Frontline:

Anti-chromatin antibodies drive in vivo antigen-specific activation and somatic hypermutation of rheumatoid factor B cells at extrafollicular sites

Robin A. Herlands1, Jacqueline William1, Uri Hershberg1,2 and Mark J. Shlomchik1,2

1 Section of Immunobiology, Yale University School of Medicine, New Haven, USA
2 Department of Laboratory Medicine, Yale University School of Medicine, New Haven, USA

A dominant type of spontaneous autoreactive B cell activation in murine lupus is the extrafollicular generation of plasmablasts. The factors governing such activation have been difficult to identify due to the stochastic onset and chronic nature of the response. Thus, the ability to induce a similar autoreactive B cell response with a known autoantigen in vivo would be a powerful tool in deciphering how autoimmune responses are initiated. We report here the establishment and characterization of a system to initiate autoreactive extrafollicular B cell responses, using IgG anti-chromatin antibodies, that closely mirrors the spontaneous response. We demonstrate that exogenously administered anti-chromatin antibody, presumably by forming immune complexes with released nuclear material, drives activation of rheumatoid factor B cells in AM14 Tg mice. Anti-chromatin elicits autoreactive B cell activation and development into antibody-forming cells at the T zone/red pulp border. Plasmablast generation occurs equally in BALB/c, MRL/+ and MRL/lpr mice, indicating that an autoimmune-prone genetic background is not required for the induced response. Importantly, infused IgG anti-chromatin induces somatic hypermutation in the absence of a GC response, thus proving the extrafollicular somatic hypermutation pathway. This system provides a window on the initiation of an autoantibody response and reveals authentic initiators of it.

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Introduction

The activation of autoreactive B cells plays a central role in the development of systemic autoimmunity. In addition to secreting pathogenic autoantibodies, autoreactive B cells can promote the activation of autoreactive T cells, which in turn can mediate tissue damage [1–4]. In lupus patients and mice genetically predis-
posed to lupus, the activation of autoreactive B cells is a selective process. Certain autoantigens, including nuclear antigens and self IgG are preferred and recurrent targets of B cell autoimmunity. Not all patients or lupus-prone mice have all the possible autoantibodies, but rather loss of tolerance to each autoantigen is stochastic [1, 5].

It is important to define the signals and autoantigens that lead to autoreactive B cell activation as well as the consequences for those B cells. Due to the heterogeneous and rare nature of autoreactive B cells, Ig-transgenic (Tg) mouse models for self or pseudo-self antigens have been invaluable. Such models have revealed early B cell tolerance checkpoints and mechanisms, including clonal deletion, receptor editing, anergy and clonal ignorance [6–14].

There has been much less success in using these models to elucidate how autoreactive B cells are activated. In part, this is due to the fact that B cells in some of the Tg models are so deeply tolerized that there is little activation even on a lupus-prone background [15–17]. However, some anti-DNA models, such as the 3H9 heavy chain-Tg [18] and knock-in [19], and the AM14 rheumatoid factor (RF) heavy (H) chain-Tg mouse model [11], which is the subject of this report, have been useful in revealing the phenotype of autoreactive B cell activation.

Although 3H9 anti-DNA B cells are anergized in normal hosts [9, 20], and AM14 RF B cells are not [21], both systems demonstrate spontaneous activation of autoreactive B cells and antibody-forming cell (AFC) formation outside of B cell follicles in the lupus-prone MRL/lpr strain [22–24]. A striking finding was that in the spleens of AM14 Tg MRL/lpr mice, RF B cells undergo somatic hypermutation (SHM) at extrafollicular sites but are not found in splenic GC [24]. AM14 Tg MRL/lpr mice have activated B cell blasts as well as plasmablasts, both of which turn over rapidly, resulting in a highly dynamic response [25, 26]. Interestingly, even with the benefit of an Ig Tg to restrict the B cell repertoire, the initiation of the response in any given animal is unpredictable [25, 26].

Despite determining the sites of activation and the nature of the responding cells, prior studies have not elucidated (1) what are the in vivo antigenic stimuli for RF B cells (or any autoreactive B cell); (2) why does a specific autoantibody response initiate stochastically; and (3) what promotes the extrafollicular pathway of SHM rather than the more conventional GC pathway. The answers to such questions will provide important mechanistic insight and presumably could also identify potential therapeutic targets.

Investigating these questions has been problematic, in part due to the nature of spontaneous autoimmunity itself. The stochastic onset of activation, without a defined starting time point, makes it difficult to determine the order of events in the process. Similarly, it is difficult to identify the autoantigens involved or the cells and signals required for propagation of a spontaneous response. We therefore concluded that a system that would allow for an experimentally controlled initiation of an autoreactive B cell response characteristic of spontaneous systemic autoimmunity would be very helpful to address such issues. The AM14 RF system is useful for this purpose, since IgG is a known part of the autoantigen and can be readily introduced to attempt initiation of the RF response. We hypothesized that providing IgG immune complexes (IC) as an RF autoantigen would lead to faithful reproduction of the spontaneous activation of autoreactive B cells in lupus-prone mice. However, previous attempts using IgG2a IC with foreign protein [24, 27] led to a GC response rather than an extrafollicular response, suggesting that a different form of IC was required to generate the latter.

A potential insight into this puzzle came from the result that, in vitro, IgG2a anti-chromatin mAb are mitogenic for AM14 B cells [28, 29], but conventional IC are not. The potent activation mediated by IgG2a anti-chromatin was subsequently shown to be MyD88-dependent, with a major role for Toll-like receptor (TLR)9 [28, 29]. The paradigm of activation through dual BCR-TLR engagement was extended in vitro to B cells that were anti-DNA and anti-Sm, two other dominant specificities of lupus, with respect to TLR9 and TLR7 [29, 30]. These studies demonstrated a unique method of activation, but only measured proliferation, not differentiation, and it was unclear how they would reflect the in vivo situation. Moreover, these cultures fail to survive beyond 2 days and thus are not suitable for investigating differentiation.

Based on the in vitro ability of IgG2a anti-chromatin mAb to cause AM14 B cell proliferation, we hypothesized that they might elicit extrafollicular activation in vivo. We tested this hypothesis by exposing AM14 RF B cells in vivo to high levels of anti-chromatin antibodies and present evidence that this does indeed lead to extrafollicular B cell activation and AFC formation in a manner that faithfully reproduces the phenotype of spontaneous RF B cell activation in MRL/lpr mice. In contrast, IgG of other specificities, whether for haptens or another self antigen, caused no detectable AFC response. Therefore, one mechanism to generate the typical extrafollicular response is via IgG anti-chromatin, which presumably forms IC in vivo with endogenous chromatin [31].

We next took advantage of this system to show that this RF AFC response to anti-chromatin antibodies can occur in autoimmune-prone MRL/lpr, young MRL/+ and even non-autoimmune BALB/c mice. Thus, we conclude that neither an autoimmune-prone environ-
ment, nor autoimmunity-related genetic defects [32] are required for these AM14 B cells to become activated. Finally, we demonstrated that this response induces clonal expansion and SHM, as originally found in AM14 B cells spontaneously activated in MRL/lpr mice [24], and in autoantibody-secreting cells in non-Tg mice [33]. Thus we have identified an important in vivo autoantigen for RF B cells and provide insight into what may stimulate the unique extrafollicular response that generates both RF and anti-DNA antibodies in lupus-prone mice.

Results

IgG2a but not IgG2b anti-chromatin antibodies elicit an RF AFC response

To try to reproduce the spontaneous extrafollicular RF response, we acutely raised the serum concentration of IgG2a anti-chromatin, an antigen for AM14 B cells, by growing the hybridoma PL2-3 i.p. in AM14 H Tg MRL/lpr mice (referred to hereafter as Tg mice, Fig. 1). PL2-3 has been shown to be mitogenic for Tg B cells in vitro by forming IC with nuclear material from apoptotic cells in culture media [28]. Mice were sacrificed 7–8 days after hybridoma injection, and serum and spleens were harvested. Because AM14 B cells become activated spontaneously in MRL/lpr mice with age [25], we used 7-wk-old and younger mice, as spontaneous activation is only detectable in mice that are a number of weeks older. We previously showed that in Tg mice, a small percentage of B cells express one of two closely related V_{k8} family light chains rearranged to J_{k4} or J_{k5}, which reconstitute the RF specificity [11, 21, 23, 24]. These B cells are specifically detected by the mAb anti-idiotype 4-44, which identifies only Tg-encoded RF B cells [11, 24].

Figure 1. Experimental design for the use of IgG2a anti-chromatin antibodies to acutely raise levels of IgG2a RF self antigen. For hybridoma treatment, mice were injected i.p. with pristane on days –10 and –3 (gray arrows), followed by hybridoma injection on day 0 (black arrow) and sacrifice on day 7. For protein treatment, mice were injected with 0.5 mg purified antibody on days 0, 2 and 5 (white arrows) and were sacrificed on day 8.

Tg MRL/lpr mice treated with PL2-3 developed large numbers of 4-44+ AFC (Fig. 2A), comparable to those generated in older mice that had undergone sponta-
neous activation [24, 25]. In contrast, the control hybridoma PL2-8, an IgG2b clonal relative of PL2-3, had no effect (Fig. 2A). Treatment with an IgG2a anti-desmoglein secreting antibody, NAK10 [34], also did not lead to generation of 4-44+ AFC (Fig. 2A). This suggested that anti-nuclear antibodies that had Fc regions that could be bound by the AM14 BCR, but not other antibodies, could drive AFC generation. Consistent with this, mice treated with a different anti-nuclear IgG2a hybridoma, the anti-DNA PA4 [35], had increased numbers of AFC (Fig. 2A).

Previously, we reported that IgG2a anti-TNP/TNP-keyhole limpet hemacyanin protein IC stimulate an RF GC response in Tg MRL/lpr animals [24, 27]. In order to determine whether in the same system a T-dependent antigen was able to drive differentiation of AM14 B cells into AFC, we generated similar protein IC from purified IgG2a anti-4-hydroxy-3-nitrophenylacetyl (NP) and NP-chicken γ-globulin (CGG) and injected them in alum into Tg MRL/lpr mice. This immunization did not generate 4-44+ AFC (Fig. 2A). Identical protein IC injected without alum into Tg MRL/lpr mice did not lead to AM14 B cell activation as measured by FACS, ELISPOT or immunohistology (data not shown).

Anti-chromatin elicits similar RF AFC responses in autoimmune-prone and non-autoimmune-prone strains

AM14 B cells become activated spontaneously in lupus-prone mice, but do not generate AFC in non-autoimmune mice [23, 24, 27]. Autoimmune-prone mouse strains have genetic defects [32, 36, 37], pro-inflammatory cytokines [38–41], and activated T cells [4, 42–44]. Furthermore, autoimmune mice may have persistent self antigen due to impaired clearance mechanisms [45]. Any of these factors could be important in the activation of AM14 B cells in response to anti-chromatin antibodies. In order to investigate how these strain-specific factors would affect the activation of AM14 B cells, we supplied PL2-3 hybridoma cells to Tg MRL/lpr, MRL/+ and BALB/c animals. All three strains of AM14 Tg mice responded with indistinguishable AFC formation (Fig. 2B). This indicates that initial AM14 B cell activation and differentiation induced by anti-chromatin antibodies does not require an intrinsic B cell defect, a pro-inflammatory environment, or an environment with enhanced cell death or turnover. As an additional control in this series of experiments, we used an anti-NP-secreting hybridoma, 23.3, that did not lead to generation of AFC, confirming that the specificity of the IgG2a antigen is important (Fig. 2B).

Although the hybridoma experiments were controlled for specificity, they could not exclude a potential effect of

Figure 3. Anti-chromatin antibodies cause RF B cell plasmablast differentiation as seen by FACS analysis. (A) Representative FACS plots of live spleen cells (y-axis “AM14” = 4-44) demonstrating that Tg MRL/lpr mice treated with PL2-3, but not PL2-8 hybridoma or protein IC, develop 4-44+/CD22lo cells, indicative of plasmablast development [26]. (B) Combined data from multiple experiments showing % of 4-44+/CD22lo cells; PL2-3, n=22; PL2-8, n=12; protein IC, n=6; *p=0.007, *p=0.0013 by Mann–Whitney U-test for PL2-3 versus PL2-8 and Protein IC, respectively. (C) Histograms of expression of CD138, CD44, CD80 and CD86 gated on live 4-44+/CD22lo (solid line) and 4-44+/CD22hi (dashed line) subsets from mice treated with PL2-3. PNA was used to identify GC cells, with 4-44+/CD22hi cells from mice treated with protein IC serving as a positive control (shaded gray).
the hybridoma itself. Therefore, we also tested the efficacy of purified PL2-3 antibody in Tg MRL/lpr and Tg BALB/c mice. Mice were sacrificed on day 8 after injections of 0.5 mg of protein on day 0, 2 and 5. PL2-3 drove AFC formation in both MRL/lpr and BALB/c strains, while control antibodies PL2-8 and Hy1.2, an anti-TNP IgG2a, did not (Fig. 2C). Thus, although hybridoma administration provides a simple and efficient method to provide high levels of anti-chromatin, the hybridoma itself is not necessary in either strain of mice.

**Anti-chromatin antibodies elicit RF plasmablasts at the extrafollicular T cell zone-red pulp border**

Spontaneous activation of AM14 B cells in MRL/lpr mice is accompanied by development of CD22lo plasmablasts [26]. Mice given PL2-3, but not PL2-8, developed a CD22lo population of AM14+ cells (Fig. 3A, B), similar to that seen in spontaneously activated AM14 cells in MRL/lpr mice [25, 26]. There was at least a fourfold induction of these cells upon treatment with PL2-3 hybridoma (Fig. 3B). This is a minimal estimate as the 4-44 CD22lo cells in mice treated with PL2-8 did not localize to the same region of the gate (Fig. 3B), and therefore probably represent background.

The CD22lo cells from PL2-3-treated mice had elevated expression of CD44 and CD138 (Fig. 3C), markers associated with plasmablast differentiation [25, 26, 46]. They also expressed increased levels of CD80 and CD86, consistent with their activated status (Fig. 3C). In mice given PL2-3, there was no evidence for GC cell differentiation in either CD22lo or CD22hi cells, as all were peanut agglutinin (PNA)-negative. We also measured PNA expression in the mice given IgG2a anti-NP and NP-CGG IC, described above. These mice did develop abundant PNA+ 4-44+ GC cells (Fig. 3C), although they developed neither 4-44+ AFC (Fig. 2A) nor 4-44+ CD22lo cells (Fig. 3A, B).

The localization of these activated cells was determined using immunofluorescence on splenic sections. Treatment with PL2-3 hybridoma or protein led to the appearance of brightly staining 4-44+ cells at the T cell zone-red pulp border, outside of the follicle (Fig. 4A, B), while PL2-8 treatment did not (Fig. 4C). Higher magnification revealed these cells to have abundant cytoplasm, a characteristic of plasmablasts (Fig. 4D). There were no GC present in mice treated with PL2-3, but there were in mice treated with IC of IgG2a anti-NP/NP-CGG (Fig. 4E–H), consistent with both ELISPOT (Fig. 2A) and FACS analysis (Fig. 3). Thus, provision of IgG2a anti-chromatin, via either hybridoma or as purified protein, led to the development of an extrafollicular plasmablast RF response without GC, a response similar to that seen in spontaneous activation of RF B cells in the Tg MRL/lpr spleen.

**Stimulation with anti-chromatin antibodies induces somatic hypermutation at extrafollicular sites**

SHM was originally thought to be restricted to GC [47–49], but we previously discovered that during the spontaneous RF response in MRL/lpr mice, SHM occurred in AM14 B cells at the T zone-red pulp border [24]. This was demonstrated by microdissections of 10–30 cells at these sites. Sequences derived from these cells often shared V-J rearrangements as well as some V region mutations, but also displayed intraclonal diversity [24]. Mathematical analysis demonstrated that the mutation rate was comparable to that found in GC [50]. Further, in those studies we could not exclude that mutation was possibly induced first at another site, such as a GC, at a previous time in these older MRL/lpr mice,
even though we did show that SHM continued at the extrafollicular site [24]. In addition, until now there was not another robust example of mutation occurring extrafollicularly, such that SHM is still widely believed to be restricted to the GC [51, 52].

Therefore, to determine whether mutation was induced, 4-44+ B cells were microdissected from splenic sections of four Tg MRL/lpr and four Tg BALB/c mice that had been treated with PL2-3 protein every other day for 2 wk (Fig. 5A). From each microdissection, called a “pick”, the DNA was amplified to determine V\textsubscript{j} sequences and mutation rates. Lists of all picks from Tg MRL/lpr and Tg BALB/c mice and their number of mutations are in Table 1 and 2. We chose to generate multiple sequences (6–18) from each pick to maximize our chances of identifying mutated sequences and being able to determine clonally related sequences in order to build genealogical trees (Fig. 5B), even though this likely resulted in some re-isolation of sequences amplified from the same cell.

Out of six total picks from Tg MRL/lpr mice, all six showed mutation with an average of 1.8 mutations per sequence (Table 1). Interestingly, Tg BALB/c mice had mutation in four of six picks, but with a lower average of

**Figure 5.** SHM occurs at extrafollicular sites of Tg MRL/lpr and Tg BALB/c mice given IgG2a anti-chromatin. (A) Immunohistochemistry of a representative microdissected region showing 4-44 B cells in a splenic section from a Tg MRL/lpr mouse treated with PL2-3 protein every other day for 2 wk. Photos are from before and after laser microdissection. Picks typically contained 10–30 4-44 B cells. Note that image quality is suboptimal due to use of foil-coated slides required for microdissection. (B) Representative genealogical trees from two Tg MRL/lpr mice (trees A2, B3 and C1) and two Tg BALB/c mice (trees E1, E2 and F4), which demonstrate ongoing mutation as reflected by intraclonal diversification. Two additional clones (B2 and F3) from these same picks had no mutation and are thus fully described in Tables 1 and 2 and not shown here. Circles at the base of trees represent germ-line sequences. Non-mutated sequences actually found in a pick are listed in this circle; when there were no germ-line isolates, the circle has no sequence identifiers. Every other open circle, or “node”, represents one or more clones with the same sequence, again as indicated by sequence identifiers. Open circles are implicit nodes required by the genealogy, although no actual sequences representing those nodes were found. Tree “branches” are the lines between nodes. The positions and types of nucleotide exchanges are listed alongside branches. * denotes an independent paralell mutation. (C) Histogram of the distribution of the number of mutations per unique sequence for all Tg MRL/lpr and Tg BALB/c picks. The distributions of mutation between MRL/lpr and BALB/c are significantly different ($p=0.005$, $\chi^2$-test for trend).
Table 1. Summary of microdissections and mutations in Tg MRL/lpr mice treated with PL2-3 hybridoma\(^a\)

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<th>Tree name</th>
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<th>Total mutations</th>
<th>Mutations/sequence</th>
<th>Total unique sequences</th>
<th>Total mutations among unique sequences</th>
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\(^a\) Sequences recovered from four Tg MRL/lpr mice treated with PL2-3 were grouped by pick and assigned to a lettered genealogical tree. Identical sequences in a pick were conservatively treated as one clone, instead of as the products of multiple cells, and were assigned to one ‘unique sequence’. Trunk mutations identify those mutations that are common to all mutated sequences.

Table 2. Summary of microdissections and mutations in Tg BALB/c mice treated with PL2-3 hybridoma\(^a\)

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\(^a\) Sequences recovered from four Tg BALB/c mice treated with PL2-3 were grouped by pick and assigned to a lettered genealogical tree (see Table 1).
0.4 mutations per sequence (Table 2). As discussed, it is likely that our sequence collection includes multiple copies of a single sequence that do not represent different cells. Although this does not affect the genealogical tree shape, it does impact the mutations/sequence calculation. Therefore, we also calculated the number of mutations per unique sequence (Table 1 and 2). We found an average of 2.8 and 0.8 mutations per unique sequence for picks from Tg MRL/lpr and Tg BALB/c mice, respectively. The distribution of mutations per unique sequence (Fig. 5C) is significantly different between the two strains (p=0.005). We previously calculated the PCR error rate of our procedure using Pfu Turbo as <0.05 mutations per sequence [24]; these current sequences were generated with Pfu Ultra, which is reported to have a threefold lower error rate (Stratagene). Thus, the observed mutation rates are far above PCR error in both MRL/lpr and BALB/c mice.

If mutation is ongoing at a site, then clonally related sequences should differ by point mutations at least some of the time. This will generate genealogic trees with branching patterns [49, 53]. At GC mutation rates of 0.5 mutations/cell/generation, each light chain will get 0.25 mutations/generation. This means that on average we can expect identical daughters approximately 75% of the time, but different ones the rest of the time. Thus picks with eight independent sequences should have at least some diversity, though identical sequences are clearly quite compatible with high mutation rates and in fact are expected.

In Fig. 5B, representative genealogic trees from PL2-3-treated Tg MRL/lpr mice and Tg BALB/c mouse are presented. In each node of the tree, all sequences containing that particular sequence are identified. Four out of eight trees from MRL/lpr mice had at least one branch, while four of the nine trees from BALB/c mice had at least one branch. Some trees had multiple branches including second-level branches (Fig. 5B, trees A2, C1 and F4). Both the significant branching in the genealogical trees and the number of mutations per sequence demonstrate that mutation was occurring at a high rate at extrafollicular sites in mice treated with PL2-3. In particular, sequences that share some but not all mutations are extremely unlikely to be generated by amplification.

Discussion

In the AM14 RF response in MRL/lpr mice, plasmablasts and activated B cell blasts proliferate and undergo SHM in the splenic T zone-red pulp border, in the absence of a detectable GC response [24]. In order to understand how and why RF B cells are activated at the T zone-red pulp border, we sought, by introducing autoantigen to RF-Tg mice, to develop a system that would reproduce these events.

In this report, we have identified IgG2a anti-chromatin antibodies as an in vivo autoantigen for RF B cells. This ligand, by itself, is sufficient to generate many characteristic features of the spontaneous RF B cell response seen in MRL/lpr mice. In contrast, other potential IgG2a-containing ligands did not. In particular, traditional immunization of AM14 Tg mice with protein-containing IC stimulated a GC response with minimal, if any, extrafollicular plasmablast formation. Thus, anti-chromatin antibodies, probably acting as IC formed with ubiquitous endogenous chromatin [31], have a unique property of stimulating RF B cells to undergo an extrafollicular, rather than a GC, response.

It has been notoriously difficult to artificially elicit disease-associated autoantibody responses in vivo. For example, immunization with DNA is relatively ineffective in generating anti-DNA antibodies [54], although bacterial DNA and DNA conjugated to certain immunogenic DNA-binding proteins can be effective [55–57]. Nonetheless, in none of these cases it was shown that the nature of the in vivo response to administered “auto-antigen” corresponded to the spontaneous response. Thus, the ability to induce the extrafollicular RF response can be useful as an in vivo method to test the requirements for autoreactive B cell activation.

For example, this system gave us the unique opportunity to stimulate the same autoreactive B cells in lupus-prone and non-autoimmune mouse strains equally, in order to test the role of genetic background. Despite the fact that B cells in MRL/lpr, MRL/+ and other autoimmune-prone strains as well as lupus patients do have intrinsic defects [58–60], we found that the elicitation of RF AFC was equally efficient in Fas-deficient and sufficient MRL strains and BALB/c mice. However, an unexpected finding were larger numbers of somatic mutations in 4-44+ B cells from Tg MRL/lpr mice compared to BALB/c mice, suggesting the intriguing possibility that regulation of mutation rate or selection could be an intrinsic strain-specific difference.

These differences in mutation levels are based on our data in a single system, and whether they relate to differences in mutation rate or rather to differences in cell proliferation and survival rates (which in turn would influence mutation content) is unknown. The disparity in mutation content could be attributed to Fas deficiency, MRL background genes, or both. It is of interest that Takahashi et al. [61] found that GC and memory B cells accumulated more mutations in Fas-deficient than Fas-sufficient mice on a C57BL/6 background. We also found more mutations in spontaneous RF+ GC of MRL/+ and MRL/lpr mice, compared to BALB/c mice [27].
The rapid development of RF AFC in response to anti-chromatin antibodies could also help to explain the phenomenon that we have previously termed “conversion”, referring to the stochastic onset of a large AFC response in AM14 Tg MRL/lpr mice [25]. This onset is accompanied by rises in serum RF and circulating RF B cells that go from background to high levels in a period of 1–2 wk [25]. It is evident from the current results that such a response can actually develop within a matter of a few days from the point at which IgG2a anti-chromatin antibodies exceed a threshold level. What in turn causes an increase in IgG2a anti-chromatin (or other antigenic antibody) beyond such threshold is not yet determined. Autoimmune mice have impaired clearance of apoptotic cells [45, 62], which could eventually lead to increased available autoantigen, and exposure to apoptotic cells can lead to autoantibody generation [63]. Interestingly, we previously found a correlation between the onset of conversion in AM14 Tg MRL/lpr mice and the appearance of IgG2a anti-chromatin serum antibodies (but not with anti-chromatin of other isotypes) [25].

The ability to stimulate the response at a known point in time also allowed us to better resolve the relationship between the mutating extrafollicular response and any potentially associated or prior GC response. Because we could see a full blown extrafollicular response in 1 wk in the absence of a concurrent GC response, the extrafollicular response and its attendant SHM do not appear to require a GC response to occur first. This is a conclusion that could not be drawn firmly from our studies of the spontaneous response [24]. Importantly, we were able to show that SHM is easily detectable within 1 wk or so after the initiation of the response, with actual onset of mutation probably several days before this time. The branching patterns and shared mutations seen among sequences derived from small clusters of cells demonstrate that mutation was occurring locally [24, 33, 64], and the presence of multiple mutations in a single V region in such young mice strongly suggests that mutation was initiated only after anti-chromatin IgG2a was provided.

One question raised by our observations is why anti-chromatin antibodies are sufficient to initiate the differentiation of RF B cells into plasmablasts. In vitro data would suggest that TLR signals are at least in part responsible. This concept is largely based on the pioneering studies by Marshak-Rothstein and colleagues [28], which demonstrate that the in vitro activation of RF B cells by anti-chromatin antibodies occurs in a MyD88-dependent manner. These signals most likely come through TLR9, and possibly TLR7 [28, 30, 65]. Our studies establish the in vivo relevance of these in vitro experiments, which relied on 2-day proliferation assays and hence did not assess differentiation.

Although in vitro this type of response is TLR-dependent, it is unclear whether this is true in vivo, as other signals such as from T cells and cytokines are likely to be present and could substitute for the TLR signal. Another complicating factor in vivo is that chromatin-containing ligands may directly activate DC and other cell types in vivo, which in turn could promote the response [66, 67], while these cells are absent in vitro. These potential contributions to the response in vivo will ultimately have to be dissected using complex approaches. Of interest in this regard, MyD88-deficient Fas-deficient mice of mixed genetic background lacked substantial serum RF [30]; however, TLR9-deficient non-Tg MRL/lpr mice do make RF (S. Christensen and M.J.S., unpublished observations), illustrating the complexity of the in vivo situation.

A related question is why activation takes place either exclusively or preferentially at the T zone-red pulp border. This is the normal site for certain types of T-independent B cell responses, although these are usually quite transient and are generally not known to undergo high-rate SHM [68–70]. As antigens such as TNP-Ficoll, which are not known to contain a TLR ligand, generate an extrafollicular response [68, 69, 71], again it is not clear what the non-exclusive role is for this family of receptors. Some data indicate that DC promote plasmablast development and survival in T-independent extrafollicular responses [67, 72]. Thus, by analogy, DC potentially could serve key roles directing the extrafollicular response. Indeed, during the spontaneous RF response in MRL/lpr mice [24], DC, which are abundant at this site in MRL/lpr animals [73], are seen juxtaposed with RF B cells. DC are also a source of BAFF, which has recently been shown to enhance antibody secretion [74], and APRIL, both of which promote plasmablast development [72] possibly via TACI [75–77]. DC located at the T zone-red pulp border could be activated via TLR, as well as Fc receptors, which can bind anti-chromatin IC [66]. DC can present undegraded antigen to B cells [78] and it was recently shown in LN that B cells can recognize unprocessed antigen displayed on DC before ever entering the follicle [79]. Against the concept that DC are important are the recent findings that CD11c+ DC are dispensable for plasmablast responses to T-independent type 2 antigens [80] and vesicular stomatitis virus infection [81]. However, these situations may differ substantially from a chronic autoimmune response.

Since IgG2a anti-chromatin by itself was sufficient to stimulate RF B cell proliferation and AFC differentiation, one might expect accompanying RF responses in autoimmune mice and patients with conditions associated with anti-nuclear antibodies [1]. Indeed, there are good examples of this. Although it is commonly believed that RF are rare in lupus, in fact a significant
fraction of lupus patients have detectable RF (27% in a recent authoritative study of 352 patients [82]). Other diseases have a high prevalence of both anti-nuclear antibodies and RF, including Sjogren's syndrome and chronic hepatitis C [83–87]. Furthermore, we have recently found a high prevalence of elevated RF levels in other lupus-prone mouse strains (manuscript in preparation). Although IgG anti-chromatin can stimulate RF B cells, it is certain that RF autoantibodies can be generated via multiple pathways [88, 89]. In systemic autoimmune syndromes there are likely other immunogenic forms of IC. These IC may include ligands for TLR other than TLR9, including TLR7 as recently shown in vitro by Lau et al. [30]. They may also include ligands for other non-TLR innate immune receptors, though this remains to be determined.

In summary we have shown that IgG anti-chromatin antibodies are sufficient to elicit a robust and rapid extrafollicular RF autoantibody response closely resembling the spontaneous RF response in MRL/lpr mice; other types of IgG led to a GC response or no response. Such a system is much more tenable than the stochastic and unpredictable activation of autoreactive B cells found in autoimmune mice [5, 25]. This system enabled us to demonstrate that an autoimmune-prone genetic background is not required for the response to take place. It also allowed us to establish that mutation occurs at this site without the need for a prior GC response. In the future, by combining IgG anti-chromatin antibody infusion with mutant mice and cell transfer approaches, we and others should be able to further dissect the mechanisms of autoreactive B cell activation and propagation.

Materials and methods

Mice

The AM14 heavy conventional IgM-only Tg [11] was backcrossed at least ten generations onto the MRL/lpr, MRL/+ and BALB/c backgrounds. All mice were housed under specific pathogen-free conditions. PCR to detect the heavy chain Tg was performed as described [11]. All studies were approved by the Yale Institutional Animal Care and Use Committee.

FACS analysis

Splenocytes were prepared and flow cytometric analysis was performed as described [23].

ELISPOT analysis

ELISPOT analysis was performed as previously described [21, 23].

Anti-chromatin hybridomas

The IgG2a and IgG2b anti-chromatin hybridomas, PL2-3 and PL2-8 [90], were obtained from Mark Monestier. For in vivo hybridoma growth, mice were injected with 0.25 mL of pristane (Sigma) on days 0 and 7. Hybridoma cells were injected (10⁷ cells) i.p. on day 10, and mice were sacrificed on day 17 or 18.

Protein purification and administration

PL2-3 protein was purified from hybridoma cells grown in serum-free media or from ascites grown in RAG-1-deficient mice. Ascites fluid was collected and clarified by centrifugation for 4 min at 7000 rpm. Protein from hybridoma supernatants was purified on a Protein G column (Pharmacia) while protein from ascites was purified by ammonium sulfate precipitation followed by purification via QAE-Sephadex (Pharmacia). Purified protein was then concentrated, filtered and tested by SDS-PAGE for purity and by anti-chromatin ELISA for quantification. PL2-3 protein was injected (0.5 mg) i.p., every other day. No gross differences were observed in the potency of protein purified from either source and they were used interchangeably.

Antibody reagents

Antibodies prepared in our laboratory as described [11] were: 4-44-biotin (anti-Id), 4-44-FITC, 4-44-Alexa 647, 4-44-Alexa 488, 4-44-Alexa 568, 160A1 (anti-CD80)-Alexa 488 and Pgp-1 (anti-CD44)-Alexa 488. The antibodies GL7 (anti-CD86)-FITC, anti-CD22.2-FITC, anti-CD22.2-PE and anti-CD138 (conjugated to PE and biotin) were obtained from BD Pharmingen. BM8-Alexa 647 (anti-F4/80) and A3-1-biotin (anti-F4/80) were obtained from Caltag. Streptavidin-Alexa 647 and streptavidin-PE (Molecular Probes) were used to detect biotinylated reagents. PNA-FITC was obtained from Vector.

Histology/immunofluorescence

Sections were prepared as described [91] and stained with antibodies described above and specified in figure legends. Nuclei were identified with 4',6-diamidino-2-phenylindole (Molecular Probes). Fluorescent images were captured on an Olympus BX-40 microscope using a SPOT-RT Slider (Scanning) digital camera. For immunohistochemistry, 4-44-FITC or 4-44-biotin antibodies along with anti-FITC-alkaline phosphatase (Molecular Probes) and streptavidin-HRP (Southern Biotechnology Associates) were developed with Fast Blue BB or 3-aminio-9-ethyl-carbazole (Sigma-Aldrich), as described previously [21].

Laser capture microdissection

Sections were cut from OCT-embedded spleens onto Leica PEN-membrane 2.0-μm slides. Staining was completed on these sections to identify extrafollicular 4-44⁺ B cells. 4-44⁺ B cell clusters (10–30 cells) were microdissected using a Leica Instruments LMD6000 and excised cells were digested over-
night at 37–55°C in 10 μL of 0.8 mg/mL proteinase K, 50 mM Tris pH 8, 50 mM KCl, 0.63 mM EDTA, 0.22% Igepal and 0.22% Tween-20.

Sequence

Sequencing was performed as described [24] except with Pfu Ultra (Stratagene) rather than Pfu Turbo. In brief, V×J4/5 rearranged sequences were amplified by nested PCR using external primers 5'-AGCTATGACATTTACGTTC-3' and 5'-AGGCCCCTCTCATTTC-3', and internal primers 5'-TTGATGACACAGTTCC-3' and either 5'-AAGTTAC-CAACAGAACCC-3' (Jc4) or 5'-TGTACITCAGTTCCAGCTCC-3' 3' (Jc5). Amplified DNA was cloned using the TOPO-Zero blunt cloning kit (Invitrogen Life Technologies) and further amplified by picking transformed colonies directly into a PCR reaction with primers M13 forward (5'-GTTTTAGTTGACAACTCACTAAAGGGA-3') and Jc4 Reverse (5'-CAGGAAACAGCTATGAC-3') provided in the kit. The PCR product was purified using the QiAquick PCR purification kit (Qiagen), mixed with the sequencing primer T3 (5'-ATTACCCCTCTAAGGGA-3') and sequenced by the Keck Biotechnology Resource Laboratory at Yale University.

Sequence analysis

Sequences of each pick were aligned to the appropriate germ-line V gene (i.e. the most similar one) found in the IMGT Ig sequence database [92]. They were always Vx8–19 or Vx8–24 combined with Jc4 or Jc5 (note that Vx8–24 has a different allele in MRL and BALB/c mice).

From these aligned sequences, phylogenetic trees were calculated using maximal parsimony criteria [93]. We developed and used a set of computer algorithms to handle the specific circumstances of Ig hypermutation analysis (manuscript in preparation). By combinatorial matching of the end regions of Vx and Jx, the algorithm determined all possible V-J junctions, including those potentially generated by P-nucleotides. This allowed us to differentiate between junctional diversity and SHM in the region of the V-J join, as those bases that could not be accounted for by any combination of the germ-line sequences considered to be mutations. Junctional diversity was then used to separate independent clones that may have been found in the same microdissection and which nonetheless used the Vx and Jx.

The computer algorithm was also used to identify cases of independent parallel mutations, which could be attributed to hybridization in the PCR amplification process. Among all the sequences we found only one independent parallel mutation, indicating that there were few if any such PCR artifacts. Since isolated independent parallel mutations occur with reasonably high frequency, particularly in hotspots, this observed single instance was not discarded in view of the absence of any other possibility of PCR hybridization in that or any other sequence set.

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