Autoimmunity Caused by Disruption of Central T Cell Tolerance

A Murine Model of Drug-induced Lupus

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Abstract

A side effect of therapy with procainamide and numerous other medications is a lupus-like syndrome characterized by autoantibodies directed against denatured DNA and the (H2A-H2B)-DNA subunit of chromatin. We tested the possibility that an effect of lupus-inducing drugs on central T cell tolerance underlies these phenomena. Two intrathymic injections of procainamide-hydroxylamine (PAHA), a reactive metabolite of procainamide, resulted in prompt production of IgM antidenatured DNA antibodies in C57BL/6xDBA/2 F1 mice. Subsequently, IgG antichromatin antibodies began to appear in the serum 3 wk after the second injection and were sustained for several months. Specificity, inhibition and blocking studies demonstrated that the PAHA-induced antibodies showed remarkable specificity to the (H2A-H2B)-DNA complex. No evidence for polyclonal B cell activation could be detected based on enumeration of Ig-secreting B cells and serum Ig levels, suggesting that a clonally restricted autoimmune response was induced by intrathymic PAHA. The IgG isotype of the antichromatin antibodies indicated involvement of T cell help, and proliferative responses of splenocytes to oligonucleosomes increased up to 100-fold. As little as 5 μM PAHA led to a 10-fold T cell proliferative response to chromatin in short term organ culture of neonatal thymi. We suggest that PAHA interferes with self-tolerance mechanisms accompanying T cell maturation in the thymus, resulting in the emergence of chromatin-reactive T cells followed by humoral autoimmunity. (*J. Clin. Invest*. 1997. 99:1888–1896.) Key words: lupus/(chemically induced) • immune tolerance • thymus gland/(immunology) • T lymphocytes • chromatin/(immunology)

Introduction

Drug-induced lupus is a side effect of long-term therapy with $>$ 40 types of medications. Procainamide poses the greatest risk for development of this syndrome with an annual incidence of $\sim$ 20% among treated patients (1). Most patients treated with procainamide develop histone-reactive antibodies and antidenatured DNA (anti-dDNA)[1] antibodies after prolonged therapy (2), whereas IgG antibodies to the (H2A-H2B)-DNA subunit of chromatin are a serologic marker in patients who develop procainamide-induced lupus (3). Numerous studies have been undertaken to explore how procainamide induces autoimmunity. Although adoptive transfer of syngeneic mouse splenocytes treated with procainamide in vitro has been reported to result in a lupus-like disease (4), no convincing autoimmune effect has been produced by direct administration of procainamide in experimental animals (5).

Several features of drug-induced lupus suggest that reactive metabolites are involved, including the chemical and pharmacological heterogeneity of lupus-inducing drugs, their largely inert nature except for their pharmacological properties and the requirement for many months of continuous exposure before appearance of autoimmunity (6). Procainamide-hydroxylamine (4-hydroxylamino-*N*-(diethylaminoethyl) benzamide) (PAHA) is a known reactive metabolite of procainamide (7–9), but its lability suggests that biosynthesis within an immune compartment would be required to generate an immunopathologic effect. Procainamide is transformed to PAHA by activated neutrophils in vitro (10–13), and indirect evidence for in vivo production of PAHA in mice (14) and humans (15) has been obtained. However, demonstration of a significant stimulatory effect of PAHA on any component of the peripheral immune system has been largely unsuccessful.

The F1 progeny of the cross between C57BL/6 and DBA/2 mice develops autoantibodies in association with experimental chronic graft-versus-host disease (16). This hybrid shows no spontaneous signs of autoimmune disease, but a subpopulation of B cells is activated when T cells from the DBA/2 parent are adoptively transferred, resulting in autoantibodies largely restricted to reactivity with chromatin (17–19). Since these are normal mice that have the genetic capacity to produce autoantibodies related to those associated with drug-induced lupus, this animal model would seem to be a good system for examining how a reactive metabolite of a lupus-inducing drug can induce autoimmunity.

In this study, we explored the possibility that PAHA induces autoimmunity be interfering with central T cell tolerance. We found that injection of PAHA into the thymus of C57BL/6 $\times$ DBA/2 F1 mice produced an autoimmune serology similar to that associated with procainamide-induced lupus. Chromatin-reactive T cell responses were detected in the spleen and in thymus organ culture after exposure to PAHA ex vivo, suggesting that loss of central T cell tolerance to chromatin underlies autoimmunity in drug-induced lupus.

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1. *Abbreviations used in this paper:* anti-dDNA, antidenatured DNA; APC, antigen presenting cell; PAHA, procainamide-hydroxylamine.

Methods

Animals. For the in vivo studies 4-wk-old C57BL/6 × DBA/2 F1 female mice were purchased from the Jackson Laboratories (Bar Harbor, ME). Older mice of this strain used as splenocyte donors for antigen presenting cells (APC) or as recipients in adoptive transfer studies as well as 2–5-mo-old BXSB males, 4-mo-old MRL/Mp-lpr/lpr males and 4-mo-old NZB × NZW F1 females were obtained from the Scripps Research Institute breeding colony. For thymus organ culture, 0–2-d-old locally bred C57BL/6 or C57BL/6 × DBA/2 F1 neonates were used along with 2–4-mo-old syngeneic donors of splenic APC.

Intrathymic injection. Injections were performed as described previously (20). In brief, 5-wk-old C57BL/6 × DBA/2 F1 mice were anesthetized by methoxyflurane inhalation and an incision was made over the lower cervical region. The thymic lobes were accessed by retracting the sternohyoideus muscle. A 20-μl aliquot of 4 mM PAHA in PBS prepared as previously described (8) was injected into each thymic lobe using a 27-gauge needle and a Tridak Stepper (Indicon Inc., Brookfield, CT). Control animals received thymic injections of PBS. Incisions were closed with silk sutures. All the control animals were also injected with PAHA into the spleen or the peritoneal cavity as indicated. Mice were subjected to a second injection by the same procedure after 2 wk. Blood was collected once or twice/week by retroorbital puncture after methoxyflurane anesthesia.

Chromatin-related antigens. Soluble chromatin in the form of oligonucleosomes (2–6-mer) were prepared from calf thymus chromatin by chromatography of micrococcal nuclease-digested chromatin through Sepharose CL-4B (Pharmacia LKB Biotechnology, Piscataway, NJ) (21). The native H2A-H2B complex was extracted from calf thymus chromatin and isolated by CM-52 (Whatman Inc., Clifton, NJ) column chromatography (22). Total histone was obtained from U.S. Biochemical (Cleveland, Ohio). The quality of these preparations has been previously demonstrated (23). S1 nuclease-digested native DNA was annealed to H2A-H2B as previously described (24). Denatured DNA was prepared by boiling DNA for 10 min followed by rapid cooling to 0°C. For in vitro use the preparations were sterilized by irradiation at 9,000 rads.

Human sera. Sera from typical patients with procainamide-induced lupus showing the characteristic IgG reactivity with (H2A-H2B)-DNA complex and serum from a normal control have been described in detail (3, 25). Serum from a patient with SLE was typical of patients showing reactivity to multiple components of chromatin including (H2A-H2B)-DNA and native DNA as previously described (26).

ELISA. Immulon 2 (Dynatech Laboratories, Inc., Alexandria, VA) microtiter plates were coated with antigen at 2.5 μg/ml, and the ELISA was performed as described (23, 24). For the antichromatin assays whole chromatin was used as the solid phase antigen. Oligonucleosomes and (H2A-H2B)-DNA were used as the soluble antigens in the competitive ELISA. Sera were generally diluted 1:200 and the bound antibodies were detected with peroxidase-conjugated anti–mouse IgG or IgM (Caltag, San Francisco, Ca). Immunoglobulin subclass-specific antibody was determined with horseradish peroxidase–conjugated anti-IgG1, -IgG2a, -IgG2b or -IgG3 (Caltag) diluted to produce a similar OD with the same amount of the respective subclass as described previously (27). Total IgG levels were determined by ELISA using anti–mouse kappa chain (Caltag) as capturing antibody and anti–mouse IgG coupled with horseradish peroxidase as detecting antibody as described (27). Generally, values shown for each data set were obtained from a single ELISA that was repeated at least once. Positive and negative control sera were included in each assay. For data determined in more than one ELISA in which precise comparisons were required, values were normalized by multiplying by the ratio of the reactivity of the positive control sera tested in both assays.

Indirect immunofluorescence staining. HEp2 12-well slides (Bion, Park Ridge, IL), mouse kidney/stomach slides (Sanofi Diagnostics, Chaska, MN) and MCF-7 cells, a human mammary epithelial adenocarcinoma (ATCC H2B 22) that was grown on coverslips and fixed in 80% methanol/20% acetone, were stained with mouse serum (1:40 dilution) followed by fluorescein-conjugated anti–mouse IgG (Caltag) and visualized with a fluorescence microscope (Olympus, Tokyo, Japan).

Proliferation assay. To assess splenocyte response to oligonucleosomes, (H2A-H2B)-DNA and histones, 0.5×10^6 viable splenocytes (as determined by trypan blue exclusion) were cultured in 0.2-ml wells in Clicks medium supplemented with 10% FCS, 4 mM glutamine, 5×10^{-5} M 2-mercaptoethanol, 100 U/ml penicillin and 100 μg/ml streptomycin (Sigma Chemical Co., St. Louis, MO). Antigen was added to a concentration of 50 μg/ml. After 1 wk, cells were collected by centrifugation, and 1×10^6 irradiated APC + 10 μg antigen were added. After 48 h 1 μCi [^{3}H]thymidine was added and cells harvested 24 h later. Incorporation of acid insoluble radioactivity was measured in a liquid scintillation spectrometer.

Spot ELISA. Antibody secretion by individual B cells from bulk splenocyte cultures was measured by spot ELISA, based on the method of Sedgwick and Holt (28). Serial dilutions of washed splenocytes starting at 2×10^6/ml in serum-free RPMI-1640 medium containing 1% albumin were added in triplicate to sells of 96-well Immulon 2 plates coated with goat anti–mouse kappa chain (Caltag) or the gelatin negative control. After 2 h at 37°C in a CO_2 incubator, plates were washed with PBS-tween and incubated for 2 h at room temperature with alkaline phosphatase-conjugated anti–mouse IgG + IgM or anti-IgG (Caltag). Colored product was developed after overnight incubation with 5-bromo-4-chloro-indoylphosphate (Sigma Chemical Co.) in 0.6% agarose at pH 10.2. Spots corresponding to the origin of antibody secretion were enumerated under a magnification of 40 and expressed per 2×10^6 cells/ml.

Cytokine measurement. IL-2 was determined by ELISA. Immulon 2 microtiter plates (Dynatech Laboratories, Inc.) were coated overnight with 100 μl of 50 ng anti–mouse IL-2 (PharMingen, San Diego, CA). After postcoating with gelatin solution, 10-fold serially diluted supernatants were added and incubated for 2 h. Bound IL-2 was detected with biotinylated anti–mouse IL-2 (PharMingen), followed by extr-avidin-alkaline phosphatase (Sigma Chemical Co.) and substrate and amplification solutions (Gibco BRL, Gaithersburg, MD) (29). A standard curve was constructed with each assay using known concentrations of IL-2 (PharMingen).

Thymus organ culture. Thymi from 2-d-old C57BL/6 mice were cultured in duplicate in transwell plates (Costar, Cambridge, MA) in 0.2 ml Iscove's medium containing various PAHA concentrations in 10% FBS, 1 mM glutamine, 1 mM pyruvate, 50 μM 2-mercaptoethanol, 100 U/ml penicillin and 100 μg/ml streptomycin. The next day the medium was replaced with fresh medium containing PAHA at the same final concentration. 24 h later each thymus was harvested, a single-cell suspension prepared and the cells were distributed into replicate wells of a 24-well tissue culture plate at 1×10^6 cells/ml, along with two- to threefold excess irradiated C57BL/6 splenocytes as APC. To triplicate wells was added either no antigen, total histones or oligonucleosomes at 50 μg/ml. After 1 wk, cells were harvested from each well, suspended in fresh medium to the same final volume and distributed into triplicate wells of a 96-well plate. APC and the same antigen preparation used during the expansion phase were added, followed by [^{3}H]thymidine 2 d later. Cells were harvested and incorporated radioactivity determined the following day.

Analysis of $CD4^+/CD8^+$ T cell subset distribution and viability. For flow cytometric analysis of thymocytes after PAHA treatment ex vivo, 1×10^6 cells from pooled lobes were incubated with anti–CD4-allophycocyanin (Caltag) and anti–CD8-phycoerythrin (Caltag) in 50 μl PBS containing 0.1% NaN_3 for 30 min at 4°C in the dark. Propidium iodide (1 μg/ml) was added to washed cells shortly before fluorescence activated cell sorter analysis to monitor viable cells. Three color data from 50–100,000 cells was collected on a FACSort® flow cytometer (Becton Dickinson, Mountain View, CA). CD4 and CD8 staining was analyzed on 10,000 viable cells (propidium iodide negative) gated around the lymphocyte window based on the extent of forward and side scatter.

Statistical analysis. Experimental and control groups were compared by Student's two-tailed *t* test.

Results

Antibody activity in sera from mice subjected to intrathymic PAHA. Within 1 wk after mice were injected with PAHA in the thymus, serum IgM anti-dDNA activity was significantly elevated compared to untreated or PBS injected controls ($P < 0.01$). As shown in Fig. 1, anti-dDNA activity at 5 wk reached a maximum of ~25-fold over the controls (1.00–1.93 OD in 11/11 PAHA-injected mice compared to < 0.05 OD in the controls), after which it began to decline. At this time (3 wk after the second PAHA injection) serum antichromatin activity was detected in 10/11 mice (Fig. 1). Antibody levels continued to rise up to 50-fold, remained elevated for 3 wk and then started to slowly decline. The range of peak antichromatin activities was similar to that of mice that develop lupus-like disease spontaneously, although the antichromatin activity in intrathymic PAHA-treated mice did not reach the level developed in older BXSB and MRL/lpr mice (Fig. 1). No antichromatin activity was detected in any of the control mice that were subjected to two intrathymic injections with PBS as well as two intraperitoneal (four mice) or intrasplenic (six mice) PAHA injections. In contrast to the anti-dDNA activity, which was exclusively IgM, only IgG antichromatin was detected. Using a subclass-specific assay, the antichromatin activity was largely limited to IgG1 (92–97%), with 3–8% of the IgG2a isotype (data not shown).

PAHA-induced antibodies showed homogeneous nuclear and chromosome staining of mitotic figures by indirect immunofluorescence (Fig. 2), typical of antichromatin reactivity with tissue culture cells. No cytoplasmic or membrane staining was apparent using transformed cells from human liver parenchyma (HEp2) or breast epithelium (MCF-7) origin. Sera from mice subjected to intrathymic PBS had no significant immunofluorescence reactivity. Using the more heterogeneous kidney/stomach section substrate, only nuclear staining was observed. Further studies on fine specificity were performed by ELISA on the individual components of chromatin including H2A-H2B, (H2A-H2B)-DNA, H3-H4, (H3-H4)-DNA, native DNA alone as well as the individual histones H1, H2A, H2B, H3, and H4. As shown in Table I for serum samples taken 39 d after the first intrathymic injection, only anti-[(H2A-H2B)-DNA] antibodies were detected, and this activity accounted for the bulk of the antichromatin antibodies. Determination of antibody specificity in sera from the intrathymic PAHA-treated mice at subsequent time points showed reactivity only

Table I. Antibody Activity to Chromatin and its Major Subunits in PAHA-treated Mice

Intrathymic agent	Mouse	IgG antibody activity by ELISA (OD)*			
		Chromatin	(H2A-H2B)-DNA	$(H3\text{-}H4)_2$-DNA	Histones
PAHA	1	1.020	0.899	−0.017‡	0.004
PAHA	2	0.965	0.756	−0.014	0.020
PAHA	3	0.799	0.237	−0.012	0.009
PAHA	4	1.062	0.549	−0.021	0.045
PAHA	5	2.254	0.820	−0.009	0.013
PAHA	6	2.580	0.998	−0.009	0.038
PAHA	7	0.005	0.002	−0.008	0.000
PBS	8	0.002	0.003	−0.006	0.001
PBS	9	0.004	0.001	−0.008	0.004

*Values are antibody binding to the indicated antigen 39 d after the first intrathymic injection. Sera were also tested for reactivity with individual histones, H2A-H2B dimer, H3-H4 tetramer, and native DNA; results were uniformly negative under conditions where positive controls were elevated (not shown). Typical results of a single assay performed twice are shown. ‡Negative values indicate that the measured OD was below the OD observed without serum.

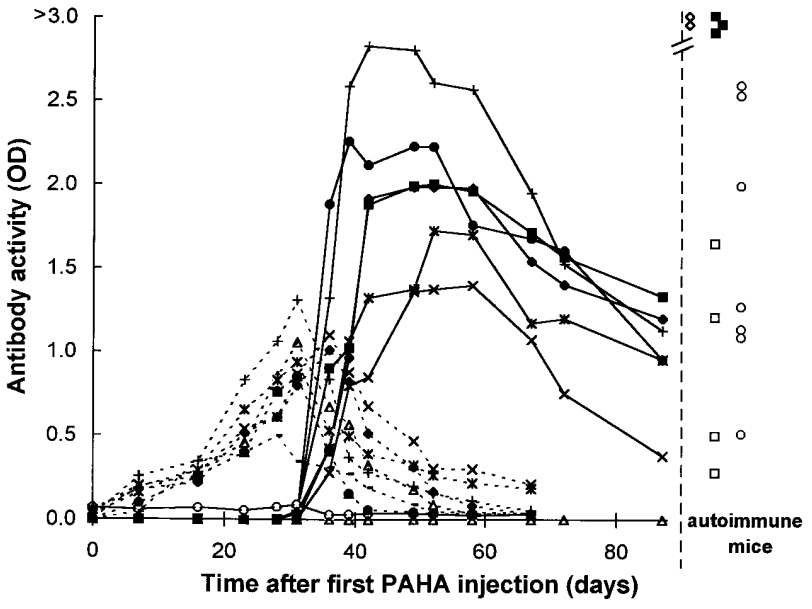

Figure 1. Serum antibody after intrathymic injection of PAHA. IgM anti-dDNA activity is shown by the dashed lines and IgG antichromatin activity is indicated by the solid lines. Antibody activity in serial serum samples of individual mice is represented by unique symbols. Results from two independent surgical procedures 6 mo apart are shown for a total of 11 experimental and 7 control mice (intrathymic PBS). Data for seven mice (three control, four experimental), which were killed 3 or 5 wk after the second intrathymic injection for evaluation of splenocyte function (Tables II and III) are not shown (IgG antichromatin activities at the day these four intrathymic PAHA mice were killed were 0.800; 0.408; 1.045; 1.978 OD). IgM anti-dDNA activity in a typical control mouse is shown (*circles*); IgG antichromatin activity in the controls was < 0.01 OD at all time points. One mouse subjected to intrathymic PAHA (*triangles*) failed to develop an antichromatin response. The panel on the right displays for comparison purposes the antichromatin activity in mice that develop spontaneous lupus-like autoimmunity. Single serum samples in 4-mo-old NZB/NZW F1 females (*circles*), 2-mo (*open squares*), and 5-mo (*filled squares*) old BXSB males and 4-mo-old MRL/lpr females (*diamonds*) are shown. The OD in this data set were normalized to that of the PAHA-treated mice. The samples plotted for convenience at ~3 OD actually had two to five times greater activity.

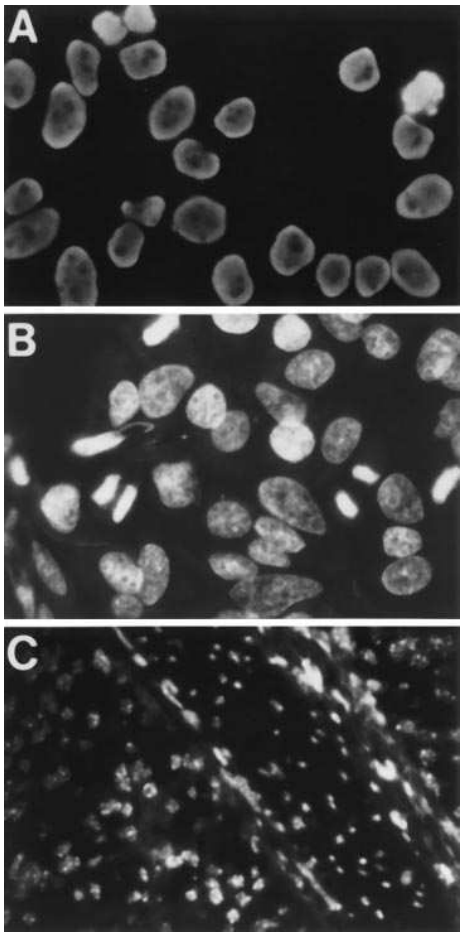

Figure 2. Indirect immunofluorescence of PAHA-induced antibodies. Typical IgG reactivity in sera 35–69 d after intrathymic PAHA is shown for HEp2 cells (*A*, initial magnification = 500), MCF-7 cells (*B*, initial magnification = 500) and a mouse kidney/stomach section (*C*, initial magnification = 400). All sera from PAHA-treated mice displayed similar staining patterns.

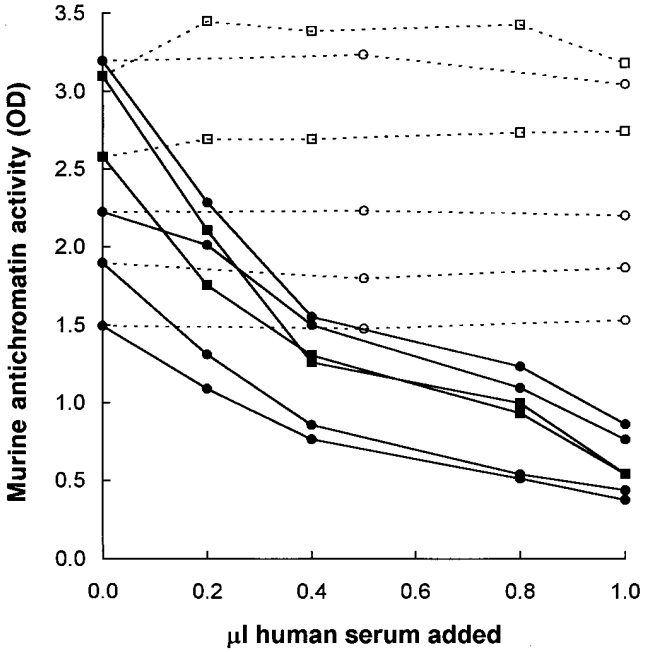

Figure 3. Competitive ELISA between murine PAHA-induced and human procainamide-induced antichromatin activity. Serum from a procainamide-induced lupus patient (*solid lines* and *filled symbols*) was used to compete for chromatin binding with mouse sera taken 35 (*squares*) or 56 (*circles*) d after PAHA injection. Serum from a healthy donor was used as a control (*dashed lines and open symbols*). Mouse serum was diluted 1:200 and human serum was added in the amounts indicated. Values are means of duplicate determinations of a typical one of two experiments.

with the (H2A-H2B)-DNA complex (data not shown) and that averaged 79±5% of the IgG chromatin binding.

Since IgG anti-[(H2A-H2B)-DNA] antibodies are also characteristic of patients with procainamide-induced lupus, blocking studies were performed to determine whether the epitopes targeted by the murine and human antibodies overlapped. As shown in Fig. 3, binding of mouse antibodies to chromatin showed a dose-dependent decrease with increasing concentrations of the human drug-induced lupus serum. The same amounts of normal human serum had no detectable effect on the mouse antichromatin activity.

Further examination of the specificity of the PAHA-induced antibodies and their comparison to chromatin-reactive antibodies arising spontaneously in autoimmune mice and in people with drug-induced and idiopathic lupus was performed by an antigen competition analysis. As shown in Fig. 4, antichromatin activity in PAHA-treated mice was remarkably sensitive to inhibition by soluble (H2A-H2B)-DNA. 65 ng of this complex, the smallest amount tested, blocked two-thirds to four-fifths of the binding to chromatin, an average of 20-fold more potency than the inhibitory capacity of oligonucleosomes, suggesting that PAHA-induced antibodies have a higher affinity for the monomeric (H2A-H2B)-DNA complex than for the same complex within the nucleosome. Of the autoimmune mice and the human patients tested, sera from the procainamide-induced lupus patients showed the most striking similarity to the intrathymic PAHA-treated mice. Inhibition of ~75% of the antichromatin activity in these patients required only 130 ng (H2A-H2B)-DNA, although, unlike the PAHA-treated mice, oligonucleosomes were an equally effective competitor. Antichromatin activity in the autoimmune mice was also inhibited by (H2A-H2B)-DNA, consistent with the demonstration of this antibody by direct binding studies (30), but ~1 μg was required to achieve 75% inhibition and oligonucleosomes were an even less effective competitor. Only oligonucleosomes could bring about 75% inhibition of the antichromatin activity in the SLE patient, consistent with the presence of a more heterogeneous set of chromatin-reactive antibodies as previously reported (26). Together, these observations indicate that antichromatin antibodies induced by intrathymic PAHA were particularly reactive with the (H2A-H2B)-DNA subunit and resembled most closely the specificity of antibodies in patients with procainamide-induced lupus.

Cellular immune responses induced by intrathymic PAHA. To determine whether intrathymic PAHA caused a generalized hyperimmune condition, Ig secretion by individual B cells from bulk splenocyte cultures was determined. Splenocytes from lupus-prone BXSB mice, known to display polyclonal B cell activation (31), were used as the positive control. As shown in Table II, a fourfold increase in total Ig-secreting cells

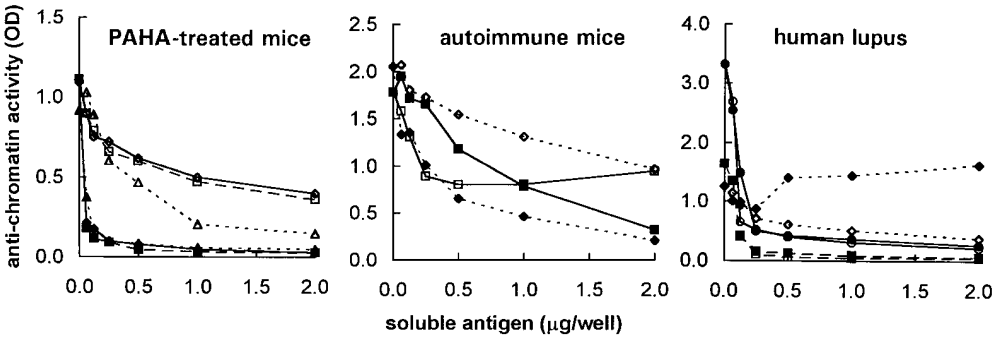

Figure 4. Inhibition of antichromatin activity by soluble antigen in a competition assay. The capacity of IgG in a diluted serum to bind chromatin-coated wells was measured in the presence of increasing amounts of soluble, competing antigen, solid symbols for (H2A-H2B)-DNA and open symbols for oligonucleosomes. The tested samples are divided into three panels, and each serum within a panel is distinguished by a unique type of line. Each of the three intrathymic-PAHA-treated mouse samples was a pool of sera 39–45 d after the first PAHA injection. The sera in the middle panel are from a 4-mo-old MRL/lpr mouse (*diamonds*) and a 2-mo-old BXSB (*squares*) mouse. The right panel depicts sera from two procainamide-induced lupus patients (*circles and squares*) and serum from a patient with SLE (*diamonds*).

and a 12-fold increase in IgG-secreting cells compared with C57BL/6 × DBA/2 mice were detected in the BXSB mouse. However, no significant increases in either the number of total Ig-secreting cells or of the IgG-specific cells were detected in mice 3 or 5 wk after the second intrathymic injection of PAHA. We also compared serum IgG levels in mice subjected to intrathymic PAHA or PBS. An age-dependent increase in the IgG levels of 4.2±0.7-fold between day 1 and day 49 was observed in control mice. The IgG levels of mice receiving intrathymic PAHA increased 3.9±2.1-fold. Thus, based on enumeration of splenic B cell activity as well as serum Ig levels, there was no detectable polyclonal B cell activation as a result of intrathymic PAHA. This conclusion is also consistent with the observation that autoantibody activity was restricted to anti-[(H2A-H2B)-DNA] (Table I).

The finding of antichromatin activity exclusively of the IgG isotype suggested T helper cell involvement, predominantly type 2 (Th2) based on the prevalence of the IgG1 subclass (32, 33). Autoreactive T cells should be detectable in the spleen, so we examined the capacity of splenocytes from PAHA-treated mice to respond to chromatin-related antigens. 3 or 5 wk after the second intrathymic injection, lymphocyte reactivity against oligonucleosomes, histones or (H2A-H2B)-DNA was determined using splenocytes expanded for 1 wk with each of these antigens. As shown in Table III, splenocytes from mice subjected to intrathymic PAHA displayed a 10–100-fold proliferative response to oligonucleosomes and a 10–16-fold response to (H2A-H2B)-DNA compared with the intrathymic PBS controls or to the proliferative response in the absence of antigen. Proliferation in response to histones was not significantly elevated in any of the mice, and there was no difference between the control and the PAHA-treated mice in the presence of only autologous antigen presenting cells.

IL-2 was detected at 2–3 ng/ml in the medium of cultured splenocytes obtained 5 wk after intrathymic PAHA and challenged with oligonucleosomes, a > 100-fold increase over the PBS controls. In contrast IL-2 in the [(H2A-H2B)-DNA]- or histone-stimulated cultures showed relatively little increase (Table III). These results suggest that T cells largely account for the splenocyte proliferative response to oligonucleosomes, whereas the (H2A-H2B)-DNA proliferative response may be due to splenic B cells. Thus, T cell reactivity against chromatin-derived antigens was present at a time point when anti–[H2A-H2B)-DNA] antibodies started to rise (Fig. 1), suggesting that autoreactive T cells appear in the periphery as a consequence of PAHA action in the thymus.

Effect of PAHA in thymic organ culture. The effect of PAHA on central T cell tolerance was tested directly by determining the capacity of PAHA to elicit chromatin-reactive T cells after PAHA treatment of thymi in organ culture. Thymi from 0–2-d-old mice were treated twice with PAHA over 2 d. As shown in Fig. 5, exposure of thymi to as low as 5 μM PAHA resulted in a subsequent 10-fold proliferative response of dissociated thymocytes to chromatin ($P < 0.01$) and a four-fold response to histones ($P < 0.05$), whereas thymocytes from untreated thymic organ cultures showed no significant response to either autoantigen. With 50 μM PAHA, thymocytes showed a 22-fold proliferative response to chromatin ($P < 0.001$). FACS® analysis immediately after the 2-d PAHA treatment of thymi showed no difference in the percentage of dead cells compared with untreated controls based on propidium iodide staining (Table IV), indicating that PAHA was not cytotoxic under these conditions. No significant change in the distribution of CD4/CD8 single or double positive thymocytes was detected, indicating that there was no global effect of PAHA on the number of thymocytes subjected to positive or

*Table II. Ig-Secretory Activity of Splenic B Cells**

Intrathymic agent	Mouse	Elapsed time	Ig-secreting cells/10^6 splenocytes	
			IgG + IgM	IgG
PBS	10	3 wk	1092±227	80±13
PAHA	11	3 wk	1100±87	147±32
PAHA	12	3 wk	1342±104	53±23
PBS	13	5 wk	2250±150	160±9
PBS	14	5 wk	2100±66	82±3
PAHA	15	5 wk	1875±195	107±8
PAHA	16	5 wk	567±76	70±13
None	BXSB	16-wk-old	4208±832	1100±90

*Mice subjected to intrathymic PAHA or PBS were killed either 3 or 5 wk after the second intrathymic injection. Data are means of triplicates±SD of two independent experiments.

*Table III. Capacity of Splenocytes to Respond to Chromatin-related Antigens**

Intrathymic agent	Mouse	Time	Proliferative response (cpm) | IL-2 response (pg/ml) to antigen			
			None	Oligonucleosomes	(H2A-H2B)-DNA	Histones
		wk				
PBS	10	3	340±360	2306±400	ND	1041±407
PAHA	11	3	139±14	22297±9244‡	ND	780±159
PAHA	12	3	184±160	83183±9558‡	ND	424±240
PBS	13	5	40±1 | 11	84±28 | 41	37±14 | 9	82±35 | 7
PBS	14	5	112±44 | 5	34±8 | < 5	137±56 | 13	48±13 | 11
PAHA	15	5	74±25 | 6	3709±1114‡ | 2371	1372±249‡ | 45	80±18 | 25
PAHA	16	5	79±12 | 4	5484±2670‡ | 3010	921±161‡ | 21	79±22 | 19

*Mice subjected to intrathymic PAHA or PBS were killed either 3 or 5 wk after the second injection. Cells were expanded and challenged with the indicated antigen, and proliferative responses were determined between days 2 and 3. Data are means of triplicates±SD of two independent experiments. A portion of the day 1 supernatant from the 5-wk cultures was saved for duplicate IL-2 determinations, the results of which are recorded to the right of the vertical line. ND, not determined. ‡Significant ($P < 0.001$) compared with the relevant PBS control(s).

negative selection. Together, these results suggest that the presence of PAHA in the thymus resulted in export to the periphery primarily of chromatin-reactive T cells.

Adoptive transfer study. Syngeneic mice were injected with chromatin-expanded splenocytes to determine whether humoral autoimmunity could be adoptively transferred with peripheral lymphocytes derived from mice subjected to intrathymic PAHA. As shown in Table V, adoptive transfer of PAHA-induced, chromatin-reactive splenocytes produced antichromatin autoantibodies in three of four mice, whereas control animals injected with chromatin-stimulated splenocytes from PBS-injected animals failed to show a detectable autoantibody response. The antichromatin response began to rise at around 21 d after transfer of activated T cells, similar to the time after the second intrathymic PAHA injection in the donor mice when these antibodies were initially detected.

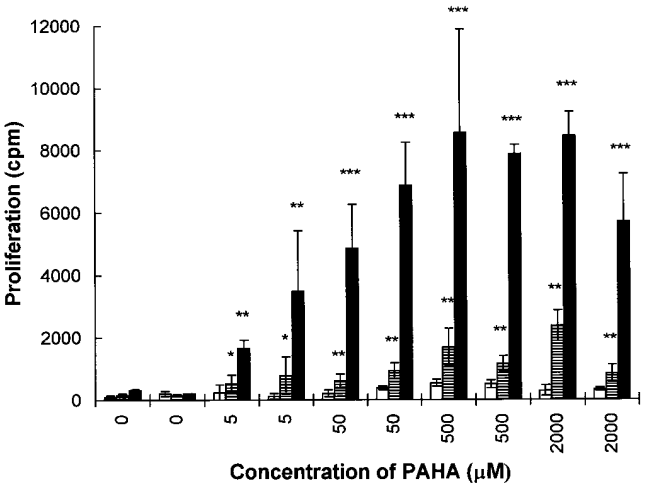

Figure 5. Effect of PAHA treatment during thymus organ culture on the appearance of thymocytes reactive with chromatin-derived antigens. After PAHA treatment thymocytes were expanded and challenged with total histones (*striped bars*), oligonucleosomes (*filled bars*), or no antigen (*open bars*). One experiment out of two with essentially the same results is shown. Data show means of triplicates±SD. *P* values are * < 0.05, ** < 0.01, *** < 0.001.

Discussion

The present study demonstrates the capacity of PAHA to produce autoimmune-like features as a result of intrathymic administration in mice. The absence of detectable polyclonal B cell activation in these mice suggests that autoantibody production was clonally restricted and antigen driven. Despite the lability of PAHA in vitro (8) and its probable transient residence in thymic tissue after the surgical procedure, PAHA treatment resulted in a delayed and relatively long-lasting loss of IgG tolerance to chromatin. In contrast, these mice showed a more immediate but transient IgM response to denatured DNA. The reciprocal relationship between the kinetics of anti-dDNA and antichromatin appearance in the serum (Fig. 1) raises the possibility that a strong T cell and chromatin drive delivered to anti–dDNA-specific B cells caused class switching and somatic mutation to B cells with antichromatin characteristics.

The serology of PAHA-treated mice has a striking resemblance to that of procainamide-treated patients. Most human subjects undergoing long-term treatment with procainamide remain asymptomatic with predominately IgM antibodies to dDNA and denatured histones, whereas the 20% of patients who develop lupus-like symptoms typically have less anti-dDNA but highly elevated IgG antichromatin (3, 25). The restriction in the immune response to the (H2A-H2B)-DNA

Table IV. Effect of PAHA in Thymic Organ Culture on Thymocyte Viability and Subset Distribution

Treatment	$CD4^+$	$CD8^+$	$CD4^+CD8^+$	Dead cells
	%	%	%	%
None	3.9±1.2	3.6±2.8	83.9±22.2	45.7±19.6
50 μM PAHA	7.4±2.9	2.7±0.6	85.1±14.1	49.7±20.0

Values are means±SD of three separate experiments.

Table V. Antichromatin Response after Adoptive Transfer of Splenocytes

Source of adoptively transferred splenocytes*	IgG antichromatin activity (OD)				
	Recipient	Day 0	Day 21	Day 29	Day 36
Mice subjected to intrathymic PAHA	No. 1	0.001	0.001	0.002	0.003
	No. 2	0.002‡	0.050‡	0.987‡	1.678‡
	No. 3	0.000	0.045	0.326	0.587
	No. 4	0.001	0.038	0.409	0.613
Mice subjected to intrathymic PBS and intrasplenic PAHA	No. 1	0.001	0.003	0.001	0.002
	No. 2	0.003	0.002	0.000	0.002
	No. 3	0.005	0.006	0.006	0.005

*5 wk after intrathymic PAHA or PBS, splenocytes from two mice subjected to each treatment were expanded for 1 wk on oligonucleosomes (Table III). After pooling and washing, 9.2×10^6 cells (63% viable) from PAHA-treated mice were injected intraperitoneally into each of four 6-mo-old C57BL/6 × DBA/2 mice; three control mice each received 11.7×10^6 splenocytes (41% viable) from PBS-treated mice. Blood was removed at the times shown and tested for IgG antichromatin at 1:40 or ‡1:200 dilution. Values shown for each serum at 1:40 dilution are after subtraction of background reactivity in the absence of antigen which ranged from 0.031 to 0.062 OD.

component of chromatin in both procainamide-induced lupus and mice injected with PAHA suggests that the immune system in both species had experienced a similar perturbation. The development of drug-induced lupus in late adulthood by disruption of central T cell tolerance would require a functioning thymus well into the sixth decade of life. It is well known that thymic involution resulting in a severe reduction in overall cellularity and thymic function occurs in late puberty. Nevertheless, bone marrow transplantation studies in aged mice demonstrated that an involuted thymus maintains its capacity to perform positive and negative selection, rejuvenating the T cell repertoire (34). We propose that transformation of circulating procainamide to PAHA, as demonstrated for neutrophils activated in vitro (10–13), may occur in the thymus by in situ activated phagocytic cells during a localized inflammatory event. Although PAHA generated in vivo by this episodic mechanism is unlikely to be physically detectable because of the reactive nature and lability of this metabolite (7, 8, 35), nitroprocainamide, a stable further oxidative product of the hydroxylamine, has been reported in the urine of procainamide-treated rats and patients (15, 36), and evidence for in vivo metabolism of procainamide to PAHA was obtained by the adoptive transfer popliteal lymph node assay (14). Although therapeutic steady-state plasma levels of procainamide are usually 15–40 μM (37) and we used 4 mM PAHA in vivo, PAHA was introduced into a thymic lobe as a single bolus in 20 μl, and we suspect that only a small proportion actually occupied thymic stroma. In fact in the better controlled in vitro environment of thymic organ culture, a 10-fold biological effect of PAHA was observed with only 5 μM PAHA.

Drug-induced lupus is generally assumed to result from interactions between the drug or its metabolite and an element of the immune system. Despite several attempts to detect a direct stimulatory effect of PAHA on lymphocytes (9, 38) or autoantibody production (39), no or only marginal changes have been reported. In the present study, PAHA also showed no capacity to directly activate the established peripheral immune system since injection of PAHA in the spleen or the peritoneal cavity had no detectable effect. Collectively, these results implicate T cells undergoing thymic maturation as the target of PAHA action. These observations are reminiscent of the capacity of semiallogeneic T cells from DBA/2 mice to induce autoantibodies (16–18) and support the view (40) that autoreactive T cells control the development of lupus-like autoantibodies.

Thymocytes reactive with self-antigen are normally either deleted or made unresponsive (anergic), and PAHA might interfere with either process. However, it is unlikely that abrogation of negative selection would be sufficient for T cells to leave the thymus in an autoreactive state since prevention of negative selection did not affect anergy in irradiated, bone marrow chimeric (41, 42), or transgenic mice (43, 44). Furthermore, failure of clonal deletion of high affinity self-reactive thymocytes in cyclosporin A–treated syngeneic mouse bone marrow chimeras (45) resulted in no (46) or only a small (47) increase of T cells bearing "forbidden" T cell receptors in the periphery. In addition, the no-antigen controls in Table III argue against an effect of PAHA on negative selection since splenocytes from the mice subjected to intrathymic PAHA failed to respond in the absence of added antigen. Therefore, global immune tolerance was preserved to the strong negative-selecting membrane self-antigens such as the minor lymphocyte-stimulating antigens that are expressed on the surface of splenic and thymic antigen presenting cells. Finally, since PAHA is cytotoxic to dividing cells in vitro under certain conditions (8, 35, 38), it is possible that intracellular macromolecules released from dying thymocytes could influence T cell selection. However, no increase in cell death was detected by exposure of thymi to PAHA ex vivo in the present studies. Furthermore, it has recently been shown that introduction of excess chromatin by intrathymic injection of polynucleosomes results in temporary suppression of the spontaneous antichromatin production in BXSB mice (20), presumably by promoting negative selection. Therefore, increased exposure to self-material in the thymus would not be expected to enhance autoantibody production.

Several groups have suggested that T cell receptor stimulation by low affinity interaction with self-antigen in the absence of costimulation accompanies T cell maturation in the thymus, rendering emerging thymocytes unresponsive to the selecting self-antigens (48, 49). T cells anergic to self- but not to foreign antigen have been shown to appear in the periphery (42, 44, 50). Interference by PAHA of anergy induction during positive selection of T cells on chromatin-derived epitopes could result in export of T cells with capacity to respond to this self-antigen. These chromatin-reactive T cells would become activated and expand in response to antigen expressed on professional antigen presenting cells. Upon interaction with B cells displaying epitopes derived from endogenous chromatin, chromatin-reactive T cells could provide help for B cell expansion, somatic mutation, and autoantibody production. The simplest interpretation of the kinetics of autoantibody appearance in the intrathymic PAHA-treated mice is that the anti–[(H2A-H2B)-DNA] specificity arose by somatic mutation from B cells with Ig receptors that recognized dDNA regions in chromatin followed by affinity maturation to (H2A-H2B)–containing structures. Chromatin, especially the (H2A-H2B)-DNA subnucleosome region, has been proposed to be an important B

cell antigen in murine (18, 19, 30, 51–53) and human (26, 54, 55) lupus-like syndromes. Alternatively, these antibodies may have no genealogical relationship and arose independently. Studies on the primary structure of monoclonal antibodies to dDNA and (H2A-H2B)-DNA derived from PAHA-treated mice should resolve this issue.

Chromatin-stimulated splenocytes from mice subjected to intrathymic PAHA induced antichromatin antibodies de novo upon adoptive transfer into normal, syngeneic mice. It is highly likely that chromatin-reactive T cells were responsible for this effect since the adoptively transferred cells produced large amounts of IL-2 in response to oligonucleosomes, and it was previously shown that only splenic T cells from the same mouse strain undergoing chronic graft-versus-host disease had capacity to induce autoantibodies in secondary recipients (56). It is interesting that the lag time between the introduction of 5.8×10^6 viable splenocytes containing chromatin-reactive T cells and the appearance of autoantibodies was essentially the same as that after injection of PAHA into the thymus in which the initial number of chromatin-reactive T cell precursors would presumably be far lower. These observations suggest that in the normal mouse the B cell repertoire includes a relatively small number of nontolerant chromatin-reactive B cells that require several weeks of stimulation by T helper cells to mutate, expand, and secrete detectable autoantibodies. The precursor frequency of B cells with autoreactivity to intracellular antigens in normal adult mice was reported to be $\sim 3\%$ of the total Ig-secreting splenocytes (57).

In addition to its significance for drug-induced lupus, our findings add weight to the view that immature T cells are selected in the thymus by low affinity interaction between the $\alpha\beta$ T cell receptor and peptidic epitopes derived from self-antigen in a process that leaves these cells nonresponsive to the selecting antigen (44, 49, 50, 58). We suspect that peptides on thymic epithelium involved in positive T cell selection are commonly derived from chromatin because of the high abundance of this material in the thymus, greater than half the macromolecular mass in a crude homogenate of calf thymus (Kretz-Rommel, A., and R.L. Rubin, unpublished observations). If epitope concentration in addition to affinity is a critical factor in the generation of the T cell repertoire as proposed (48, 59–61), appearance of T cells reactive with an array of chromatin-derived epitopes might be the most sensitive indicator of the disruption of central T cell tolerance. In our recent studies, exposure of T cell clones to micromolar concentrations of PAHA prevented induction of anergy in an in vitro system that may reflect the events accompanying positive selection of T cells on self-antigen in the thymus (62). The general importance of this mechanism is suggested by the high precursor frequency of chromatin-reactive T cells in murine (63) and human lupus (64) and by the ubiquitous presence of antichromatin antibodies in lupus induced by several drugs (3, 65), in idiopathic SLE (26, 54, 66), and in murine models of SLE (30, 51).

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