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Depletion of B Cells in Murine Lupus: Efficacy and Resistance

Anupama Ahuja,* Jonathan Shupe,* Robert Dunn,† Michael Kashgarian,‡ Marilyn R. Kehry,† and Mark J. Shlomchik2*

In mice, genetic deletion of B cells strongly suppresses systemic autoimmunity, providing a rationale for depleting B cells to treat autoimmunity. In fact, B cell depletion with rituximab is approved for rheumatoid arthritis patients, and clinical trials are underway for systemic lupus erythematosus. Yet, basic questions concerning mechanism, pathologic effect, and extent of B cell depletion cannot be easily studied in humans. To better understand how B cell depletion affects autoimmunity, we have generated a transgenic mouse expressing human CD20 on B cells in an autoimmune-prone MRL/MpJ-Fas1pr (MRL/lpr) background. Using high doses of a murine anti-human CD20 mAb, we were able to achieve significant depletion of B cells, which in turn markedly ameliorated clinical and histologic disease as well as antinuclear Ab and serum autoantibody levels. However, we also found that B cells were quite refractory to depletion in autoimmune-prone strains compared with non-autoimmune-prone strains. This was true with multiple anti-CD20 Abs, including a new anti-mouse CD20 Ab, and in several different autoimmune-prone strains. Thus, whereas successful B cell depletion is a promising therapy for lupus, at least some patients might be resistant to the therapy as a byproduct of the autoimmune condition itself. The Journal of Immunology, 2007, 179: 3351–3361.

Systemic lupus erythematosus (SLE) is characterized by autoantibodies against a diverse array of nuclear Ags (1). Our understanding of the disease process has been enabled by murine models of lupus, such as New Zealand Black × New Zealand White F1 (NZB/W), NZM, and MRL/MpJ-Fas1pr (MRL/lpr) (2, 3). Similar to humans, MRL/lpr mice produce autoantibodies to nuclear components, develop nephritis, arthritis, and skin lesions.

Both T and B cells play a role in SLE (4–6). Due to the presence of autoantibodies in renal and other lesions, lupus was thought to be an exclusively immune complex-mediated disease (1), with B cells secreting autoantibodies and T cells providing help to B cells. However, a broader view of pathogenesis of lupus has recently emerged, involving multiple interactions between T and B cells (2, 7–9).

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3 Abbreviations used in this paper: SLE, systemic lupus erythematosus; AFC, Ab-forming cell; ADCC, Ab-dependent cellular cytotoxicity; ANA, anti-nuclear Ab; BAC, bacterial artificial chromosome; DC, dendritic cell; FO, follicular; GN, glomerulonephritis; hCD20, human CD20; IN, interstitial nephritis; LN, lymph node; mCD20, murine CD20; mLN, mesenteric LN; MZ, marginal zone; NP, (4-hydroxy-3-nitrophenoxy)acetyl; NZB/W, New Zealand Black × New Zealand White F1; PerC, peritoneal cavity; RA, rheumatoid arthritis; RF, rheumatoid factor; SLT, secondary lymphoid tissue; Sm, Smith Ag; Tg, transgene.

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on T cells found in peripheral blood (20), suggesting an influence of B cells on T cell activation, as in the case of mice deficient of B cells from birth.

Animal models are ideal for evaluating B cell depletion mechanisms and efficiency, and determining how disease modification comes about. Nonetheless, murine models have generally lagged patient investigation, due to the lack of effective techniques to deplete B cells. However, using similar strategies, Gong et al. (21) and our group have been developing mice that express human CD20 (hCD20) as part of a transgene (Tg) comprised of a large genomic fragment containing the hCD20 locus. Indeed, as Gong et al. (21) have published (and see below), this strategy works well to direct tissue-specific expression of hCD20 on murine B cells. Moreover, Gong et al. (21) have elegantly demonstrated the efficacy, kinetics, and subset specificity of B cell depletion in C57BL/6 mice. Tedder and colleagues (22) have produced a number of depleting Abs to murine CD20 (mCD20), and have published similar results.

In this study, we report crossing our hCD20 Tg onto the MRL/lpr strain and the efficacy and impact of B cell depletion on clinical disease. We found that treatment of Tg MRL/lpr mice with high doses of depleting Ab for prolonged periods of time led to substantially reduced B cell frequencies. Depletion of B cells in turn led to a reduction in T cell activation and amelioration of clinical disease, formally demonstrating that B cell depletion affects disease, the finding that resistance to depletion in autoimmune mice is a general feature and is not specific to a given depleting Ab. In addition to demonstrating that B cell depletion affects disease, the finding that B cells from autoimmune animals are quite resistant to such depletion has therapeutic implications for patients in whom we generally only measure loss of B cells in PBL.

Materials and Methods

Mice

A 168-kb human bacterial artificial chromosome (BAC) clone, RP11-729B4 (a gift from T. Tedder, Duke University, Durham, NC), carrying the gene for hCD20 was purified, as described (23), and injected at 1–2 ng/μl by Genomics Services Transgenic Mouse Service into pronuclei of C57BL/6j × SJL/j F1 embryos. Tg mice were backcrossed >7 generations onto MRL/lpr, MRL+/+ (The Jackson Laboratory), and BALB/c (Charles River Laboratories) backgrounds and maintained as heterozygous. Four founders were identified using four sets of PCRs to ensure the presence of the entire BAC in the Tg mice. Two of these PCRs amplify regions within the hCD20 gene (exon 2 and 3′ untranslated region), and the other two amplify sequences near the two ends of the BAC (5′ BAC and 3′ BAC). The sequence of oligonucleotides is as follows: exon 2 F, 5′-CACAAGGGTAAGACTGGCCCCCCATC-3′ and R, 5′-ATATACAAGCCCCAAAACAAAAG-3′; 3′ untranslated region, F, 5′-GGCTTGGCATGGATGAC-3′ and R, 5′-AGGCCTTGTAGAAAGATTGGA-3′; 5′ BAC, F, 5′-ATTGGTTGAGTCTCAGATG-3′ and R, 5′-GGGAAAACATAATTGCCCTC-3′; 3′ BAC, F, 5′-GTGGAGTGCTTGCTGAT-3′ and R, 5′-AATTGCCCTGAGATTGGCTC-3′. Additionally, BALB/c from one founder are presented. NZB/W mice were purchased from Jackson Laboratories.

Antibodies

For FACS analysis, mAbs against murine CD5 (53.7), CD8 (TIB105), CD21 (7G6), CD44 (IM47), CD45 (RA3-682), CD62L (Mel14), CD90.2 (30H12), IgM (RS5.1), IgM (AF-678), IgD (AMS15), and FcR (2.4G2) were prepared, as described previously (24). Abs against CD11b (Mac-1), CD11c (HEL), CD19 (ID3), CD20 (H27), CD22 (Cy3.1), CD23 (B3B4), CD69 (H1.2F3), and goat anti-mouse IgG2b (R1-23) were purchased from BD Pharmingen. Abs against CD4 (GK1.5; eBioscience), IgD (11-26.2a; Biogene), streptavidin-PE/ Cy7 (eBioscience), streptavidin-Al647 (Southern Biotechnology Associates), and peanut agglutinin-FITC (Vector Laboratories) were purchased. For B cell depletion in Tg mice, three anti-hCD20 mAbs, rituximab (Genentech) (25), 2H7 (mouse IgG2b; hybridoma was a gift from E. Clark, University of Washington, Seattle, WA) (26), and SB9 (an engineered IgG2a derivative of 2B8, the mouse parent of rituximab) were used. A mAb against mCD20 (18B12) was generated by immunizing CD20+/− mice (27) with CD20-transfected cell lines (30.08.18 and 70Z23) and CD20-peptide-keyhole limpet hemocyanin conjugate. Splenocytes were plated with NS-1 myeloma cells. One hybridoma (18B12, IgG1k) was specific for CD20+ NS-1 myeloma and wild-type B6 splenocytes, with no reactivity toward CD20− splenocytes. All anti-CD20 mAbs were purified from culture supernatants by affinity chromatography on protein A-Sepharose, concentrated using Amicon filters and filter sterilized. Control mice were treated with total mouse IgG (Rockland), as specified.

Immunodepletion

Mice were injected i.p. with mAbs in sterile PBS using dosing schedules indicated in figure legends, with the exception that B6 mice were injected s.c. The extent of depletion was evaluated by both FACS and one hybridoma (18B12, IgG1k) against CD20+ B cell percentages by FACS. Residual B cells in treated mice were plotted as percentage of B cells in control mice. MRL/lpr mice, due to massive lymphoproliferation, exhibit large variability in cell numbers (8), mandating the use of B cell percentages as the basis for assessment of depletion.

Analysis of clinical disease

For assessment of renal disease, kidneys were bisected and one-half was fixed in formalin, embedded in paraffin, and stained with either HE or periodic acid-Schiff stains. Sections were scored for IN and glomerulonephritis (GN) by a renal pathologist (M. Kashgarian). Proteinuria was assessed using Albustix (Bayer). Skin disease was scored, as described (28).

Determination of serum Ig isotype levels

A multiplex assay kit (Beadlyte mouse IgG isotyping kit; Upstate Biotechnology) was used as per the manufacturer’s instructions and quantitated on a Bio-Plex analyzer (Bio-Rad).

Determination of autoantibody profiles

Rheumatoid factor (RF) and anti-Smith Ag (Sm) Abs were determined by ELISA, as described (28, 29). Serum titers of Ab to (4-hydroxy-3-nitrophenylacetyl) (NP) were determined, as described (30). Anti-nuclear Abs (ANA) were also quantitated by indirect immunofluorescence of fixed Hep-2 cells (Antibodies) on coded samples, as described (28). Sera from C57BL/6 and old MRL/lpr mice were negative and positive controls.

Statistical analysis

The Mann-Whitney two-tailed U test was used unless otherwise stated.

Results

Generation of hCD20 Tg mice

To develop a model system for ablation of B cells, we generated mice transgenic for hCD20 using a BAC carrying the hCD20 gene. Tg mice on an MRL/lpr background showed high expression of hCD20 specifically on splenic B cells (Fig. 1A, top panel), whereas both T cells and macrophages lacked expression. Small numbers of cells that were double positive for hCD20 and Thy1.2 or Mac-1 are most likely due to doublets on the FACS machine because essentially all hCD20+ cells are CD22+ (data not shown) and hence are authentic B cells. Similar expression was seen in lymph node (LN), peritoneal cavity (PerC), and PBL (data not shown). Similar patterns and levels of expression of hCD20 were seen in Tg mice of the BALB/c
and MRL+/+ backgrounds (data not shown). The expression of hCD20 on splenocytes and PBL of Tg mice was ~7-fold lower compared with that on the B cells from human PBL, probably reflecting differences in transcriptional regulation between mice and humans as well as the heterozygous state of the Tg (19). We have, therefore, generated a mouse model that expresses hCD20 specifically on mature B cells.

B cells in autoimmune-prone strains are resistant to depletion

Depletion of B cells in Tg BALB/c is more efficient compared with Tg MRL/lpr. We initially attempted to deplete B cells with rituximab. However, there was limited depletion of B cells in Tg MRL/lpr mice with doses ranging from 50 μg to 15 mg/wk (data not shown). In contrast, in Tg BALB/c mice, B cells could be depleted more efficiently, with ~90% depletion in mesenteric LN (mLN) and PBL and 76% depletion in spleen on day 14 (data not shown). This is consistent with the extent of depletion obtained by Gong et al. (21) in similarly generated hCD20 Tg mice on the C57BL/6 background. The resistance of B cells to depletion with rituximab could potentially be due to generation of Abs against the human C region of rituximab (data not shown). Therefore, we subsequently used a murine anti-hCD20 mAb, 2H7, which binds an epitope similar to that bound by rituximab.

To find a dose of 2H7 that depleted B cells in both Tg BALB/c and Tg MRL/lpr mice, we treated cohorts of 8-wk-old mice with 4 mg/wk 2H7, given twice per week, for 2 wk (Fig. 2). This resulted in >90% depletion of splenic B cells in BALB/c (Fig. 2, A and C). The residual B cells were mainly of follicular (FO) phenotype (CD21low, CD23high, Fig. 2A). Similar depletion was achieved in mLN and PBL (Fig. 2C). However, in Tg MRL/lpr, this dose resulted in only a 20% depletion of splenic B cells (Fig. 2, B and C). FO, marginal zone (MZ) (CD21high, CD23low), and T2 (CD21high, CD23high) B cells were all resistant to depletion. Interestingly, B cells from mLN and PBL were more amenable to depletion in Tg MRL/lpr mice (72% mean depletion in both tissues). Although due to unavailability of sufficient mice and purified mAb, these preliminary cohorts comprised of only two
mice each, similar data were obtained in subsequent experiments (see below). In fact, Tg BALB/c mice had greater B cell depletion even with a single dose of 100 μg of 2H7 given i.v. than did Tg MRL/lpr mice treated with 4 mg/wk 2H7 given i.p. (data not shown).

Incomplete depletion of B cells, independent of autoimmune-prone strain or mAb

Depletion in Tg MRL+/+ mice using 2H7. Resistance to B cell depletion in Tg MRL/lpr mice may be specifically due to the lpr defect and/or an inherent feature of the attendant systemic autoimmune disease. To test the role of lpr, we treated 18-wk-old Tg MRL+/+ females with either 1 or 2 mg/wk 2H7 for 2 wk (Fig. 3A). As observed in Tg MRL/lpr mice, B cells from both mLN and PBL were relatively susceptible to 2H7 treatment (74 and 95% depletion, respectively, with 1 mg/wk dose), but splenic B cells were refractory (25% depletion at either dose) despite the absence of lpr defect in these mice.

Depletion in Tg MRL/lpr mice using anti-hCD20, 8B9. It seemed possible that the resistance to depletion was specific to the depleting Ab. To probe this further, we compared depletion efficiency of 2H7 with another anti-hCD20 mAb, 8B9, a mouse IgG2a version of rituximab. Seven- to 9-wk-old Tg MRL/lpr mice were treated with 4 mg/wk of either 2H7 or 8B9 for 2 wk (Fig. 3B). Compared with 2H7, 8B9 was better, in general, at eliminating B cells from the PBL, mLN, and PerC compartments. However, the two mAbs were comparable in mediating only a partial depletion of splenic B cells (~30–45% depletion), suggesting that failure of depletion is not specific to 2H7.

Depletion in MRL/lpr using anti-mCD20. To further strengthen this conclusion, we used 18B12, a novel murine IgG1 anti-mCD20, to deplete B lymphocytes in autoimmune mice. Six-week-old MRL/lpr females were treated with either 1 or 3 mg/wk 18B12 for 2 wk. With either of the doses tested, B cells from PBL and mLN were depleted >70%, but B cells from PerC and spleen were resistant to depletion (33 and 20% depletion, respectively, even with 3 mg/wk; data not shown). In contrast, B cells could be depleted efficiently in nonautoimmune-prone BALB/c and C57BL/6 mice even with a single dose of 250 μg/mouse of 18B12 (PBL, ~97%; LN, ~90%; spleen, ~76%; PerC, ~38%; data not shown). These data further substantiate the notion that B cells from autoimmune-prone mice are harder to deplete compared with nonautoimmune-prone mice, regardless of the Ab used.

Depletion in MRL+/+ using anti-mCD20. We also further investigated the function of lpr in resistance to depletion using 18B12. Eight-week-old (young, prediseased; Fig. 3C) and 7-mo-old (old, early disease; Fig. 3D) MRL+/+ females were treated with either 1 or 3 mg/wk of 18B12, administered twice per week, for 2 wk. IgG-treated females and age-matched BALB/c females served as controls. In all groups, >90% of the B cells were eliminated from mLN and PBL, whereas PerC B cells were largely resistant to depletion using 18B12. However, in line with the 2H7 data, splenic B cells from MRL+/+ mice were more resistant to depletion compared with BALB/c, despite very high levels of circulating excess 18B12 in these mice (for most mice >80 μg/ml at the 1 mg/wk dose and >200 μg/ml for the 3 mg/wk dose; data not shown). Even at the highest dose of 3 mg/wk, a maximum of 50% of splenic B cells could be depleted in young MRL+/+ mice compared with 65% in BALB/c mice (p = 0.0286). In older mice, this difference was further exacerbated, with only 25% depletion of...
splenic B cells in MRL/+ compared with >70% depletion in BALB/c ($p = 0.0159$).

**Depletion in NZB/W using anti-CD20.** The above data suggest that B cell resistance to depletion is likely to be a feature of autoimmune disease per se. To further support this, we analyzed depletion of B cells in another autoimmune-prone strain, NZB/W, using 18B12. We used the same dosing strategy as described above for MRL/+ mice in either 10-wk-old (young, prediseased; Fig. 3E) or 20-wk-old (old, early disease; Fig. 3F) females. IgG-treated females and age-matched BALB/c females treated with 18B12 served as controls. Results from NZB/W mice paralleled those from MRL/+ shown above. B cells from both mLN and blood were just as readily depleted as in BALB/c (at either age), whereas B cells from spleen and PerC from NZB/W mice were more resistant to depletion. Furthermore, there was a statistically significant difference in depletion in older NZB/W (<40%) compared with BALB/c (>70%, $p = 0.0286$ for 1 mg/wk and $p = 0.0159$ for 3 mg/wk). Serum of treated mice had high levels of 18B12, ruling out that an insufficient amount of 18B12 was injected (data not shown).

**High doses of anti-CD20 mAb for extended durations can overcome resistance to depletion in autoimmune-prone mice**

**Ab dose optimization.** Efficient depletion of B cells in Tg BALB/c mice demonstrated the potential of the B cell depletion model system. Therefore, we felt that optimization and/or prolongation of the treatment protocol might overcome the resistance to B cell depletion in autoimmune mice, allowing us to pursue the original goal of our work. Toward this end, we treated 6-wk-old Tg MRL/lpr mice with 4, 10, or 15 mg/wk 2H7 for 4 wk (Fig. 4), with a starting dose on day 0 that was twice that of subsequent doses. We followed the depletion of circulating B cells by serially bleeding the mice (Fig. 4A). Although minor differences in depletion were observed between the three doses on day 7, by day 14 all doses reached a plateau and yielded a similar effect, with only 3–4% of circulating B cells remaining. On day 28, at the time of sacrifice, B cells from both mLN and PerC were depleted efficiently (>90%), but splenic B cells continued to be resistant (~20–30% depletion; Fig. 4B). Eighty-three percent of residual B220+, Thy1.2+ splenocytes from treated mice could be easily stained with anti-mouse IgG2b, compared with ~4% in untreated mice, suggesting that these cells were coated with 2H7 (Fig. 4C).

**Long-term high-dose treatment.** Improved B cell depletion in the previous experiment, particularly in mLN and PBL, suggested that a higher dose of Ab given for an even longer duration might overcome the resistance to depletion. To test this, we treated Tg MRL/lpr females with 10 mg/wk 2H7 (twice per week), starting at 6 wk of age, for either 7 wk ($n = 4$) or 10 wk ($n = 12$) (Fig. 5). Age-matched, untreated Tg- and Tg+ females were controls, along with smaller cohorts ($n = 2$) of Tg- females given total mouse IgG and Tg+ females ($n = 3$) given 2H7 mAb. Because there was no detectable effect of the treatment on B cell percentages and disease in all control mice, data from these mice are not shown in Fig. 5.

Remarkably, treating the mice for 10 wk led to ~85% depletion of splenic B cells, breaking the barrier observed previously. Even a 7-wk treatment led to >50% depletion of splenic B cells. With either protocol, there were <10% of B cells remaining in mLN, PBL, and PerC (Fig. 5A).

Although we achieved substantial depletion of B cells with this treatment regimen, it was still not complete. Again, this was not due to insufficient amounts of administered Ab. High background observed in older MRL/lpr mice of this age precluded detection of 2H7 binding by staining with anti-mouse IgG2b. Instead, we tested for saturation of 2H7 binding sites by incubating the B cells from treated mice with additional fluorescently labeled 2H7 in vitro. The residual splenocytes from 10-wk-treated mice showed no binding with optimal amounts of exogenously added 2H7-PE (data not shown), indicating saturation of all binding sites by in vivo administered 2H7 or that these cells no longer expressed hCD20, which is unlikely in view of the fact that in other strains residual B cells expressed hCD20, but were completely coated with anti-CD20 (Fig. 4C) and that CD20 is not generally known to be modulated by Ab binding (31).

We further analyzed residual B cells to determine whether depletion differed for particular subsets. FO, MZ, and T2 B cells depleted similarly in 10-wk-treated mice (Fig. 5B). In contrast, with 7-wk treatment, both FO and MZ subsets depleted to 55% and T2 cells depleted to only 30% of controls (data not shown). In C57BL/6 mice, MZ B cells were reported to be resistant to depletion after short-term treatment (21). B1a, B1b, and B2 peritoneal cells depleted almost completely (Fig. 5A and data not shown). Additionally, germinal center B cells were relatively refractory to treatment (data not shown; 58% depletion compared with 85% depletion of total splenic B cells with 10-wk treatment).

Of relevance to autoantibody production, on average only 20%...
and DC populations remained unchanged in treated mice (data not shown). However, commensurate with depletion of B cells, a major proportion of splenocytes, the percentage of total Thy1.2+ T cells in spleens of treated mice, increased by 33% (data not shown). An increase in T cell percentages was not evident in LN, probably due to the already large accumulation of T cells compared with B cells in LN of MRL/lpr mice with age (8).

**Effect of B cell depletion on T cell activation.** Based on genetically deficient mice, B cells are believed to enhance the accumulation of activated (CD44high, CD261high) and memory (CD44high, CD62Llow) phenotype T cells in MRL/lpr mice, in an age-dependent manner (8). However, the phenotype may be different with B cell depletion during disease, because B-deficient mice have irreversible changes in lymphoid architecture (32, 33). In addition, once certain types of T cells are activated, they may not need B cells for maintenance. We therefore evaluated T cell activation in spleens of treated mice (Fig. 6A). Notably, in both the 7- and 10-wk-treated mice, the percentage of naive phenotype (CD44low, CD261low) CD4+ T cells increased with treatment by 134% (Fig. 6B, p = 0.0041), whereas there was a 10% decrease in activated (p = NS) and memory (p = 0.0057) CD4+ T cell populations. Changes in activation status of CD8+ T cells with treatment followed the same trend for naive (24% increase) and memory (14% decrease) CD8+ cells, but not activated CD8+ cells (23% increase; data not shown).

**Amelioration of clinical disease after B cell depletion.** We evaluated several clinical findings that MRL/lpr mice develop spontaneously with age, including GN and IN, proteinuria, and dermatitis. Histologically, IN was present in kidneys of almost all mice; however, the degree of infiltration was markedly reduced in 2H7-treated mice. Untreated mice typically showed several large areas of infiltration (Fig. 7, A and B), whereas there were much smaller and fewer foci of infiltration in age-matched treated mice (Fig. 7, D and E). Moreover, compared with treated mice (Fig. 7F), glomeruli in untreated mice (Fig. 7C) were larger and hypercellular, with proteinaceous deposits, marked thickening of capillary loops, and obliteration of lumens. The extent of IN and GN was blinded scored from 0 to 3 for all the mice (Fig. 7G). IN was significantly reduced even after 7 wk of treatment (p = 0.0286). The reduction in IN was more pronounced with 10-wk treatment (p = 0.0023). Although there was a trend toward reduced GN with 7-wk treatment, the reduction became highly significant after 10-wk treatment (p = 0.0003). This difference may be due to the smaller cohort and the lesser degree of disease among controls in the 7-wk treatment group, which were younger at sacrifice (13 wk).

Urinary protein levels were low even in untreated mice from the 7-wk cohort, because these mice were too young to manifest...
proteinuria (Fig. 7H). In contrast, there was a significant reduction of proteinuria in mice treated for 10 wk (p = 0.0092, one-tailed Mann-Whitney U test). Similarly, severity of dermatitis was significantly reduced with 10-wk treatment (p = 0.0382, one-tailed Mann-Whitney U test) with 2H7 (Fig. 7I). Again, for the 7-wk treatment cohort, at the time of sacrifice, neither the untreated control nor the treated mice had any dermatitis, due to their age.

**Reduction in total Ab, as well as autoantibody titers in serum.**

Disease in MRL/lpr mice is characterized by hypergammaglobulinemia as well as ANA. Despite the fact that the majority of plasma cells do not express CD20, we found that with 10-wk treatment, there was a 2-fold reduction in serum levels of total IgG1 (p = 0.0015) and IgG2a (p = 0.0002) as well as 1.5-fold reduction in IgG3 levels (p = 0.0338) (Fig. 8A). The serum levels of IgM (data not shown) and IgG2b (Fig. 8A) were unaffected, the latter possibly due to measurement of residual 2H7, which could not be excluded from the assays.

Remarkably, there was a significant decrease (>6-fold) in serum RF levels of mice treated for 10 wk (p = 0.0051; Fig. 8B). Serum RF levels remained low in the 7-wk group due to their relatively young age. There was no significant change in titers of anti-Sm Abs (Fig. 8C). Most of the mice had low titers of anti-Sm, with the exception of two untreated mice. We also measured anti-NP Ab levels to control for cross-reactivity of MRL/lpr serum, but found minimal anti-NP Abs in either treatment group (data not shown).

Sera from all mice showed homogeneous nuclear staining of Hep-2 cells, consistent with the presence of anti-chromatin/anti-DNA Abs.
FIGURE 8. Reduction in serum IgG and autoantibody levels as a result of B cell depletion. Serum titers of IgG isotypes (A), RF (B), and anti-Sm (C) were measured after 7 (n = 4) or 10 (n = 12) wk of treatment. Horizontal lines represent median values. *, p < 0.05; **, p < 0.01; ***, p < 0.002 by Mann-Whitney U test. D, Representative serum ANA (1/50 dilution) from untreated (left panel) and treated (right panel) mice. White arrows indicate cells in metaphase that exhibit positive chromatin staining. Magnification ×40. E, ANA were blindly scored for intensity. Horizontal lines represent median values. *, p < 0.05; ***, p < 0.0001 by Mann-Whitney U test.

(Fig. 8D). Additionally, equatorial staining of chromosomes in metaphase (shown by white arrows in Fig. 8D), indicative of anti-chromatin Abs, was observed in all the mice. However, the fluorescent intensity of Hep-2 cell nuclei, scored blindly from 0 to 4 (Fig. 8E), was significantly reduced in mice treated for either 7 wk (p = 0.0286) or 10 wk (p = 0.0001).

Discussion

Methods to target B cells to treat autoimmune diseases are currently being evaluated in patients, most notably via the depleting anti-CD20 drug rituximab (14, 15, 17, 18, 34). This therapy is already approved for certain RA patients, and anecdotal evidence suggests efficacy in some, but not all SLE patients. These exciting results have provided a strong rationale for developing a better understanding of how B cell depletion works in SLE, and how it should be applied to patients. Animal models have an important role because of limited ability to assess mechanism and efficacy in humans. In this study, we report three contributions in this direction. First, we have developed methods to deplete B cells in lupus-prone mice, using either novel hCD20 Tg mice or a new anti-mCD20, 18B12. Second, and unexpectedly, we found that in MRL/lpr mice it was difficult to deplete B cells in SLT, even though PBL appeared well depleted. Two other lupus-prone strains were also resistant to B cell depletion, suggesting this is a general feature of murine lupus. Finally, we were substantially able to overcome this problem, although it required higher doses and longer treatment periods than anticipated, to deplete B cells in adult mice and observe the effects on disease.

Not surprisingly, the results we obtained in depleting B cells in normal strains of mice were similar to those reported previously (21, 22). The picture was completely different in MRL/lpr mice. Even substantial doses of mAb did not achieve >20% depletion in the spleen of MRL/lpr mice, whereas it resulted in ∼90% depletion in BALB/c. Indeed, we found that even larger doses had little incremental impact on MRL/lpr splenic B cells, unless maintained for long periods. Resistance to depletion was independent of depleting Ab and applied to all three lupus-prone mouse strains tested. Thus, relative inability to deplete B cells seems to associate generally with autoimmunity.

Splenic B cells were most resistant to depletion, regardless of strain. Although LN and PBL were more easily depleted, they were still relatively resistant, particularly in the MRL/lpr strain. One limitation in generalizing these findings is that we focused on high doses and long treatment times in efforts to impact the spleen. It is possible that with lower doses or shorter treatment duration, greater differences would emerge in LN and PBL. In normal strains, depletion in spleen and other SLT depends on the same FcRs (21, 22). It could be that different accessory cells are required to eliminate opsonized B cells in spleen and LN. Supporting this, in normal mice, splenic MZ cells were more resistant than FO B cells, which could be reversed by mobilizing the MZ cells with anti-integrin Abs (21). Alternatively or in addition, the unknown factors that interfere with depletion during autoimmunity may be differentially present in the spleen vs other SLT.
A more general question is why autoimmune-prone mice are resistant to cellular depletion in the first place. It is unlikely due to a lack of adequate levels of depleting Ab. We presented evidence of high degrees of opsonization using several assays, including detection of hCD20 mAb coating of residual B cells as well as lack of accessible sites for binding of excess anti-hCD20 on residual B cells. Moreover, for anti-mCD20, we measured substantial levels of circulating excess Ab in the serum of treated mice. This interpretation is also in keeping with the fact that there was little dose-response effect going from 4 to 15 mg/wk/mouse. Because we had the same results with multiple different preparations of 2H7, 8B9, and 18B12, it is unlikely that defects in a particular mAb or preparation are an explanation.

To understand the basis for resistance to B cell depletion in autoimmune mice, we need to consider the mechanisms of rituximab action that are proposed based on studies in monkeys and in vitro cultures, namely, Ab-dependent cellular cytotoxicity (ADCC), complement-dependent cytotoxicity, or apoptosis (31). The majority of in vivo data in mice indicate a dominant role for FcR-mediated clearance of anti-CD20-opsonized cells by macrophages (21, 35). In this connection, the anti-mCD20 Ab 18B12 depleted B cells effectively in C5-deficient mice (data not shown), ruling out involvement of lytic complement in mice. Therefore, autoimmune mice are defective in either apoptosis induction or ADCC; defects in ADCC could either be B cell intrinsic or B cell extrinsic.

It seems most likely that a B cell-intrinsic defect resulting in refractoriness to depletion is either inherited along with autoimmune predisposition and/or is a consequence of development of the autoimmune state. We favor the latter explanation for several reasons. The location of a B cell can determine its susceptibility to elimination by anti-CD20 (21); there is no reason to think that recirculating FO B cells would modulate their intrinsic resistance depending on where they happened to migrate, spleen vs LN. MRL/lpr mice, which have more severe autoimmunity, appear more difficult to deplete than MRL+/+ mice, which are otherwise genetically identical except for Fas. However, Fas itself is unlikely to be an important signal for depletion, given the known mechanisms of CD20-mediated B cell depletion (22, 36). Therefore, inherited genetic predisposition by itself cannot explain this. Similarly, even within the same strain, it is harder to deplete B cells in older mice. A likely explanation is that older mice manifest more age-dependent autoimmune effects than younger mice. Although we favor a role for acquired defects resulting from autoimmunity, it remains possible that intrinsic resistance to apoptosis, known to be part of an autoimmune phenotype, could at least contribute partially to our findings.

In this regard, given the role of macrophages in the clearance of cells (21), it is interesting to suggest that defects in phagocytic cells, such as macrophages, could contribute to inefficient B cell deletion (37–39). Although some of these defects were thought to be primary, whether they caused or were the result of autoimmunity was difficult to discern. In addition, although macrophages of young autoimmune mice were deficient in clearing apoptotic cells, they were more efficient in FcγR-mediated clearance of opsonized RBCs (40). Polymorphism in FcγRIII has been linked to clinical response in FO lymphoma patients and B cell depletion in lupus patients (41, 42). However, there are no known common FcγR polymorphisms shared among resistant strains (MRL and NZB/W) that are not shared among susceptible strains (BALB/c and C57BL/6). Because the presence of immune complexes is a common feature of systemic autoimmunity in mice and humans, we suggest that a constitutive excess of immune complexes may inhibit macrophage function, most likely in an FcγR-dependent manner. Intravenous Ig, which has been used to treat disorders of accelerated cellular clearance such as autoimmune thrombocytopenia and anemia, may function in a similar manner (43).

Another possible mechanism of resistance, acquired or intrinsic, is that elevated levels of B cell activating factor of TNF family could contribute to enhanced B cell survival. Such elevated levels are found in both human and murine lupus (44), and are induced by B cell depletion in RA patients (45). Moreover, in normal mice, combined treatment with anti-CD20 and blocking BR3-Ig leads to more complete depletion, albeit the two reagents target different subsets (21).

Given the fact that B cells in MRL/lpr, MRL+/+, and NZB/W strains were all resistant to depletion, it is tempting to speculate that at least in some lupus patients, and possibly in patients with other autoimmune syndromes, depletion is not as efficient as therapeutically desirable. In fact, there is no accepted dose or even appropriate therapeutic endpoint in such diseases. Depletion in the blood can be measured, but our data suggest that this could be misleading with respect to depletion in SLT. Even in nondiseased primates, depletion in blood exceeds that in tissues (25). Interestingly, there is anecdotal evidence that some SLE patients are more difficult to deplete of B cells and do not achieve full depletion (15). Moreover, not all patients respond, and it could be that failure to respond represents failure to sufficiently deplete B cells. Our study provides a rationale to think that in these patients, longer courses with higher doses may be useful or even required to achieve clinical response.

Indeed, despite the virulent nature of lupus in MRL/lpr mice and the fact that complete depletion most likely took weeks of treatment to achieve, there were strong effects on disease. Of note, we saw reductions in both presumed Ab-mediated and Ab-independent aspects of disease. There was a clear amelioration of GN, in which Ab most likely plays an important role. Commensurately, there was reduced proteinuria in treated mice. This was accompanied by substantial reductions in ANA intensity along with more modest declines in IgG levels. Nonetheless, these results need to be interpreted in the context of numerous studies that have unlinkd ANA from GN (46–48). In view of this, and the minimal effects of 2H7 treatment on some types of Ab (see below), it seems likely that effects on GN are in part independent of Ab.

Considering that the majority of Ab-forming cells (AFCs) do not express CD20, reductions in Ab levels are mechanistically informative. Although we cannot rule out an effect of early treatment in preventing development of long-lived plasma cells, we would argue that reductions in certain Ab levels are likely to be due to depletion of plasmablast/cell precursors, consistent with the idea of a substantial contribution of short-lived AFCs to the autoantibody pool. We have shown that RF B cells in MRL/lpr mice (49) are derived from short-lived plasmablasts, and a majority of AFCs in spleens of NZB/W mice are also short-lived (50). In addition, depletion of GC B cells, the source of long-lived plasma cells, was incomplete (only 50%), at a level inconsistent with explaining large reductions in the certain Ab levels. In this regard, the differential effect of B cell depletion on particular specificities and isotypes is of interest. Notably, RF levels (as predicted from the RF Tg mice (49)), as well as the ANA were rather markedly affected, suggesting that a substantial proportion of these specificities, if not all, is derived from short-lived rather than long-lived AFCs. Similarly, the 2- to 3-fold drops in IgG1 and IgG2a levels in 10 wk from initiation of B cell depletion suggest that a proportion of these switched isotypes is also generated via short-lived AFCs presumably in an extrafollicular reaction (49). These data should be
Effects on T cell-mediated aspects of disease were also evident, as follows: there were reduced IN and dermatitis, both of which are characterized by T cell-rich infiltrates. In accord with this, there were significant increases in the naive T cell populations and decreases in the memory phenotype T cell populations in spleen. Yet, these increases were not dramatic, suggesting that B cells are not required for the maintenance of at least some memory phenotype T cells once they are generated. In contrast, in mice of similar age that lacked B cells from birth, there was a 10-fold reduction in the numbers of memory CD4 T cells (8). Another possibility is that depletion was not complete enough to fully extinguish the effects of B cells on T cells. Although these issues need to be further addressed in future studies, our data do indicate that ongoing activation of T cells and T cell-mediated disease can be affected by B cell depletion, in line with reduction of activation markers on T cells in blood of B cell-depleted patients (20).

Some other aspects of our studies should be extended in the future. Although we were able to carry out two substantial depletion protocols of 7- and 10-wk duration, we started both of these at ~6 wk of age (with mice being 13 or 16 wk old at the time of sacrifice). Although we would ideally like to treat older mice, it remains unclear whether this is even feasible. In this connection, the degree that B cells were resistant to depletion even at 6 wk of age was striking. This is consistent with the idea that the disease process had already begun at this age, even if overt tissue damage was not yet evident. Thus, we feel that early treatment of the MRL/lpr model most likely resembles in some respects treatment of early lupus or less-severe lupus in patients.

Despite our best efforts, B cell depletion was still not complete. Data on splenic depletion at the 4-, 7-, and 10-wk time points suggest that maximal depletion may have only been achieved for the last 2-3 wk of the 10-wk study. Although with only partial depletion at 7 wk we observed definite effects on IN, ANA intensity, and T cell activation, these effects may have been more pronounced with continued treatment. In any case, it is reasonable to conclude that partial depletion can lead to partial remission, a point that is relevant for clinical application.

In sum, our studies have shown that B cell depletion impacts both Ab- and T cell-mediated aspects of disease. We have documented and explored a potentially general problem of resistance to therapy, which should be very informative for clinical trial design and interpretation. Future studies in the mouse will focus both on the mechanisms of treatment resistance and approaches to circumvent it, as well as on other treatment regimens, including transient therapy. Efforts to understand resistance to depletion are ongoing. If ways to surmount it are discovered, this will make possible interpretable studies of B cell depletion in more advanced disease and may have additional relevance to treatment strategies in patients.

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Drs. Dunn and Kehry are employees of Biogen-Idec.

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