorporated into newly synthesized DNA, where it covalently binds the methyltransferase that methylates cytidine in the newly synthesized DNA strand when 5-methyldeoxycytidyldeoxyguanosine is recognized on the parent strand (18). Pca and Hyd bind DNA (19–21), and may interfere with the action of the methyltransferase, resulting in hypomethylation of the newly synthesized strand.

Our findings support the contention that hydralazine and procainamide induce a lupus-like syndrome by causing T cell autoreactivity. In the chronic graft-versus-host model of lupus, CD4+ T cells responding to self-MHC determinants in the host cause a lupus-like disease (22), and the autoreactive cells induced by inhibitors of DNA methylation may mediate a similar process. Since DNA methylation inhibitors alter cellular phenotype and function by inducing gene expression, it is reasonable to propose that T cell autoreactivity induced by these agents is mediated by a similar mechanism.

It remains to be determined whether other agents that induce gene expression, such as viruses or ultraviolet light (23), could also induce autoimmunity by activating certain genes determining T cell autoreactivity. Identification of the specific genes activated by DNA methylation inhibitors will be crucial in testing their role in T cell autoreactivity.

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