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*J Immunol* 1998; 160:2263-2271;

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Maternal Ig Mediates Neonatal Tolerance in Rheumatoid Factor Transgenic Mice but Tolerance Breaks Down in Adult Mice

Haowei Wang and Mark J. Shlomchik

We have recently demonstrated that B cell deletion occurs in the bone marrow of IgH* high affinity anti-IgG2α (RF) transgenic mice. Here we demonstrate via genetic crosses that the source of IgG2α is the mother, thus establishing a transplacental mechanism that ensures tolerance to developmentally expressed Ags. Since maternal IgG can mediate tolerance in young mice, whether tolerance is maintained or, instead, autoimmunity ensues after weaning was investigated. We find that deletion remits abruptly in these RF transgenic mice beginning at 2 to 3 wk postweaning, and some degree of autoreactivity can be observed thereafter for weeks to months. The mechanism of sustained expression of autoreactive RF B cells in normal mice is unclear as yet, but a plausible mechanism is that once self-reactive cells are present, the antibody they secrete markedly reduces the autoantigen levels, presumably allowing further development, rather than deletion, of newly arising B lineage cells. The phenotype of these RF transgenic mice suggests a positive feedback mechanism that tends to perpetuate autoimmunity once it has been established. If such a mechanism were to exist in autoimmune animals, it could have important implications for the establishment and maintenance of B and T cell tolerance in chronic autoimmune diseases.

Rheumatoid factors (RFs) are autoantibodies that recognize IgG (1). They are prominent in rheumatoid arthritis and several other autoimmune diseases (2, 3), where they are often isotype switched and somatically mutated (4–6). Disease-associated RFs may also be pathogenic (7–11). In addition, a significant, but transient, IgM-RF response frequently accompanies secondary immune responses in normal animals, although normal individuals generally have low constitutive RF levels (12–14). These latter RFs are not pathogenic and may even be protective (15). Thus, RFs are found constitutively in autoimmune disease and can be elicited in healthy individuals. This partial tolerance phenotype (16) along with the potential for both pathogenicity and protection make understanding the regulation of RFs particularly interesting. For this reason we have been studying tolerance and activation of RFs in transgenic mouse models. We have recently shown that in normal mice, a moderate to low affinity RF is not tolerized, whereas a high affinity RF is tolerized in the bone marrow (BM) (17–19).

Our results from these transgenic mice raised the question of the origin of the deleting Ag. IgG, the presumed tolerogen, can exist in soluble form in plasma, milk, and secretions and can be passed through the placenta and to neonates through the milk. It also can exist in membrane form on IgG-expressing B cells and as IgG bound to Fc receptors (FcR). Since we previously observed deletion of RF B cells in weanling mice, we speculated that maternally derived IgG could mediate deletion. To test this idea, in the present report we have exploited the allotype specificity of our RF transgenic system. Crosses in which the mother can or cannot donate tolerogenic IgG2α were used to provide genetic evidence that maternal Ab mediates deletion in young mice. This conclusion, based on genetics, was corroborated by direct infusion experiments.

Since maternal IgG is a relevant tolerogen, this, in turn, raised the issue of whether autoimmunity would ensue once maternal IgG donation was ended by weaning. Therefore, we next studied the maintenance of tolerance with age and found that deletion in our transgenic mice is not stable; it remits several weeks after mice are weaned and in some mice is re-established later in life. Remarkably, the reversal of deletion occurs over a very brief time period. After induction, an “autoimmune” state in which the autoantibody is expressed at detectable levels can persist for months. Further evidence is presented suggesting that deletion is an interplay among Ag levels, B cell numbers, and autoantibody levels, all of which may modulate each other. The fluctuating nature of deletion and autoantibody expression in these mice may be an important model for the abrupt onset of expression of autoantibodies that occur in spontaneous autoimmunity (20, 21). The fact that the autoimmune state tends to be self-perpetuating has important implications for understanding the pathophysiology of chronic autoimmune disease, including whether apparent central tolerance defects are primary ones or occur secondarily to the clearance of tolerizing autoantigen by previously secreted autoantibodies.

Materials and Methods

Mice

20.8.3 transgenic mice were constructed and bred as previously described (17). For standard propagation, mice were backcrossed to either BALB/c (Ig heavy chain allotype a (IgHα)) or CB.17 (IgHβ) with the male in each mating carrying the transgenes. Various additional matings were set up as described in Table I. Mice were typed by PCR for transgenes as described and for allotype by either PCR or Southern blot as described below.

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Received for publication June 23, 1997. Accepted for publication November 18, 1997.

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1 This work was supported by the National Institutes of Health (P01 AI/AR 36529), the Arthritis Foundation, a Hulda Irene Duggan Arthritis Investigatorship (to M.S.), and a Richard Gershon Postdoctoral Fellowship (to H.W.).

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3 Abbreviations used in this paper: RF, rheumatoid factor; BM, bone marrow; FcR, Fc receptors; IgH, immunoglobulin heavy chain allotype a; Sm, Smith autoantigen.

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0022-1767/98/$02.00
IgG2a a source for infusion into 20.8.3 transgenic mice, with B6/\textit{lpr}/IgH a mouse serum was selected as a passive soluble C57Bl/6(B6)/IgH a and B6/\textit{scid}/IgH b mice were used in lieu of serum at the concentrations indicated in Materials and Methods.

PCR to distinguish IgH a and IgH b allotypes

A single pair of oligonucleotides was designed based on the BALB/c \(\gamma_{2a}\) sequence: \(5\text{-}\text{TGTCCTTGTACATTCCGAG-3}\) and \(5\text{-}\text{TCTGCTTACGACTTAC-3}\). These oligos amplify a 179-bp fragment for the IgH a allotype and a 201-bp fragment for the IgH b allotype due to an insertion sequence polymorphism. These fragments were resolved on a 3% NuSieve agarose gel (FMC, Rockland, ME). Reaction conditions were as described with a 1.5 mM final MgCl 2 concentration. Cycling conditions were 95°C for 5 min (one cycle); 95°C for 30 s, 48°C for 30 s, and 72°C for 45 s (10 cycles); 95°C for 30 s, 48°C for 30 s, and 72°C for 30 s (25 cycles); and 72°C for 5 min (one cycle). Cycling was performed on an Omnigene Thermal Cycler (Labnet, Woodbridge, NJ) using tube control.

FACS

FACS on spleen, blood, and bone marrow cells was performed as previously described (17, 18).

ELISA

Serum Ab level ELISA assays were determined as previously described (17, 18).

Serum infusion

C57Bl/6(B6)/\textit{lip/IgH a} mouse serum was selected as a passive soluble IgG2a a source for infusion into 20.8.3 transgenic mice, with B6/\textit{lip/IgH b} serum serving as a negative control. B6/\textit{lip/IgH a} and B6/\textit{lip/IgH b} mice 3 mo or older, were bled about once per week, and sera from multiple bleeds were pooled. The IgG2b concentration in serum was measured by ELISA using a monoclonal IgG2b as a standard. An amount of pooled serum containing 1 mg of IgG2b was used as one infusion dose in a 200-µl volume diluted in PBS. PBS alone was used as an infusion control. In each set of experiments, mice received IgG2a-derived serum, IgG2b-derived serum, or PBS. IgG2b measurement was used to standardize the doses, as we did not have an assay available that would measure IgG2a a and IgG2a b equivalently. Mice were infused every other day, and PBL FACS and serum sampling for IgG2a and RF assays were performed once after each three-dose infusion. Infusions were continued for 3 or 4 wk, then the mice were killed and checked by splenocyte FACS. In one experiment either IgG2a a (23.3) or IgG2a a (15G5) protein G-purified mAbs obtained from ascites raised in \textit{scid} mice were used in lieu of serum at the concentrations indicated in Results and figure legends.

Statistics

Differences between various groups were determined using the Mann-Whitney unpaired \(U\) test as computed by StatView 4.5 for Macintosh (Abacus Concepts, Berkeley, CA). A value of \(p < 0.05\) (two-tailed test) was considered significant.

Results

Genetic evidence of the maternal origin of deletion-mediating IgG

Previously we demonstrated that 3- to 5-wk-old IgH a 20.8.3 transgenic mice born to BALB/c (IgH a) mothers manifested deletion of RF B cells (19). (Our analysis of deletion in the BM of 20.8.3 mice showed that it actually had features of receptor editing and developmental arrest; for simplicity, we will refer to this process herein as deletion.) We speculated that deletion was mediated by maternally derived Ig; alternatively, endogenous IgG2a a or circulating IgG2a a-expressing B cells in the young transgenic progeny could have provided sufficient Ag to mediate tolerance. Therefore, to establish the origin(s) of deletion-mediating Ag, we set up crosses to generate progeny of IgH a/b mothers that were endogenously IgH a (b, baby; a, mother) or of IgH b mothers that were endogenously IgH b (a, baby; b, mother; see Table I).

The deletion phenotype in these mice entirely segregates with the IgH maternal allotype. This is depicted in three ways, all of which provide the same general conclusion. In the left panel of Figure 1, the percentage of splenocytes that retain the RF specificity is shown, which demonstrates a marked reduction in mice with IgH a mothers compared with IgH b. Since this analysis could be confounded if IgG2a a Ag caused IgM receptor modulation or competed with the detecting reagent, in the center panel we show B220+/k c cells (which are always >90% of total B220 + cells). Again, the same picture is seen. Finally in the right panel, the fraction of B220 + cells that have the RF specificity is shown. This is a good indicator of deletion, since B cells that do not longer bind IgG2a a because they have edited their receptors or coexpress and endogenous Ig gene(s) are preserved, while B cells that continue to express the RF specificity are deleted. By any of these analyses, IgH a babies born to IgH b mothers deleted RF B cells to the same extent as positive control mice entirely of IgH a origin. As these progeny have no endogenous capacity to produce IgG2a a, deletion in these mice is entirely attributable to maternally derived IgG2a a. Conversely, IgH a/b babies born to IgH b mothers did not delete and had percentages (and numbers, not shown) of RF B cells similar to those in control mice that were entirely of IgH b origin. Thus, the endogenous capacity to express IgG2a a is not required for deletion in these young mice and is insufficient to mediate it. Similar data were obtained for a second founder line (not shown). From these results we conclude that maternal IgG can mediate deletion.

Infusion experiments directly demonstrate the role of serum Ab in deletion

The results of the above genetic crosses predicted that soluble IgG2a a should mediate deletion when injected into IgH b recipients. This prediction stems from the fact that the mothers that did (IgH a) and did not (IgH b) have progeny with deletion differed only at the IgH locus. Since the 20.8.3 RF that provided the V genes for the transgenic is specific for IgG2a a, and since the IgH locus represents the only known difference between BALB/c and CB.17, IgG2a a is almost certainly the active factor being transferred only by IgH a mothers. Further, since soluble IgG is known to be transferred across the placenta and in milk, soluble IgG2a a is most likely the form of Ag mediating deletion in the crosses. To formally establish this point, we injected serum or IgG2a a mAbs derived from IgH a or IgH b mice into young IgH a transgenics.

Table I. Mating strategies to generate transgenic progeny with and without maternal IgG2aa a donation and with and without endogenous capacity to produce IgG2aa a

<table>
<thead>
<tr>
<th>Designation</th>
<th>Allotype a of Mouse</th>
<th>Allotype and Transgenotype a of Mother</th>
<th>Allotype and Transgenotype b of Father</th>
<th>Maternal IgG2aa a</th>
<th>Endogenous Capacity to Produce IgG2aa a</th>
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</thead>
<tbody>
<tr>
<td>a mother/a baby</td>
<td>a/a</td>
<td>a/a</td>
<td>a/a</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>a mother/b baby</td>
<td>b/b</td>
<td>a/b</td>
<td>b/b</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>b mother/a baby</td>
<td>a/b</td>
<td>b/h</td>
<td>a/b</td>
<td>No</td>
<td>Yes</td>
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<tr>
<td>b mother/b baby</td>
<td>b/b</td>
<td>b/h</td>
<td>b/b</td>
<td>No</td>
<td>No</td>
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</table>

* Allotype is given as “a” or “b” for each allele, separated by a “/”.
  * Transgenotype is indicated by a “+” or “−” after the allotype designation.
  * Other progeny were also generated in this mating but were excluded by PCR typing for allotype as described in Materials and Methods.
In the first sets of experiments, serum isolated from either IgH\textsubscript{a} (B6/lpr\textsubscript{a}/IgH\textsubscript{a}) or IgH\textsubscript{b} (B6/lpr\textsubscript{b}) donors was infused into IgH\textsubscript{b} transgenics in an attempt to cause deletion. The donor mice were chosen because the hypergammaglobulinemia characteristic of these strains provided an enriched source of IgG\textsubscript{2a} and so that this preparation might mimic the situation of emerging RF B cells in an autoimmune animal, which would have circulating immune complexes. In addition, these congenic mice differ only in their IgH allotype; thus, any differences in effects would be attributable to IgH gene products and not to other factors in crude serum preparations. As shown in Figure 2\textit{A}, this protocol induced complete disappearance from the blood of detectable RF-expressing lymphocytes within 1 wk (the first time point examined). Deletion in PBL was essentially maintained for the 3-wk protocol ($p < 0.01$ comparing IgH\textsubscript{a}-infused mice to PBS- and IgH\textsubscript{b}-infused mice for wk 1, 2, and 4; $p = 0.07$ for wk 3) and for an additional week thereafter and was observed in spleen at the termination of the experiment (Fig. 2\textit{B}). The average serum IgG\textsubscript{2a} level in recipients at this time was 84 mg/ml. We consider this to be deletion, as demonstrated previously in intact IgH\textsubscript{a}-expressing mice, since the percentages of total B220\textsubscript{1} cells in PBL were also significantly reduced in these serum-infused mice ($p < 0.01$ comparing IgH\textsubscript{a}-infused mice to combined other groups at wk 1–3). A similar result for B220\textsubscript{1} cells was seen in spleen at the 4 wk point.

Although serum from B6/lpr mice was a convenient source of large amounts of IgG\textsubscript{2a} required for the above experiments, one drawback of this approach is that this serum preparation could contain immune complexes that could efficiently delete RF B cells. Thus, these data do not necessarily bear on the effects of normal IgG, as might occur in a nonautoimmune animal. To address this issue, we injected mAbs of either the IgG\textsubscript{2a}\textsubscript{a} or IgG\textsubscript{2a}\textsubscript{b} type into IgH\textsubscript{b} transgenics; these mAbs highly purified from tissue culture would not contain immune complexes. Further, these mAbs both have the same irrelevant specificity (anti-nitrophenyl hapten), the Ag for which would not be expressed in the recipients. As with serum injections, we observed deletion at both doses of IgG\textsubscript{2a} tested, but not with IgG\textsubscript{2a} (Fig. 2\textit{B}).

Multiple independent mice show re-expression after weaning

Since maternally derived IgG\textsubscript{2a} can mediate B cell deletion in 20.8.3 transgenic mice, this raised the question of whether tolerance would continue or autoimmunity would ensue postweaning. We therefore determined the extent of deletion in spleen and PBL as mice aged. Figure 3 shows the analysis of splenocytes from two independent founder lines. Beginning about 5 to 6 wk of age and progressing through 7 to 8 wk of age, the percentages of transgenic B cells in spleen and PBL were markedly increased, in contrast to the decreases observed in serum-infused mice.
with those in 3- to 4-wk-old mice (Fig. 3A; \( p < 0.01 \) comparing 3- to 4-wk-old mice to 6-, 7-, and 8-wk-old mice; Fig. 3B; \( p < 0.05 \) comparing 3- to 4-wk-old mice to every other week). This pattern is identical in both founder lines. At later ages in at least some of the mice, expression of RF B cells continued, while in other mice, deletion was re-established. In the Warren line (Fig. 3B), continued expression with age was somewhat more prevalent than that in the Wendy line (Fig. 3A).

Residual B cells in older mice do not express the RF specificity

It was possible that in older mice the apparent lack of RF B cells was due to a blockage of the RF receptor by IgG2a rather than to true disappearance. This issue is further raised by the fact that it was due to a blockage of the RF receptor by IgG2a rather than to receptor down-modulation.

It was possible that in older mice the apparent lack of RF B cells appears modulated in either nondeleting (A, B, Warren line) or fully deleting (C, Wendy line). Among IgH\(^{a}\) mice, wk 6, 7, and 8 are significantly different from wk 3 to 4 (\( p < 0.01 \)); all other weeks are not. IgH\(^{a}\) mice have significantly fewer RF B cells than IgH\(^{b}\) mice (\( p < 0.05 \)) at all weeks except 6, 7, 9 to 15, and 16 to 20. Warren line. All age groups of IgH\(^{a}\) mice differ from wk 3 to 4 (\( p < 0.05 \)). Differences between IgH\(^{a}\) and IgH\(^{b}\) mice were significant (\( p < 0.05 \)) at all weeks:

- \( p < 0.05 \) comparing 3- to 4-wk-old mice to every other week.
- \( p < 0.05 \) comparing 3- to 4-wk-old mice to every other week.
- \( p < 0.05 \) comparing 3- to 4-wk-old mice to every other week.

FIGURE 3. Deletion remits and can be re-established as IgH\(^{a}\) transgenic mice age. Splenocytes or PBL from a large cohort of IgH\(^{a}\) transgenic mice and IgH\(^{b}\) controls were analyzed by FACS, and the deletion index was determined for each sample. Data from spleen and PBL are pooled because the separate data each had the same pattern. Each dot represents an individual IgH\(^{a}\) mouse; for clarity, only the average of the IgH\(^{a}\) controls (□) at each age group is shown with SD bars. Columns are means of the IgH\(^{a}\) mice. A, Wendy line. Among IgH\(^{a}\) mice, wk 6, 7, and 8 are significantly different from wk 3 to 4 (\( p < 0.01 \)); all other weeks are not. IgH\(^{a}\) mice have significantly fewer RF B cells than IgH\(^{b}\) mice (\( p < 0.05 \)) at all weeks except 6, 7, 9 to 15, and 16 to 20. Warren line. All age groups of IgH\(^{a}\) mice differ from wk 3 to 4 (\( p < 0.05 \)). Differences between IgH\(^{a}\) and IgH\(^{b}\) mice were significant (\( p < 0.05 \)), partly deleting (D, Wendy line). Among IgH\(^{a}\) mice, wk 6, 7, and 8 are significantly different from wk 3 to 4 (\( p < 0.01 \)); all other weeks are not. IgH\(^{a}\) mice have significantly fewer RF B cells than IgH\(^{b}\) mice (\( p < 0.05 \)) at all weeks except 6, 7, 9 to 15, and 16 to 20. Warren line. All age groups of IgH\(^{a}\) mice differ from wk 3 to 4 (\( p < 0.05 \)). Differences between IgH\(^{a}\) and IgH\(^{b}\) mice were significant (\( p < 0.05 \)).

Residual B cells in deleting mice cannot bind IgG2a. Splenocytes from an IgH\(^{a}\) transgenic mouse (A and D) or from two 10-wk-old IgH\(^{a}\) transgenic mice were stained as follows. Upper row. Cells were incubated with 50 \( \mu \)g/ml IgG2a\(^{a}\) mAb (dark line) or were not treated (light line). After washing, cells were stained with a fluoresceinated anti-IgG2a antiserum (Southern Biotech) and with biotin-anti-IgM\(^{a}\), which was subsequently developed with streptavidin-phycoerythrin. Histograms show the anti-IgG2a fluorescence of IgM\(^{+}\) gated cells (x-axis). Note that IgH\(^{a}\) RF cells (A) bind IgG2a\(^{a}\) (dark line) and show increased fluorescence compared with unexposed cells (light line), as expected. The positive gate is set for 1% background of cells not exposed to IgG2a\(^{a}\), which determines that 69% of IgM\(^{+}\) cells are positive. In contrast, in B, only about 33% of IgM\(^{+}\) cells bind IgG2a, and in C, practically none do. This demonstrates that these IgM\(^{+}\) cells are not RF B cells, since they do not bind saturating amounts of IgG2a\(^{a}\). Bottom row. The same cells were stained directly with biotinylated IgG2a (revealed with streptavidin-phycoerythrin; x-axis) and fluoresceinated anti-IgM\(^{a}\) (y-axis). Percentages shown near each region are of IgM\(^{+}\)-positive cells, which facilitates comparison to the upper row. Note the concordance between the presence of RF B cells as defined by direct binding of biotin-IgG2a and the binding of excess IgG2a revealed with anti-IgG2a. In B and E, only some of the cells are positive in each assay; in C and F, practically none are positive in each assay. All staining was conducted in the presence of excess 24G.2 (rat anti-mouse FcR) to block nonspecific binding.

FIGURE 4. Residual B cells in deleting mice cannot bind IgG2a. Splenocytes from an IgH\(^{a}\) transgenic mouse (A and D) or from two 10-wk-old IgH\(^{a}\) transgenic mice were stained as follows. Upper row. Cells were incubated with 50 \( \mu \)g/ml IgG2a\(^{a}\) mAb (dark line) or were not treated (light line). After washing, cells were stained with a fluoresceinated anti-IgG2a antiserum (Southern Biotech) and with biotin-anti-IgM\(^{a}\), which was subsequently developed with streptavidin-phycoerythrin. Histograms show the anti-IgG2a fluorescence of IgM\(^{+}\) gated cells (x-axis). Note that IgH\(^{a}\) RF cells (A) bind IgG2a\(^{a}\) (dark line) and show increased fluorescence compared with unexposed cells (light line), as expected. The positive gate is set for 1% background of cells not exposed to IgG2a\(^{a}\), which determines that 69% of IgM\(^{+}\) cells are positive. In contrast, in B, only about 33% of IgM\(^{+}\) cells bind IgG2a, and in C, practically none do. This demonstrates that these IgM\(^{+}\) cells are not RF B cells, since they do not bind saturating amounts of IgG2a\(^{a}\). Bottom row. The same cells were stained directly with biotinylated IgG2a (revealed with streptavidin-phycoerythrin; x-axis) and fluoresceinated anti-IgM\(^{a}\) (y-axis). Percentages shown near each region are of IgM\(^{+}\)-positive cells, which facilitates comparison to the upper row. Note the concordance between the presence of RF B cells as defined by direct binding of biotin-IgG2a and the binding of excess IgG2a revealed with anti-IgG2a. In B and E, only some of the cells are positive in each assay; in C and F, practically none are positive in each assay. All staining was conducted in the presence of excess 24G.2 (rat anti-mouse FcR) to block nonspecific binding.

either incubated, or not, with saturating (50 \( \mu \)g/ml) concentrations of IgG2a\(^{a}\), then washed and stained with anti-IgG2a antiserum (FITC) and anti-IgM\(^{a}\) (biotin). In this scheme, additional circulating IgG2a should enhance, rather than block, detection. The IgM\(^{a}\)-expressing IgH\(^{a}\) splenocytes that were not incubated with IgG2a\(^{a}\) were considered negative or background; after incubation with IgG2a\(^{a}\), however, these were revealed with anti-IgG2a, demonstrating our ability to detect RF B cells by this staining technique (Fig. 4A). A similar percentage of cells was detected by our standard technique, using anti-IgM\(^{a}\) and biotinylated IgG2a\(^{a}\) (Fig. 4D). In two different IgH\(^{a}\) mice, different levels of deletion were seen by this technique. In one animal, some of the B cells (32.6%) were detected by this technique, but others failed to stain any more than the IgH\(^{a}\)-negative control. The percentage of B cells detected matches closely the percentage detected in our standard staining technique (45.4%; Fig. 4E). A similar picture was seen for the second animal, in which nearly all cells did not display RF activity, as they were not stained even after incubation with saturating IgG2a\(^{a}\) (only 1.5% positive; Fig. 4C). Again, the standard technique detected a similar percentage of cells (1%; Fig. 4F). Note also in Figure 4, D through F, that surface IgM levels did not appear modulated in either nondeleting (D), partly deleting (E), or fully deleting (F) mice. From these analyses we confirm the true absence of RF B cells in late deleting mice.

Serial bleeds demonstrate the rapid onset of expression

The fact that at any age some of the mice failed to express RF B cells could mean that some mice always remain tolerant or could
reflect the fact that these particular mice were assayed before the point at which they would have expressed or after they had again down-regulated expression. To distinguish these possibilities and to better define the time course of the switch between deletion and expression in individual mice, we performed weekly analysis of PBL by FACS on a cohort of 11 IgHa transgenic mice (Wendy line) starting at 4 wk of age. Three patterns of expression emerged (Fig. 5). Five of the 11 mice showed transient and low level expression of B cells between 5 and 6 wk of age, returning to a deletion phenotype by 7 wk. Three mice, however, expressed large percentages (nearly as much as IgHb controls) of RF B cells, and this expression persisted up to 25 wk of age. A third pattern, seen in two mice, was high level expression, followed by an eventual return to deletion by 25 wk. These results are remarkable for several reasons. First, about half the mice never expressed significant frequencies of RF B cells, supporting the idea of heterogeneity among mice as opposed to sampling time variation in explaining the data presented in Figure 3. Second, the transition from complete deletion to peak expression occurred in all mice over the space of a single week. Finally, half the mice manifested persistent expression, including three that expressed from wk 6 through 25, indicating that at least for some mice, expression of RF autoantibody is self-perpetuating. This corroborates the phenotypes shown in Figure 3. As will be discussed in detail, these patterns of expression are predicted by a positive feedback model with two metastable and self-reinforcing states: deletion and expression.

**Artificial maintenance of passive IgG2a postweaning prevents expression**

The most likely explanation for the late onset of expression of RF B cells is the natural clearance of maternally derived serum IgG2a after weaning. If this were the case, one would predict that artificial maintenance of serum IgG2a levels postweaning would abrogate the expression of RF B cells. To determine whether this was, in fact, the case, we again turned to infusion of passive IgG, although in this case with the aim of maintaining deletion in IgHb mice rather than inducing deletion in IgHb mice. At the start of the protocol, all 3-wk-old IgHb recipient mice had few circulating RF B cells, as expected (Fig. 6, day 0). However, within 2 to 3 wk, mice receiving either PBS or IgHb-type serum had low, but above background, levels of circulating RF B cells, the expression of which became more prominent at 3 and 4 wk into the experiment, also as expected. In contrast, mice that received IgHa-derived serum never had significant numbers of detectable RF B cells for the duration of the experiment ($p < 0.05$ comparing IgHb serum-infused group to PBS- and IgHb-infused groups at wk 2, 3, and 4). There were also significant differences in the percentages of total B220+ cells ($p < 0.05$) at all weeks except wk 2 (not shown).

**Relationship between IgG2a levels and RF expression in individual mice of different ages**

The results presented thus far demonstrate an interplay among autoantigen, RF B cells, and possibly soluble IgM-RF secreted by RF B cells. To better understand these interactions, we simultaneously examined the serum IgG2a and RF levels in mice of various ages and correlated these with the frequency of RF B cells in spleen. These data are shown in Figure 7. As expected, in young (3- to 5-wk-old) mice, relatively high serum IgG2a levels were generally associated with low levels of RF B cells as well as little RF expression. In particular, very low RF B cell frequencies were associated with IgG2a concentrations of 55 μg/ml or more. This suggests a rough estimate of the concentration required to cause deletion in vivo.

In the 6- to 8-wk-old cohort (Fig. 7, second row), the picture was quite different. Whereas, at 3 to 5 wk nearly all mice had IgG2a levels >10 μg/ml and $<3\%$ (average, 0.9%) of splenocytes with RF specificity, at 6 to 8 wk most mice had undetectable (<0.1 μg/ml) IgG2a, with 59 of 61 mice having $<10$ μg/ml. Among the mice in the 6- to 8-wk-old cohort with undetectable IgG2a, many had a substantial percentage of splenocytes with RF specificity (average, 6%; ranging up to 25%). Conversely, 6- to 8-wk-old mice that have even a low but detectable level (1–10 μg/ml) of IgG2a did not have RF B cells (average, 0.4% of splenocytes). Thus, at the age when tolerance is switching to expression, two
different phenotypes emerge: mice with very low IgG2a, many of which already express significant numbers of RF B cells, and mice that still have high IgG2a (albeit at lower levels than 3- to 5-wk-old mice) and have very few RF B cells. This phenotypic divergence is even more accentuated in older mice (Fig. 7, bottom row, and see below). This reciprocal relationship of IgG2a and RF B cells is as predicted by a positive feedback switch. Similarly, a positive feedback switch predicts our observation that there are few if any mice with intermediate values for IgG2a and frequency of RF B cells.

A last issue in the dynamic process of RF deletion and expression is the cause of late deletion (see Figs. 3A and 5 at time points beyond 8 wk). Late deletion does not always occur (it is less prominent in the Warren than in the Wendy line; Fig. 3), and its timing is variable (Figs. 3 and 5). We hypothesized that late deletion reflects activation of endogenous IgG2a production, which, in turn, could rely on environmental exposure. To examine this possibility, we determined IgG2a levels in older mice and, in fact, found very high levels in many older deleting mice (Fig. 6, bottom panels). In addition, several IgHβ transgenics that were born to IgHαβ mothers, which uniformly experience early deletion (Fig. 1), were examined at later time points. As expected from their inability to produce endogenous IgG2aβ, late deletion in these mice was not observed (not shown). These data are consistent with the idea that late deletion results from re-emergence of serum IgG2aβ.

Discussion

The results presented here have implications for three related areas: the role of maternal Ags in causing neonatal tolerance, the role of soluble Ag in mediating B cell clonal deletion, and a hypothetical mechanism by which B cells can mediate interclonal positive feedback, thereby propagating and exacerbating autoimmunity.

Using a series of reciprocal genetic crosses, we have established that maternally derived IgG plays a critical role in neonatal tolerance of high affinity RF B cells. Mice without maternal IgG2aβ but with the endogenous capacity to express IgG2aβ still do not delete, whereas in the converse situation, mice with maternal IgG2aβ but without the endogenous capacity to express do, in fact, delete. These results provide evidence for the role of maternal Ag in censoring the neonatal B cell repertoire of cells that would react with a developmentally expressed Ag.
Examples of this phenomenon as it relates to B cells and Ags other than IgG are rare in the literature. This could mean that RF is a special case. However, in principle one would expect maternal donation of other developmentally expressed Ags through either the placenta or milk to cause B cell tolerance. Therefore, we believe that the lack of similar data in the literature may reflect a lack of suitable systems for detecting and distinguishing B cell tolerance.

T cells, on the other hand, are known to be susceptible to transplacental Ag-mediated tolerance, as has been elegantly shown in an HBVe-Ag transgenic model (22–24) as well as for mouse mammary tumor virus (25–27). Similarly, Geiger et al. demonstrated that tissue-specific expression of transgenic SV40 T Ag led to tolerance if this expression began early in ontogeny but led to autoimmunity if it began later (28–30). If early expression of some Ags is indeed important to establish tolerance, then maternal transfer of adult developmental Ags, such as IgG, may be one means to prevent autoimmunity of both T and B cells. Further work on this and similar systems would be needed to address the general relevance of this mechanism.

Initial establishment of tolerance via maternally derived IgG may be an important factor in the prevention of RF production in nontransgenic mice and in humans. In this regard, literature from the 1960s regarding serum normal agglutinins is reminiscent of the situation in 20.8.3 transgenic mice (31, 32). These workers found that neonatal humans did not make RF-like Abs to maternal allootypes at 6 mo of age or younger, but often did so at older ages. They speculated that this related to initial self-tolerance, followed by an immunization by the maternal IgG as the neonate aged. Our 20.8.3 data are consistent with this phenomenon. In contrast to 20.8.3, in the case of humans, the roles of B cell vs T cell tolerance could not be discerned.

One implication of the role of the maternal IgH allotype in causing deletion is that soluble IgG is mediates deletion. The alternative, that maternal surface IgG2a-positive B cells are being passed to the fetus, seems unlikely. In this regard, two other groups have investigated the negative regulatory effects of IgG. Weigle and colleagues, in classic tolerance experiments, injected high doses of soluble human deaggregated IgG and found both B and T cell tolerance (33). These results differ from ours in that the Ag was actually foreign, deaggregated IgG had a unique effect (whereas our autoimmune serum with immune complexes also did), and the mechanism of tolerance was not shown. More recently, Tighe et al. have demonstrated deletion after injection of soluble, deaggregated human IgG into mice that were expressing a transgenic, high affinity human RF. In this case, the deletion resembled activation-induced cell death (34). The idea that soluble Ag mediates deletion is further supported by our experiments in which soluble injected IgG either caused deletion in mice that had been expressing or maintained deletion in weanlings. It has been suggested that soluble Ags or Ags with low valency would cause anergy, whereas membrane-bound or highly multivalent Ags would cause deletion (35–38). The dominant role of valency has been emphasized, since even very low affinity reactions have caused deletion when the Ag is membrane bound, whereas the interaction of soluble lysozyme with a transgenic B cell Ag receptor causes anergy even though it is of high affinity. Up to now, the only precedent for a soluble endogenous Ag causing deletion is DNA (39, 40). Soluble DNA, however, may be more akin to a membrane-bound Ag, since it is highly polymeric. One problem in interpreting our finding is that IgG can bind to FcRs and could mediate deletion in this form as a membrane-bound Ag. Experiments are in progress to distinguish the direct role of soluble IgG in mediating deletion from indirect effects. In any case, we doubt that the effects are exclusively due to either immune complexes or the V region specificity, since a purified mAb specific for an irrelevant hapten readily caused deletion.

In our view, the most striking feature of the 20.8.3 transgenics is the dynamic nature of the B cell tolerance. After an initial phase of deletion that is caused by maternal IgG, a second phase ensues during which deletion fails or is incomplete. The onset of this second phase is due to the decay of maternal IgG after weaning, and it can be delayed by providing passive IgG2a at this point (Fig. 6). This second phase is then perpetuated, for weeks to months, by the subsequent suppression of serum IgG2a by the autoreactive RF. The low levels of serum IgG2a and high levels of serum RF during this time (Fig. 7) are also consistent with this interpretation. Whether B cells are specifically activated during this interval or whether serum levels reflect tonic secretion of IgM by naïve B cells remains to be determined.

The appearance of self-specific RF B cells at 6 to 7 wk of age is quite sudden, taking less than a week to reach its peak (Fig. 5). Since in the absence of RF, the half-life of IgG2a is about 3 wk, the abrupt onset suggests a steep threshold effect for tolerance and/or a positive feedback loop that accelerates the process. Positive feedback, at least, seems likely, since we have recently shown that RF, when present, does contribute to decreased serum IgG2a levels in vivo (19). Thus, one could envision that as the serum IgG2a level falls to a threshold level of around 55 μg/ml, a fraction of B cells that would have been tolerized at higher IgG2a levels escapes and contributes to serum RF. This RF, in turn, accelerates the disappearance of serum IgG2a, leading to an increased rate of tolerance escape and completing the positive feedback loop. The kinetics of RF expression are consistent with this. Serum IgG2a levels are on the order of 100 μg/ml at weaning age. Assuming a normal 3-wk half-life of IgG2a (since RF is not present at weaning), one would predict the initiation of RF expression, when serum levels drop below 55 μg/ml, to be at about 6 wk of age. The observed onset is, in fact, 6 to 7 wk of age.

The sudden appearance of particular autoantibodies is also a characteristic feature of both murine and human lupus (20, 21, 41, 42). Why this occurs is unknown. However, it is interesting to speculate that a positive feedback mechanism similar to that demonstrated here is operating, particularly in the chronic phase of the disease. Such a positive feedback mechanism would also account for why, once tolerance is broken for a particular Ag, it may be hard to re-establish. This mechanism may, in fact, apply more to autoantigens present in low concentrations, such as DNA and chromatin or ribonucleoproteins, than to a high concentration Ag such as RF. However, it may apply to RF as well, since the tolerance threshold for disease-related RFs, such as the RF transgenic AM14 (18), must be quite high (e.g., >100 μg/ml); thus, even reductions in effective concentrations of highly expressed Ags could result in an alteration in the population of B cells that escape tolerance in the BM.

Autoantibodies are known to evolve both specificity and affinity; the pattern of autoantibody specificities progressively spreads within a particle (43, 44), and the affinities of these tend to increase (45, 46). Both of these could be affected by a putative positive feedback circuit. Regarding the spreading of autoantibody specificities, there could be several explanations for why loss of tolerance to one epitope on a particle subsequently leads to loss at other epitopes. It could in part be due to the enhanced immunogenicity of the particle or to the loss of T cell tolerance, leading to the activation of nontolerant B cells. However, in view of our results, we propose that autoantibodies themselves may mediate cross-talk between B cell clones by clearing or blocking autoantigens, thus preventing them from mediating tolerance of newly emerging B
cell clones in the bone marrow. This would provide an additional mechanism for how, once autoantibodies to one particular epitope on an autoantigen appear, they are often accompanied or followed by autoantibodies to multiple epitopes.

Affinity maturation, the second major feature of autoimmune progression, could also be accounted for in two ways, each of which could be influenced by positive feedback. First, it is clear that somatic hypermutation and Ag-driven selection operate on established, activated B cell clones to yield higher affinity mutants (4, 45–48). While selection on mutants is manifest, it is puzzling to explain how competition-driven Ag selection would operate for self-Ags that are at high concentration, such as IgG. Feedback by pre-existing autoantibodies could drive competition, however, by causing clearance or epitope blocking of high concentration Ags.

A second possible mechanism for affinity maturation is the effect of autoantigen clearance on B cell deletion, as indicated by our results. In particular, the prolonged presence of autoreactive high affinity RF B cells that occurs for several weeks after the initial period of deletion is accompanied by relatively high autoantibody levels as well as low autoantigen levels. During this interval, newly emerging B cells from the BM are not tolerized (not shown) but instead develop, thus maintaining the peripheral pool for a period of weeks or more (Fig. 3). By analogy, we propose that in lupus, initial autoantibodies will cause clearance or blockage of autoantigen. Concurrently, more avid B cells that would have been deleted at higher (i.e., normal) autoantigen levels will instead mature and will then become activated in the periphery. This predicts that as autoimmunity progresses, even unmutated autoantibodies will begin to appear that have high affinity and lupus-specific characteristics, such as the ability to bind dsDNA. In fact, such Abs have been found in both human and murine lupus (46, 49–54) and have otherwise been difficult to fit into a model that requires affinity maturation.

In summary, our results strongly support the idea that as autoimmunity progresses, even unmutated autoantibodies will cause clearance or blockage of high concentration Ags. Concurrently, more avid B cells that would have been deleted at higher (i.e., normal) autoantigen levels will instead mature and will then become activated in the periphery. This predicts that as autoimmunity progresses, even unmutated autoantibodies will begin to appear that have high affinity and lupus-specific characteristics, such as the ability to bind dsDNA. In fact, such Abs have been found in both human and murine lupus (46, 49–54) and have otherwise been difficult to fit into a model that requires affinity maturation.

**References**


**Acknowledgments**

We thank Joe Craft, Ann Haberman, Mark Mamula, and Martin Weigert for critical reading of the manuscript.


