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Visualizing the Onset and Evolution of an Autoantibody Response in Systemic Autoimmunity

Jacqueline William,* Chad Euler,* Elizabeth Leadbetter,† Ann Marshak-Rothstein,† and Mark J. Shlomchik²*

The onset of systemic autoimmunity is variable, making it difficult to identify early events. In this study, we show in rheumatoid factor (RF) Ig-transgenic autoimmune-prone mice that the appearance of RF B cells in blood signifies the onset of RF B cell activation in spleen, providing a novel window into the initiation of an autoantibody response. This allowed us to study the early and late phases of spontaneous induction of the B cell autoimmune response. Using this approach we showed that extensive Ab-forming cell generation in spleen, accompanied by somatic hypermutation, occurred despite the lack of an early germinal center response. The onset of the RF response correlated with the levels of IgG2a-containing immune complexes but not total IgG2a. By identifying the time of onset in individual mice, we were able to track progression of disease. We found remissions of RF Ab-forming cell production in some mice, suggesting that at the clonal level, chronic autoantibody responses are dynamic and episodic, much like acute pathogen responses. Surprisingly, there was little accumulation of long-lived plasma cells in bone marrow of mice with long-standing RF responses in spleen. These studies are among the first to define the early events of a spontaneous B cell autoimmune response. The Journal of Immunology, 2005, 174: 6872–6878.

Systemic autoimmune diseases result from activation of autoreactive B cells that in turn leads to autoantibody (autoAb) production and T cell activation (1). In healthy individuals, these B cells are regulated. Deciphering how regulation breaks down in disease is particularly difficult, because the initial loss of tolerance is followed by tissue destruction which can lead to further inflammation and secondary effects in a positive feedback loop. Therefore, immune dysregulation at the end stage of disease could be quite different from that which occurred during the initial loss of self-tolerance. Most of what we know in patients and mice comes from the study of established disease. Furthermore, because the onset of spontaneous systemic autoimmunity is not predictable from individual to individual (2), it has been difficult to study early events in the loss of self-tolerance.

Ig transgenic (Ig-Tg) mouse models have been instrumental in studies of normal B cell self-tolerance and its loss during disease. The first models used Ig-Tg specific for model self-Ags, like hen egg lysozyme and MHC class I. These demonstrated several regulatory mechanisms, including receptor editing, deletion, and energy (3–5). To determine which of these mechanisms break down in autoimmune disease, these Ig-Tgs were crossed onto lupus-prone genetic backgrounds; however, the Tg self-reactive B cells remained tolerant even in older mice (6–8). Although the selection of model autoantigens in these systems allowed for the control of autoantigen expression, which is critical for interpretation of tolerant phenotypes, the inability of these same systems to reveal how tolerance breaks down might have been because the chosen self-Ags were not those targeted in lupus. In systemic autoimmune diseases, a small number of autoantigens are the predominant targets: chromatin, ribonucleoproteins, and IgG (9, 10). These autoantigens most likely have properties that render them better targets for B cell responses when immune regulation is abnormal.

Indeed, Ig-Tg models that focus on disease-related autoantigens, like anti-DNA and anti-Sm, did reveal a loss of tolerance in Tg B cells in autoimmune-prone mice (11–14). However, there were limitations to these initial studies that require further exploration. First, since these autoantigens are ubiquitous, the regulation of the autoreactive B cells in the absence of autoantigen could not be studied. Second, in most of these models it was difficult to define the status of the precursors of the autoAb-secreting cells and thereby determine whether loss of tolerance was due to breakdown of deletion, anergy, or stimulation of nontolerant but self-reactive B cells. Finally, these studies generally did not identify early events and distinguish them from later ones.

We have been focusing on an Ig-Tg model with IgG-specific (rheumatoid factor, RF) B cells. RF autoAbs are found at high levels in rheumatoid arthritis, Sjögren’s syndrome, in some patients with systemic lupus erythematosus (15), as well as in Fas-deficient lpr mice (16, 17). In AM14 Ig-Tg mice, the H chain confers specificity for the Fc portion of IgG2a when paired with an endogenous or Tg-encoded Vκ8 L chain (18). AM14 binds only to IgG2a of the α allele; thus, RF B cells can be studied in the presence or absence of a defined and measurable autoantigen using IgH congenic mice. The AM14 model is unique in combining the design advantages of the earlier model autoantigen Tg mice with the features of authentic disease specificity of the autoAb. Using AM14 Tg mice, we initially found that, in contrast to most other models, the RF B cells develop normally and are immunocompetent in normal IgHα mice (19), a phenotype referred to as “clonal ignorance.”

Abbreviations used in this paper: autoAb, autoantibody; Tg, transgenic; RF, rheumatoid factor; GC, germinal center; AFC, Ab-forming cell; IC, immune complex; PNA, peanut agglutinin.

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3 Abbreviations used in this paper: autoAb, autoantibody; Tg, transgenic; RF, rheumatoid factor; GC, germinal center; AFC, Ab-forming cell; IC, immune complex; PNA, peanut agglutinin.
To determine how RF B cells become activated on an autoimmune-prone background, we crossed the AM14 Tg onto the MRL/lpr strain (20). RF B cells in spleens of older H chain IgTg MRL/lpr mice form clusters at the T zone-red pulp borders and undergo somatic hypermutation at this site; they are only very rarely found in germinal centers (GCs) (21). Critically, by comparing the IgH\(^\text{H}^\text{lpr}\) and IgH\(^\text{H}^\text{Tg}\) Tg mice, we demonstrated that activation of autoreactive B cells required the presence of the nominal autoantigen. These results showed that regulation of RF B cells, which normally occurs in the periphery, becomes impaired in MRL/lpr mice. These studies further suggested that ignorant B cells are precursors to autoantigen-driven RF-secreting cells.

However, previous studies analyzed older autoimmune mice that had lost tolerance for an undefined time period. A kinetic analysis has been lacking, and thus there was little direct insight from these studies into the requirements for initiation of autoimmunity and how the response subsequently matures. In this study, we investigate how the tolerant state initially becomes disrupted in MRL/lpr AM14 Tg mice. To do this, we developed a novel strategy to identify the onset of spontaneous autoimmunity without sacrificing the mouse. We screened the PBL of H chain Ig-Tg mice for the appearance of RF cells and showed that this correlated with the presence of RF Ab-forming cells (AFCs) in spleen. This strategy then allowed us to define the initial events and types of B cell response (e.g., GC or AFC) generated when B cell tolerance is lost then allowed us to define the initial events and types of B cell response (e.g., GC or AFC) generated when B cell tolerance is lost.

For IgG2a and antichromatin ELISAs, Immulon 2 plates (Dynex) were used as secondary Abs. IgG2a ICs were measured using a C1q binding assay previously described (25), with anti-IgG2a as the secondary Ab.

**Materials and Methods**

**Mice**

The AM14 H Tg is a conventional IgM-only Tg. AM14 H Tg mice (18) were backcrossed at least 10 generations onto the MRL/lpr background. All mice were housed under specific pathogen-free conditions.

**Cell isolation and FACS analysis**

Cells were enriched from PBL using Lymphocyte Separation Medium (Cellgro) according to the instructions and resuspended in FACS buffer (PBS/1% FCS/0.05% sodium azide). Splenocytes were prepared as described elsewhere (22). FACS analysis of PBL and splenocytes was performed as described previously (22). Propidium iodide was used to exclude dead cells.

**Reagents**

Abs prepared in our laboratory as described (18) were: 4-44-biotin (anti-Id), 4-44-FITC, 4-44-Alexa Fluor 647, 4-44-Alexa Fluor 488, 30H12-biotin (anti-CloS0.2), and C363-29B-FITC (anti-CD3). DS1-PE (anti-mouse IgM) was obtained from BD Pharmingen. Peanut agglutinin (PNA)-biotin (anti-CD90.2), and C363-29B-FITC (anti-CD3). DS1-PE (anti-mouse IgM) was obtained from BD Pharmingen. Peanut agglutinin (PNA)-biotin (Intergen) and streptavidin-PE (Molecular Probes) were used to detect biotinylated reagents.

**Histology**

Sections were prepared and stained exactly as described previously (23).

**ELISAs, ELISPOT assays, and IC detection**

ELISPOTs and ELISAs were performed as described elsewhere (19, 22). For IgG2a and antichromatin ELISAs, Immulon 2 plates (Dynex) were coated with anti-IgG2a (Southern Biotechnology Associates) or purified calf thymus chromatin (24), respectively. Anti-\(\kappa\)-alkaline phosphatase and anti-IgG2a-alkaline phosphatase (Southern Biotechnology Associates) were used as secondary Abs.

**Results**

The generation of RF-secreting AFCs in AM14 MRL/lpr mice is variable in onset

AM14 H Tg mice have a small proportion of B cells that express an appropriate V\(\kappa\)8 L chain and therefore have the anti-IgG2aa RF specificity. We previously showed that among older IgH\(^\text{H}^\text{lpr}\) AM14 H Tg Fas\(^{br}\) mice, there was a large increase in RF AFCs compared with older IgH\(^\text{H}^\text{H}\) mice or very young IgH\(^\text{H}^\text{H}\) mice (Ref. 22 and our unpublished observation). However, there was substantial variability in AFC numbers from mouse to mouse even in the older mice, implying stochastic factors in the onset and/or expansion of the RF response. We therefore wanted to detect mice at the earliest stages of the RF response to identify the factors that triggered onset and how the response evolved from this point. We began by investigating the onset of RF AFC formation in young mice. We found that RF B cells in spleens of H Tg MRL/lpr mice younger than 8 wk were resting, their phenotype resembling that of RF cells from IgH\(^\text{H}\) mice (data not shown and see below); these mice had relatively few RF AFCs, as identified by the anti-Id Ab 4-44 (Fig. 1A). In contrast, in IgH\(^\text{H}\) Tg mice, all cohorts older than 9 wk included mice with large numbers of RF AFCs. Although the average frequency of AFCs among older cohorts was also higher than the 5- to 8-wk cohort, each of the older cohorts included a substantial fraction of animals with few AFCs (Fig. 1A). Because age alone was not a consistent predictor of RF AFC formation, we could not base subsequent studies on age-based cohorts.

**Individual AM14 MRL/lpr mice have abrupt increases in the frequency of RF-expressing cells in the blood**

To develop another method to identify mice in the early stages of RF B cell activation, we screened cohorts of H Tg MRL/lpr mice using FACS of PBL to detect the appearance of circulating RF-expressing cells (18). In IgH\(^\text{H}\) mice of all ages, 4-44\(^{-}\) cells typically constituted <2% of PBL (Fig. 1B), as expected from the frequency of B cells that should express suitable V\(\kappa\)8 genes (26). Similarly, IgH\(^\text{H}\) mice at 9 wk of age typically had low frequencies of 4-44\(^{-}\) cells in PBL, as shown by the mouse depicted in Fig. 1C. However, in this same mouse 2 wk later, there was a dramatic increase in 4-44\(^{-}\) cells (Fig. 1D). We refer to the onset of increased RF B cells in PBL as “conversion” because it resembles classic seroconversion. Mice with a frequency of 4-44\(^{-}\) cells in PBL >3% were classified as “converted” since control IgH\(^\text{H}\) mice that lack the autoantigen very rarely had frequencies of >3% (data not shown). By screening 119 H Tg MRL/lpr mice every 2 wk, we were able to construct a cumulative incidence curve for conversion (Fig. 1E). At ~14 wk of age, 50% of the mice had converted, roughly paralleling the fraction of mice in the 13- to 15-wk cohort that had elevated numbers of RF AFCs in spleen (Fig. 1A). However, these data did not indicate whether the level of RF B cells in PBL would predict the state of tolerance or autoimmunity in the spleen.

**Increase in RF-expressing cells in PBL predicts the presence of large numbers of RF-expressing cells in spleen**

To determine whether conversion in PBL was indeed a good marker for initial loss of B cell tolerance in spleen, splenocytes from individual mice were analyzed either before conversion or at various times after (Fig. 2, A and B). Nonconverted mice had relatively few 4-44\(^{-}\) AFCs/10\(^6\) splenocytes (320 ± 80, mean ± SEM), whereas mice sacrificed within 2 wk of conversion had an average seven times more AFCs (2040 ± 433/10\(^6\), \(p < 0.0001\).
Using >500 per 10^6 as a cutoff (mean + 2 SEM of nonconverts), the positive and negative predictive values of the PBL assay for determining splenic RF B cell activation were 91 and 82%, respectively. Similar conclusions were reached when considering the total number of 4-44^+ AFCs per spleen (Fig. 2B). Spleen sections demonstrated that nonconverted mice had very few darkly staining 4-44^+ cells (Fig. 3A), whereas recent converts had numerous dark-staining 4-44^+ cells (Fig. 3B). Thus, the assay considering the total number of 4-44^+ AFCs per spleen (Fig. 2B). Spleen sections demonstrated that nonconverted mice had very few darkly staining 4-44^+ cells (Fig. 3A), whereas recent converts had numerous dark-staining 4-44^+ cells (Fig. 3B). Thus, the assay.
of PBL for increased frequency of 4-44+ cells is a method to predict the onset of autoimmunity in the spleen without having to sacrifice the mouse. We next used this approach to determine the order of events that occur after the onset of autoreactive B cell activation in the spleen.

Kinetics of the GC response

We previously demonstrated that somatic hypermutation in the spleen can occur in areas outside of the GCs, but because we studied older animals without any information concerning the duration of RF B cell activation, we noted that we could not distinguish whether mutating cells had previously taken part in a GC response (21). Comparing GC formation before and after conversion, we found that 4-44+ GCs generally do not precede the onset of autoimmunity, with onset signaled by increased numbers of 4-44+ cells in the PBL and AFCs in the spleen (Figs. 2C and 3E). More than 80% of spleens in both recent converts (<2 wk after conversion, n = 31) and nonconverts (n = 24) had no 4-44+ GCs in a single plane of section and only two nonconverted mice and one recently converted mouse had three or more 4-44+ GCs per section (Fig. 2C). Older converts also had very few splenic 4-44+ GCs (21). Importantly, no difference in the number of 4-44+ GCs was observed between nonconverts and recent converts, arguing against a relationship between GC formation and generation of splenic RF AFCs. Since most of the mice that had recently initiated RF AFC reactions had few if any GCs, we conclude that a local GC reaction is unlikely to be necessary for the development of the RF AFC reaction in the spleen.

Evolution of the RF response

To determine how the RF autoimmune response evolved with time, we studied mice at various times after conversion. The spleens of mice sacrificed at conversion and those sacrificed at later points were histologically similar in that 4-44+ cells accumulated at the T zone-red pulp border (Fig. 3, B–D). However, in a number of the mice that had been converted for longer than 2 wk, there were striking accumulations of 4-44+ cells extending into the red pulp and/or the T cell zones, affecting the bulk of the spleen. To more rigorously ascertain whether these 4-44+ clusters continue to expand with age, mice were grouped into recent and old converts (≤2 wk or >2 wk after conversion). The number of splenic 4-44+ AFCs almost doubled in old converts compared with recent converts (Fig. 2B, p = 0.0131). However, the spleens of old converts have only slightly higher proportions of 4-44+ AFCs than recent converts (Fig. 2A). Therefore, the size and/or number of 4-44+ clusters increases with time although the density of the 4-44+ AFCs remains constant. Surprisingly, neither recent nor old converted AM14 MRL/lpr mice had higher numbers of bone marrow 4-44+ AFCs than nonconverts (Fig. 2D). Thus, RF B cell activation does not promote the accumulation of many RF plasma cells in bone marrow.

The RF response can fluctuate following initial conversion

Although lupus in humans can relapse and remit, there has been little investigation of this in an animal model beyond serial serological analysis (27, 28) and thus little insight into whether an initial autoimmune response (vs clinical disease) can be controlled at least temporarily. It is tempting to assume that once initiated, autoAb responses always progress due to the constitutive presence of autoantigen. To assess this, at 2-wk intervals we performed FACS of PBL from a cohort of converted mice. We found that the frequency of 4-44+ cells among PBL often fluctuates substantially after initial conversion, as exemplified by the three converted mice tracked for 6 wk following conversion shown in Fig. 4A. To assess whether reductions in the frequency of RF B cells in PBL were paralleled by a fall in the number of AFCs in spleen, 12 converted mice were sacrificed when levels of 4-44+ cells in the blood had declined from their previous peak at least 2 wk after initial detection of conversion. Although the spleens of these mice usually had...
more $44^+$ AFCs than nonconverts, only 7 (60%) of 12 of these mice had splenic $44^+$ AFCs above the cutoff of 500 per $10^6$ (Fig. 1B), compared with 91% of mice sacrificed at conversion with $>500$ per $10^6$ AFCs (Fig. 1A, $p = 0.0297$). We conclude that some mice do have periods of remission after the onset of B cell autoimmunity. Conversely, 14 of 15 converts sacrificed $>2$ wk after initial conversion, but when $44^+$ cells in PBL were still high, had AFCs $>500$ per $10^6$. Thus, persistence of $44^+$ cells in PBL continued to predict an active AFC response in the spleen.

**Converted mice have higher levels of IgG2a immune complexes than nonconverted mice**

Since age and genetics alone do not predict the initiation of autoimmunity, other factors must play a role. A number of factors could influence the initiation of the AM14 RF B cell response, including the presence of autoantigen, availability of T cell help, and reduction of potential regulatory influences from B or T cells. In these studies, we focused on analyzing the potential RF Ags, and reduction of potential regulatory influences from B or T cells. Could influence the initiation of the AM14 RF B cell response, extending earlier in vitro data (25, 29) by implicating a unique role for chromatin-containing ICs in activating RF B cells in vivo. Taken together, our work provides a comprehensive window into the early events of the spontaneous activation of an autoAb response.

**Assay of PBL identifies mice in the early stages of autoimmunity**

The presence of RF B cells in PBL was indicative of the initiation of RF B cell activation, differentiation, and expansion in the spleen (Fig. 2). In contrast, age alone was a poor predictor of autoimmunity in individual mice, although the prevalence of conversion increased with age. The RF B cells in blood have unique markers, but in many respects resemble and may be derived from the plasmablasts in the spleen (43). Although the origin and identity of the circulating RF B cells is not clear, they seem similar to the circulating plasmacytoid cells that correlate with exacerbations in lupus patients (33, 34). Possibly, a similar process to that observed in MRL/lpr spleens occurs in secondary lymphoid tissues of systemic lupus erythematosus patients and causes the subsequent release of plasmablasts into the blood during exacerbations. In these patients, the specificities of B cells in PBL are not yet clear.

**Histologic nature of the early response**

The paucity of RF-expressing GCs, even in early converts, along with a lack of correlation between the number of such GCs and conversion or age, strongly suggests that the extrafollicular response proceeds independently of any GC response. Thus, it is unlikely that RF cells have to pass through a GC to initiate somatic hypermutation at the T zone-red pulp border.
The activation of RF B cells differs from the activation of anti-DNA B cells in the 3H9 model, albeit that the 3H9 studies did not necessarily characterize the earliest events in the response. Anti-DNA-secreting B cells were predominantly seen in the outer T cell zone (12). It is less clear whether GCs were a part of the anti-DNA response. Notably, 3H9 anti-DNA B cells on a wild-type background were anergic and sequestered in the T zone-B zone border, unlike the follicular distribution of the immunocompetent RF B cells (35, 36). If these anergic B cells are the precursors of activated anti-DNA B cells, then the difference in the state of the precursor cells could underlie the different phenotypes in the autoimmune mice. High-affinity anti-DNA B cells are normally edited in the bone marrow, but either a failure or modification of this process leads to the accumulation of autoreactive B cells coexpressing κ- and λ-chains in the marginal zone (37). Our results thus demonstrate that different autoAb responses can evolve with distinct features.

Although the expansion of RF B cells in spleen occurs at the T zone-red pulp border early on, the same site that is dominant at later stages, there are differences between early and late stages. In early converts, RF B cell expansion often appeared to be focal. Even at these focal sites, somatic hypermutation was active and each site had a distinct clonal makeup. In contrast, in later converts RF B cells were found at most, if not all, white pulp cords. The finding of multiple clones participating in a focal response early on indicates that the presence of RF B cells is not the limiting factor in conversion; rather, some events or cell types at a local site limit activation. Although availability of autoantigen in the proper form is likely a limiting factor (see below), it would be expected to be present throughout the spleen as it circulates in plasma. An interesting candidate for a locally limiting factor would be autoreactive T cells with appropriate specificity to help the RF B cells.

Factors that correlate with the onset of autoimmunity

Even though the onset of autoimmunity is unpredictable, key precipitating factors should be present at the time of onset. Faulty clearance of autoantigens has been linked genetically to predisposition for autoimmune disease (38). However, until now there has not been a report of direct correlation with the onset of autoimmunity and the levels of a putative autoantigen. In AM14 Tg MRL/lpr mice, only levels of IgG2a ICs and IgG2a anticromatin, but not total IgG2a correlated with onset of RF B cell activation. This suggests that the form of the autoantigen is important; RFs including AM14 are known to bind well to ICs and ICs can elicit a transient RF response in normal mice (25, 39). Finding that IgG2a anti-chromatin correlates with in vivo RF B cell activation adds to the in vitro data showing that AM14 B cells are activated remarkably well by ICs made with IgG2a anti-chromatin Abs (29). It is likely that other types of ICs can also activate RF B cells in vivo under the right circumstances. Our findings implicating chromatin-containing ICs in activation of RF B cells provides further evidence for the theory that autoantigen levels and the form of autoantigen play critical roles in the initial loss of B cell tolerance in the periphery. They also help to further define the true autoantigen for RF B cells.

Evolution of B cell autoimmunity

Autoimmune disease tends to be a chronic condition, but whether specific autoimmune responses and clones continue indefinitely once initiated is not at all clear. We used the initial identification of converted mice to ask whether the initial activation of RF B cells was always followed by progressive autoimmunity. Somewhat to our surprise we found spontaneous remissions in a fraction of the animals (Fig. 4B). Our results suggest that, even in genetically predisposed animals, regulatory forces could operate to bring an initial loss of tolerance under control. Alternatively, the conditions required to precipitate autoimmunity may fluctuate at the outset and may fall below a threshold needed to sustain the response.

Although the sites of RF B cell expansion do not seem to change, the extent of the response in the spleen increases over time. This was evidenced both histologically and by measuring the numbers of RF AFCs, which double in older vs. early converts. Nonetheless, there seem to be inherent limits to the extent of the response, possibly explained by limitations in the size of available niches for plasmablasts in spleen (40). One might have expected that a substantial proportion of long-term autoimmunity would derive from long-lived AFCs. It was therefore surprising that there was no significant accumulation of RF AFCs in the bone marrow following conversion (Fig. 2D). Since long-lived plasma cells are thought to derive from GCs, the paucity of bone marrow AFCs may be secondary to the lack of GCs in spleen (41). Indeed, in related work we have found that classical plasma cells were quite rare in spleens of converted mice and that most AFCs were plasmablasts (43). Given the lack of accumulation of substantial RF AFCs in bone marrow with time, it seems likely that most of these plasmablasts do not serve as precursors for longer-lived plasma cells that reside in the bone marrow. Interestingly, it was recently shown that in NZM2410 lupus-prone mice, AFCs accumulate in spleen and do not migrate to bone marrow (42).

Conclusion

Identification of the early events, sites of activation, and potential inciting autoantigens all have implications for our understanding of spontaneous systemic autoimmune disease and strategies for its treatment. In this study, we have presented a model system along with a paradigm for investigation of early events and triggers for the activation of autoreactive B cells in the periphery. We plan to use this system to test the roles of specific molecules and cell interactions and to screen for ways to prevent early activation or abort it. In addition, we hope that this approach will be extended to other systems.

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Disclosures

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