

Treating Activated CD4⁺ T Cells with Either of Two Distinct DNA Methyltransferase Inhibitors, 5-Azacytidine or Procainamide, Is Sufficient to Cause a Lupus-like Disease in Syngeneic Mice

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Abstract

Human antigen-specific CD4⁺ T cells become autoreactive after treatment with various DNA methylation inhibitors, including 5-azacytidine, procainamide, and hydralazine. This suggests a mechanism that could contribute to the development of some forms of autoimmunity. In this report we have asked whether T cells treated with DNA methylation inhibitors can induce autoimmunity. Murine CD4⁺ T cells were treated with 5-azacytidine or procainamide and were shown to respond to syngeneic antigen-presenting cells, similar to CD4⁺ human T cell clones treated with these drugs. Functional characterization demonstrated that cells treated with either drug spontaneously lysed syngeneic macrophages and secreted IL-4, IL-6, and IFN- γ . Adoptive transfer of 5-azacytidine- or procainamide-treated cells into unirradiated syngeneic recipients induced an immune complex glomerulonephritis and IgG anti-DNA and antihistone antibodies. These experiments demonstrate that T cells treated with either of two distinct DNA methyltransferase inhibitors are sufficient to induce a lupus-like disease. It is possible that the lysis of macrophages, together with the release of cytokines promoting B cell differentiation, contributes to the autoantibody production and immune complex deposition. These results suggest that environmental agents that inhibit DNA methylation could interact with T cells *in vivo* to produce a lupus-like illness, a mechanism that could have relevance to drug-induced and idiopathic lupus. (*J. Clin. Invest.* 1993. 92:38–53.) Key words: lupus • DNA methylation • T cell • procainamide • 5-azacytidine

Introduction

It is generally accepted that autoreactive T cells are responsible for some autoimmune diseases, but the mechanisms causing autoreactivity are controversial. Conventional models postulate that autoreactive T cells arise by failure of thymic deletion or in response to cross-reactive antigens (1–5). However, recently it was reported that under certain conditions antigen-specific T cells can become autoreactive. Normally, CD4⁺ T cells respond to antigen presented by accessory cells such as

macrophages (M ϕ),¹ but are not activated by M ϕ alone (6). However, if first treated with DNA methyltransferase inhibitors, antigen-specific CD4⁺ T cells can be activated by self determinants on autologous M ϕ without antigen (7–9). This autoreactivity is accompanied by changes in gene expression (10, 11) and is presumably due to altered expression of one or more genes regulated in part by mechanisms associated with DNA methylation. Autoreactivity has been documented after treatment of cloned (7, 9) and polyclonal (8) human T cells using structurally unrelated DNA methylation inhibitors such as 5-azacytidine (5-azaC), procainamide (Pca), and hydralazine (7–9, 12). The reactivity to self determinants resembles an alloreactive response (7). In mice, adoptive transfer of semiallogeneic CD4⁺ cells produces a chronic graft-versus-host (GvH) disease with some features of systemic lupus erythematosus (SLE) (13, 14). The similarity of the autoreactive cells to the cells producing chronic GvH disease, together with the well-established observation that Pca and hydralazine can cause a lupus-like disease (15), led us to propose that T cells made autoreactive with DNA methylation inhibitors might induce a disease with features of murine chronic GvH or human drug-induced lupus.

In this report, we have asked whether T cells made autoreactive with DNA methyltransferase inhibitors have pathological significance and can induce autoimmunity. The results demonstrate that adoptive transfer of activated, CD4⁺ T cells treated with either 5-azaC or Pca will cause an immune complex glomerulonephritis, IgG deposition at the dermal-epidermal junction, and IgG anti-DNA and antihistone antibodies. These experiments support the hypothesis that inhibiting methylation of newly synthesized T cell DNA with chemicals such as Pca or perhaps other environmental agents may contribute to the pathogenesis of lupus-like diseases.

Methods

T cell isolation and culture. The conalbumin-reactive cloned T cell line D.10.G4.1 (H-2^k) (16) was obtained from the American Type Culture Collection (Rockville, MD) and maintained according to their directions. Alloreactive CD4⁺ T cell lines were obtained by treating splenocytes from female DBA/2 mice (Charles River, Cambridge, MA) (H-2^d) with anti-CD8 (Becton Dickinson & Co., Sunnyvale, CA) plus complement (Pel-Freez Biologicals, Brown Deer, WI) as described (8). 10⁶ cells were then cultured with 10⁶ irradiated (2,000 rad)

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1. Abbreviations used in this paper: APC, antigen-presenting cells; 5-azaC, 5-azacytidine; BUN, blood urea nitrogen; dC, deoxycytosine; d^mC, deoxymethylcytosine; dsDNA, double-stranded DNA; GAM, goat anti-mouse; GvH, graft-versus-host; M ϕ , macrophages; Pca, procainamide; ssDNA, single-stranded DNA.

C57BL/6 (Charles River) (H-2^b) splenocytes in 2 ml RPMI supplemented with 10% FCS (HyClone Laboratories Inc., Logan, UT), 2 mM glutamine, 40% IL-2-containing supernatant (17), 100 IU/ml penicillin, 100 µg/ml streptomycin, and 5×10^{-5} M 2-mercaptoethanol, using flat-bottomed 24-well plates (Nunc; from Gibco Laboratories, Grand Island, NY). After 7–10 d the cells were restimulated with $0.5\text{--}1.0 \times 10^6$ irradiated C57BL/6 splenocytes and treated with 5-azaC (Sigma Chemical Co., St. Louis, MO or Aldrich Chemical Co., Milwaukee, WI) or Pca (Sigma Chemical Co.) the next day. 6 d later the cells were tested for autoreactivity or given in adoptive transfer. At this time point, staining with anti-CD4-FITC (Becton Dickinson & Co.) or anti-CD8-FITC (Coulter Corp., Hialeah, FL) followed by cytofluorography on a flow cytometer (EPICS C, Coulter Corp.) (8) demonstrated that the cells were usually $\geq 96\%$ CD4⁺. In some experiments the cells were maintained for longer periods by restimulation with irradiated C57BL/6 splenocytes every 7–10 d. Con-A-activated T cell lines were similarly generated by culturing CD8-depleted DBA/2 splenocytes with 5 µg/ml Con A (Pharmacia LKB Biotechnology Inc., Uppsala, Sweden). These lines were also maintained in IL-2-containing media and restimulated every 7–10 d with 1 µg/ml Con A and $0.5\text{--}1.0 \times 10^6$ irradiated syngeneic splenocytes. Con A-activated lines were treated with 5-azaC 3 d after restimulation.

Measurement of T cell proliferative and cytotoxic responses. Proliferation assays were performed as described (7), using irradiated splenocytes as antigen-presenting cells (APC). To test cytotoxic responses, peritoneal Mφ were obtained using thioglycolate broth (18, 19). This procedure routinely gives preparations containing 80–90% Mφ (18, 19), and morphological analysis demonstrated similar percentages in these preparations. These cells were labeled with ⁵¹Cr and used as targets (11, 20). Percent cytotoxicity was calculated as [(experimental – background release)/(total incorporation – background release)] × 100 (20). Where indicated, mAbs to Ia^d (Becton-Dickinson & Co.), H-2K^d (Pharmingen, San Diego, CA), Ia^k (Pharmingen), or to CD11a (LFA-1 α) (M17/4.2; obtained from the American Type Culture Collection) were added to the cultures. These monoclonals were used at concentrations equal to or greater than those recommended by the manufacturer to completely saturate the relevant cells.

Adoptive transfers. For adoptive transfer experiments, 5×10^6 viable treated or untreated cells were given intravenously to unirradiated young (< 12 wk) female DBA/2 mice. Viability was assessed by trypan blue exclusion and was similar in treated and untreated cells. Where indicated, the 5-azaC-treated cells were killed by incubation at 60°C for 30 min before adoptive transfer; killing was verified by trypan blue exclusion.

Laboratory analysis. Phycoerythrin-conjugated mAbs to CD4 were purchased from Becton Dickinson & Co. (San Jose, CA), and FITC-conjugated antibodies to CD8 and Thy-1.2 were purchased from Coulter Corp. mAbs specific for murine CD11a were isolated from the supernatant of the M17/4.2 hybridoma line (21) obtained from the American Type Culture Collection. The anti-CD11a antibodies were used with the FITC-conjugated F(ab')₂ fragment of goat anti-mouse (GAM) Ig (Cappel Laboratories, West Chester, PA) in indirect immunofluorescence assays as previously described (11). In all experiments negative controls included cells stained with FITC-conjugated nonspecific mouse Ig (Coulter Corp.) alone, and experiments using indirect staining also included FITC-conjugated GAM Ig. The stained cells were analyzed on a flow cytometer (model EPICS C or ELITE; Coulter Corp.) using previously described protocols (8). Whole blood leukocyte counts were determined using a particle counter (model ZF; Coulter Corp.) and hemoglobin concentrations were determined using the cyanmethemoglobin method and a hemoglobinometer (Coulter Corp.). Serum blood urea nitrogen (BUN) determinations were made using the urease method, and creatinine determinations using the peroxidase method, both analyzed on an Ektachem DT system (Eastman Kodak Co., Rochester, NY). Leukocyte counts, hemoglobin, BUN, and creatinine determinations were performed by the University of Michigan Unit for Laboratory Animal Medicine using equipment standardized and calibrated daily. Urine was monitored for blood and pro-

tein using Chemstrips (Boehringer Mannheim Corp., Indianapolis, IN).

Cytokine analysis. ELISA kits for IL-4 and IL-6 were purchased from Endogen Inc. (Boston, MA) and ELISA kits for IFN-γ from Gibco Bethesda Research Laboratories (Gaithersburg, MD). The assays were performed according to the manufacturers' directions. The minimum concentration detected by the IL-4 ELISA is 5 pg/ml, 15 pg/ml for the IL-6 ELISA, and 50 pg/ml for IFN-γ. IL-2 bioassays were performed using CTLL, obtained from the American Type Culture Collection and maintained according to the protocols provided by the American Type Culture Collection, or D.10.G4.1. IL-2 activity was determined using proliferation assays as previously described, with recombinant IL-2 or MLA-144 supernatant as positive controls (22).

Histological analysis and immunofluorescence. Tissue was prepared and stained with hematoxylin and eosin or FITC-conjugated antisera specific for IgG, IgM, IgA, and C₃ (Sigma Chemical Co.) using standard procedures. Histological changes were graded using the lupus renal pathology scoring system (23). Tissues were prepared for electron microscopy as previously described (24) and examined with a transmission electron microscope (model 400T; Philips Electronic Instruments Co., Mahwah, NJ).

Autoantibody assays. For radioimmunoprecipitation assays, total serum protein was adjusted to 0.2 µg/ml then incubated with single- or double-stranded [³H]DNA (New England Nuclear, Boston, MA) and precipitated with antisera to mouse Ig (25). Initial ELISAs for single- (ss) and double-stranded (ds) DNA ds were performed as described, with and without inhibition by purified ss- and dsDNA to confirm specificity (25). In later studies, anti-ssDNA and anti-dsDNA were measured by coating Immulon 4 plates (Dynatech Laboratories, Inc., Chantilly, VA) with 100 µl purified single-stranded calf thymus DNA (Sigma Chemical Co.) or purified pUC18 plasmid (25 µg/ml in 0.01 M PBS, pH 7.2) for 18 h at 4°C. The plates were then washed five times with PBS 0.5% Tween 20 (Sigma Chemical Co.) and then sealed with 5% BSA in PBS with 0.1% gelatin for 2 h at 23°C. The serum samples were then incubated for 18 h at 4°C, washed five times as before, then 100 µl horseradish peroxidase-conjugated goat anti-mouse heavy- and light-chain specific antisera was added at 1:2,500 final dilution (Gibco Bethesda Research Laboratories) for 2 h at 23°C. The plates were then washed five times, and were developed using o-dianisidine (Sigma Chemical Co.) as previously described (26). OD was measured at 405 nm using a spectrometer (model Titertek Multiskan; Flow Laboratories, McLean, VA). Positive controls always included pooled NZB/W sera. A 1:100 dilution of this sera produced an OD of 0.178±0.084 (mean±SD of 12 experiments, range 0.060–0.288), which was well within the linear range of the instrument. To measure antihistone antibodies, identical assays were used, except that plates were coated with purified calf thymus histone proteins (type II-s; Sigma Chemical Co.). A 1:100 dilution of NZB/W sera produced an OD of 0.170±0.073 (mean±SD of 10 experiments, range 0.061–0.279) in these assays. Where indicated, purified ssDNA or histone proteins were added with the antisera for 30 min before adding the antisera to the well. To measure antithymocyte and antierythrocyte antibodies, antisera was incubated with DBA/2 thymocytes or erythrocytes for 30 min at 4°C, and then washed three times with PBS containing 1.0% horse serum and 0.1% sodium azide, and then stained with GAM-FITC (Cappel Laboratories) for 30 min at 4°C. The cells were then washed three times, fixed in 1% Formalin, and then analyzed on a EPICS C or ELITE flow cytometer as described above.

Determination of DNA deoxycytosine (dC) and deoxymethylcytosine (d^mC) content. EL-4 cells were grown in RPMI/10% FCS supplemented with penicillin, streptomycin, and glutamine as described above. DNA was isolated and hydrolyzed to nucleosides using DNase I, phosphodiesterase, and alkaline phosphatase as described (9). Total dC and d^mC content were determined by reverse-phase HPLC using previously described protocols (27), and results are presented as the mean±SD of duplicate determinations.

Statistical analysis. The difference between means was compared using Student's *t* test. The difference between groups was tested using

chi-square analysis with Yates' correction for small sample numbers or by analysis of variance, using Systat Inc. (Evanston, IL) software.

Results

5-azaC-treated murine CD4⁺ cells respond to syngeneic APC. Since previous experiments studied human lymphocytes, initial experiments determined if 5-azaC also induced autoreactivity in murine T cells. The conalbumin-reactive cloned line D.10.G4.1 (H-2^k) was treated with 5-azaC and then challenged with irradiated H-2^k APC. Fig. 1 *a* confirms that optimal activation of untreated D10 cells requires APC and conalbumin.

Treatment with 5-azaC significantly increased the response to APC alone. To test the generality of this phenomenon, similar experiments were performed using short-term T cell cultures. DBA/2 (H-2^d) splenocytes were cytolytically treated with anti-CD8 and complement, activated with irradiated C57BL/6 (H-2^b) splenocytes, and expanded with IL-2 and restimulation with irradiated C57BL/6 splenocytes. The cells were treated with 5-azaC, and 6 d later were challenged with irradiated syngeneic splenocytes. The response of 2×10^4 untreated cells to 10^5 APC alone was $2,522 \pm 677$ cpm. This relatively low proliferative response seen in the untreated cells presumably represents the syngeneic mixed lymphocyte reaction (28) or a response to xenogeneic proteins in the culture media. In contrast,

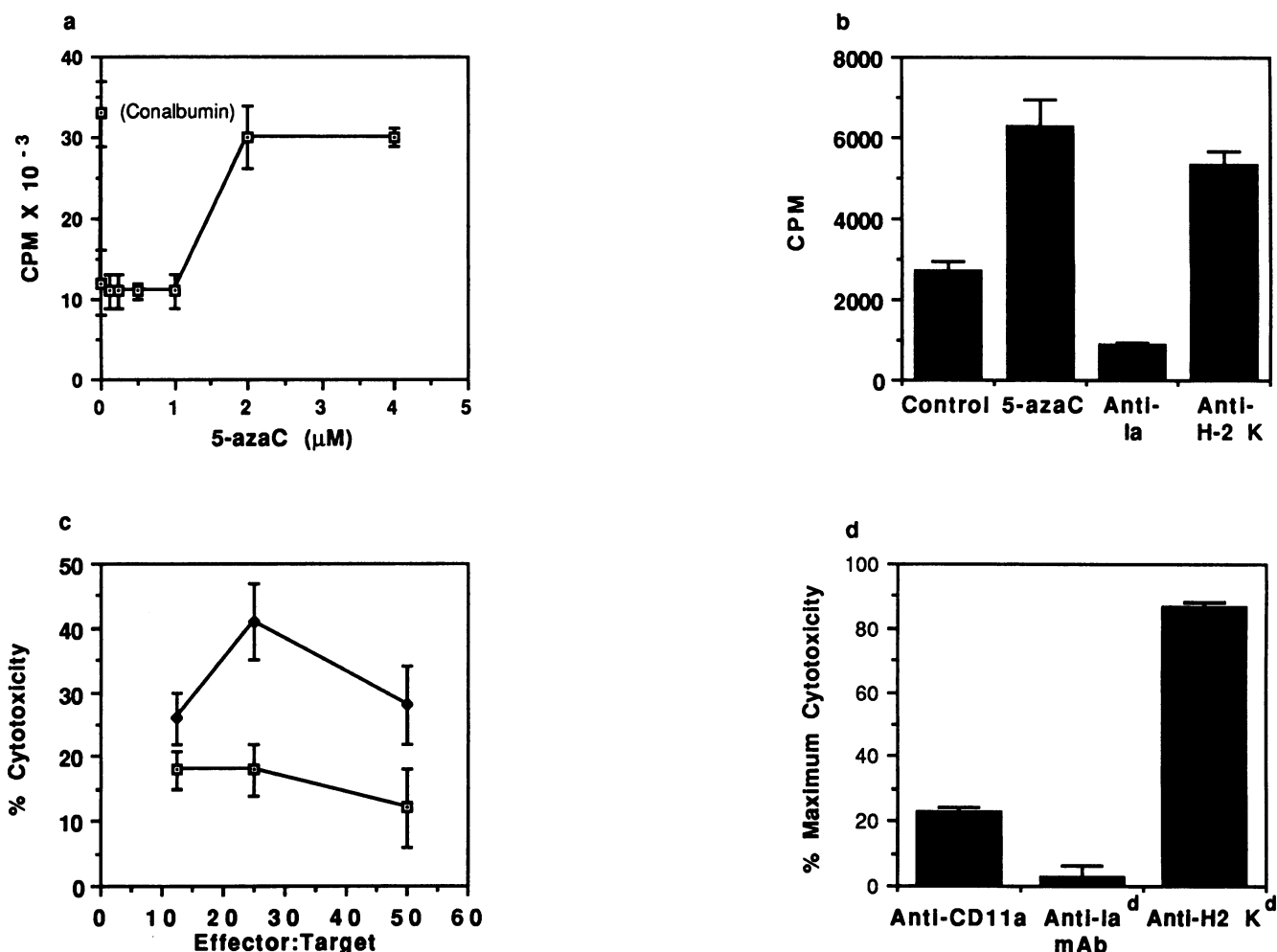


Figure 1. Effect of 5-azaC on CD4⁺ murine T cells. (a) D.10.G4.1 (H-2^k) cells were treated with the indicated concentrations of 5-azaC. 6 d later the cells were washed and 5×10^4 responder cells challenged with 8×10^4 irradiated (2,000 rad) H-2^k APC. Proliferation was measured by [³H]TdR incorporation, and results represent the mean \pm SEM of six determinations. The response of untreated cells to APC + conalbumin is also shown. ($P < 0.005$, untreated vs. $4 \mu\text{M}$ 5-azaC). (b) CD4⁺, proliferating, alloreactive DBA/2 cells were treated with 5-azaC, and 6 d later 2×10^4 treated (5-azaC) or untreated (Control) cells were washed and challenged with 10^5 irradiated DBA/2 APC. Proliferation was measured by [³H]TdR incorporation, and results represent the mean \pm SEM of triplicate determinations. Where indicated, mAbs to Ia^d or H2-K^d were added at 100 ng/ml to cultures containing 5-azaC-treated T cells and syngeneic APC. Tritiated thymidine incorporation by the T cells cultured alone was < 600 cpm in all cases, and the response of the untreated cells to 10^5 irradiated C57BL/6 cells was $5,637 \pm 780$ cpm (mean \pm SEM). ($P < 0.01$, control vs. 5-azaC, and $P < 0.001$, 5-azaC vs. anti-Ia). (c) CD4⁺ alloreactive DBA/2 T cells were treated with 5-azaC as in b. 6 d later treated or untreated effector cells were washed and cultured with 2×10^4 ⁵¹Cr-labeled syngeneic peritoneal exudate cells (Mφ) at the indicated ratios. 16 h later ⁵¹Cr release was determined. The results represent the mean \pm SEM of replicates of six. Open squares, untreated cells; filled diamonds, treated cells ($P < 0.01$ at 25:1). (d) CD4⁺ alloreactive DBA/2 cells were treated with 5-azaC, and then cultured with ⁵¹Cr-labeled syngeneic Mφ as in c, but at an effector/target ratio of 25:1. The indicated mAbs were added at a final concentration of 1–2 $\mu\text{g}/\text{ml}$. Results represent the mean \pm SEM of two independent experiments, each performed in triplicate or quadruplicate, and are expressed relative to maximum cytotoxicity ($50 \pm 17\%$, mean \pm SEM of the two experiments) ($P < 0.02$, anti-H-2^K vs. anti-CD11a).

identical stimulation of 5-azaC-treated cells gave $18,397 \pm 338$ cpm (mean \pm SEM above background, $P < 0.001$). Fig. 1 b shows a second experiment, in which the autoreactive response was inhibited by mAb to class II but not class I MHC determinants, similar to human T cells (7, 8). In serial repeats of these experiments with various T cell lines, optimal autoreactivity was usually induced with 0.125–2.0 μ M 5-azaC. The variability in optimal concentration could be attributed in part to differences between T cell lines as previously described (7).

CD4⁺ T cells have recently been shown to lyse stimulating M ϕ (20, 29–31). To confirm the effect of 5-azaC, the alloreactive T cells were treated with 5-azaC and tested for syngeneic M ϕ killing (Fig. 1 c). Untreated cells demonstrated low-level (10–18%) cytolysis, which may be analogous to the syngeneic mixed lymphocyte reaction. The significance of this low level response is uncertain because adoptive transfer experiments, presented below, demonstrated no pathological effects induced by these cells. However, similar to results obtained using proliferation assays, the 5-azaC-treated cells demonstrated significantly greater killing, with a maximum at an effector/target ratio of 25:1. In two repeats of this experiment, little to no killing was seen at effector/target ratios $< 5:1$, and killing did not increase beyond that which occurred at an effector/target ratio of 25:1, even at ratios up to 100:1. M ϕ lysis was inhibited by mAb to class II better than to class I MHC determinants (Fig. 1 d), similar to the proliferative response, and anti-LFA-1 (anti-CD11a) also inhibited cytotoxicity, similar to other reports (32).

Effect of 5-azaC-treated CD4⁺ T cells in vivo. We next examined the effect of untreated and 5-azaC-treated T cells on syngeneic hosts, using the same alloreactive CD4⁺ cells. Healthy, unirradiated DBA/2 mice were given one to three intravenous injections of 5×10^6 5-azaC-treated cells. Experiments in the GvH system have shown that adoptive transfer of $3\text{--}5 \times 10^6$ cells is necessary to induce GvH disease (33). Controls included age- and sex-matched DBA/2 mice given one to five injections of the same number of untreated (control) T cells or 12 weekly intravenous injections of HBSS. The results of urinalyses on these mice are shown in Table I. None of the mice receiving control cells or HBSS developed hematuria, and the majority showed zero to trace proteinuria. Rarely proteinuria of 30 mg/dl was observed in the control groups, but this was never sustained, and on repeat testing was always negative to trace. In contrast, a majority of mice receiving 5-azaC-treated cells developed significant proteinuria, defined as ≥ 30 mg/dl present for ≥ 2 wk, and hematuria, defined as ≥ 50 erythrocytes/ μ l on at least one occasion. The maximum proteinuria was ≥ 100 mg/dl in the majority, and maximum hematuria was ≥ 250 erythrocytes/ μ l. The urinary abnormalities were usually first seen 7–14 d after injection of the cells. Hematuria was seen between weeks 1 and 3, and lasted 7–14 d. In contrast, proteinuria of ≥ 30 mg/dl was more persistent, lasting up to ≥ 7 wk in the majority. Hematuria is somewhat unusual in murine models of renal disease (34, 35), so the urinary sediment was characterized further. Fig. 2 shows photomicrographs of urine specimens from mice with active hematuria. Fig. 2 A shows an uncentrifuged urine specimen on a hemocytometer and demonstrates the presence of > 250 erythrocytes/ μ l. Fig. 2, B–D shows various cellular casts, indicative of renal disease.

Additional controls included DBA/2 mice given three injections of heat-killed 5-azaC-treated cells or freshly isolated,

Table I. 5-azaC-treated T Cells Induce Proteinuria and Hematuria

Treatment [†]	Injections per mouse	Number of mice studied	Number of mice with*	
			Hematuria	Proteinuria
HBSS	12	5	0/5	0/5
Control T cells		20	0/20	0/20
Experiment 1	1	3	0/3	0/3
Experiment 2	2	10	0/10	0/10
Experiment 3	3	4	0/4	0/4
Experiment 4	5	3	0/3	0/3
5-aza C-treated				
T cells		20	16/20	16/20
Experiment 1	1	5	3/5	4/5
Experiment 2	2	10	8/10 [§]	7/10 [§]
Experiment 3	3	5	5/5	5/5
Heat-killed,				
5-aza C-				
treated T cells	3	6	0/6	0/6
5-aza C-Treated,				
unstimulated				
CD4+ T cells	3	6	0/6	0/6

* Results are expressed as the number positive/total number of mice per group.

[†] 5×10^6 CD4⁺, proliferating, alloreactive DBA/2 T cells or an equal volume of HBSS were administered intravenously to healthy, young (< 12 -wk-old) female DBA/2 mice. Serial injections were spaced ≥ 2 wk apart. Urinalyses were scored as positive if they demonstrated ≥ 30 mg/dl protein for ≥ 2 wk and positive for hematuria if ≥ 50 erythrocytes/ μ l were found. Where indicated, the cells were treated with 5-azaC as described in Fig. 1. In some experiments, 5-azaC-treated cells were killed by incubation at 60°C for 30 min. Unstimulated CD4⁺ cells were obtained as described in Methods and were treated with 5-azaC for 3 h before adoptive transfer.

[§] $P < 0.005$ by chi-square analysis relative to controls receiving two injections of untreated cells.

^{||} $P < 0.001$ by chi-square analysis relative to controls receiving three injections of 5-azaC-treated cells.

unstimulated CD4⁺ splenocytes treated with 5-azaC for 3 h before adoptive transfer. These controls were performed in parallel with mice receiving three injections of proliferating, 5-azaC-treated or untreated cells (Table I). The 5-azaC-treated, unstimulated cells were tested for autoreactivity in assays similar to those described above and did not demonstrate greater responsiveness to syngeneic APC than did untreated, unstimulated cells. None of the mice receiving heat-killed 5-azaC-treated cells, unstimulated 5-azaC-treated cells, or untreated proliferating cells developed hematuria or proteinuria. In contrast, all mice receiving three injections of viable 5-azaC-treated, proliferating cells developed active urinary sediments. These results suggest that viable, proliferating, 5-azaC-treated T cells are required to produce the urinary abnormalities, and that activation or 5-azaC-treated alone is not sufficient.

Histological studies were performed on 10 mice given one to five injections of control cells and 14 mice given one to five injections of 5-azaC-treated cells (Fig. 3). Histological analysis was usually performed 2 wk after the last injection of cells. All mice that received treated cells and developed abnormal urinalyses had evidence for a proliferative glomerulonephritis. The

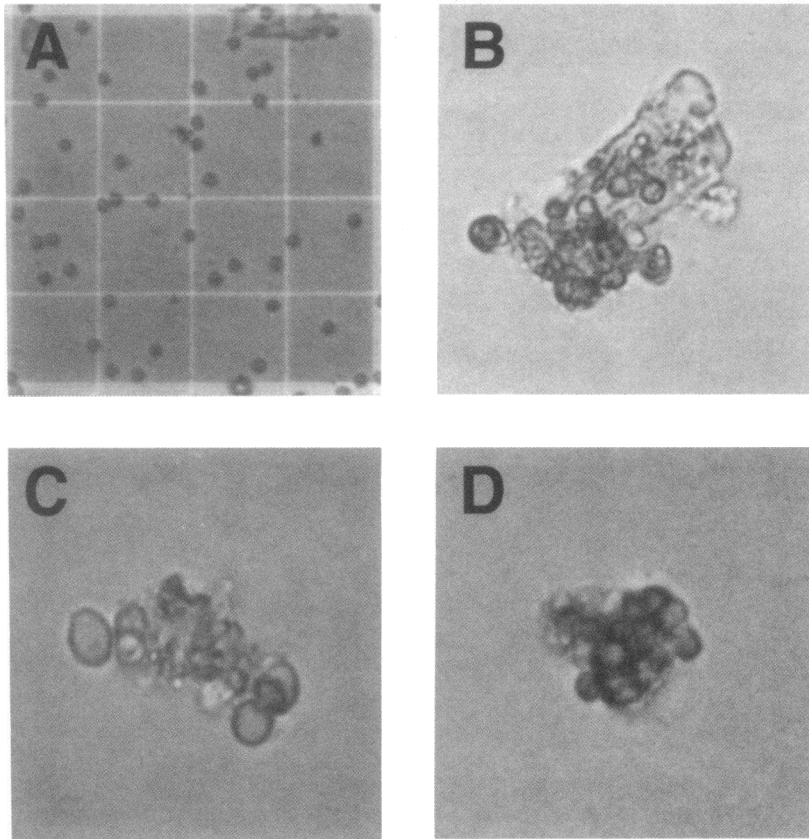


Figure 2. Photomicrographs of urinary sediments from mice receiving 5-azaC-treated, proliferating cells. (A) Erythrocytes on a hemocytometer. The grid is $0.2 \times 0.2 \text{ mm}^2$ and the chamber is 0.1 mm deep. $\times 250$. (B–D) Cellular casts $\times 750$. The casts shown in B and C appear to contain leukocytes, and the cast shown in D contains erythrocytes.

glomerular alterations included variable mesangial proliferation, increased matrix deposition, and variable neutrophilic infiltration (see also Figs. 9 and 10). Some focal scarring and fibrosis was observed in mice receiving multiple injections of 5-azaC-treated cells. Using the lupus renal pathology scoring system (21), the activity index was 3–4+ and chronicity index was 2–3+. In contrast, none of the mice that received untreated cells developed abnormal renal histology. Additional studies were performed on three mice receiving heat-killed, 5-azaC-treated cells and three mice receiving unstimulated, 5-azaC-treated cells. No histological abnormalities were observed in mice receiving these control injections.

Immunofluorescence was performed on kidney sections from four mice given 5-azaC-treated cells and four mice given untreated T cells. Each group received injections at week 0 and 2 and were killed at week 4. The mice receiving 5-azaC-treated cells had active hematuria but the mice receiving untreated cells did not. The mice that received 5-azaC-treated T cells had significantly greater glomerular IgM, IgG, and IgA deposition than did control mice (Fig. 4). C_3 deposition was also seen in glomeruli from mice receiving 5-azaC-treated but not untreated cells (data not shown). The immunoglobulin and complement deposition was mesangial in distribution. In addition, IgG deposition was seen at the dermal–epidermal junction, resembling a lupus band test (Fig. 5). The glomerular immunoglobulin deposition correlated with active hematuria, and no difference in immunofluorescence was seen between mice receiving 5-azaC-treated or control cells once the hematuria resolved. However, the histological changes of increased mesangial matrix and hypercellularity persisted up to 12 wk after injection of the cells. No specific abnormalities were observed

in the lungs, liver, spleen, thymus, brain, joints, muscle, or salivary glands of mice receiving treated cells. One mouse developed bowel ischemia and peritonitis secondary to mesenteric vasculitis after five injections of 5-azaC-treated cells (data not shown). However, the significance of this lesion in a single mouse is uncertain.

Effect of 5-azaC-treated $CD4^+$ T cells on clinical laboratory parameters. Whole blood leukocyte counts and hemoglobin concentrations as well as serum BUN and creatinine were measured in the group of mice receiving three injections of 5-azaC-treated or untreated cells. Determinations were made during times of active hematuria. No significant differences in BUN, creatinine, or hemoglobin determinations were observed, although a mild leukopenia (5.7 ± 0.6 vs. $8.6 \pm 0.9 \times 10^3/\mu\text{l}$, treated vs. untreated, $P < 0.05$) was seen in mice receiving 5-azaC-treated cells. Total numbers of splenocytes and the percent Thy 1.2⁺, Ig⁺, CD4⁺, and CD8⁺ splenocytes were the same in both groups.

Evidence for anti-DNA antibodies was sought. Serum was obtained every 4 wk from five mice given one injection of 5-azaC-treated cells and four mice given five injections of 5-azaC-treated cells. Controls included three mice receiving 1 injection of untreated cells, three mice receiving 5 injections of untreated cells, and three mice that received 12 injections of HBSS. Sera were tested for anti-ssDNA and anti-dsDNA antibodies by radioimmunoprecipitation (Fig. 6). Overall, five of nine mice given 5-azaC-treated cells developed significant amounts of anti-ssDNA antibodies (> 2 SD above negative controls) by 12 wk, but not at earlier time points. No difference was seen between mice receiving one or five injections. Similar results were found using an ELISA specific for ssDNA (data

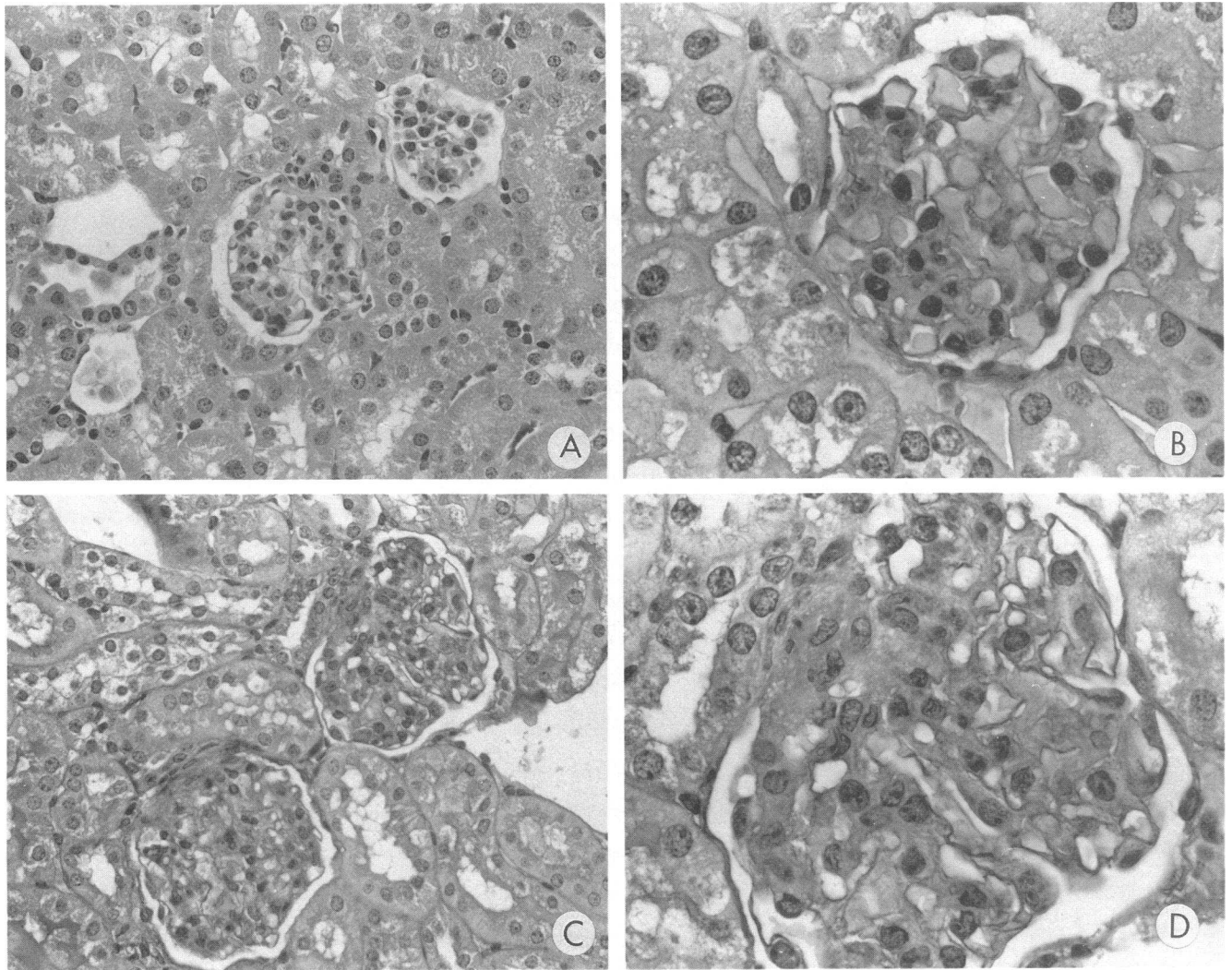


Figure 3. Histological analysis of kidneys from mice given 5-azaC-treated or untreated T cells. DBA/2 mice were given five intravenous injections of 5×10^6 untreated or 5-azaC-treated CD4⁺ DBA/2 cells as described in Table I. 2 wk after the last injection the mice were killed and kidneys were removed for histological analysis. (A) Representative section from the kidney of a mouse receiving untreated cells. The kidneys appear normal with no inflammation or scarring. $\times 750$. (B) A high power view of a normal glomerulus is shown. Note the widely patent capillary loops and normal cellularity of the mesangial region. $\times 2,250$. (C) Two glomeruli from a mouse receiving 5-azaC-treated cells. The glomeruli appear hypercellular with increased amounts of mesangial matrix. $\times 575$. (D) High power view of a glomerulus from a mouse receiving 5-azaC-treated cells. $\times 1,820$. (Hematoxylin and eosin).

not shown). In contrast, none of the nine control mice developed anti-DNA antibodies. No mice in either group developed anti-dsDNA antibodies. In addition, none of the mice receiving heat-killed cells or unstimulated, 5-azaC-treated cells developed anti-DNA antibodies. For all anti-DNA antibody determinations, total serum immunoglobulin was measured by ELISA to exclude the possibility that the autoantibodies detected were the result of polyclonal B cell activation. Total serum immunoglobulin was not significantly different between the various groups, suggesting that marked hypergammaglobulinemia is not a part of this autoimmune disease.

Effect of 5-azaC-treated, mitogen-activated CD4⁺ T cells in vivo. The initial studies described above used alloreactive T cells because large numbers of cells could easily be obtained, and specificity could be demonstrated. However, it is possible that the ability to induce autoimmunity is restricted to all-reactive cells. To exclude this, similar experiments were per-

formed using CD4⁺ DBA/2 T cells activated with Con A. We have previously reported that mitogen-activated T cells also become autoreactive after 5-azaC treatment (8). Female DBA/2 mice were given a total of six injections of 5×10^6 5-azaC-treated (five mice) or untreated (five mice) Con A-activated cells, again administered approximately every other week, and the mice were monitored for hematuria and proteinuria. As before, all mice receiving 5-azaC-treated cells developed hematuria, proteinuria, and cellular casts, identical to those seen in the urine of mice receiving 5-azaC-treated, all-reactive cells. After 4 mo, sera were obtained for autoantibodies, and the mice were killed for histological analysis. Kidneys from mice receiving treated, but not untreated, cells demonstrated a proliferative glomerulonephritis identical to that seen with 5-azaC-treated all-reactive cells (data not shown). Total IgG concentrations were determined in sera from all 10 mice. Once again, no significant difference was observed (1.12 ± 0.09 vs.

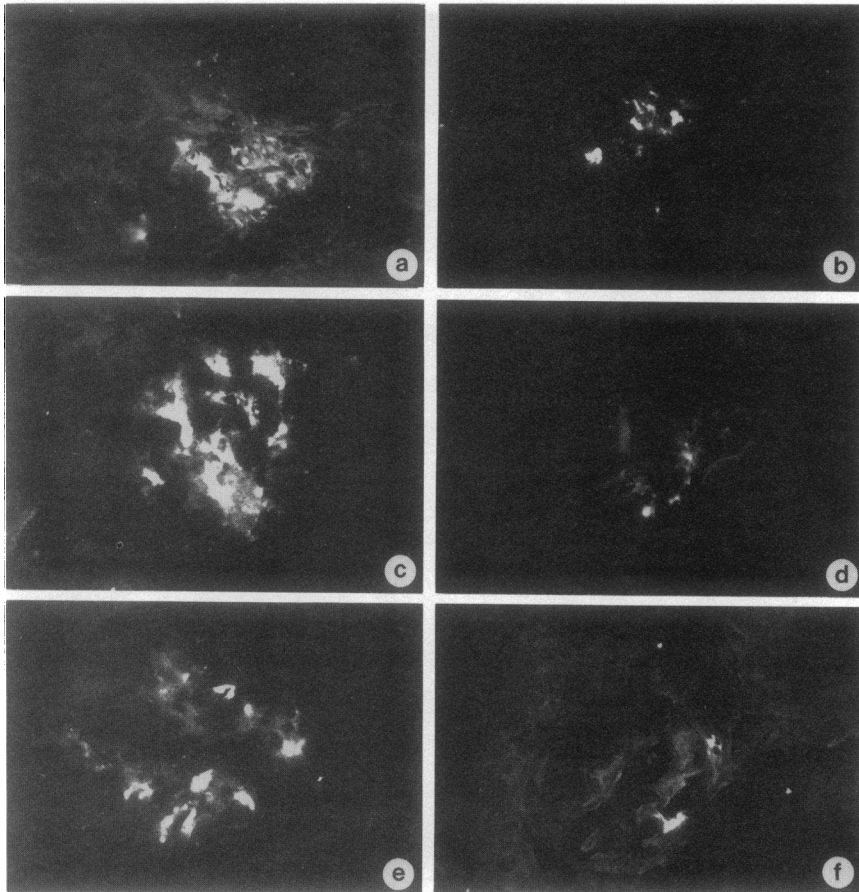


Figure 4. Immunofluorescence of glomeruli from mice receiving 5-azaC-treated or untreated T cells. DBA/2 mice received two intravenous injections of 5×10^6 5-azaC-treated (a, c, and e) or untreated (b, d, and f) cells 2 wk apart, as in Fig. 2. 2 wk after the second injection kidneys were removed, sectioned, and stained with FITC-conjugated antisera specific for IgG (a and b), IgM (c and d), or IgA (e and f). There is 3–4+ immunoglobulin deposition in the glomeruli from mice receiving 5-azaC-treated cells compared with minimal deposition in the control animals. $\times 400$.

1.14 \pm 0.025 mg/ml, untreated vs. 5-azaC-treated) between the groups receiving the Con A-activated cells. Fig. 7 shows the results of the ELISAs for IgG antibodies to ssDNA as well as to histones. Mice receiving 5-azaC-treated cells (numbers 1–5) developed significantly greater amounts of anti-ssDNA and antihistone antibodies than mice receiving untreated cells ($P < 0.001$ for each). Lesser amounts of antihistone antibodies were produced than anti-ssDNA antibodies relative to controls of pooled sera from female NZB/W mice ($P < 0.01$). Since

total IgG concentrations were the same in both groups, in these experiments protein concentrations were not normalized. To confirm specificity, purified ssDNA or histone protein was added to the respective ELISAs. The ELISAs for anti-ssDNA and histone antibodies were significantly inhibited ($P < 0.001$ for each). It is noted that the titers obtained in this experiment were significantly less than the NZB/W controls, whereas the titers shown in Fig. 6 approximate the titer of controls. This could be due to a difference in titer between the pooled NZB/W

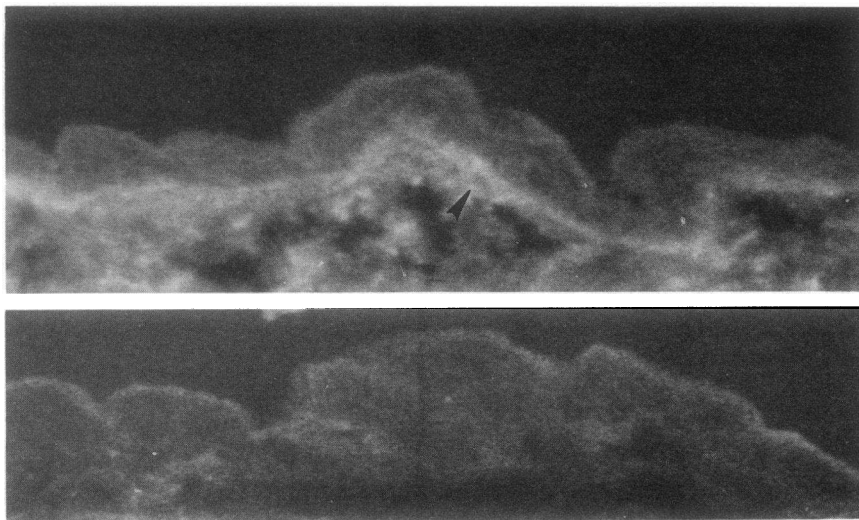


Figure 5. Immunofluorescence of skin from mice receiving 5-azaC-treated or untreated T cells. Skin specimens from the mice described in Fig. 2 were stained with FITC-conjugated anti-IgG. The mice received 5-azaC-treated cells (top) or untreated cells (bottom). Note the presence of a lupus band at the dermal-epidermal junction, indicated by an arrow in the top panel. $\times 800$.

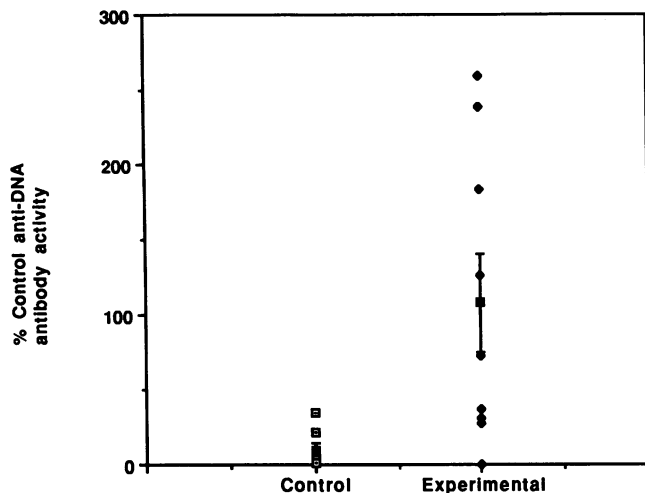


Figure 6. Measurement of anti-DNA antibodies. Sera were obtained from nine mice receiving 5-azaC-treated cells (*Experimental*) or nine mice receiving control injections (*Control*). Total serum protein was adjusted to 0.2 $\mu\text{g}/\text{ml}$ and then incubated with single-stranded [^3H]DNA and precipitated with antisera to mouse Ig. Positive controls consisted of sera from 8-mo-old (SWR \times NZB) F_1 mice, and negative controls consisted of pooled sera from healthy, young SWR and AKR mice. Results were calculated as (sample - negative control)/(positive control - negative control) $\times 100$. Each point represents the mean of two experiments, each performed in duplicate. Boxes with error bars represent the mean \pm SEM of the data points. The difference between groups was significant ($P < 0.01$ by *t* test).

SWR and NZB/W sera used as controls in the two experiments or to the fact that both IgG and IgM were measured in the first experiments whereas only IgG was measured in the second set of experiments.

Comparison of the effects of 5-azaC and Pca treatment on $CD4^+$ T cells. The results presented above suggest that dividing $CD4^+$ T cells treated with a DNA methyltransferase inhibitor are sufficient to induce a lupus-like disease in DBA/2 mice. However, it is possible that effects of 5-azaC other than DNA methyltransferase inhibition cause the changes in the T cells. To further confirm the effects of DNA methyltransferase inhibition on $CD4^+$ T cells, an unrelated DNA methyltransferase inhibitor, Pca, was used. Pca has recently been shown to decrease T cell $d^m\text{C}$ content (9) and inhibit DNA methyltransferase (12) in human T cells. The effects of Pca on murine T cells were established first.

To determine if Pca inhibits murine T cell DNA methylation, EL-4 cells were treated with 1, 10, or 100 μM Pca, and 4 d later total DNA $d^m\text{C}$ content was determined. $d^m\text{C}$ represented $4.61 \pm 0.02\%$ of total untreated EL-4 DNA $d\text{C}$, whereas the $d^m\text{C}$ content of EL-4 cells treated with 10 and 100 μM Pca was 4.38 ± 0.01 and $4.35 \pm 0.01\%$ ($P = 0.003$). This represents a decrease of 0.26% or $\sim 160,000$ methylated bases (36). Similar to our previous report (37), the decrease caused by 1 μM Pca was smaller and not statistically significant. Controls in which EL-4 cells were treated with 0.25 μM 5-azaC demonstrated a 0.30% decrease in overall $d^m\text{C}$ content.

The effects of Pca and 5-azaC on murine T cells were then compared. $CD4^+$, alloreactive DBA/2 anti-C57BL/6 T cells were treated with 5–50 μM Pca and 6 d later were tested for reactivity to syngeneic and allogeneic APC. Fig. 8 *a* compares the proliferative response of treated and untreated T cells to syngeneic APC. In this figure, the autoreactive response is ex-

pressed relative to the response to the same number of allogeneic APC. Significant autoreactivity ($P < 0.01$) is seen at all concentrations tested, similar to the results observed in cloned human T cells (9, 37). Fig. 8 *b* compares killing of syngeneic $M\phi$ by untreated T cells and T cells similarly treated with 50 μM Pca. Untreated cells again caused low level killing approximating that shown for untreated cells in Fig. 1. Pca-treated cells demonstrated significantly ($P < 0.01$) greater killing, which was inhibited with anti-CD11a and anti-Ia^d but not anti-Ia^k antibodies. In the experiment shown, complete inhibition was not achieved using the anti-Ia^d mAb. However, a second experiment performed under identical conditions gave complete ($0 \pm 0.7\%$) inhibition. The variability in the results may be attributed to the normalization of the data to the spontaneous ^{51}Cr release from the $M\phi$. These mAb inhibition results are also similar to those shown in Fig. 1.

5-azacytidine has recently been reported to increase CD11a expression on the surface of human T cells, which may contribute to the development of autoreactivity by stabilizing the low affinity interaction between the T cell receptor and Ia determinants. This increase could not be attributed to DNA synthesis inhibition and was presumably due to inhibition of DNA methylation (11). Subsequent studies have shown that Pca causes an identical increase in CD11a expression without affecting DNA synthesis (reference 38; K. S. O'Rourke, D. Power, and B. C. Richardson, manuscript in preparation). Therefore it was important to determine if a similar effect was induced by 5-azaC and Pca in murine T cells. In Fig. 8 *c*, the alloreactive $CD4^+$ T cell line was treated with 5-azaC or Pca, and 6 d later CD11a expression was compared on treated and untreated cells. The peak CD11a immunofluorescence of the untreated cells was 4. In contrast, the peak immunofluorescence was 65 in the 5-azaC-treated cells. The Pca-treated cells demonstrated two peaks, one at ~ 10 and one at 65. This suggests that both Pca and 5-azaC can induce a > 10 -fold increase in CD11a expression on a subset of the cells, similar to that seen in human T cells (11). To test whether altered cell cycle kinetics contribute to increased LFA-1 expression in murine cells, the alloreactive T cell lines were treated for 6 d with 100 μM hydroxyurea, and then LFA-1 expression and cell cycle kinetics were compared between treated and untreated cells. Using propidium iodide fluorescence to examine cellular DNA content, hydroxyurea was found to decrease the percent of cells in S and G_2M compared with untreated controls ($71 \pm 1.25 \pm 1.5 \pm 1$ vs $83 \pm 1.14 \pm 1.3 \pm 0.1$, $G_0G_1:S:G_2M$ in untreated vs. treated, mean \pm SD of duplicate determinations). However, LFA-1 expression decreased by 40% on the treated cells, arguing against an effect of DNA synthesis inhibition on increased LFA-1 expression. In addition, concentrations of 5-azaC that increased LFA-1 expression had no significant effect on murine T cell cycle kinetics ($74 \pm 3.17 \pm 5.8 \pm 2$, $G_0G_1:S:G_2M$).

Since human $CD4^+$ T cells treated with 5-azaC and Pca secrete cytokines capable of promoting B cell differentiation into antibody-secreting cells (8), the effects of 5-azaC and Pca on $CD4^+$ T cell cytokine secretion were compared. Fig. 8 *d* compares IL-4, IL-6, and IFN- γ secretion by stimulated, alloreactive T cells to cytokine secretion by the same cells after treatment with 0.25 μM 5-azaC or 50 μM Pca. No significant differences were seen in IL-6 and IFN- γ secretion between untreated, 5-azaC- and Pca-treated cells. In contrast, 5-azaC- and Pca-treated cells secreted more IL-4 than the untreated controls ($P < 0.05$, untreated vs. Pca-treated). Evidence for

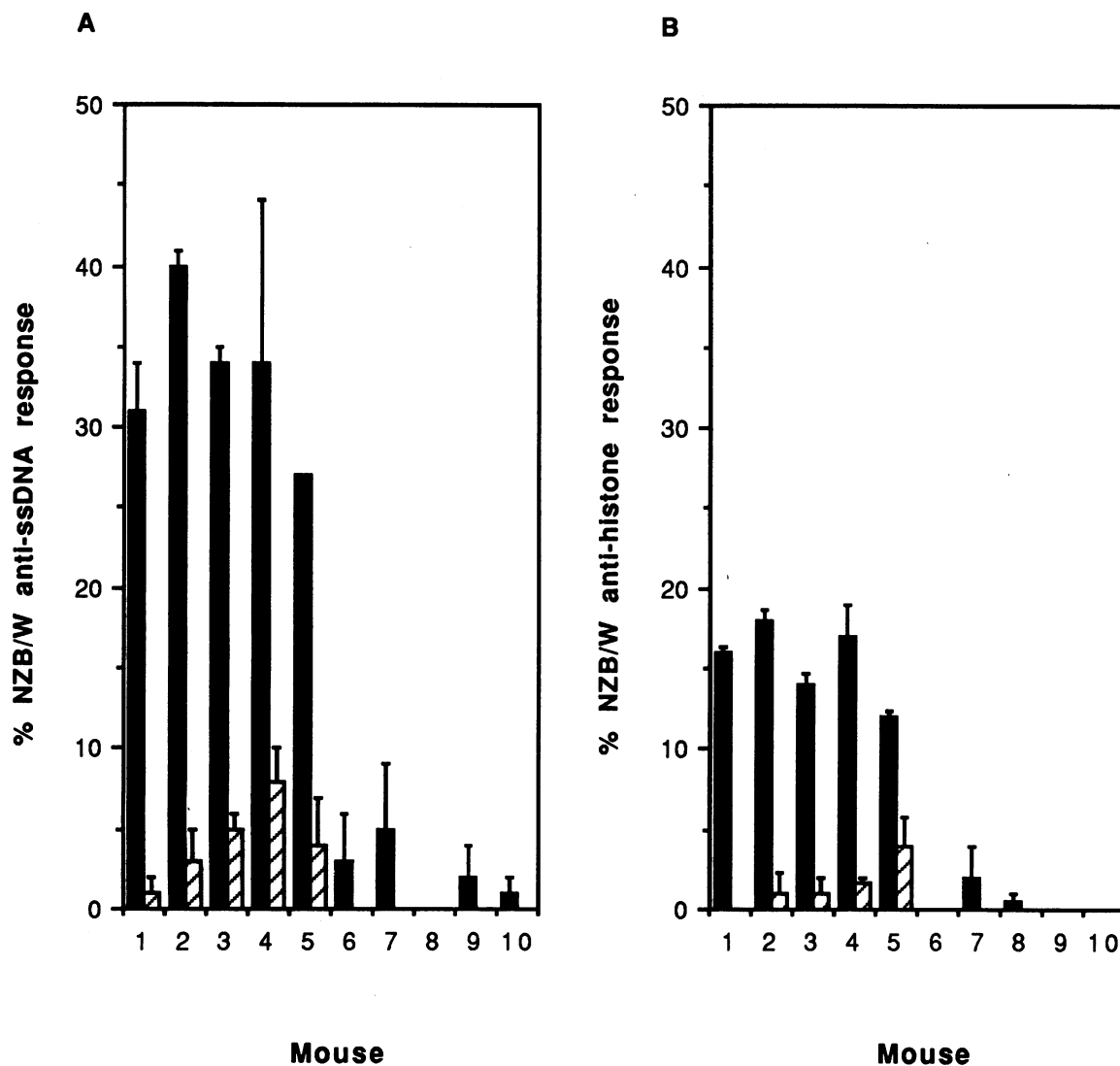


Figure 7. Anti-ssDNA and antihistone antibodies in mice receiving Con A-activated T cells. Female DBA/2 mice received six injections of 5×10^6 5-azaC-treated (mice 1-5) or untreated (mice 6-10) Con A-activated CD4⁺ T cells. After 4 mo, sera were harvested, diluted 1:100, and tested for IgG antibodies to ssDNA and histone proteins using ELISAs. For each experiment a standard curve was performed using pooled sera from NZB/W female mice. Results are expressed relative to the amount of NZB/W sera producing the same OD on the standard curve, using the formula: % response = [(reciprocal dilution test sera)/(reciprocal dilution NZB/W sera)] \times 100, and represent the mean \pm SEM of duplicate determinations. (A) Anti-ssDNA antibodies. The hatched bars represent sera tested in the presence of purified ssDNA (5 μ g/ml), and the solid bars represent sera tested without specific inhibitor. (B) Antihistone antibodies. Again, the hatched bars represent sera tested in the presence of purified histone proteins (5 μ g/ml) and the solid bars represent sera tested without inhibitor. Inhibitors were only used in sera from mice 1-5 in each group.

IL-2 secretion was also sought. Although newly established lines could be shown to secrete significant amounts of IL-2, this appeared to decrease with time. At the time of adoptive transfer, significant IL-2 secretion was usually not detectable and was not induced with 5-azaC or Pca.

The effects of Pca and 5-azaC-treated cells *in vivo* were then compared. Unirradiated, female DBA/2 mice received a total of six injections of 5×10^6 alloreactive cells treated with 50 μ M Pca or 0.25 μ M 5-azaC. Controls included six identical injections of untreated alloreactive cells or six injections of unstimulated, CD4⁺ DBA/2 T cells treated for 4 h with 50 μ M Pca. Each group contained five mice. The injections were given intravenously as before and were administered over 5-6 mo. The mice were tested for hematuria, proteinuria, histological

evidence of glomerulonephritis, and autoantibodies as before. All 10 mice receiving activated, Pca- or 5-azaC-treated cells developed hematuria, proteinuria, and cellular casts identical to those seen in the first groups of mice receiving 5-azaC-treated cells, whereas none of the mice receiving untreated, activated cells or unstimulated Pca-treated cells developed urinary abnormalities. 1 of the 5 mice receiving Pca-treated cells died in the 4th month of the experiment, whereas none of the 10 control mice died. After 6 mo the mice were killed. Histological analysis of the kidneys from mice receiving Pca (Fig. 9) and 5-azaC-treated proliferating cells again demonstrated hypercellular glomeruli with increased mesangial matrix, identical to that previously shown (Fig. 3), and immunofluorescence again demonstrated increased glomerular IgG deposition rela-

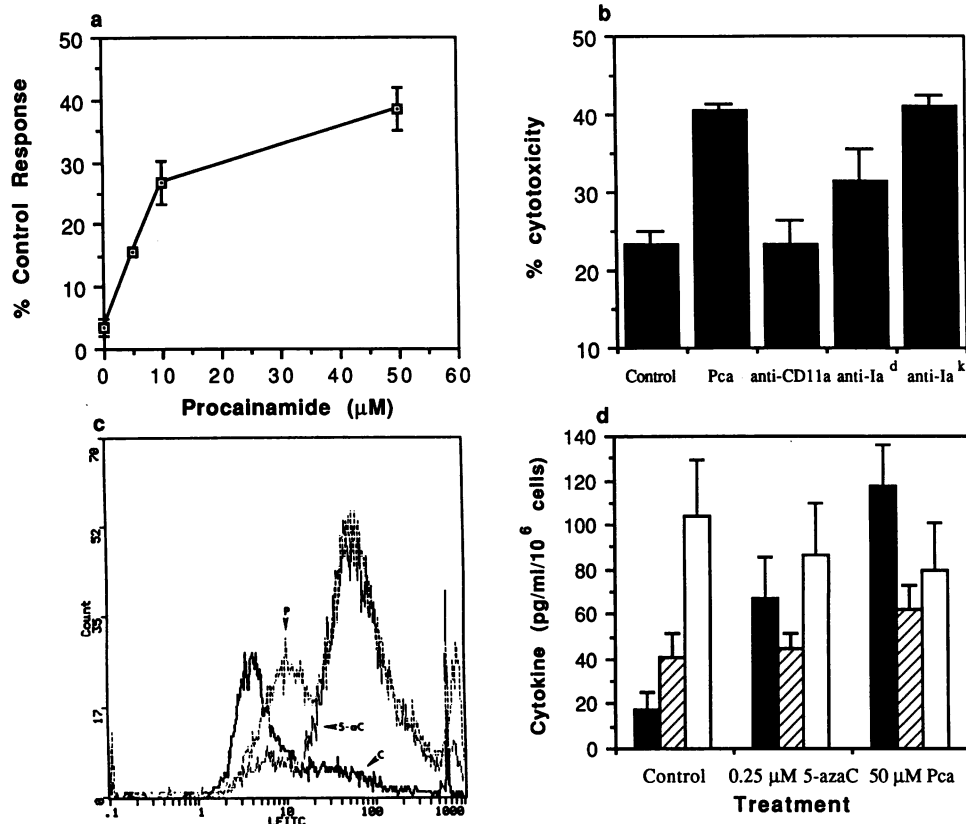


Figure 8. Effect of Pca on CD4⁺ T cells. (a) CD4⁺, proliferating, alloreactive DBA/2 T cells were treated with the indicated concentrations of Pca, and 6 d later 5×10^4 T cells were challenged with 10^5 irradiated C57BL/6 or DBA/2 APC. Proliferation was measured as described in Fig. 1 b, and results are presented as (response to DBA/2 APC - ³H incorporation by T cells cultured alone)/(response to C57BL/6 APC - ³H incorporation by T cells cultured alone) $\times 100$. Each point represents the mean \pm SEM of quadruplicate determinations. The response to the allogeneic stimulators ranged from 63,418–139,359 cpm ($106,086 \pm 15,834$, mean \pm SEM). (b) The same alloreactive T cells were treated with 50 μ M Pca and 6 d later untreated (Control) cells were challenged with ³¹Cr-labeled syngeneic M ϕ , using an effector/target ratio of 25:1 and the protocol described in Fig. 1. Where indicated, anti-CD11a, anti-Ia^d, or anti-Ia^k were added at 100 ng/ml. Each point represents the mean \pm SEM of triplicate determinations. (c) Alloreactive CD4⁺ T cells were treated with 0.25 μ M 5-azaC or

50 μ M Pca as described in Figs. 1 and 8 a. 6 d later untreated (C), 5-azaC-treated (5-aC), and Pca-treated (P) cells were stained with anti-CD11a and GAM-FITC, and then analyzed by flow cytometry. In this figure the number of cells is plotted in the y-axis and the log green fluorescence is plotted on the x-axis. Negative controls included cells stained with FITC-conjugated mouse Ig alone and GAM Ig-FITC alone, and no significant difference was seen. (d) Alloreactive CD4⁺ T cells were treated with the indicated concentrations of 5-azaC or Pca, and 6 d later were cultured for 18 h at $1-2 \times 10^6$ /ml in RPMI, 10% FCS. The concentration of IL-4 (solid bars), IL-6 (hatched bars), and IFN- γ (open bars) in the supernatant was then determined by ELISA. The results represent the mean \pm SEM of two experiments for IL-4 and IL-6 and of three experiments for IFN- γ . Each determination was performed in duplicate.

tive to controls, identical to that shown in Fig. 4 (data not shown). Fig. 10 shows electron micrographic pictures of glomeruli from mice receiving untreated and Pca-treated T cells. Mesangial electron-dense immune deposits, neutrophils, and endothelial cell disruption with fibrin deposition are seen in glomeruli from mice receiving Pca-treated, but not untreated, cells. Identical changes were observed in glomeruli from mice receiving 5-azaC-treated cells (data not shown).

Sera were then tested for total IgG concentration and IgG antihistone and anti-ssDNA antibodies and compared with the same pooled NZB/W sera used in Fig. 7. DBA/2 mice receiving untreated alloreactive T cells had 0.98 ± 0.1 mg/ml IgG, and mice receiving unstimulated, Pca-treated T cells had 1.00 ± 0.09 mg/ml. Mice receiving 5-azaC-treated alloreactive cells had 1.10 ± 0.07 mg/ml, and mice receiving Pca-treated alloreactive cells had 1.11 ± 0.1 mg/ml IgG (mean \pm SEM of the four or five mice in each group). These differences were not significant ($P > 0.05$) and approximate those of normal mice (35). Fig. 11 compares the titer and specificity of IgG anti-ssDNA and antihistone antibodies in mice receiving Pca- and 5-azaC-treated alloreactive cells. Fig. 11 a demonstrates that mice receiving Pca- or 5-azaC-treated, activated cells develop significantly ($P < 0.01$ and $P < 0.02$, respectively, relative to mice receiving untreated, activated cells) greater amounts of

anti-ssDNA antibodies than controls. In this experiment the titers approximate those of the NZB/W controls and are greater than those found using the Con A-activated cells. This is most likely due to the longer duration of this experiment (6 vs. 4 mo), because earlier bleedings from this group of mice demonstrated lower anti-ssDNA titers (data not shown). Fig. 11 b shows that the mice receiving Pca- ($176 \pm 50\%$, $P < 0.02$) or 5-azaC- ($46 \pm 7\%$, $P < 0.05$) treated cells develop more anti-histone antibodies than controls ($26 \pm 0.4\%$ for stimulated untreated and $9 \pm 3\%$ for unstimulated, Pca treated). Interestingly, mice receiving Pca-treated cells made greater amounts of anti-histone antibody than mice receiving 5-azaC-treated cells ($P < 0.05$). The reason for this is not apparent, but is reminiscent of the specificity of autoantibodies in humans receiving long-term Pca therapy (39).

Fig. 11 c compares the anti-ssDNA titer of sera from representative mice receiving Pca- or 5-azaC-treated cells with pooled sera from female NZB/W mice and demonstrates that the titers are comparable. Fig. 11 d demonstrates that the titers of antihistone antibodies from representative mice receiving Pca-treated cells are comparable to titers from NZB/W mice, whereas mice receiving 5-azaC-treated cells appear to make relatively less antihistone antibodies, confirming the results shown in Fig. 11 b. Control sera from mice receiving Pca-

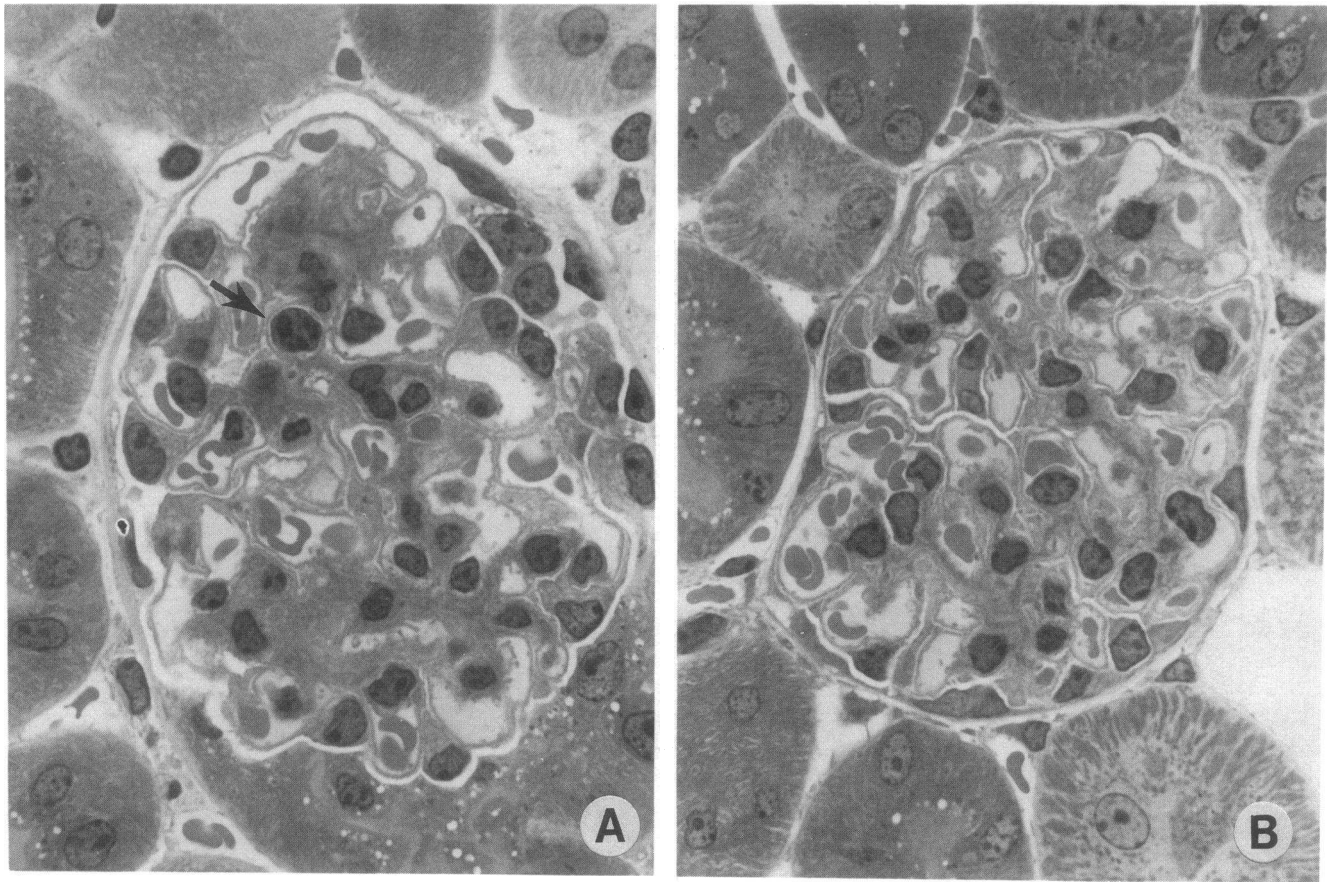


Figure 9. Light microscopic analysis of glomeruli from mice receiving Pca-treated T cells. 1- μ m thick sections stained with toluidine blue illustrate the characteristic glomerular alterations seen in mice receiving six injections of 5×10^6 proliferating, alloreactive CD4⁺ T cells treated with 50 μ M Pca compared with mice receiving six similar injections of untreated but otherwise identical cells. In mice receiving Pca-treated cells (A), the glomeruli appear hypercellular with increased numbers of cells present in the mesangium, along with occasional neutrophils present in glomerular capillary loops (arrow). There is also an increased amount of mesangial matrix. A representative glomerulus from an animal receiving untreated cells is illustrated in B. There is normal cellularity, no increase in mesangial matrix, and no evidence of neutrophils. $\times 400$.

treated, unstimulated cells were also studied at dilutions of 1:100 and 1:300. At a dilution of 1:300, the OD of the anti-ssDNA assay was 0.021 ± 0.009 (mean \pm SD of the ODs from the five mice, each performed in duplicate) above background, and the average OD of the antihistone assays was less than background. In contrast, in this assay, a 1:100 dilution of NZB/W sera gave ODs of 0.134 ± 0.006 and 0.119 ± 0.004 in the anti-ssDNA and antihistone ELISAs, respectively, and serial dilutions of the NZB/W sera only reached levels comparable to 1:300 dilutions of the control sera at dilutions of 1:1,600–1:3,200.

Fig. 11, e and f demonstrate that purified ligands inhibit the ELISAs, confirming specificity. In Fig. 11 e it may be seen that complete inhibition was not achieved using sera from a mouse receiving Pca-treated cells. This may be due to higher titers of antibody in this animal, since 5 μ g of purified ssDNA gave 74% inhibition in a second animal from this group.

In additional experiments, evidence for antithymocyte and antierythrocyte antibodies was sought, similar to those reported in chronic GvH disease (14). However, again using female NZB/W sera as a positive control (35) and flow cytometry to test for Ig binding, no evidence for significant amounts of antithymocyte or antierythrocyte antibodies were found. IgG anti-dsDNA antibodies were also sought, and evidence for

small amounts were found in mice receiving Pca-treated cells (OD 0.016 ± 0.002 vs. 0.028 ± 0.005 , untreated vs. Pca-treated, $P < 0.05$). However, the titer of these antibodies was $< 25\%$ that of pooled sera from NZB/W mice, and the significance of this low titer autoantibody is uncertain.

Discussion

The goal of these studies was to determine if T cells treated with DNA methyltransferase inhibitors could induce an autoimmune disease. The results demonstrate that activated, syngeneic CD4⁺ T cells treated in vitro with either 5-azaC or Pca are sufficient to induce glomerulonephritis and anti-ssDNA and antihistone antibodies in unirradiated DBA/2 mice, a strain that does not normally develop autoimmune diseases. To the best of our knowledge, this is the first description of an autoimmune disease being induced by T cells treated in vitro with either of these agents.

Initial experiments asked whether autoimmunity could be induced using the well-characterized DNA methylation inhibitor 5-azaC. Adoptive transfer of CD4⁺, proliferating cells treated with this drug induced hematuria, proteinuria, cellular urinary casts, mesangial immune complex deposition, a proliferative glomerulonephritis, IgG deposition at the dermal-epi-

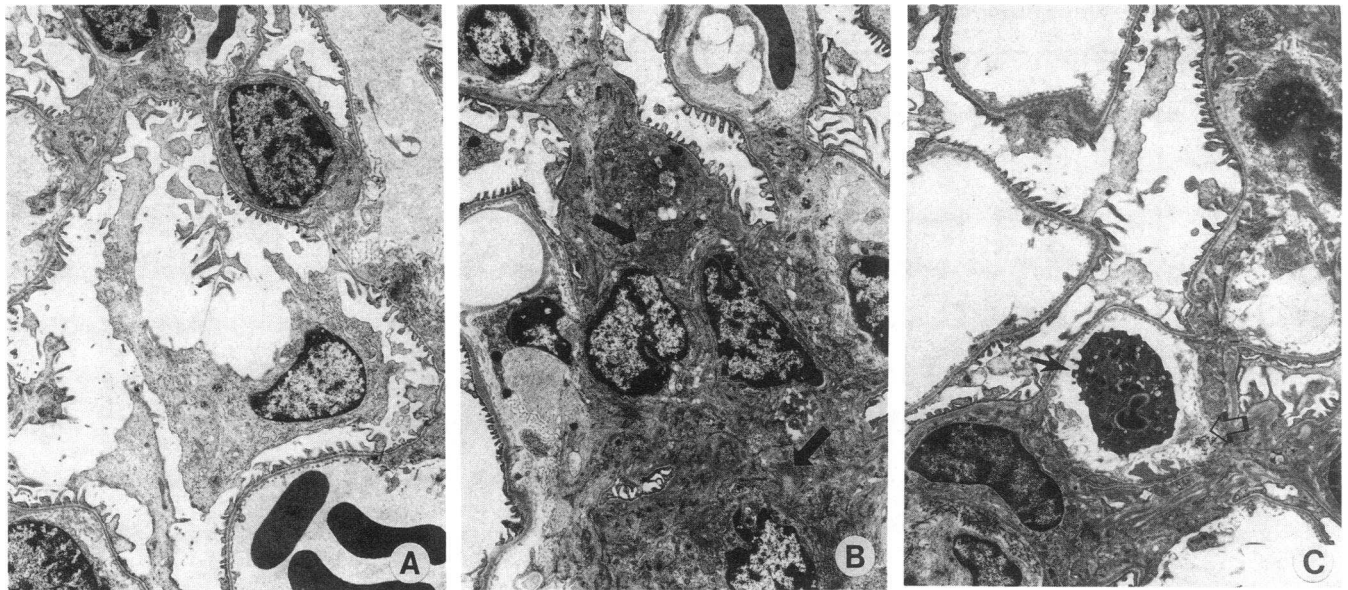


Figure 10. Electron microscopic analyses of glomeruli from mice receiving Pca-treated T cells. *A* illustrates normal glomerular ultrastructure, which was found in kidneys from animals receiving untreated T cells as described in Fig. 9. By comparison, *B* and *C* are representative of glomerular ultrastructural alterations in kidneys from mice receiving Pca-treated cells. As shown in *B* there is increased mesangial matrix with electron-dense deposits (*arrows*) consistent with immune deposits. *C* illustrates a neutrophil in a glomerular capillary loop (*filled arrow*) with injury to the glomerular endothelial cell in that location with fibrin deposition (*open arrow*). Transmission electron micrographs: *A* and *B*, $\times 3000$; *C*, $\times 6,700$.

dermal junction, and anti-ssDNA antibodies. Thus, evidence for an autoimmune disease was found, and the autoimmune disease most closely resembled human lupus. Clinically, the disease appeared to be relatively mild, with no significant change in serum BUN or creatinine, no marked anemia, and only 1 mouse out of 20 dying from complications possibly related to the treatment. The lack of effect on BUN and creatinine is consistent with the mesangial pattern of immune complex deposition (40). The hematuria was also self-limited, perhaps reflecting the transient nature of the autoreactivity induced by 5-azaC (7). However, despite the fact that the renal disease was relatively mild, it remains significant that changes were induced in a strain that does not normally develop autoimmunity. Others have shown that multiple genes contribute to the development of murine lupus (41). It is possible that the mild nature of the disease reflects in part a lack of the genes required for full and severe manifestations of lupus in DBA/2 mice. Similar studies in strains predisposed to lupus might answer this question. Alternatively, since the cell lines were polyclonal, it is possible that only a subset of the treated cells are responsible for the disease and that transfer of greater numbers of pathogenic cells may induce more severe disease. Similar studies using cloned T cell lines could answer this question.

Experiments were performed exploring requirements for induction of the lupus-like disease. Others have reported that an immune complex glomerulonephritis and autoantibodies can be induced by the adoptive transfer of semiallogeneic CD4⁺ T cells in the chronic GvH model of autoimmunity (13, 14), suggesting that in some fashion alloreactive CD4⁺ T cells, used in the initial studies, may be unique in the ability to induce autoimmunity. Therefore CD4⁺ T cells activated with Con A were similarly treated with 5-azaC and injected into syngeneic recipients. Identical results were found. This demonstrates that cells capable of inducing renal disease and autoanti-

bodies after 5-azaC-treated can be found in CD4⁺ T cell lines activated by either stimulus. Again, it is possible that a specific CD4⁺ T cell subset, defined by cytokine secretion or by antigenic specificity, is required and present in both lines. Future studies will examine this possibility.

5-azaC could cause the autoimmunity by altering gene expression in proliferating T cells, but it is also possible that inadvertent coadministration of 5-azaC with the transferred cells contributes to the *in vivo* abnormalities. However, transfer of 5-azaC as a mechanism seems unlikely because the cells were treated 6 d before adoptive transfer and 5-azaC is unstable in aqueous media (42). Furthermore, the cells were thoroughly washed before transfer. Finally, heat-killed 5-azaC-treated cells and unstimulated, 5-azaC-treated cells failed to induce autoantibodies or renal disease. For these reasons, the disease is most likely due to an effect of 5-azaC on T cells, rather than carryover of the drug.

At the concentrations used, the major effect of 5-azaC is hypomethylation of newly synthesized DNA (43, 44). DNA methylation is one of the mechanisms regulating gene expression (45), and in many systems hypomethylation of regulatory sequences has been shown to correlate with increased gene transcription (45). By inhibiting DNA methylation, 5-azaC can alter gene expression (46). It seems reasonable to postulate that changes in DNA methylation are responsible for the increased CD11a expression observed in this and other studies (11, 38) and that the autoreactivity induced is also due to altered gene expression. This would explain why treatment of proliferating cells is required to produce the increase in LFA-1, autoreactivity, and autoimmune disease reported in this and other studies (7, 8, 11).

To further examine whether DNA methylation inhibition could be responsible for the autoimmunity, the effects of 5-azaC and Pca were compared. Pca was shown to decrease T cell

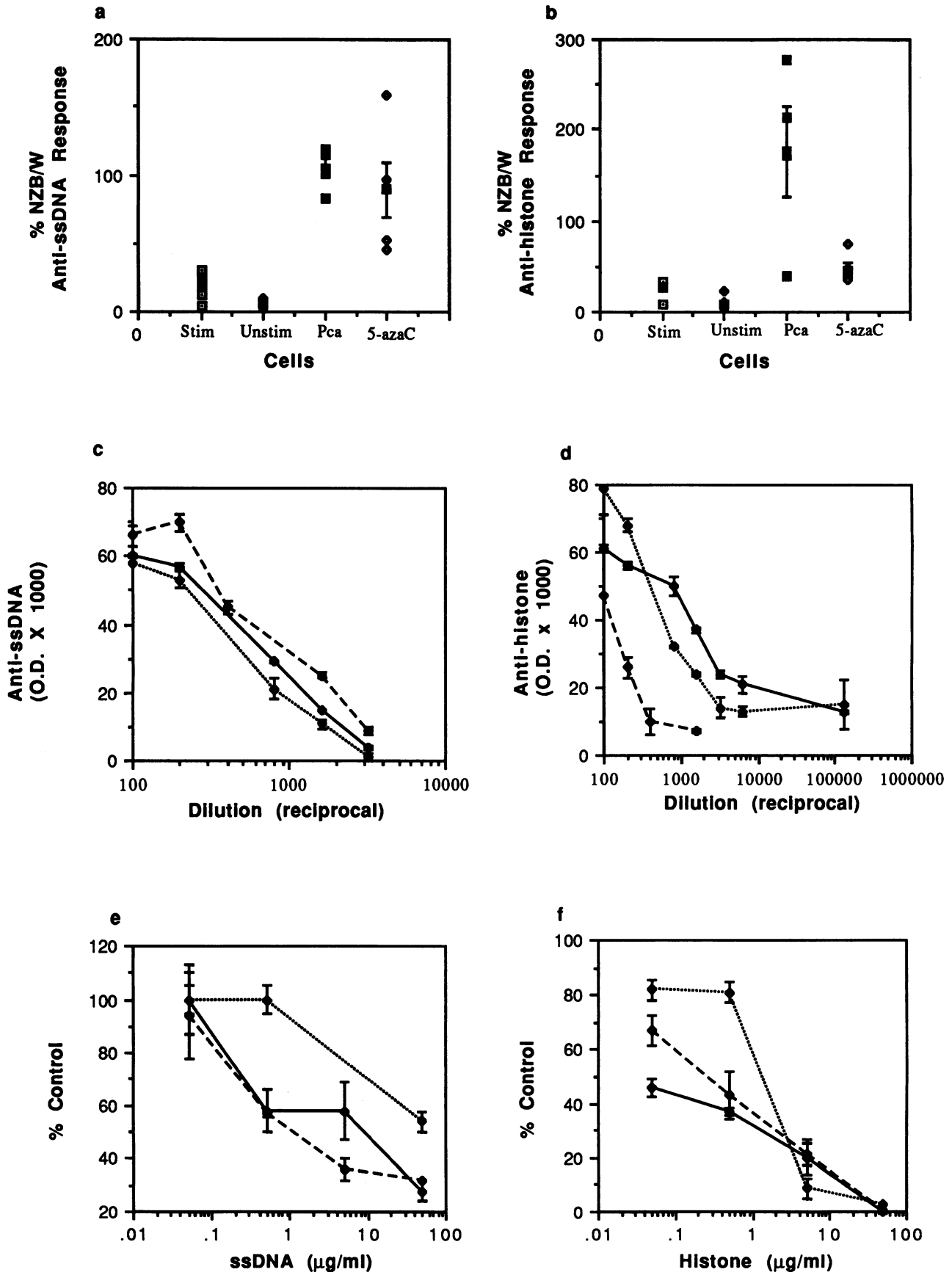


Figure 11. Autoantibody titers and specificity from mice receiving 5-azaC- and Pca-treated cells. DBA/2 mice received a total of six injections of 5×10^6 untreated, $0.25 \mu\text{M}$ 5-azaC-treated, or $50 \mu\text{M}$ Pca-treated alloreactive CD4^+ T cells or unstimulated CD4^+ T cells treated with $50 \mu\text{M}$ Pca. Sera were obtained after 5–6 mo. (a) Sera from untreated, alloreactive cells (*Stim*, $n = 5$), unstimulated Pca-treated cells (*Unstim*, $n = 5$),

d^mC content, increase CD11a expression, and induce autoreactivity in murine T cells, suggesting that 5-azaC and Pca have identical effects on proliferating murine as well as human T cells at the DNA, protein, and functional levels. It should be noted that these murine studies differ from the human studies in that polyclonal cells were used in most of the murine experiments. This makes it difficult to exclude the possibility that the increased CD11a expression and the autoreactivity induced could result from selection of T cell subsets. However, the previous results demonstrating identical effects on cloned human T cells and the present demonstration that cloned murine T cells also become autoreactive after 5-azaC-treated suggests that the changes observed are due to alterations in the treated cells rather than subset selection.

Adoptive transfer of Pca-treated T cells was then shown to induce glomerulonephritis and autoantibodies, similar to 5-azaC-treated T cells. Again, treatment of proliferating T cells was required. Interestingly, Pca-treated T cells induced greater amounts of antihistone antibodies than did 5-azaC-treated cells. Since the other effects of these drugs were identical, this discrepancy is surprising. It is possible that metabolites of Pca, released from the treated T cells, favor the development of antihistone antibodies, as proposed by Uetrecht et al. (47).

These experiments demonstrate essentially identical effects of Pca and 5-azaC on CD4⁺ T cells, and the treated T cells have nearly identical effects on syngeneic hosts. The elements common to both agents are DNA methylation inhibition, altered gene expression, and autoreactivity induction. We propose that inhibiting DNA methylation in CD4⁺ T cells alters expression of certain genes involved in T cell activation, inducing autoreactivity, and have now demonstrated that the autoreactive cells are sufficient to cause a lupus-like disease. This hypothesis implies that environmental agents or other signals inhibiting T cell DNA methylation or altering expression of the crucial genes in vivo may play a role in triggering lupus. In this context it is significant that T cells from patients with active lupus have hypomethylated DNA (27), increased LFA-1 expression (11), and a subset that appears to be autoreactive (11), suggesting that a similar mechanism may be involved in idiopathic human SLE.

The mechanism by which treated cells might induce autoimmunity was also explored. The chronic GvH model demonstrates that semiallogeneic CD4⁺ cells responding to self determinants on host APC can induce anti-DNA antibodies, antihistone antibodies, and immune complex renal disease (13, 14). 5-azaC- and Pca-treated human CD4⁺ T cells were previously shown to respond to self determinants on Mφ (7-9) and, in this report, 5-azaC- and Pca-treated murine CD4⁺ cells were also shown to respond to syngeneic APC. Since this autore-

active response to syngeneic APC resembles that of semiallogeneic T cells to host APC in the chronic GvH model, it seems reasonable to propose that this autoreactive response could contribute to the autoimmune disease induced by these cells. The effects of the autoreactive cells on syngeneic Mφ and the pattern of cytokines secreted by the treated cells were therefore examined to learn how the autoreactive cells might induce autoimmunity.

Mφ killing was observed using 5-azaC- and Pca-treated cells. The Mφ death could result from specific killing or the nonspecific release of cytotoxic molecules by cultured T cells. However, the cytotoxic responses were inhibited with mAb to class II MHC determinants as well as LFA-1, arguing against nonspecific cytotoxicity and supporting a mechanism requiring cell-cell contact similar to the Ia-dependent APC killing frequently caused by CD4⁺ T cells (48, 49). The Mφ killing is interesting and deserves comment. Recent reports indicate that CD4⁺ T cells can specifically lyse stimulating Mφ (20, 29-31). Work from this laboratory has confirmed these observations and demonstrates that the Mφ undergo apoptosis, or programmed cell death, after activating T cells (24). It seems likely that the same mechanism is involved in the Mφ killing caused by the autoreactive response described in these studies.

The Mφ killing may have relevance to the effects induced in vivo by the treated T cells. Mφ in the liver and spleen remove immune complexes from the circulation. It is possible that killing of Mφ could impair immune complex clearance, leading to deposition in tissues such as the kidney and skin. In addition, lysis of Mφ could release intracellular molecules such as histones and DNA, contributing to the development of autoantibodies.

In contrast to the cytolytic effect observed when treated T cells were cultured with syngeneic Mφ, human T cells made autoreactive with 5-azaC do not kill autologous B cells, but rather induce B cell differentiation into immunoglobulin-secreting cells, most likely by cytokine secretion (8). The lack of a cytotoxic effect on B cells may be explained by a recent report demonstrating that B cells express the Bcl-2 gene (50), which confers resistance to apoptotic death (51). In the present report, some of the secreted cytokines were analyzed. The treated cells were shown to synthesize IL-4, IL-6, and IFN-γ in amounts comparable to other short-term T cell cultures (52). IL-4 secretion increased significantly after 5-azaC and Pca treatment, raising the possibility that DNA methylation may contribute to the regulation of this cytokine. However, since the cells were polyclonal, other explanations are possible. No IL-2 secretion was detected, but this was not unexpected since synthesis of this cytokine has been reported to decrease over time in polyclonal cells cultured in IL-2 (53).

Pca-treated alloreactive cells (*Pca*, $n = 4$), or 5-azaC-treated alloreactive cells (*5-azaC*, $n = 5$) were tested for IgG anti-ssDNA antibodies as described in Fig. 7. Results are expressed relative to a standard curve of pooled NZB/W sera as before and represent the mean of duplicate determinations. The filled rectangle with error bars represents the mean±SEM of the data points in each column. (b) The same sera were tested for IgG antihistone antibodies, using the methods described in a. (c) Sera from NZB/W mice (*solid line*), or representative mice receiving Pca (*dotted line*) or 5-azaC- (*dashed line*) treated cells were tested for anti-ssDNA antibodies at the indicated dilutions. Results represent the mean±SEM of duplicate determinations. (d) Sera from the same mice shown in c were tested for antihistone antibodies at the indicated dilutions. Results represent the mean±SEM of duplicate determinations. (e) Sera from NZB/W mice (*solid line*) or representative mice receiving Pca (*dotted line*) or 5-azaC- (*dashed line*) treated cells were tested for anti-ssDNA antibodies at a dilution of 1:100 in the presence of the indicated concentrations of purified anti-ssDNA. Results represent the mean±SEM of duplicate determinations and are normalized to results performed without inhibitor. (f) The same sera shown in e were tested for antihistone antibodies in the presence of the indicated concentrations of purified histone protein. Results again represent the mean±SEM of duplicate determinations and are normalized to results performed without inhibitor.

The secretion of IL-4, IL-6, and IFN- γ could contribute to the development of autoimmunity. Numerous reports document the ability of these cytokines to promote B cell antibody synthesis (for review see reference 54). In addition, each of these cytokines has been implicated in the pathogenesis of lupus (55–57). It is possible that secretion of these cytokines in lymph nodes, together with intracellular antigens released by lysed M ϕ , could promote autoantibody synthesis and secretion by adjacent B cells. Since previous experiments demonstrated that cytokines secreted by similar autoreactive CD4⁺ cells could promote polyclonal B cell activation (8), and other murine models of lupus have significant polyclonal B cell activation and hypergammaglobulinemia (35), it is somewhat surprising that mice receiving the drug-treated cells did not also develop hypergammaglobulinemia. This may be due to the transient nature of the drug-induced autoreactivity (7) and is consistent with the relatively mild disease induced.

An alternative explanation for how the treated cells induce autoimmunity is that the host mounted an immune response to novel determinants present on the treated cells and this response in some fashion contributed to the disease. This seems unlikely, since heat-killed, 5-azaC-treated cells failed to induce renal disease and autoantibodies. However, it remains possible that a heat labile determinant on the cells was destroyed by this treatment. Proof that the autoreactive response contributes to the disease will require selectively reversing the autoreactivity, perhaps by using mAbs specific for those molecules important to or responsible for the autoreactive response.

In contrast to the lupus-like disease described in this report, Yoshida et al. (58) reported that parenteral 5-azaC diminishes the massive lymphadenopathy and the lupus-like syndrome that spontaneously develop in MRL/lpr mice. This suggests an apparent contradiction. However, Yoshida et al. found no inhibitory effect of 5-azaC on BXSB mice, another strain spontaneously developing lupus. It is likely, as Yoshida et al. point out, that the beneficial effect of 5-azaC in MRL/lpr mice may be due to the inhibition of the massive lymphoproliferation that occurs in this strain (58). This is consistent with the known inhibitory effect of 5-azaC on DNA synthesis (59).

In summary, these experiments support the hypothesis that T cells made autoreactive with DNA methylation inhibitors can induce a lupus-like autoimmune disease. Since agents such as Pca, hydralazine, and ultraviolet light inhibit DNA methylation and induce T cell autoreactivity (9, 38, 60; and manuscript in preparation), and patients with idiopathic lupus have T cells with hypomethylated DNA (27), it is possible that similar mechanisms are involved in 5-azaC-induced murine lupus, in human drug-induced lupus, and some forms of idiopathic SLE.

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