A Disease-related Rheumatoid Factor Autoantibody Is Not Tolerized in a Normal Mouse: Implications for the Origins of Autoantibodies in Autoimmune Disease

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Summary

We have analyzed B cell tolerance in a rheumatoid factor (RF) transgenic mouse model. The model is based on AM14, a hybridoma originally isolated from an autoimmune MRL/lpr mouse that has an affinity and specificity typical of disease-related RFs from this strain. AM14 binds to immunoglobulin (Ig)G2a of the "a" allotype (IgG2a^a) and not to IgG2a^b. Thus, by crossing the transgenes onto an IgH^a (BALB/c) background or to a congenic IgH^b (CB.17) background, we could study the RF-expressing B cells when they were self-specific (IgH^a) or when they were not self-specific (IgH^b). These features make the AM14 model unique in focusing on a true autoantibody specificity while at the same time allowing comparison of autoreactive and nonautoreactive transgenic B cells, as was possible in model autoantibody systems such as anti–hen egg lysozyme. Studies showed that AM14 RF B cells can make primary immune responses and do not downregulate sIgM, indicating that the presence of self-antigen does not induce anergy of these cells. In fact, IgH^a AM14 transgenic mice have higher serum levels of transgene-encoded RF than their IgH^b counterparts, suggesting that self-antigen–specific activation occurs even in the normal mouse background. Since AM14 B cells made primary responses, we had the opportunity to test for potential blocks to self-reactive cells entering the memory compartment. We did not find evidence of this, as AM14 B cells made secondary immune responses as well. These data demonstrate that a precursor of a disease-specific autoantibody can be present in the preimmune repertoire and functional even to the point of memory cell development of normal mice. Therefore, immunoregulatory mechanisms that normally prevent autoantibody production must exert their effects later in B cell development or through T cell tolerance. Conversely, the data suggest that it is not necessary to break central tolerance, even in an autoimmune mouse, to generate pathologic, disease-associated autoantibodies.

N egative regulation of self-reactive B cells, "tolerance," can occur at multiple points during B cell development (1–6). In the bone marrow (BM)[1], newly formed self-reactive cells can be eliminated ("clonal deletion") (1, 6, 7), modified by further rearrangement of the Ig receptor ("receptor editing") (8, 9), or inactivated to prevent Ag-specific responses ("clonal anergy") (3, 4, 10). Self-reactive cells escaping to the periphery can also undergo deletion on encountering self-Ag in organs such as the liver (11). Further, self-reactive cells that are stimulated by Ag may be subject to a less-defined form of negative regulation during

memory cell differentiation accompanying a primary immune response (12–15). It is also clear that some nominally self-reactive B cells may not be tolerized at all (16–19), a state which has been called "clonal ignorance" (20). Negative regulation of self-reactive B cells could also be accomplished less directly, for example, via T cell tolerance or by efficiently downregulating ongoing antiself responses. It is not clear which, if any, of these regulatory mechanisms break down during systemic autoimmune disease, although the multistep nature of self-tolerance argues against a general breakdown.

Why some self-reactive B cells are subject to one or another of these forms of negative regulation or are not regulated at all is not clear. Whether the self-Ag is soluble or membrane bound may play a role (1, 4, 7). Affinity for self-

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[1]Abbreviations used in this paper: BM, bone marrow; HEL, hen egg lysozyme; Tgic, transgenic.

Ag must also play a role (5, 21). Qualities of the self-Ag that dictate the timing and site of encounter, availability, and valency of interaction with self-reactive B cells could also be important. To understand why certain self-reactive B cells are subject to particular forms of tolerance is critical, in view of the fact that specific self-Ags are targets in systemic autoimmunity, and that these targets differ among various systemic autoimmune diseases (22). For example, anti-dsDNA is a hallmark of SLE (23, 24) while RFs are most common in rheumatoid arthritis (25, 26). In addition, in SLE patients or in MRL/lpr/lpr mice, only a fraction of individuals make anti-Sm (22, 24, 27). Therefore, most lupus patients are tolerant of their own Sm, at least at the B cell level. The selective loss of self-tolerance that characterizes systemic autoimmune diseases may reflect the different levels of B cell tolerance that occur for different autoAbs. Our long-term goal is to understand why and how selective loss of self-tolerance occurs in autoimmune disease.

In this regard, the recent work of Goodnow and colleagues (1, 4, 5) using an anti-hen egg lysozyme (HEL)/ HEL Tgic system, and Nemazee and colleagues (2, 7) using an anti-MHC class I transgenic (Tgic) system may not be indicative of disregulation in autoimmune disease. Although their studies have verified the existence of autonomous B cell tolerance and have revealed both mechanisms and sites of B cell tolerance (9, 28–31), the specificities studied do not have any obvious correlate among actual autoAbs. Therefore, these models do not help to explain selectivity of autoAb production and may not be representative of actual autoAb-producing B cells. To address some of these concerns, several groups have generated Tgic mouse models using V genes from spontaneously generated autoAb-producing cell lines isolated from autoimmune mice (8, 32–36). Tgic models using real autoAgs have revealed that at least certain self-specific B cells can be censored from the preimmune repertoire via either deletion or anergy. For example, DNA-specific B cells can be deleted, anergized, or edited (8, 32, 33, 36), while antierythrocyte B cells are variably activated, anergized, or deleted (34). However, interpretation of these models is also limited for two reasons: first, as in the facultative autoAb Tgics, in both the DNA and erythrocyte systems, high affinity mutated autoAbs have been put back into the germline and so developing B cells may not mimic the fate of the original unmutated precursors that were first activated. Second, clear interpretation of data is hampered by constitutive autoAg expression, which precludes Ag-negative controls possible in the anti-HEL or anti-class I systems.

To ask whether the B cell precursors of a bona fide autoAb are tolerized in a nonautoimmune mouse while addressing the concerns inherent in previous models, we created the AM14 RF Tgic model (16). In the AM14 model, rearranged V genes from an RF (specific for IgG2a^a but not IgG2a^b) originally isolated from an MRL/lpr/lpr mouse, have been placed in the germline. AM14 has a typical moderate affinity and has few if any somatic mutations, and so is a logical candidate for a germline or near-germline au-

toAb. Most importantly, the specificity for IgG2a^a allows us to generate Ag-negative (IgH^b) and autoAg-expressing (IgH^a) mice by crossing onto appropriate backgrounds.

In earlier work (16), we determined that AM14 B cells were not subject to clonal deletion in a nonautoimmune BALB/c background. In the present work, we show that these cells are not subject to the second major form of B cell tolerance—anergy. We also show that such B cells can participate in memory responses. Thus, these self-specific RF B cells nonetheless appear fully immunocompetent in a normal mouse. We conclude, in contrast to earlier suggestions based on artificial autoAb Tgics (4, 37), that neither central nor peripheral tolerance mechanisms are responsible for regulating bona fide RF autoAb precursors in normal mice. Instead, these results suggest that events leading to B cell autoimmunity act at later stages either in aberrant B cell activation or in failure to appropriately downregulate activated autoreactive B cells.

Materials and Methods

Mice

Tgic mice were constructed as described (16). H chain Tgics were bred onto the CB.17 (IgH^b) background. Originally, two H chain Tgic lines were described and found to be similar. All work reported here is on one of those lines. L chain Tgics were bred onto both the CB.17 and BALB/c (IgH^a) backgrounds. At the 18th BC generation, L chain mice of both types were intercrossed to obtain homozygous Tgics (as confirmed by test crosses) which were maintained by continuous intercrossing and which were used in all experiments. To obtain RF-specific double Tgic mice, male H chain Tgics were bred to female L chain Tgics of either the BALB or CB.17 background. Thus HL double Tgics were obtained with either the IgH$^{a/b}$ (experimental) or the IgH$^{b/b}$ (control) background. Mice were genotyped by PCR as described (16). Genotypes were reconfirmed in all experimental mice using either a specific antiidiotype assay or PCR of recut tails or both. BALB/c mice were obtained from the Jackson Laboratory (Bar Harbor, ME) or from our own breeding colony which had been established with breeding pairs from the Jackson Laboratory. CB.17 mice were from our colony derived from breeding pairs obtained from Taconic Farms, Inc. (Germantown, NY). All mice were maintained in our specific-pathogen–free barrier facility. The H and L chain Tgic nucleus colony was maintained in microisolator cages.

ELISA and ELISA Spot Assays

ELISA assays for IgM, IgM^a, IgG2a, and RF were performed as described (16). A sandwich anti-Id ELISA and ELISAspot assay were used to unambiguously distinguish Tg-derived RF from background RF derived from non-Tgic cells. Two antiidiotypes, both specific for the Tg-encoded HL combination, were derived as previously described, and purified and biotinylated as described below (16). For ELISA, plates (Immulon 2; Fisher Scientific Co., Pittsburgh, PA) were coated with 8 μg/ml of 4-44 overnight in BBS, washed, and blocked with 1% BSA/PBS. Serially diluted samples were incubated in duplicate for 1 h, followed by washing, detection with 4G7-biotin (0.25 μg/ml in 1% BSA/PBS), washing, incubation with streptavidin-alkaline phosphatase (Southern Biotechnology, Birmingham, AL) diluted 1:1,500, and finally

development with p-nitrophenylphosphate 1 mg/ml in 0.05 M Na carbonate/10 mM MgCl₂. Plates were read on an EL390 microplate spectrophotometer (Bio-Tek Instruments, Inc., Burlington, VT) and concentrations determined using Deltasoft (Biometallics, Princeton, NJ) by comparison to standard curves on each plate. ELISAspot was carried out similarly, except that either 4G7 (8 μg/ml) or goat anti–mouse IgM antiserum (10 μg/ml; Southern Biotechnology) was used to coat plates and 4-44-biotin used to detect (0.6 μg/ml). Splenocyte single cell suspensions were made in RPMI 1640 (GIBCO-BRL, Gaithersburg, MD)/2.5% FCS and were plated directly onto coated, blocked plates in triplicate. Serial dilutions were carried out in the plate. Cells were incubated for 4.5–6 h. in a total volume of 200 μl/well at 37° C. All washes were done with PBS/1% Tween 20 (3×) followed by dH20 (2×) between each step. Final development was with 80% bromochloroindoyl phosphate solution (Sigma Chemical Co., St. Louis, MO) in 0.6% low melting point agarose (FMC Corp. Bioproducts, Rockland, ME). Spots were read using a dissecting microscope. All wells from a single assay were read at the same sitting. Data were analyzed by extrapolating from dilutions that yielded 20–150 spots in a well or from wells which contained the greatest number of spots if <20.

FACS® Analysis

FACS® analysis was performed as described (16).

Antibodies and Ags

Antibodies were obtained and purified as described (16). Purified Abs were biotinylated as described (16). TNP-KLH was prepared according to (38). KLH was originally obtained from Calbiochem Corp., La Jolla, CA. Before derivitization, high molecular weight KLH was isolated by ultracentrifugation.

Transfer Immunization

Cell Preparation. Single cell suspensions were prepared from spleens of 12-wk-old RF Tgic or 11–14-wk-old bone marrow chimeras. Splenocytes were depleted of T cells by incubating with J1J (anti-Thy-1) culture supernatant, followed by treatment with guinea pig complement (GIBCO-BRL). T-depleted splenocytes (referred to as B cells in the text) were washed into PBS just before injection. 8–10-wk-old BALB/c T cell donors were immunized with 100 μg KLH in CFA (GIBCO-BRL) subcutaneously at the tailbase and right footpad 1 wk before killing. Cells were teased from inguinal and popliteal lymph nodes and maintained in media containing 40 μg/ml DNAse (Sigma Chemical Co.). Lymph node cells were depleted of B cells and some macrophages by incubation with MKD6 (anti-I-A^d) and J11D (anti-HSA) supernatants followed by treatment with rabbit complement (Cedarlane Laboratories Ltd., Hornby, Canada). B-depleted LN cells (referred to as T cells in the text) were washed into PBS supplemented with 20 mM Hepes (GIBCO-BRL) and 2.5% ACD-A (Baxter-Fenwal, Deerfield, IL) just before injection. Aliquots of B and T cell–enriched preparations were analyzed by FACS® (using anti-Ly1 to identify T cells and anti-B220 to identify B cells) to confirm depletion of target cell populations, and enumerate Id⁺ B cells (using the AM14 ant-id 4-44). Generally, T-depleted preparations had <2% residual T cells and B-depleted populations had <3% residual B cells.

Antigen Preparation. Hy1.2 mAb (IgG2a^a anti-TNP) or control C4010 (IgG2a^b anti-TNP) was incubated with TNP-KLH for 1 h at 37°C to allow formation of Ag–Ab complexes. Each

dose of cell/Ag mixture contained 125 μg Hy1.2 or C4010 and 31.2 μg TNP-KLH.

Injections. Each recipient received 2 × 10⁶ T-depleted splenocytes from Tgic donors or, in the case of BM chimera donors, 0.8 × 10⁶ Id+ B cells. All recipients received 4–6 × 10⁶ T cells and Ag or control immunogen as indicated in figures and text. Sublethally irradiated (550 R) 8-wk-old BALB/c and 7–9-wk-old CB.17 recipients were given the cell/Ag mixture via i.v. injection in the lateral tail vein, within 6 h of irradiation. Cells and Ag were mixed just before injection. Recipients were given acidified water until being killed.
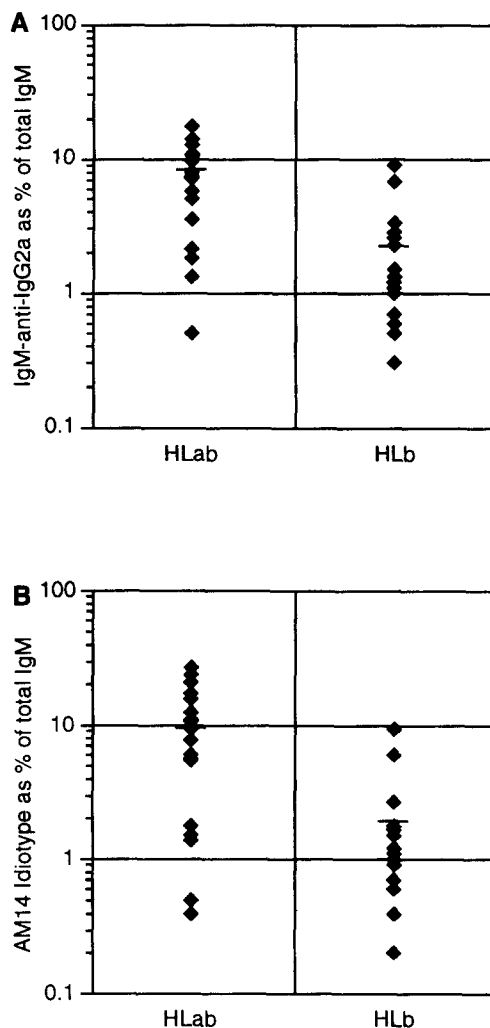
Figure 1. RF Tgics that express the IgG2a^a autoantigen have higher levels of spontaneous serum RF. RF Tgic mice of IgH^a/b (HL^a/b) or IgH^b (HL^b) genotypes were bled and serum IgM, IgM anti-IgG2a^a, and AM14-Id were determined in micrograms per milliliter based on standard curves derived from an irrelevant IgM and a transfectoma expressing an IgM identical to the Tg IgM. The values of the ratio of IgM anti-IgG2a^a/IgM total (A) or AM14-Id/IgM total (B) are shown, with each point representing a single mouse. Horizontal bars in each column denote the means. For each ratio, HL^a/b animals have higher serum levels indicative of the Tgic protein (four to five time higher on average). The two ratios were highly correlated among individual mice (not shown). Non-Tgic, L only, or H only mice generally had values <0.5% (not shown).
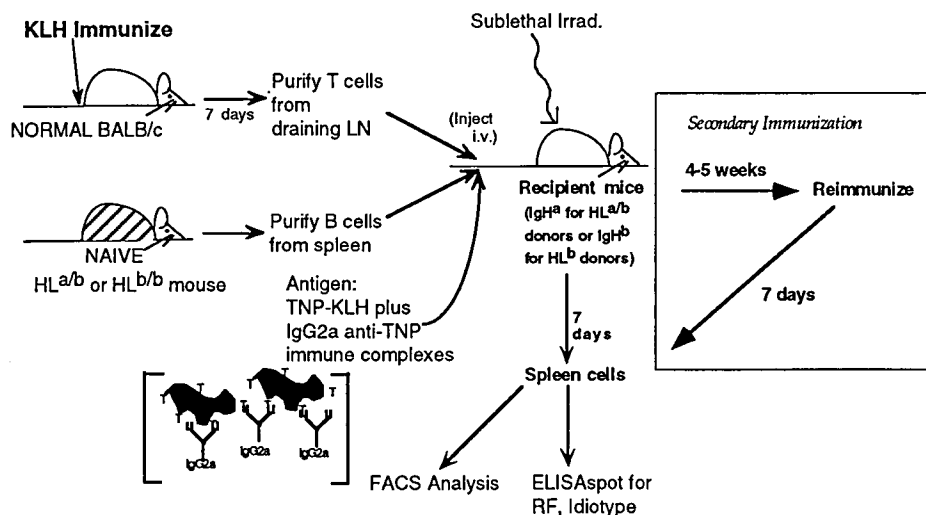
Figure 2. Transfer immunization protocols. Shown is a diagram of the transfer immunization schemes for primary and secondary (*box*) immunization, as described more fully in Materials and Methods. T denotes TNP, as derivitized onto the KLH carrier protein.

Bone Marrow Chimeras

BM was harvested from femurs, tibias, and humeri of 9-wk-old BALB/c, 14-wk-old CB.17 and individual 8–13-wk-old RF Tgic mice. Aliquots from each Tgic donor were analyzed by FACS® to confirm presence of Id⁺ B cells. Cells from BALB/c and a/b Tgic were pooled in various ratios, as were CB.17 and b/b Tg cells. A total of 1.4×10^7 bone marrow cells in PBS were injected i.v. into each allotype-matched 9-wk-old BALB/c or

Figure 3. FACS® analysis of primary transfer immunization of Tgic RF B cells. Mice prepared and immunized as described in Materials and Methods and Fig. 2 were killed 7 d after immunization and aliquots of spleen cells were analyzed by FACS®. 2% probability plots are shown and percentages of total live cells (as determined by propidium iodide dye exclusion) in each gate are indicated. The ordinate shows expression of the AM14 (4-44) idiotype and the abscissa total surface IgM. Each plot is from a different mouse from the same experiment which compared the absence of autoantigen (IgHᵇ, *A* and *B*) to the presence of autoantigen (IgHᵃ, *C* and *D*) and the use of IgG2aᵃ immunogen (*A* and *C*) to control IgG2aᵇ immunogen (*B* and *D*). Note that variability in the frequency of IgM⁺/Id⁻ (R2 gate) cells was seen in multiple recipients in multiple experiments regardless of treatment group. We suspect that this represents variable autoreconstitition of endogenous B cells 7 d after sublethal irradiation.

10–14-wk-old CB.17 recipient, irradiated (650 R) 6 h earlier. Recipients were kept in microisolator cages and provided with acidified water until being killed.
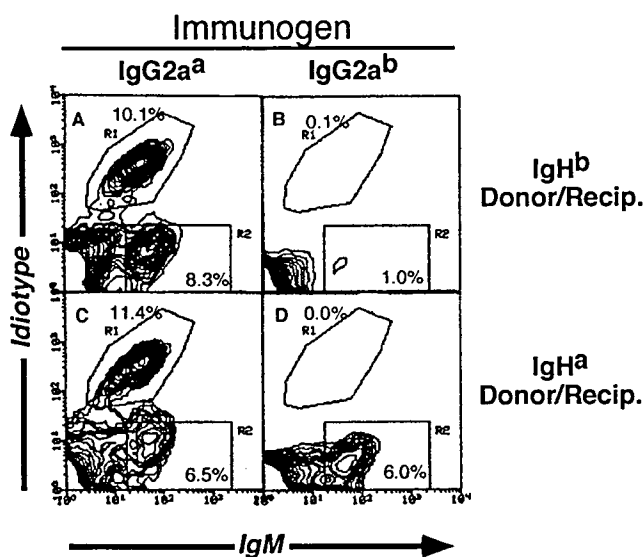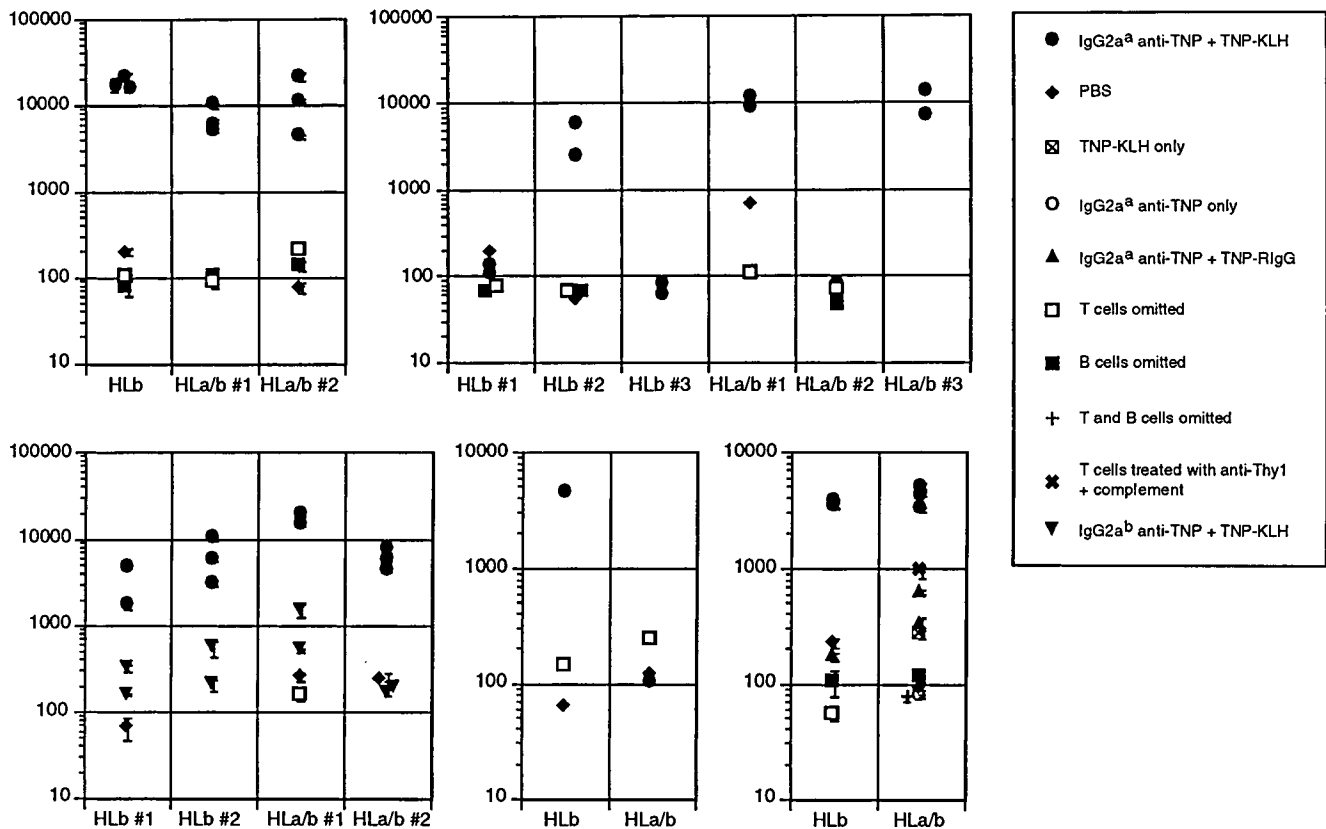
Results

Autospecific RF B cells Are Not Anergic

We have shown that AM14 RF B cells are not deleted in IgG2aᵃ-expressing mice (16). To determine the functional capacity of these potentially autoreactive B cells, we assessed both spontaneous serum Ab and the response of Tgic B cells to immunization.

Serum AutoAb. We compared the level of serum IgM-RF and AM14-idiotype in control IgHᵇ (lacking autoAg) and IgHᵃ/ᵇ AM14 Tgic mice from two different founder lines (16). Fig. 1 summarizes the data from multiple individual mice from one of the founders. Measured as either IgM–anti-IgG2aᵃ RF or as AM14-idiotype, spontaneous serum Tgic Ig was significantly higher in the IgHᵃ/ᵇ mice. Similar data were obtained for the other founder (reference [16] and not shown). Finding higher RF levels in IgHᵃ/ᵇ compared to IgHᵇ/ᵇ mice is contrary to the expectation of anergy in IgHᵃ/ᵇ mice. However, it does not by itself establish that RF B cells in IgHᵃ/ᵇ mice are immunocompetent.

Immunization. To directly test the capacity of such cells to participate in primary immune responses, we established a transfer immunization assay. T cells were primed to KLH in normal animals, and transferred into sublethally irradiated recipients, along with naive Tgic B cells and TNP-KLH + IgG2aᵃ anti-TNP Ag (Fig. 2). Immune responses of Tgic B cells were assayed by FACS® (Fig. 3) and ELISA spot (Fig. 4) analysis of recipient splenocytes. No difference was seen between Ag-positive (IgHᵃ/ᵇ donors into IgHᵃ recipients) and Ag-negative (IgHᵇ/ᵇ donors into IgHᵇ recipients) transfers. In each case, responding cells could comprise up to 20% of recipient spleen, implying a large degree of proliferation (Fig. 3 and data not shown). Such cells also had increased forward scatter, consistent with activation

Figure 4. ELISAspot analysis of primary transfer immunization of Tgic RF B cells. Mice prepared and immunized as described in Materials and Methods and Fig. 2 were killed 7 d after immunization and aliquots of spleen cells analyzed by ELISAspot for IgM⁺/AM14-Idᵃ Ab-forming cells. Each panel shows the result of an independent experiment. Each column shows recipients derived from a single donor mouse; under each column is given the Tg type (HL) and IgH type (either b or a/b) of the donor. Treatment groups are indicated by different symbols (see key). All groups received T and B cells as outlined in Fig. 2, except as denoted in the key to the symbols. In some treatment groups multiple recipients received the same treatments and are represented by individual symbols. Error bars shown for each individual recipient are the SEM of triplicate determinations; many error bars are smaller than the symbols and are obscured. All data are expressed as IgM⁺/AM14-Id⁺ AFC/spleen. In cases where no spots were seen at the highest cell concentration on ELISAspot plates, the upper 95% confidence limit of detection sensitivity was taken as the result.

(not shown). Large increases in the number of spot-forming cells were also evident. Controls performed using either the T cell Ag alone (TNP-KLH), the B cell Ag alone (IgG2a anti-TNP) or complexes of the B cell Ag with an irrelevant T cell Ag (IgG2a anti-TNP + TNP-rabbit-IgG) (Fig. 4; see legend) all gave background responses. Together, these data show that B cell activation, proliferation, and differentiation were unaffected by the presence or absence of autoantigen during the development and immunization of the B cells.

Memory Responses

Since chronic autoantibody responses in autoimmune individuals resemble memory responses, a candidate stage for regulation of autoreactive cells is memory cell differentiation. As autoreactive AM14 B cells made primary immune responses, this provided the opportunity to test for regulation of secondary responses. We modified our primary adoptive transfer assay as follows (Fig. 2): Instead of killing animals 1 wk after immunization, we waited 4–6 wk

and reimmunized them with immune complexes (or PBS) i.v. in saline. Few spontaneous antibody-forming cells and Tg-idiotype expressing B cells were detected in mice given PBS 4–6 wk after primary immunization and assayed 1 wk after PBS injection (Fig. 5). Similarly, AM14-Id positive B cells were undetectable (<0.1% of splenic cells) in the mice that received PBS in lieu of the second immunization (data not shown). This demonstrated that the primary response had concluded during the 4–6 wk wait and that the vigorous secondary response was not due to a large number of residual precursor cells. Mice given a second injection of immune complexes made strong immune responses, with large numbers of double-Id⁺ antibody-forming cells (Fig. 5) and detectable Tg-idiotype expressing splenocytes (data not shown). Again, no difference was observed between autoreactive (i.e., from IgHᵃ/ᵇ donors transferred to IgHᵃ recipients) and nonautoreactive (IgHᵇ donors transferred to IgHᵇ recipients) Tgic B cells. Specificity controls (see Fig. 5) demonstrated that the secondary response is Ag specific and T cell dependent. Thus, we conclude that autoreactive RF

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B cells are not blocked from functional memory development even in normal mice.

Mixed Bone Marrow Chimeras

Presumably owing to allelic exclusion by the IgM transgene, serum IgG2a^a levels are on average fivefold lower in adult (8–16 wk) RF Tgic mice (40 μg/ml) compared to BALB/c mice (200 μg/ml) (16). It was therefore possible that the Ag concentration in AM14 Tgic mice was below the level required to induce tolerance. To assess the effects of higher IgG2a^a levels on AM14 B cells, we constructed radiation chimeras by reconstituting normal mice with mixtures of Tgic and normal bone marrow. In such chimeras, RF Tgic B cells develop as a minority population amidst normal B cells and these normal B cells should restore near-normal levels of serum IgG2a^a Ag. In addition, any effects due to a preponderance of Tgic B cells, such as lack of competition for follicular niches (31), would be negated in such mixed chimeras.

To obtain a population of 5–15% Tg-expressing B cells 6–10-wk after transfer a 10–20:1 ratio of Tg:normal marrow was required (Fig. 6 A). Repopulation of the splenic B cell compartment was indistinguishable in antigen-negative (IgH$^{b/b}$ + CB.17 BM → CB.17) or antigen-positive (IgH$^{a/b}$ + BALB/c BM → BALB/c) chimeras. As expected, given the high frequency of non-Tgic B cells, most of the recipient mice achieved normal IgG2a^a serum levels at the time of assay. Time course analysis of serum IgG2a^a levels further suggested that chimeric mice had near-normal levels (i.e., >100 μg/ml) of IgG2a^a for at least several weeks before killing (Fig. 6 B). Thus, the chimeras did in fact provide an environment for naturally derived high Ag levels. Reconstitution was equivalent in IgH^a or IgH^b recipients. Thus, even when the Tgic B cells were a minority population, no deletion was seen.

We tested B cells isolated from mixed BM chimeras for the characteristics of anergy using the transfer immunization assay. In two separate experiments with multiple independent chimeric mice as B cell donors, we found equivalent responses of RF Tgic B cells in either the antigen-positive or antigen-negative context (Fig. 7). We conclude that even at IgG2a^a autoAg levels in the range for a normal BALB/c, self-reactive RF B cells make normal primary immune responses and do not appear anergic.
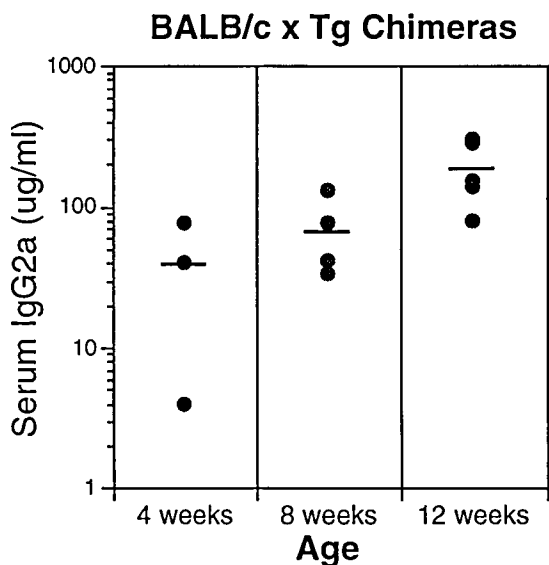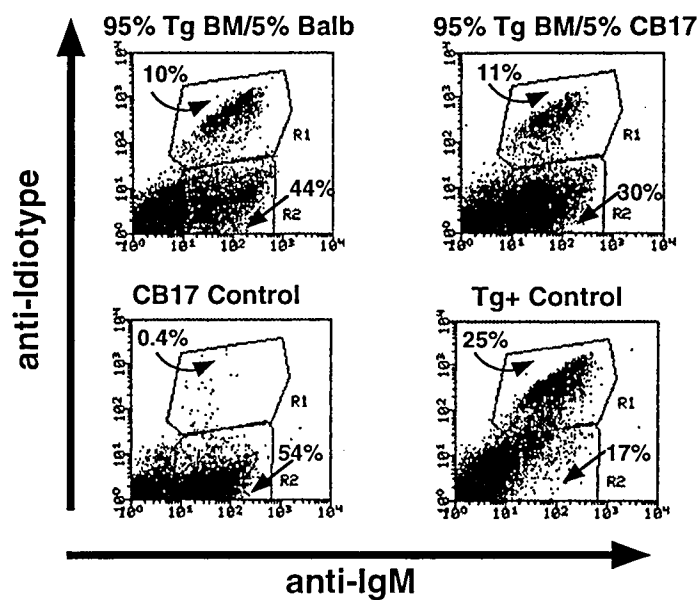
Discussion

AM14 RF Tgics, unlike previous autoAb Tgics (1, 2, 4, 5, 7, 8, 32–36), show that disease-related, self-specific B cells can be immunocompetent in a nonautoimmune mouse. AM14 Tgic B cells were not anergic as determined by an assay system that closely resembled the one used by Goodnow and colleagues (4) to define anergy in Tgic B cells. Nor do AM14 B cells exhibit receptor downregulation in the presence of autoAg, as is seen in anergic anti-HEL B cells (16). Furthermore, serum levels of Tgic Ig (assayed either as RF or idiotype) are elevated, not reduced, in mice

that had the autoAgic IgG2a^a. Anti-HEL/sHEL double-Tgic mice had much reduced levels of Tgic Ig (4). Indeed, the elevated RF levels in Ag-positive RF Tgics in comparison with Ag-negative controls, suggest specific self-Ag–dependent activation of Tgic B cells and further support the idea that autospecific RF B cells are immunocompetent. Although we have not yet defined the details of such activation, we suspect that endogenous IgG2a^a-driven activation is limited to a minority of cells since only ~10% of serum IgM in IgH$^{a/b}$ mice is of Tg origin (vs. 2% in IgH^b mice) even though >95% of splenic B cells express the AM14 Id by FACS®.

As the RF autoAb Tgic system demonstrates a primary immune response, we can examine whether the constitutive presence of autoantigen selectively blocks a secondary autoAb response. This question is of interest because natu-
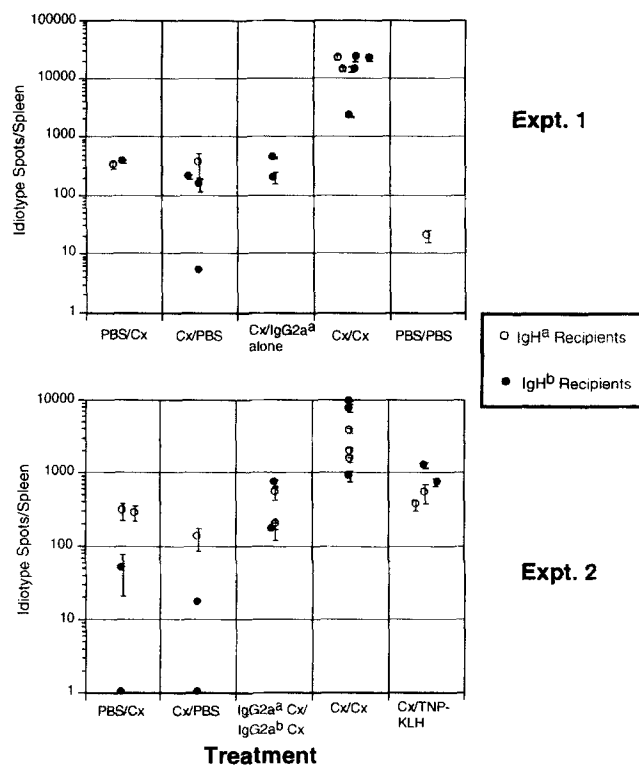
Figure 5. ELISAspot analysis of secondary immunizations. Initially, primary transfer immunizations were carried out as for Fig. 4 except that recipients received splenocytes pooled from three to four Tgic donor mice. 4–6 wk after primary immunization, recipients were reimmunized (as detailed in the box in Fig. 2). Results of two independent experiments are shown. Each column shows a separate treatment protocol as indicated by primary immunization/secondary immunization. Cx is IgG2a^a anti-TNP + TNP–KLH immune complexes. In the second experiment one group received IgG2a^a complexes first and IgG2a^b complexes as secondary immunization. IgH^a donor/recipient results are indicated in open circles and IgH^b donor/recipient results in closed circles. Error bars show SEM for triplicate determinations from each recipient mouse. All data are expressed as AM14-Id$^+$ AFC/spleen, as determined using the double idiotype assay described in Materials and Methods. This assay gives similar results to the IgM/Id assay but is more specific and less sensitive, yielding absolute values of 1 or more orders of magnitude fewer total cells than the IgM/Id assay.

95% Tg BM/5% Balb

10%

R1

44%

R2

95% Tg BM/5% CB17

11%

R1

30%

R2

CB17 Control

0.4%

R1

54%

R2

Tg+ Control

25%

R1

17%

R2

anti-Idiotype

anti-IgM

BALB/c x Tg Chimeras

Serum IgG2a (ug/ml)

1000

100

10

1

4 weeks   8 weeks   12 weeks

Age

Figure 6. Generation and characterization of mixed BM chimeras. (A) shows FACS® analysis of splenocytes of representative mixed BM chimeras 8 wk after BM transplant. Above each panel is indicated the donor inoculum. Note that a 20:1 ratio of Tg:normal BM yielded a 1:3 to 1:4 ratio of B cells in spleen 8 wk later. No difference was seen between potentially autoimmune (BALB/c IgH^a) and control (CB.17 IgH^b) transfers. (B) Serum IgG2a^a levels in mixed BM chimeras derived from BALB/c and Tg donors. Normal serum IgG2a levels in 8–10-wk-old mice in our colony are ~100 µg/ml (reference and data not shown). Note that BM chimeras reach near normal levels at 8 wk and normal levels at 12 wk after transplant.

ral RF responses in normal mice are transient, are mainly restricted to IgM, and do not demonstrate other characteristics of memory. In contrast, RF responses in autoimmune mice resemble secondary immune responses. If autoAb-producing B cells were blocked from the memory pathway in normal mice, but not autoimmune mice, this might provide an explanation for secondary autoimmune responses only in autoimmune mice.

To test for tolerance in the secondary response, we adapted our transfer immunization assay as shown in Fig. 2. By this protocol we found secondary responses in both Ag-negative controls and in Ag-positive experimental mice. Thus, we found no evidence that physiologic autoAg impedes the development of memory cells. As yet, we have not been able to assay events which often correlate with memory, such as isotype switch and somatic mutation. Such assays are difficult in the IgM Tgic setting. Nonethe-

less, we have shown that RFs can satisfy the fundamental definition of memory: functional persistence of the secondary response. The observation of secondary AM14 responses in spite of the presence of autoAg is relevant to hypotheses that posit a window of particular sensitivity to self-Ag tolerance at the memory differentiation stage of Ag-driven B cell development (12, 14, 39–42). It has also recently been shown that ongoing germinal center reactions can be aborted by infusion of large amounts of Ag, a result which has been interpreted to demonstrate sensitivity to tolerization during memory B cell differentiation (12, 13, 43). The fact that we did not observe physiologic inhibition of secondary response development in the presence of autoAg may reflect the specific conditions of our assay. In our case modest (albeit physiologic) levels of autoAg were present constitutively while in passive administration of self-Ags large doses were injected at particular time points.
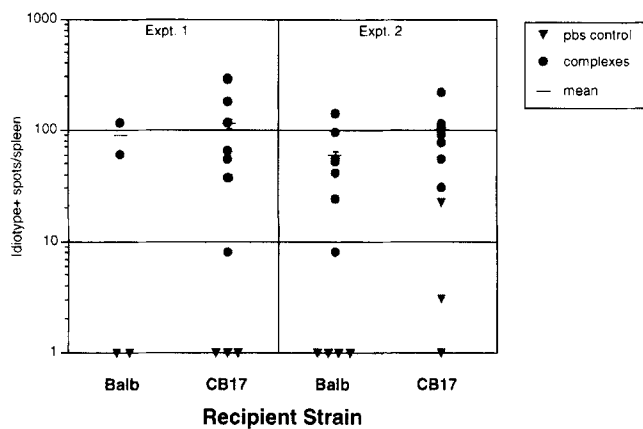
Figure 7. ELISAspot analysis of transfer immunization of splenocytes derived from mixed BM chimeras. Data are represented as in Fig. 5. Two independent experiments are shown. Each column shows either an IgH^a donor/recipient pair (BALB/c) or an IgH^b pair (CB.17). There is no significant difference between the two types in either experiment. PBS-immunized mice that showed no spots in any of triplicate wells are indicated as having one spot per spleen.

Of the autoreactive Ig Tgic systems that have been described, the AM14 system is novel in design and results. Systems that demonstrated anergy or deletion used facultative autoantigens which are not characteristic targets of spontaneous autoimmunity. Systems that used classical SLE targets, such as DNA or RBC, also seemed to show deletion or anergy, but in these cases, the interpretation was not straightforward, since the autoAg is inevitably constitutive. In our RF system we have introduced self-Ag as a novel Ag (in IgH^b controls) or as a self-Ag, and in this case observed neither anergy nor deletion.

Why is autoreactive RF not tolerized? One possibility is the affinity of the interaction between RF Ab and the IgG-Fc. The moderate affinity of AM14 for IgG2a^a is distinct from the Abs represented in the other models: Anti-HEL and anti-Class I Abs are high affinity Abs derived from hyperimmunized mice, and classic autoAb Tgics such as 3H9 (8, 32) were constructed with highly mutated V genes coding for Abs known to have undergone significant affinity maturation. Thus, although 3H9 appears to be deleted and/or edited (8, 32), the fate of the much lower affinity unmutated precursor (that bound only ssDNA) is as yet unknown. The role of affinity cannot be considered in isolation. AutoAg concentration and valency must also be considered. IgG2a concentrations in AM14 Tgics are much higher than soluble lysozyme concentrations in the HEL model and therefore would be expected to compensate to some degree for the lower affinity of AM14. In fact, the products of the estimated affinity and molar concentration of Ags for each of the two systems are similar (16). Perhaps AM14 is near an affinity threshold required for anergy, or the in vivo valency of lysozyme is greater than IgG2a, thereby mediating more cross-linking. In this regard, AM14 mice may be similar to anti-Class I mice that expressed small amounts of soluble Class I autoAg or low levels of soluble

HEL (5, 11). These mice did not show reduced serum levels of anti-Class I or anti-HEL, but whether the B cells were indeed anergic was not directly investigated in the case of anti-Class I.

Another characteristic that may distinguish the regulation of particular autoAbs is the nature of the target autoAg. In particular, the site and timing of expression and the conformation of a target Ag could affect whether B cells specific for it are tolerized. In the case of RF, the fact that the Ag (IgG2a) can be complexed with foreign proteins is relevant. This complex Ag would attract activated Th cells to RF B cells and these could break or prevent tolerance. In addition, B cells have FcRγII, which may be coligated when B cells bind immune complexes via sIg. Since coligation of FcRγII and sIg can inhibit activation during immunization (44), it is possible that a similar signal could inhibit tolerance. Presumably, these mechanisms may also explain why RF is a dominant autospecificity. In any case, an understanding of why certain Ags are preferred targets in various autoimmune disease will be critical to understanding the pathogenesis, and knowing that an RF B cell is not centrally tolerized is an important step in this direction. Experiments in progress examining RFs with higher affinity should distinguish whether affinity for or nature of the target Ag explains the lack of tolerance of the AM14 RF.

That self-specific AM14 B cells are immunocompetent shows that regulation of autoAbs in normal mice does not simply depend on central tolerance or peripheral anergy. Several studies have demonstrated that the normal preimmune repertoire does contain self-reactive B cells, including RFs and anti-DNA, and that they can be activated under certain conditions (e.g., LPS) (17, 18, 45, 46). Whether these cells are in fact the precursors of autoAb-secreting clones has not been established, but here we demonstrate that a clone which is known to be relevant can develop in the normal preimmune repertoire. It thereby suggests that at least some of these self-specific RF clones in the normal preimmune repertoire are likely to be the precursors to disease-related autoAbs. This scenario may apply to other autoAb specificities as well.

A corollary to this point is that in autoimmune mice, a failure of central tolerance need not occur to generate activated autoreactive B cell clones. This raises the issue of which regulatory mechanisms do prevent autoreactive B cell clonal expansion in normal mice. Recent work of Rathmell and colleagues (47) suggests that autoimmunity can result from the failure to eliminate anergic B cells through a Fas-based mechanism. Our results show that non-anergic B cells may also be precursors for autoAbs. Based on AM14 model, we hypothesize that systemic autoimmune disease is a consequence of defects either in T cell tolerance or at later stages of B cell regulation: for example, aberrant activation of and/or failure to appropriately downregulate an anti-self response. This fits well with emerging data on the expression of Fas in B cells that suggests a role for Fas in apoptosis of B cells once activated in

the periphery (48–50). It is also consistent with observations that the lpr defect does not grossly affect central deletion or anergy (51, 52).

A view of the selective origin of pathologic autoAb in disease that is consistent with all of these data is as follows: The precursors of cells that secrete pathologic autoAb would not be tolerized in either normal or autoimmune mice. Autoimmune disease could ensue if, once activated, these clones somatically mutate, are selected by autoAg, and ultimately yield high affinity pathogenic autoAbs. Autoimmune individuals may be particularly prone to activate such normally quiescent cells and/or may be impaired in the ability to downregulate them once activated.

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