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*J Immunol* 1999; 162:813-820; ;
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Persistence of Autoreactive T Cell Drive Is Required to Elicit Anti-Chromatin Antibodies in a Murine Model of Drug-Induced Lupus

Anke Kretz-Rommel and Robert L. Rubin

Long-term treatment with procainamide and numerous other medications is occasionally associated with the development of drug-induced lupus. We recently established a murine model for this syndrome by disrupting central T cell tolerance. Two intrathymic injections of procainamide-hydroxylamine (PAHA), a reactive metabolite of procainamide, into (C57BL/6 × DBA/2)F1 mice resulted in the appearance of chromatin-reactive T cells and anti-chromatin autoantibodies. The current study explores in this model the role of autoreactive T cells in autoantibody production and examines why autoantibodies after a single intrathymic drug injection were much more limited in isotype and specificity. Injection of as few as 5000 chromatin-reactive T cells into naive, syngeneic mice induced a rapid IgM anti-denatured DNA response, while injection of at least 100-fold greater number of activated T cells was required for induction of IgG anti-chromatin Abs, suggesting that small numbers of autoreactive T cells can be homeostatically controlled. Mice subjected to a single intrathymic PAHA injection after receiving splenic B cells from an intrathymic PAHA-injected syngeneic donor also developed anti-chromatin Abs, but adoptive transfer of similarly primed T cells or of B cells without intrathymic PAHA injection of the recipient failed to produce an anti-chromatin response. However, anti-chromatin Abs developed after a single intrathymic PAHA injection in Fas-deficient C57BL/6-lpr/lpr mice, suggesting that activation-induced cell death limited autoimmunity in normal mice. Taken together, these results imply that chromatin-reactive T cells produced by intrathymic PAHA created a B cell population primed to somatically mutate and Ig class switch when subjected to a heavy load or second wave of autoreactive T cells. The Journal of Immunology, 1999, 162: 813–820.

Drug-induced lupus is the prototype for an aseptic systemic autoimmune disease caused by a known environmental agent. The drug procainamide poses the greatest risk for developing this syndrome (1) probably through the action of its reactive metabolite, procainamide-hydroxylamine (PAHA) (2–4). Most patients undergoing prolonged procainamide therapy develop anti-denatured DNA (dDNA) (5) and anti-histone Abs (6), whereas IgG Abs to the (H2A-H2B)-DNA subunit of chromatin are a serologic marker in patients who develop symptomatic procainamide-induced lupus (7). We recently established a murine model exhibiting these hallmark autoimmune features of drug-induced lupus (8). In this animal model, a single injection of PAHA into the thymus of (C57BL/6 × DBA/2)F1 mice resulted in the rapid appearance of IgM anti-dDNA and anti-histone Abs; after a second intrathymic PAHA injection, chromatin-reactive T cells were detected in the spleen, and IgG anti-chromatin Abs rose to levels typically seen in spontaneous models of murine lupus. Chromatin-specific T cells from (SWR × NZB)F1 mice were shown to provide helper activity in vitro for autoantibody production to DNA, histones, and histone-DNA complexes (9, 10), and to accelerate glomerulonephritis when adoptively transferred into syngeneic preautoimmune mice (9). It seems likely, therefore, that the chromatin-reactive T cells that appeared in the periphery after intrathymic injection of PAHA may direct autoantibody induction in this system as well. However, in spontaneous models of murine lupus, it is generally believed that multiple genetic defects are required for autoimmunity to develop (11, 12), and direct demonstration that chromatin-reactive T cells alone are sufficient to induce anti-chromatin Abs in mice with normal genetic backgrounds is lacking.

The finding that autoimmunity developed by introduction of PAHA only in the thymus and not the periphery strongly suggested that disruption of central T cell tolerance underlies the observed phenomena. However, why two intrathymic PAHA injections were required for appearance of anti-chromatin Abs is unclear. It is possible that the autoimmunity-inducing effector function of chromatin-reactive T cells that emigrated to the periphery after a single intrathymic injection of PAHA was inhibited by peripheral tolerance mechanisms such as induction of anergy (13), down-regulation of the TCR or its coreceptors (14), and/or clonal elimination by activation-induced cell death (15).

The current study evaluates the role of chromatin-reactive T cells in eliciting autoantibodies by adoptive transfer of a T cell line into naive syngeneic recipients. We also address the question of why the autoimmune response after a single intrathymic PAHA injection was limited to IgM autoantibodies, whereas only after a second intrathymic injection were true autoantibodies of the IgG isotype produced. The overall results indicate that chromatin-reactive T cells were necessary and sufficient to drive the autoantibody response, but peripheral tolerance mechanisms involving Fas...
could be overcome only if the animal was subjected to an abundant level or second wave of chromatin-reactive T cells.

Materials and Methods

Animals

(C57BL/6 × DBA/2)F1 female mice were purchased from The Jackson Laboratory (Bar Harbor, ME). For examining the effect of age on the autoimmune response, mice of 4 to 8 wk of age were obtained in the same shipment. Female C57BL/6, C57BL/6-lpr/lpr, and AJ mice were obtained from The Scripps Research Institute (La Jolla, CA) breeding colony.

Intrathymic injections

Injections were performed as described previously (16). Unless otherwise indicated, 5-wk-old (C57BL/6 × DBA/2)F1 mice were injected under anesthesia in each thymic lobe with 20 μl of 4 mM PAHA in PBS. Control animals received thymic injections of PBS. Incisions were closed with silk sutures. In some experiments, mice were subjected to a second injection 2 wk later. Blood was collected once or twice per week by retroorbital puncture under methoxyflurane anesthesia.

Isolation of T and B cells

Splenocytes were harvested and the T cell population was prepared by cytotoxic lysis of B cells using anti-mouse B220 (clone RA3-6B2; Caltag, San Francisco, CA) and rabbit C (Accurate Chemical & Scientific, Westbury, NY). The splenic B cell population was obtained by lysing T cells with anti-mouse α/β TCR (clone H57-597; Caltag, San Francisco, CA) and rabbit C. Purity of the populations was determined by FACScan analysis, as shown in Results.

FACS analysis of B and T cells

For flow-cytometric analysis of the enriched splenic B and T cell preparations, 1 × 10^7 cells were incubated with either FITC-coupled polyclonal anti-mouse IgM (Caltag) or anti-CD3 (PharMingen, San Diego, CA) in PBS/0.1% NaN3. After washing, cells were analyzed with a FACSort flow cytometer (Becton Dickinson, Mountain View, CA). Staining patterns of 10,000 cells within the lymphocyte gate based on the extent of forward and sideward scatter were determined.

Expansion of chromatin-reactive T cells

Splenocytes harvested 21 days after a second intrathymic PAHA injection were cultured in Clicks medium supplemented with 10% FCS, 4 mM glutamine, 5 × 10^−3 M 2-ME, 100 U/ml penicillin, and 100 μg/ml streptomycin (Sigma, St. Louis, MO) in 24-well plates with the addition of 10% supernatant of Con A-activated rat splenocytes and 50 μg/ml soluble (H1-depleted) chromatin (prepared as previously described (8, 17)). T cells were expanded by weekly dilution to 2 × 10^6 cells/well and addition of 50 μg/ml chromatin and 5 × 10^6 fresh syngeneic splenocytes irradiated at 3000 rad to serve as APC.

Adoptive transfer studies

A total of 5 × 10^6 primary B or T cells or 500 to 5 × 10^6 T cells expanded on chromatin for 2 mo was injected in 200 μl PBS into the tail vein. Some mice also received intrathyamic PAHA the following day. To control for autoantibody production due to nonspecific T cell help, AJ mice were injected i.v. with 6 × 10^6 12-11 cells, a Th type I clone derived from an A/J mouse and specific for human γ-globulin (HGG) (18). Similar to chromatin-reactive T cell lines, 12-11 cells were maintained by addition of APC, Ag, and 10% supernatant of Con A-activated rat splenocytes, as described (19). 12-11 cells and chromatin-reactive T cell lines were rested for 8 to 10 days after Ag stimulation before adoptive transfer. Two days before injection of HGG-specific T cells, AJ mice also received i.p. 200 μg HGG in CFA (Difco, Detroit, MI). Some AJ mice only were immunized with HGG in Freund’s adjuvant. Mice were serially bled weekly.

ELISA for Abs to HGG, histone, dDNA, and chromatin

Immulon 2 (Dynatech Laboratories, Alexandria, VA) microtiter plates were coated with Ag at 2.5 μg/ml, and the ELISA was performed as described (17, 20). Sera were diluted 1/200, unless indicated otherwise, and the bound Abs were detected with peroxidase-conjugated anti-mouse IgG or IgM (Caltag).

Proliferation assay

To determine the proliferative response to chromatin, 5 × 10^6 primary splenocytes were cultured in 1 ml Clicks complete medium supplemented with 50 μg soluble chromatin. After 1-wk expansion, cells were harvested, and 1 × 10^6 fresh irradiated splenocytes as APC and 10 μg chromatin/well were added to replicate wells of 96-well plates. After 48 h, 1 μCi [3H]thymidine was added and cells were harvested 18 h later. Incorporation of radioactivity was measured in a liquid scintillation spectrometer.

Results

Capacity of chromatin-reactive T cells to induce autoantibodies in vivo

We previously demonstrated that detection of chromatin-reactive T cells in the spleen coincided with the appearance of antichromatin Abs 3 wk after the second injection of PAHA into the thymus of (C57BL/6 × DBA/2)F1, mice (8). To determine the capacity of chromatin-reactive T cells to elicit an autoimmune response, we expanded with soluble chromatin splenic T cells from a mouse subjected to two intrathymic PAHA injections and adoptively transferred these chromatin-reactive T cells into otherwise untreated syngeneic mice. At the time of transfer, the T cell line showed a proliferative response to chromatin with a stimulation index of 10 (data not shown). Naïve mice in groups of three received i.v. injections of 500 to 5 × 10^6 cells.

Fig. 1A shows the autoantibody activity at various time points after adoptive transfer of incremental numbers of chromatin-reactive T cells. IgM anti-dDNA Ab activity was unaffected by injection of 500 T cells, but became elevated in mice that had received as few as 3000 T cells and was detected as early as 7 days after the transfer. No clear IgM anti-dDNA dose response with increasing numbers of chromatin-reactive T cells was observed, and the Ab activity declined after 3 wk. IgG anti-dDNA and anti-histone Abs were only detected in mice that had received at least 0.5 × 10^6 cells, and a period of up to 2 wk after adoptive transfer was required for these Abs to appear. IgG anti-chromatin Abs were not detected until 3 wk after the transfer, and only mice receiving 0.5–5 × 10^6 cells displayed these Abs. The lack of a time-dependent increase in anti-dDNA activity and the requirement for much greater numbers of T cells to elicit IgG anti-chromatin, dDNA, and histone Abs suggests that some form of homeostatic regulation was operating to control the autoimmune response in this system.

To test whether the production of autoantibodies after adoptive transfer was dependent on chromatin-specific T cells and not just the result of nonspecific cytokine effects due to activated T cells within the B cell microenvironment, autoantibodies were measured in mice undergoing a strong Ab response to a foreign Ag. A/J mice immunized with HGG displayed a very robust immune response to this Ag, since sera had to be diluted 20,000-fold to quantify this activity (Fig. 1B). However, this immune response was not accompanied by anti-chromatin or anti-histone Ab production. Nevertheless, a small but significant increase in IgM and IgG anti-dDNA Ab titers was detected. Injection of a HGG-specific T cell clone 2 days before HGG immunization did not enhance the anti-HGG response presumably because the B cell response to endogenous HGG-specific T cells could not be boosted above the level achieved by immunization alone. In addition, the HGG-specific T cells had little effect on the anti-dDNA response and had no capacity to induce anti-chromatin Abs, indicating that the chromatin specificity of the T cells used for adoptive transfer in Fig. 1A was required for the anti-chromatin Ab production.

Importance of age at the time of intrathymic injection

The adoptive transfer studies pointed to the importance of the quantity of autoreactive T cells for eliciting IgG autoantibodies. It is possible, therefore, that the previously observed requirement for two intrathymic injections of PAHA to elicit IgG anti-chromatin Abs (8) may simply reflect inadequate numbers of autoreactive T cells.
cells produced after a single PAHA injection. However, the mice in these studies were only 5 wk old when they received the first intrathymic injection, and 7 wk old when they received the second injection. This is a significant age difference, and raises the possibility that natural developmental maturation of B cells or T cells between 5 and 7 wk of age accounted for the more robust, IgG-dominated autoantibody response after the second injection.

To test whether the age at which mice are injected with PAHA is important for production of anti-chromatin Abs, we subjected mice of 5, 6, 7, 8, and 9 wk of age to a single dose of intrathymic PAHA. None of these mice developed anti-chromatin Abs. Interestingly, significant differences with regard to the anti-histone or anti-dDNA responses were detected in the different age groups. As shown in Fig. 2A, mice of all ages developed an IgM anti-dDNA response. However, in 5-wk-old mice, IgM anti-dDNA Ab quickly declined, whereas in older mice it reached higher levels and remained elevated at least 10 wk after intrathymic injection of PAHA. The IgM anti-histone response was not induced in mice of 5 wk of age, as previously reported (8), but was detectable after intrathymic injection of 7-wk-old mice, in which it remained elevated for at least 10 wk (Fig. 2B). IgM anti-histone Ab levels in mice injected at 8 or 9 wk of age were not as elevated as in the 7-wk-old mice, although they were significantly higher 4 wk after injection compared with the mice injected at 5 wk. Taken together, with regard to the IgM anti-histone and the IgM anti-dDNA response, there was a trend in older mice to produce a more robust...
and more persistent response to intrathymic PAHA. In addition to the IgM Ab responses, IgG autoantibodies appeared in older mice, but these were limited to anti-histone Abs (Fig. 2C), and no IgG reactivity to dDNA or chromatin was detectable. Failure of the autoimmune response to develop reactivity with native epitopes on chromatin is consistent with the data in Fig. 1, suggesting that autoimmunity was controlled by a peripheral tolerance mechanism when only one injection of PAHA was introduced in the thymus of normal mice.

To examine at the cellular level the basis for this apparent immune down-regulation, the responsiveness of splenocytes to chromatin after one versus two PAHA injections was compared. Three mice were sacrificed 2 or 5 wk after one intrathymic PAHA injection or 3 wk after the second intrathymic PAHA injection. To detect a chromatin-specific response, it was necessary to expand the splenocyte population for 1 wk on chromatin, as described in Materials and Methods. As summarized in Table I, a >10-fold T cell response to chromatin was observed 2 wk after a single PAHA injection, but chromatin-reactive T cell activity declined to near baseline by 5 wk postinjection. Splenocyte proliferative response to chromatin 3 wk after the second intrathymic PAHA injection was only slightly higher than the chromatin response 2 wk after the first injection, but chromatin-reactive T cells in double-injected mice remained elevated at least 5 wk after the first injection. These observations suggest that the persistence of the T cell response is critical for the appearance of anti-chromatin Abs.

**Primming effect of first intrathymic PAHA injection**

The preceding data suggest that to develop an IgG anti-chromatin response, two intrathymic injections of PAHA may be needed to sustain sufficient numbers of chromatin-reactive T cells, thereby overcoming the natural homeostatic regulation of this autoimmune response. In addition, the first intrathymic PAHA injection may have resulted in some form of priming in an immune compartment such that a qualitative change in B and/or T cells made these lymphocytes more prone to express autoreactive specificities. To test the importance of B cell or T cell priming, 5-wk-old (C57BL/6 × DBA/2) F1 mice were subjected to one or two intrathymic PAHA injections and the importance of B cell or T cell priming, 5-wk-old (C57BL/6 × DBA/2) F1 mice were subjected to one or two intrathymic PAHA injections. As summarized in Table I, a >10-fold T cell response to chromatin was observed 2 wk after a single PAHA injection, but chromatin-reactive T cell activity declined to near baseline by 5 wk postinjection. Splenocyte proliferative response to chromatin 3 wk after the second intrathymic PAHA injection was only slightly higher than the chromatin response 2 wk after the first injection, but chromatin-reactive T cells in double-injected mice remained elevated at least 5 wk after the first injection. These observations suggest that the persistence of the T cell response is critical for the appearance of anti-chromatin Abs.

![Table I. Proliferative response to chromatin of splenocytes from (C57BL/6 × DBA/2) F1 mice subjected to one or two intrathymic PAHA injections](image)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>First Infection (wk)</th>
<th>Second Infection (wk)</th>
<th>Spleen Harvest (wk)</th>
<th>Proliferative Response (cpm) (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td>5</td>
<td>None</td>
<td>7</td>
<td>756 ± 386 534 ± 180</td>
</tr>
<tr>
<td>PAHA</td>
<td>5</td>
<td>None</td>
<td>7</td>
<td>672 ± 35 7990 ± 8313</td>
</tr>
<tr>
<td>PAHA</td>
<td>5</td>
<td>None</td>
<td>10</td>
<td>310 ± 111 889 ± 610</td>
</tr>
<tr>
<td>PAHA</td>
<td>5</td>
<td>7</td>
<td>10</td>
<td>358 ± 15 12509 ± 7435</td>
</tr>
</tbody>
</table>

**FIGURE 3.** FACS analysis of splenocytes used for adoptive transfer. A, Anti-CD3 staining of B cell preparation before (bold line) and after (dotted line) lysis with anti-Thy-1.2 + C, and B, Anti-IgM staining of the T cell preparation before (bold line) and after (dotted line) lysis with anti-B220 + C.

4. only those animals that had received B cells derived from a PAHA-injected animal as well as an intrathymic PAHA injection developed IgG anti-chromatin Abs. Mice that had received T cells from a PAHA-injected animal with or without intrathymic PAHA injection of the recipient failed to develop anti-chromatin Abs. Similarly, if B cells from PAHA-treated mice were transferred into mice without subsequent intrathymic PAHA, no anti-chromatin Abs developed. The inability of adoptively transferred, primed T cells to induce anti-chromatin Abs was not due to nonspecific suppression effects or failure of the intrathymic injections because the IgM anti-dDNA response in these mice associated with one intrathymic PAHA injection developed to a similar extent as that in the group that received primed B cells or no cells at all (Table II). Taken together, these results suggest that intrathymic PAHA in the donor created a population of B cells primed to rapidly somatically mutate and Ig class switch to secrete IgG anti-chromatin Abs when subjected to a second wave of chromatin-reactive T cells.

**Response of Fas-deficient mice to intrathymic PAHA**

We suspected that the disappearance of the T cell response to chromatin 5 wk postinjection and failure to produce an IgG Ab response to chromatin after a single intrathymic PAHA injection might be due to homeostatic regulation of the immune response by some form of peripheral tolerance. Loss of T cell activity by activation-induced cell death has been described for Ag-engaged T cells in vitro as well as in vivo (15, 21–23), and the Fas/Fas ligand system is likely to mediate this process since most activated CD4+ Th cells are killed by Fas engagement (24). If Fas-mediated killing were responsible for controlling the immune response in this system, a single PAHA injection into the Fas-deficient C57BL/6-lpr/
lpr mice might be sufficient to induce an anti-chromatin response. As shown in Fig. 5A, similar to the observations in Fas-intact mice, anti-dDNA Abs were detectable 1 wk after intrathymic PAHA injections into C57BL/6-lpr/lpr mice and reached a maximum after 2 wk before slowly declining. However, in contrast to Fas-intact C57BL/6 mice (Fig. 5B), C57BL/6-lpr/lpr mice subjected to only one intrathymic PAHA injection developed a delayed but generally strong IgG anti-chromatin response in seven of nine animals, first detectable 30 to 42 days after PAHA injection. There was considerable variability in the magnitude of the response among the treated mice, but the anti-chromatin activity was remarkably long-lasting (at least 9 wk) and reached very high levels in three mice during a 3- to 4-wk period. Three of the seven anti-chromatin-producing mice exhibited declining Ab levels after 7 wk, but titers were still at 2 OD after 70 days. Of the six intrathymic PBS-treated controls, two mice showed slightly elevated anti-chromatin autoantibody levels, but this activity was not detectable until 9–10 wk after intrathymic injection, when the mice were approximately 15 wk old. Female C57BL/6-lpr/lpr mice were reported to have substantial lymphoproliferation by 18 wk (25), at which time IgG anti-DNA Abs (26) and IgM rheumatoid factor (27) were detected. Therefore, the appearance of low level anti-chromatin and anti-dDNA activity in a minority of the control mice is consistent with the development of a delayed autoimmune response in these mice.

Table II. Anti-dDNA activity in (C57BL/6 × DBA/2)F1 mice after adoptive transfer of a B or T cell-enriched preparation of splenocytes from syngeneic mice subjected to one intrathymic injection of PAHA

<table>
<thead>
<tr>
<th>Cells Transferred</th>
<th>Intrathymic Injection</th>
<th>IgM Anti-dDNA Activity (OD) After Intrathymic Injection (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>day 0</td>
</tr>
<tr>
<td>T cells None</td>
<td>0.163 ± 0.133</td>
<td>0.499 ± 0.257</td>
</tr>
<tr>
<td>T cells PAHA</td>
<td>0.284 ± 0.238</td>
<td>0.811 ± 0.491</td>
</tr>
<tr>
<td>B cells None</td>
<td>0.301 ± 0.097</td>
<td>0.260 ± 0.203</td>
</tr>
<tr>
<td>B cells PAHA</td>
<td>0.293 ± 0.128</td>
<td>1.893 ± 0.311†</td>
</tr>
<tr>
<td>None PAHA</td>
<td>0.066 ± 0.065</td>
<td>0.935 ± 0.761*</td>
</tr>
</tbody>
</table>

* Intrathymic injection of the cell recipient. Donor mice were subjected to intrathymic PAHA 2 wk prior to harvesting and pooling splenocytes.

p-values vs day 0 of the same group are <0.05 (*), <0.02 (†), <0.01 (‡), and 0.001 (§) using Student’s two-tailed t test.

FIGURE 4. Anti-chromatin activity in (C57BL/6 × DBA/2)F1 mice after adoptive transfer of either a B cell- or T cell-enriched preparation (Fig. 3) of syngeneic splenocytes pooled from mice that had received one intrathymic PAHA injection 2 wk earlier. A total of 5 × 10⁶ cells was injected i.v. into mice that received one intrathymic injection of either PAHA or PBS the following day. Solid lines depict the response of three mice receiving primed B cells + intrathymic PAHA. All other groups, including mice receiving primed T cells or receiving T or B cells without intrathymic PAHA injection of the recipient, showed no response (dashed lines at 0 OD).

FIGURE 5. Effect of mutated Fas on the capacity of PAHA to induce anti-dDNA and anti-chromatin Abs. A, A single intrathymic injection of PAHA was performed on groups of three to six C57BL/6-lpr/lpr mice on four different occasions to obtain sufficient 5-wk-old mice for this study. The IgG anti-chromatin response in individual mice is shown for a total of nine PAHA-injected mice (filled symbols, solid lines) and six control mice subjected to intrathymic PBS (open symbols, solid lines). For clarity, the IgM anti-dDNA activities were averaged for the nine PAHA-treated mice (solid diamond, dashed line) and the six PBS-treated mice (open triangle, dashed line). Error bars are SD. B, One or two intrathymic injections with PAHA were performed on C57BL/6 control mice. The IgG anti-chromatin response in each of four mice receiving a single PAHA injection (filled symbols, solid lines), and the average IgM anti-dDNA response of these four mice (dashed line) are shown. One mouse received two PAHA injections 2 wk apart as a positive control (IgG anti-chromatin reactivity shown in open symbols, solid line). Error bars are SD.
mice is most likely related to spontaneous production of these Abs associated with aging in C57BL/6-lpr/lpr mice. Overall, these results suggest that in the absence of Fas expression, activation-induced cell death no longer limits the capacity of chromatin-reactive T cells produced from a single intrathymic injection of PAHA to provide helper activity for generation of an IgG anti-chromatin response.

Discussion

It is widely believed that autoantibody production in systemic lupus erythematosus and murine lupus requires CD4+ T cell help (28). Whereas in normal mice autoreactive T cells are absent or present in only very low numbers, disruption of central T cell tolerance by introduction of PAHA into the thymus resulted in the release of chromatin-reactive T cells into the periphery. Activated chromatin-reactive T cells were sufficient to induce anti-chromatin Abs when adoptively transferred into naive mice. However, rather high numbers of T cells (at least 0.5 × 10^6) were required to induce this response; with introduction of fewer chromatin-reactive T cells, the autoimmune response was limited to IgM anti-dDNA and/or anti-histone Abs. These results are consistent with an active down-regulation of autoreactive T cells, and only when this homeostatic control is overwhelmed by excess bolus of T cell help can the putative Ig class switching and Ab affinity maturation in the B cell compartment take place.

The autoimmune serology induced by injection of 5–50 × 10^3 chromatin-reactive T cells was similar to that in response to a single injection of intrathymic PAHA, dominated by IgM anti-dDNA and anti-histone Abs. However, autoantibodies induced by one intrathymic PAHA injection in 5-wk-old mice declined to negligible levels over the subsequent 5 wk, and chromatin-reactive T cells were nearly undetectable by this time, consistent with the view that homeostatic regulation of Th cells prevented further maturation of the immune response. When mice a few weeks older were given intrathymic PAHA, a more robust and long-lasting immune response developed, which included IgM and IgG anti-histone Abs, but Abs to native epitopes on chromatin still failed to develop. Only by reinjecting PAHA 2 wk after the first injection to produce a second wave of chromatin-reactive T cells emerging from the thymus were chromatin-reactive lymphocytes detectable in the spleen 3 wk later. At this time, IgG anti-chromatin Abs dominated the serology of these mice, similar to that observed in mice adoptively transferred with excess chromatin-activated T cells. These results are consistent with the view that development of a full-blown autoimmune response requires the sustained presence of autoreactive T cells. Whether the autoantibody production was due only to a sustained quantity of chromatin-reactive T cells, or qualitative changes in cytokine production were also important, would require further studies. Nevertheless, the capacity of chromatin-reactive T cells alone to generate a variety of autoantibodies reactive with chromatin components demonstrates that these T cells are responsible for the bulk of the serologic changes associated with intrathymic PAHA.

The finding that IgM anti-dDNA and anti-histone Abs were readily induced by single intrathymic PAHA injections is surprising because Ab induction limited to the IgM isotype is traditionally associated with Th-independent responses. It is possible that some unknown immunogen mimicking dDNA, a PAHA-dDNA complex, or a poly/oligoclonal B cell activator was produced by PAHA administration. However, since transfer of 5–50 × 10^3 chromatin-reactive T cells in the absence of PAHA also resulted in a very fast induction of IgM anti-dDNA Abs, the action of chromatin-reactive T cells alone is sufficient to account for the IgM-dominated B cell response. However, it is also possible that anti-dDNA appearance resulted in part from bystander cytokine production due to independently activated T cells within the microenvironment of the B cells, since this autoantibody activity appeared to increase in association with an anti-HGG response induced by immunization. Nevertheless, these results strongly suggest that the rapidly appearing anti-dDNA response is dependent on Th cells, and it is likely that dDNA-specific B cells serve as the APC, presenting chromatin-derived epitopes to chromatin-specific T cells. There is increasing evidence that upon Ag uptake through their B cell receptor, B cells can present Ag, possibly even to naive T cells (29, 30). We suspect that the in vivo Ag in this process is some form of partially degraded chromatin derived from normal dead cell debris and likely to contain localized regions of dDNA that serves as the initial B cell epitope.

Abs to dDNA are widely distributed, including various rheumatic diseases, chronic infectious diseases, and liver diseases (reviewed in Refs. 31 and 32). In addition, there is substantial evidence that anti-dDNA Abs are part of the normal immune repertoire (33) and have been described as natural autoantibodies (34). Anti-dDNA Abs have no or few somatic mutations and can be considered germline encoded (32). These specificities are not deleted or anergized during B cell development (35), presumably because ssDNA regions in chromatin are rare or transient. Thus, dDNA is viewed by the immune system as a foreign Ag, and the ease in eliciting an immune response to dDNA (36, 37) is consistent with this view.

Structural studies of anti-DNA mAbs at the nucleotide sequence level have allowed construction of genealogical trees, implying that anti-native DNA autoantibodies evolve by stepwise somatic mutation from the germline (38–40) by an Ag-driven, T cell-dependent process. The specificity of the immediate precursors of native DNA-specific B cells is unknown and widely debated (41–43). In a similar way, it is possible that affinity maturation and Ig class switching of dDNA-specific B cells may convert them to an IgG anti-chromatin specificity if sufficient T cell help is available. This view is supported by the observation that as anti-dDNA activity decreased 2–3 wk after the second intrathyhmic PAHA injection in (C57BL/6 × DBA/2)F1 mice or 3–5 wk after a single injection in C57BL/6-lpr/lpr mice, IgG anti-chromatin Abs began to appear, suggesting dDNA-specific B cells had undergone somatic mutation to react with native epitopes on chromatin. The adoptive transfer studies directly demonstrated that B cells from a mouse subjected to a single intrathyhmic PAHA injection had a pronounced propensity to produce anti-chromatin Abs in recipients that also received one intrathyhmic PAHA injection, consistent with the view that peripheral B cells had undergone a qualitative change after exposure to one wave of autoreactive T cells. The qualitatively different effect of the second injection cannot be explained by age-related maturation of the B cell repertoire, since single injections into mice of different ages did not produce an anti-chromatin response. Sequencing the expressed Ig genes in B cell hybridomas produced from these mice should reveal whether there is a genealogical relationship between anti-dDNA and anti-chromatin Abs.

Significant differences were observed between 5- and 9-wk-old mice with regard to the level and duration of IgM anti-dDNA and anti-histone responses. These differences might be related to maturation of the B cell repertoire in the periphery (44, 45) or to possible changes in the thymocyte repertoire, as suggested by the periodic influx of precursor T cells into the thymus (46). Although there was no difference between 5- and 7-wk-old mice in the magnitude of the splenocyte response to chromatin, more subtle
changes in the immune repertoire in the B and/or T cell compartment may underlie the age-related features of the intrathymic PAHA-induced immune response.

The immune system seems to be able to avoid humoral autoimmunity when exposed to relatively small numbers of autoreactive T cells. Even under the conditions in the current study of two intrathymic PAHA injections that resulted in a strong anti-chromatin response, Ab levels eventually declined after approximately 3 mo. Maintenance of B cell tolerance is consistent with several experimental systems in which autoreactive T cells occur in the absence of autoantibody production (47) and the existence of low levels of autoreactive T production in healthy individuals (48). It appears that the continuous presence of autoreactive T cells was necessary for induction or maintenance of a sustained IgG autoantibody response. A similar conclusion was reached by Förster et al. (49), who demonstrated that IgG autoantibodies against SV40 Tag spontaneously arose in double transgenic mice that expressed an autoantigen in the absence of T cells.

Several mechanisms for securing peripheral T cell tolerance have been demonstrated, such as induction of anergy, down-regulation of the TCR or its coreceptors, and clonal elimination by activation-induced cell death (reviewed in Ref. 50). Some form of down-regulation of the TCR or its coreceptors, and clonal elimination by activation-induced cell death cannot down-regulate peripheral lymphocytes (51). In the current study, we tested the role of Fas in limiting autoimmunity after one intrathymic PAHA injection of mice with a normal genetic background. Indeed, C57BL/6-lpr/lpr mice produced a robust anti-chromatin Ab response after only a single intrathymic PAHA injection, strongly suggesting that activation-induced cell death was the principal mechanism in normal mice of peripheral immune tolerance in this system. However, even in Fas-deficient mice, the PAHA-induced anti-chromatin activity eventually decreased in one-half of the treated animals. Although T and B cells in these mice are not subject to Fas-mediated activation-induced cell death, presumably they do not have an unlimited life span. In the absence of PAHA, no naive chromatin-reactive Th cells are generated, causing autoantibody secretion by B cells to eventually decline. Nevertheless, the Fas-deficient mice produced up to 6 times higher anti-chromatin levels after a single intrathymic PAHA injection compared with Fas-intact mice after two injections, and even those mice with declining Ab activity exhibited anti-chromatin levels exceeding those in double injected normal mice after 70 days.

The present study demonstrates that IgM anti-dsDNA Abs can be readily elicited if chromatin-reactive T cells are present in the periphery, whereas prolonged Th presence was required to induce IgG anti-chromatin Abs in a normal mouse. Recent studies using mice double transgenic for a TCR and its cognate Ag expressed only in the periphery are consistent with the view that the development of spontaneous autoimmunity requires a large number of autoreactive T cells (49, 52) or high expression of costimulatory molecules on APC (52–54). The current study demonstrates that this phenomenon is not unique to transgenically manipulated mice or limited to rare, organ-specific Ags. Systemic autoimmunity to chromatin, a ubiquitous self Ag, was limited to DNA and histone components when only low numbers of autoreactive T cells were present, but peripheral tolerance to native epitopes on chromatin failed in the presence of substantial numbers of or repeated exposure to autoreactive T cells.

References