Immunity, Vol. 19, 837–847, December, 2003, Copyright ©2003 by Cell Press

Activation of Autoreactive B Cells by CpG dsDNA

Gregory A. Viglianti,[1,*] Christina M. Lau,[1,3] Timothy M. Hanley,[1,3] Benjamin A. Miko,[1] Mark J. Shlomchik,[2] and Ann Marshak-Rothstein[1]
[1]Department of Microbiology
Boston University School of Medicine
Boston, Massachusetts 02118
[2]Departments of Laboratory Medicine and Immunobiology
Yale University School of Medicine
New Haven, Connecticut 06520

Summary

The proliferative response of autoreactive rheumatoid factor (RF) B cells to mammalian chromatin-containing immune complexes (ICs) results from the sequential engagement of the B cell receptor (BCR) and Toll-like receptor 9 (TLR9). We have used ICs constructed from anti-hapten antibodies and defined haptenated dsDNA fragments to determine the form of mammalian DNA that mediates this process. Despite their relatively low abundance in mammalian DNA, we found that inclusion of hypomethylated CpG motifs in these ICs was necessary for effective activation. In the absence of antibody, the same fragments could efficiently stimulate low-affinity hapten-specific and DNA-reactive 3H9 B cells, but not RF B cells. These results extend the BCR/TLR9 coengagement paradigm to a second major class of autoreactive B cells, further confirm the critical role of the BCR in chromatin ligand delivery to TLR9, and implicate hypomethylated CpG motifs as ligand elements necessary for the initiation of systemic autoimmune disease.

Introduction

Systemic lupus erythematosus (SLE) and other autoimmune diseases are characterized by the production of antibodies specific for a relatively limited set of autoantigens (Plotz, 2003). DNA, chromatin-associated proteins, ribonucleoproteins, and autologous IgG are among those molecules that consistently emerge as prime targets in systemic diseases such as SLE and rheumatoid arthritis, both in human patients and in animal models (Cohen and Eisenberg, 1991; Kotzin, 1996). A number of B cell receptor (BCR) transgenic (Tg) mouse lines have provided useful experimental tools to better understand the factors that link these particular autoantigens to disease. The MRL-lpr derived AM14 strain is one example (Hannum et al., 1996). AM14 rheumatoid factor (RF) producing B cells recognize autologous IgG2a with relatively low affinity. On a nonautoimmune prone genetic background, AM14 B cells persist in the periphery as mature naive B cells, but on an autoimmune-prone Fas-deficient background, they convert to actively proliferating autoantibody producing plasmablasts (Hannum et al., 1996; Wang and Shlomchik, 1999; William et al., 2002). Thus they constitute a suitable prototype to study the events that lead to the activation of potentially autoreactive peripheral B cells. In vitro, they proliferate vigorously when bound through the BCR to immune complexes (ICs) containing IgG2a$^{a/j}$ mAbs specific for autoantigens such as DNA, intact nucleosomes, or histones but not when bound by ICs containing IgG2a^{a} complexed with proteins or carbohydrates (Leadbetter et al., 2002).

One common feature of the stimulatory mAbs is that they can complex with chromatin released into culture fluids from dead cells to form multivalent DNA-containing ICs (chromatin ICs). Supporting the idea that DNA is an essential component of the antigen is the finding that DNase I blocks the ability of these mAbs to stimulate AM14 B cells (Leadbetter et al., 2002). In addition, stimulation by these ICs requires the Toll-like receptor (TLR) adaptor protein, MyD88, and is inhibited by agents that block TLR9 signaling, either by preventing endosome acidification or directly inhibiting TLR9 (Leadbetter et al., 2002). Although TLR-9 is known to mediate responsiveness to hypomethylated CpG-containing bacterial DNA (Bauer et al., 2001; Hemmi et al., 2000), mammalian DNA has not been considered an efficient ligand for TLR9 (Messina et al., 1991; Yamamoto et al., 1992). How then can mammalian DNA be an essential bioactive component of ICs that stimulate cells through TLR-9-mediated signaling? One possibility is that coengagement of the BCR somehow relaxes the stringency of TLR9 recognition for canonical CpG motifs and/or hypomethylated CpG dinucleotides (Wang and Krieg, 2003). Another possibility is that the chromatin released into the culture fluids is particularly enriched for hypomethylated CpG stretches of DNA. A better understanding of the mechanism by which chromatin ICs stimulate B cells should provide important insights into the initiation and propagation of systemic autoimmunity and, potentially, new therapeutic approaches. In this regard, there are two fundamental questions. First, is signaling initiated through BCR crosslinking required, or is the BCR merely a conduit to deliver the TLR9 ligand to the appropriate intracellular compartment? Second, what is the true nature of the ligand?

In order to better dissect the mechanisms responsible for this BCR/TLR9-dependent activation scheme, it is critical to identify the key properties of stimulatory ligands, both in terms of their structural ability to stimulate TLR9 as well as their capacity to crosslink the BCR. Unfortunately, our attempts to subfractionate chromatin and add it back to cultures containing chromatin-reactive antibodies have inevitably been confounded by continuously released chromatin of indeterminate size and composition from apoptotic cells. As an alternative strategy, we have now synthesized a series of defined haptenated-dsDNA fragments that can be recognized by IgG2a$^{a/j}$ mAbs, and compared the capacity of these DNA fragment ICs and spontaneous chromatin ICs to bind and stimulate AM14 B cells. We find that only those

*Correspondence: gviglian@bu.edu
[3]These authors contributed equally to this work.

ICs containing DNA with canonical hypomethylated CpG motifs can stimulate AM14 B cells. These fragments have also proven to be a powerful tool for demonstrating that chromatin IC stimulation includes an active role for the BCR in the signaling cascade. Finally, these same fragments have allowed us to directly test the relevance of BCR/TLR9 coengagement to other BCR Tg models, including low-affinity NP/NIP-specific and 3H9 DNA-reactive lines. The results confirm the general applicability of our model to non-RF autoreactive B cells. Together these data indicate that hypomethylated CpG motifs in mammalian DNA play an important role in systemic autoimmune diseases, and suggest that environmental and genetic factors that increase the availability of this material to the immune system can contribute to disease onset.

Results

Binding of Monomeric and Multimeric IgG2a to IgG2a-Specific B Cells

AM14 B cells proliferate vigorously in response to chromatin ICs that spontaneously form between chromatin-reactive mAbs, such as PL2-3, and DNase 1 sensitive antigens present in "spent" culture media. In contrast, protein ICs formed between anti-hapten mAbs, such as Hy1.2, and haptenated proteins, such as TNP-BSA, fail to stimulate AM14 B cell proliferation (Leadbetter et al., 2002). One possible explanation for this apparent disparity is that the protein ICs simply bind to AM14 B cells less efficiently than the chromatin ICs, due to differences in the overall size and/or configuration of the two types of complexes. To address this issue, we compared the binding of monomeric mAb (PL2-3 + medium or Hy1.2 + medium), preformed chromatin IC (PL2-3 preincubated with spent culture supernatants), and protein IC (Hy1.2 + TNP-BSA) to either non-Tg, AM14 Tg, or 20.8.3 Tg B cells (Wang and Shlomchik, 1997) (Figure 1A). The BCRs expressed by both Tg lines recognize IgG2a$^{a/j}$ antibodies; however AM14 was derived from a mouse with systemic autoimmune disease and the 20.8.3 line was derived from a mouse immunized with allotype-disparate IgG2a. AM14 binds IgG2a with a binding constant ($K_d = 1.7 \times 10^{-6}$) typical of the RF antibodies isolated from an MRL/lpr mouse (Jacobson et al., 1994), while 20.8.3 binds IgG2a with an affinity that is 2 to 3 logs higher than that of the AM14 receptor (unpublished data; Wang and Shlomchik, 1997). As might be expected, AM14 cells bound monomeric IgG2a only slightly better than non-Tg cells, while both the protein ICs and chromatin ICs bound quite effectively. By comparison, the higher affinity 20.8.3 cells bound the monomeric mAbs almost as well as the ICs. Notably, as measured by mean fluorescent intensity, the protein IC and chromatin IC bound AM14 and 20.8.3 cells to a comparable extent, suggesting that the great differences in their stimulatory capacity for AM14 B cells is not attributable to differences in their ability to bind the BCR on these cells. These staining data indicate that chromatin IC and protein IC form multivalent antigens that bind much more avidly to the low-affinity AM14 receptor than monomeric IgG2a; however, multivalency is much less essential for the 20.8.3 receptor.

Protein ICs Stimulate High-Affinity RF B Cells

To prove that the protein ICs were sufficiently multivalent to stimulate B cells with antigen binding constants that approach the standard affinity for foreign antigen, it was important to compare the proliferative responses of the AM14 and 20.8.3 B cells stimulated by the various configurations of IgG2a. B cells isolated from non-Tg and Tg mice were cultured for 2 days with Hy1.2 (monomeric IgG2a), Hy1.2+TNP-BSA (protein IC), or PL2-3 (chromatin IC), and proliferation was assessed by ^{3}H-thymidine incorporation (Figure 1B). Even though monomeric Hy1.2 bound to the 20.8.3 cells quite effectively, it failed to stimulate either AM14 or 20.8.3 B cells to proliferate. By contrast, protein ICs that failed to activate AM14 B cells were found to activate 20.8.3 cells remarkably well, especially when compared to a standard F(ab)'$_2$ anti-IgM reagent. Nevertheless, chromatin IC stimulated the 20.8.3 cells much more effectively than protein IC; 0.01 μg/ml of chromatin IC IgG2a induced the same level of proliferation as 1.0 μg/ml of protein IC IgG2a. These results demonstrate that even though monomeric IgG2a and the protein IC bind comparably to the 20.8.3 B cells, a proliferative response can only be induced by a form of the antigen that can effectively crosslink the B cell receptor. Moreover, the nature of the antigen in the ICs is critical even with the higher affinity receptor, as chromatin ICs stimulate 100-fold more effectively than protein ICs. FcγRII-deficient 20.8.3 B cells responded to both protein and chromatin ICs to a similar extent as the FcγRII-sufficient 20.8.3 B cells, demonstrating that stimulation by these ICs is not adversely affected by engagement of the FcγRII.

Construction of Defined DNA ICs

These data demonstrate that chromatin ICs provide a remarkably potent stimulus for both low-affinity and high- affinity IgG2a-specific B cells. One question raised by these observations is whether there is anything unique about the chromatin present in the culture fluids, presumably released from apoptotic cells, which might distinguish it from mammalian chromatin purified from intact live cells. To begin this analysis, DNA purified from the supernatants of cultured total MRL/+ spleen cells was separated by gel electrophoresis and resolved into a DNA ladder characteristic of internucleosomal cleavage during apoptosis. The chromatin fragments ranged in size from 146 bp mononucleosomes to polynucleosomes greater than 10 kb in length (Figure 1C). Based on the intensity of ethidium bromide staining relative to DNA standards, we estimated that the cell-free supernatant contained approximately 5 μg/ml of DNA. We initially attempted to compare the immunostimulatory activities of chromatin prepared from various sources but found that this approach was limited by the high background resulting from the continuous release of apoptotic chromatin from the responder population. We therefore switched to a strategy that involved the synthesis of double-stranded DNA products that could be haptenated and then bound by monoclonal IgG2a^a anti-hapten antibodies.

We isolated a 660 bp EcoRI/BamHI DNA restriction fragment containing an array of 50 CpG motifs from the

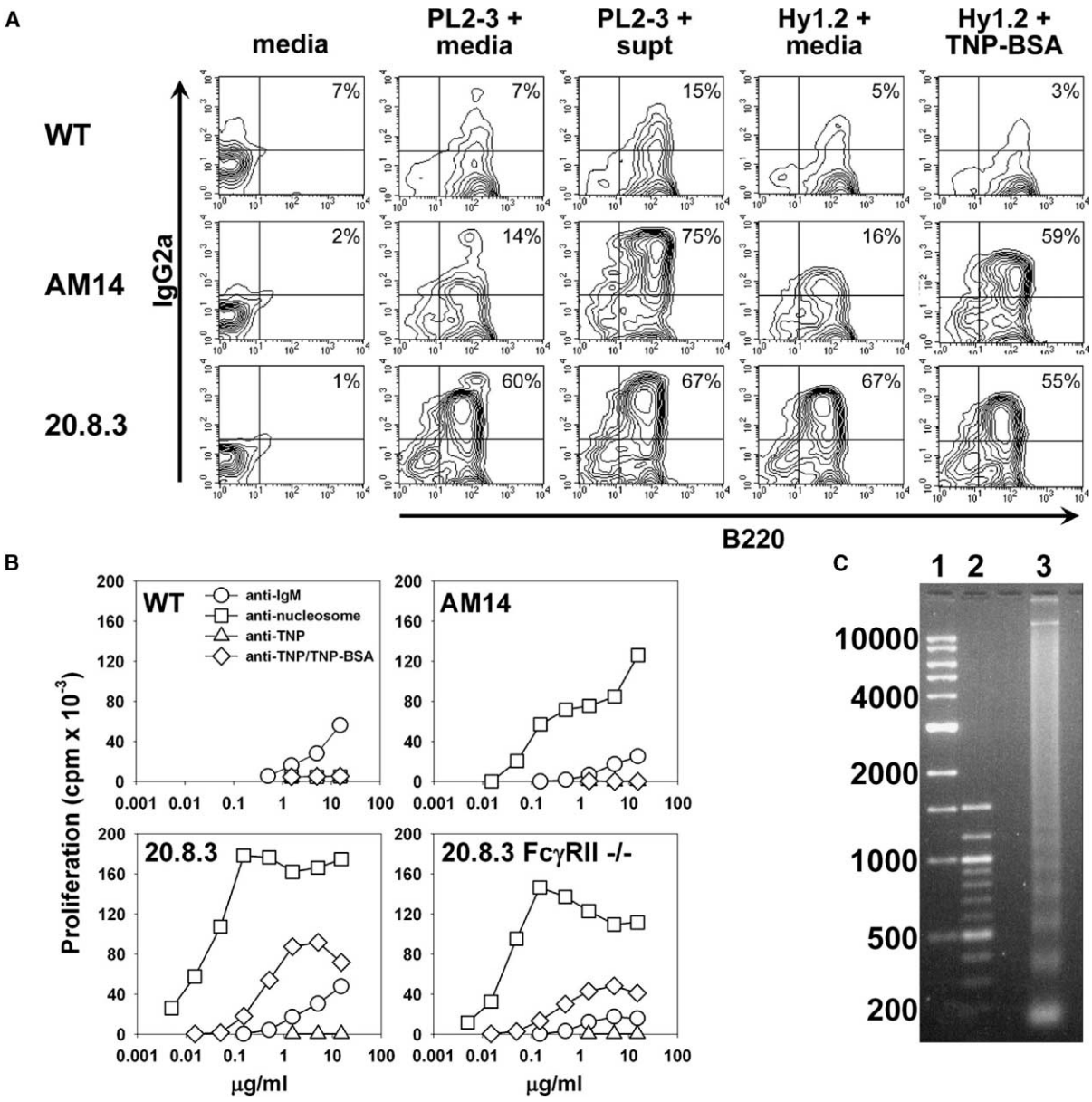

Figure 1. Chromatin-Containing but Not Protein-Containing Immune Complexes Stimulate AM14 B Cells

(A) The anti-nucleosome mAb, PL2-3, or the anti-TNP mAb, Hy1.2, were added either alone or as preformed ICs (PL2-3 + spent cell supernatant; Hy1.2 + TNP-BSA [4:1 mass ratio]) to either wild-type, low-affinity RF (AM14), or high-affinity RF (20.8.3) splenic B cells. Antibody/IC binding to B cells from WT, AM14, or 20.8.3 mice was detected by staining for both IgG2a and B220.

(B) Proliferation of wild-type, AM14, 20.8.3, and 20.8.3 FcγRII-deficient B cells in response to varying concentrations of anti-IgM F(ab′)$_2$, anti-nucleosome (PL2-3), or anti-TNP (Hy1.2) mAbs, or anti-TNP/TNP-BSA ICs (Hy1.2/TNP-BSA).

(C) Chromatin content of splenic B cell culture supernatants (size standards, lanes 1 and 2; chromatin-associated DNA, lane 3).

plasmid pMCG50 (Krieg et al., 1998). The CpG dinucleotides in this fragment, referred to as CG50, are in an optimal sequence context (GACGTT) to activate murine TLR9. The overhanging 5′ ends of the CG50 fragment were repaired with Klenow polymerase in the presence of 5-(3-aminoallyl)-dUTP (aa-dUTP) and the amine groups on the incorporated aa-dUTP residues were derivatized using succinimidyl ester conjugated nitroiodophenyl (NIP). This procedure added 2 NIP hapten groups to one end of the fragment and 1 NIP hapten group to the other end, resulting in NIP2-CG50-NIP. These fragments were mixed with the anti-NIP mAb, 23, to form DNA-fragment ICs.

Hapten-DNA-Fragment ICs Stimulate AM14 B Cells by a TLR9-Dependent Pathway

We found that anti-NIP/NIP2-CG50-NIP ICs stimulated AM14 B cell proliferation and that proliferation was dependent on the concentration of both the mAb and the NIP-DNA (Figure 2A). Neither the DNA nor the mAb alone

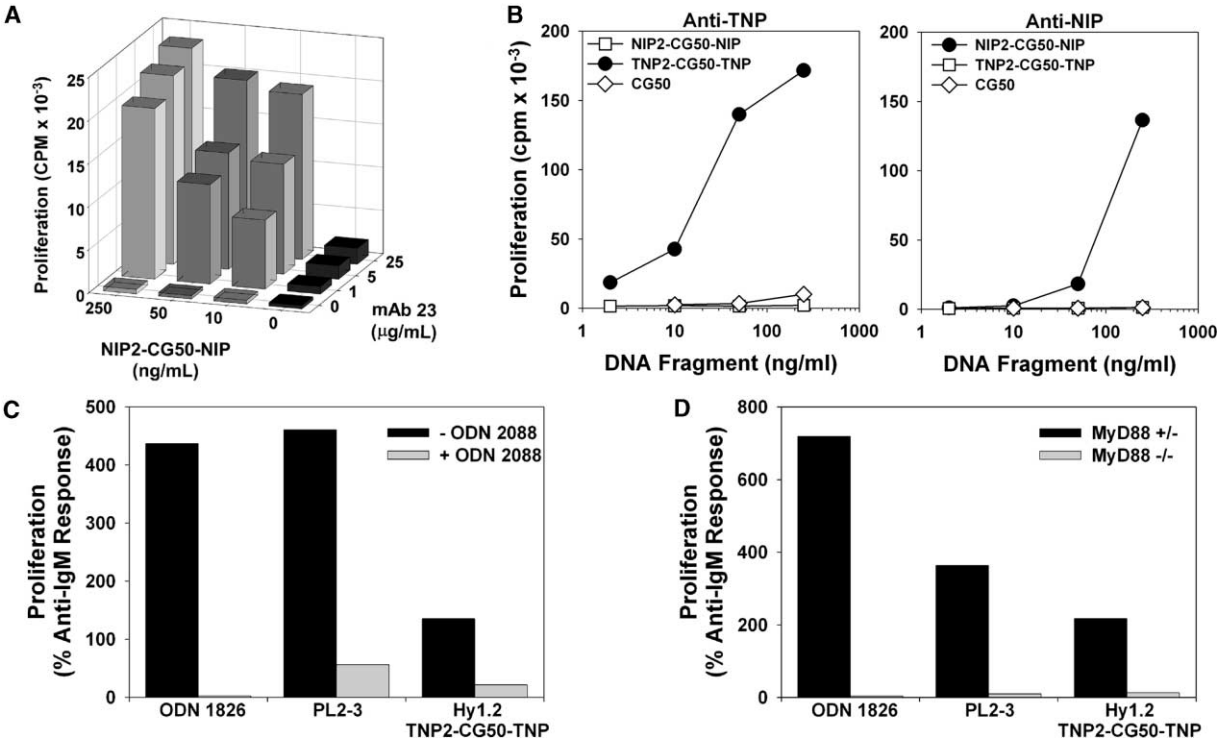

Figure 2. DNA Fragment Immune Complexes Stimulate AM14 B Cells through a TLR-Dependent Mechanism

(A) Proliferation of AM14 B cells in response to ICs formed between varying concentrations of a NIP-conjugated DNA fragment containing 50 immunostimulatory CpG motifs (CG50) and the anti-NIP mAb, 23.

(B) Proliferation of AM14 B cells in response to ICs formed between unconjugated or NIP- or TNP-conjugated CG50 and either the anti-TNP mAb, Hy1.2 (left), or the anti-NIP mAb, 23 (right).

(C) Proliferation of AM14 B cells preincubated for 1 hr with the inhibitory CpG sODN 2088 (4 μg/ml) before the addition of either the stimulatory sODN 1826 (1 μg/ml), PL2-3 mAb, or Hy1.2 mAb and TNP-conjugated CG50 (250 ng/ml). The ability of the various ligands to stimulate proliferation of AM14 B cells was compared with the stimulation induced by anti-IgM $F(ab')_2$.

(D) Proliferation of AM14 B cells from either $MyD88^{-/-}$ or $MyD88^{+/-}$ mice that were stimulated with sODN 1826, or PL2-3, or Hy1.2/TNP2-CG50-TNP ICs.

could effectively stimulate proliferation, showing that the immunostimulatory activity of the ICs was not due to either monovalent antibody-dependent signaling through the BCR or endotoxin contamination of either of the reagents. Moreover, NIP2-CG50-NIP failed to stimulate B cell proliferation when combined with the anti-TNP mAb, Hy1.2, even though Hy1.2/TNP2-CG50-TNP ICs stimulated very effectively. These data indicate that independent engagement of the BCR does not augment the immunostimulatory activity of uncomplexed DNA fragments (Figure 2B).

To determine whether DNA fragment ICs actually activate through TLR9, we stimulated AM14 B cells either in the presence or absence of the TLR9 inhibitory oligodeoxynucleotide (sODN) 2088 (Krieg et al., 1998). We found that sODN 2088 inhibited DNA fragment IC-induced proliferation by ~80%, similar to the level of inhibition obtained when the cells were stimulated with PL2-3/chromatin ICs (Figure 2c). As expected, 2088 inhibited the response to the immunostimulatory CpG sODN, 1826, and had no effect on the response to crosslinking of the BCR by anti-IgM (Leadbetter et al., 2002; data not shown). We also asked whether MyD88 was required for the response to these fragment ICs by comparing the responses of AM14 $MyD88^{+/-}$ and AM14 $MyD88^{-/-}$ B cells. DNA fragment ICs were potent activators of the $MyD88^{+/-}$ cells but they failed to activate the $MyD88^{-/-}$ cells (Figure 2d). The $MyD88^{-/-}$ cells also failed to respond to either sODN 1826 or to PL2-3/chromatin ICs. As expected, they responded normally to anti-IgM $F(ab)'_2$ fragments, indicating that their ability to proliferate in response to BCR crosslinking was not impaired.

DNA Fragment ICs Can Stimulate without Extensive Crosslinking of the BCR

Together the above experiments demonstrate that BCR-mediated uptake of DNA fragments that would otherwise remain extracellular facilitates stimulation of AM14 B cells in a TLR9-dependent fashion. However, the exact role of the BCR in this process is unclear. Does the BCR simply serve as a shuttle between the plasma membrane and TLR9, or does crosslinking of the BCR actually contribute to the activation process? Although the CG50 fragment could at most have a coupling ratio of 3 hapten groups/molecule, in theory it should still be possible to form multivalent complexes. To examine the role of BCR crosslinking more rigorously, we prepared CG50 fragments conjugated at only one end with a single TNP group (TNP-CG50). The stimulatory activity of the mono-

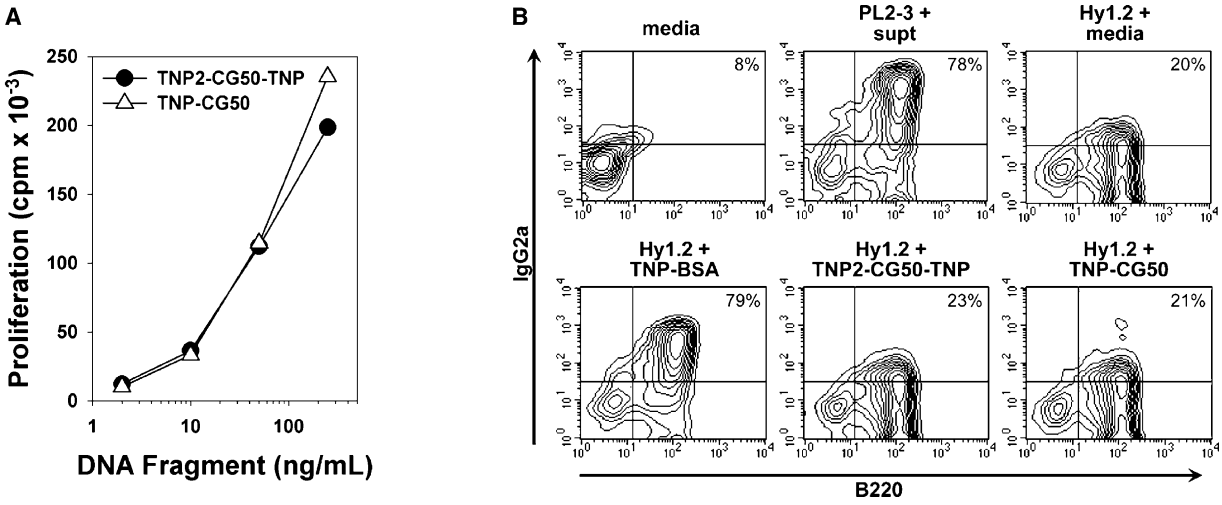

Figure 3. Monohaptenated DNA Fragment Immune Complexes Stimulate AM14 B Cells

(A) Proliferation of AM14 B cells in response to ICs formed between Hy1.2 and varying concentrations of TNP-conjugated CG50 DNA fragments containing either a single (TNP-CG50) or three (TNP2-CG50-TNP) hapten groups.

(B) Binding of preformed chromatin ICs (PL2-3 + spent cell supernatant), monomeric IgG2a (Hy1.2 + media), protein ICs (Hy1.2 + TNP-BSA), and DNA fragment ICs (Hy1.2 + TNP2-CG50-TNP or Hy1.2 + TNP-CG50; each containing 250 ng/ml DNA), to AM14 B cells detected by staining for both IgG2a and B220.

substituted CG50 was then compared to the trisubstituted CG50 described above (TNP2-CG50-TNP). We reasoned that if crosslinking of the BCR were required, then the activity of the TNP-CG50 fragment ICs should be substantially less than that of the TNP2-CG50-TNP fragment ICs. However, we found that the mono- and trisubstituted fragments elicited comparable responses (Figure 3A), consistent with the premise that BCR crosslinking is not required for BCR-mediated enhancement of proliferation by these ligands.

Moreover, flow cytometry showed that both the monosubstituted and the trisubstituted fragment ICs were inefficiently bound by the low-affinity AM14 B cells, suggesting that neither was likely to induce extensive receptor crosslinking (Figure 3B). In the same experiment, comparable levels of IgG2a incorporated into either chromatin ICs or protein ICs were much more effectively bound by these B cells. These data indicated that the DNA component of the fragment ICs was a more important determinant of their stimulatory activity than their ability to crosslink the BCR. However, we did consistently find that "natural" chromatin ICs were approximately 2- to 5-fold more stimulatory than the DNA fragment ICs (Figure 2C); the difference may reflect the contribution of BCR-initiated signaling events that are a consequence of a higher valency of the chromatin ICs.

DNA Sequence and Methylation Are Critical Factors in Fragment IC Activation

Given the paramount role of the DNA component, it was important to determine whether the DNA sequence contributed to the stimulatory capacity of the fragment IC. To evaluate the role of CpG motifs, we compared the activities of ICs formed with three different TNP-haptenated DNAs. These included either (1) the 660 bp TNP2-CG50-TNP fragment; (2) a 726 bp fragment derived from the promoter region of HIV-1 that contained 25 CpG dinucleotides, 12 of which are flanked by a 5′ C or a 3′ G and are therefore predicted to be either poorly immunostimulatory or perhaps inhibitory [TNP2-HIV(CG+)-TNP]; and (3) a 629 bp fragment derived from the HIV-1 gag gene that contained no CpG dinucleotides [TNP2-HIV(CG–)-TNP]. Only the CG50 fragments induced a strong proliferative response (Figure 4A). Although ICs formed with both of the HIV-1-derived DNAs induced modest AM14 B cell proliferation at high concentrations, they were at least 100-fold less active than the fragment containing canonical CpG motifs. These findings demonstrate that sequence is a critical factor with regard to the stimulatory capacity of both fragment ICs and chromatin IC.

The CG50 fragments were isolated from plasmids grown in a dam^-/dcm^- strain of *E. coli* and all the cytosines and adenosines were therefore unmethylated. To determine whether hypomethylation was necessary for stimulatory activity, we methylated the TNP2-CG50-TNP fragment in vitro with the CpG methylase, SssI. This procedure effectively eliminated hypomethylated CpGs, as shown by the complete resistance of these fragments to the restriction endonuclease HpyCH4IV (Figure 4B). We found that methylation of the fragment completely blocked its ability to stimulate proliferation (Figure 4B). This finding confirms that hypomethylated, CpG-containing DNAs are a necessary component of the immunostimulatory DNA fragment ICs. Importantly, the HIV-1 and CG50 DNA fragment ICs all bound to AM14 B cells equivalently, indicating that differences in their immunostimulatory activity were not due to differences in their ability to bind to the BCR (Figure 4C).

Since we found that anti-hapten mAbs could augment the immunostimulatory activity of haptenated CG50 DNA fragments, we asked whether they could also augment the activity of haptenated, restriction enzyme digested murine DNA. We found that ICs formed between

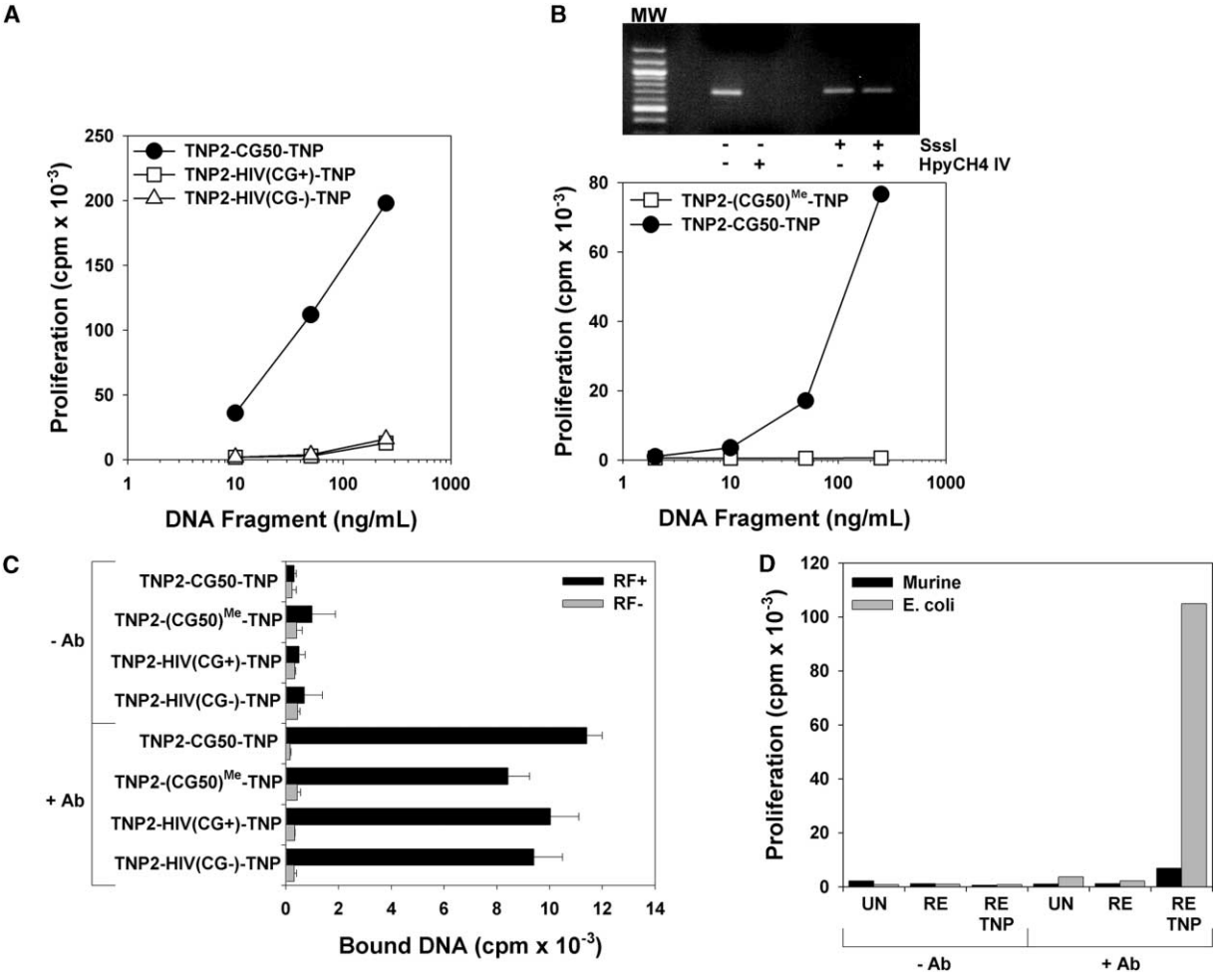

Figure 4. DNA Sequence and Methylation Are Critical Factors in DNA Fragment Immune Complex Activation of AM14 B Cells

Proliferation of AM14 B cells in response to ICs formed between Hy1.2 and varying concentrations of either TNP-conjugated CG50 DNA (TNP2-CG50-TNP), a TNP-conjugated DNA fragment containing 25 noncanonical CpG motifs (TNP2-HIV(CG+)-TNP), or a TNP-conjugated DNA fragment lacking CpG dinucleotides (TNP2-HIV(CG-)-TNP). (B) Digestion of unmethylated or in vitro methylated (SssI) TNP-conjugated CG50 DNA with the methylation sensitive restriction endonuclease, HpyCH4 IV (top). Proliferation of AM14 B cells in response to ICs formed between Hy1.2 and varying concentrations of either unmethylated or methylated TNP-conjugated CG50 DNA (bottom). (C) Binding of ^{32}-P labeled TNP-conjugated DNA fragments (250 ng/ml) to either AM14 RF^+ or RF^- B cells either alone or as ICs with the anti-TNP mAb, Hy1.2. (D) Proliferation of AM14 B cells in response to undigested (UN), DdeI digested (RE), or DdeI digested and TNP-conjugated mouse or *E. coli* DNA (RE TNP) (all at 250 ng/ml), either alone (-Ab) or as ICs with Hy1.2 (+Ab) was measured by ^{3}H-thymidine incorporation.

TNP-conjugated, DdeI digested mouse genomic DNA fragments and Hy1.2 stimulated AM14 B cell proliferation approximately 10-fold above the media control. In contrast, similar ICs formed using *E. coli* DNA fragments stimulated proliferation approximately 160-fold (Figure 4D). Importantly, neither mouse nor *E. coli* DNAs at the concentrations used (250 ng/ml) were active in the absence of antibody. These results indicate that mouse genomic DNA is only weakly immunostimulatory even when it is taken up by cells via the BCR and suggest that certain features of apoptotic chromatin must render this form of autoantigen particularly immunogenic.

DNA Fragments Can Activate Low-Affinity B Cells Directly

To determine whether low-affinity B cells, other than AM14 B cells, can be activated by the haptenated CG50 DNA fragment, we examined its effect on B cells from two additional Tg mouse lines. These mice, VH186.2 and VH186.2L, express heavy chain Tgs that pair with endogenous λ chains to form NIP/NP-specific receptors of medium or low affinity, respectively. Similar to our findings with AM14 B cells, we found that splenic B cells from both VH186.2 and VH186.2L proliferated in response to NIP2-CG50-NIP fragments but not to NIP-BSA (Figure 5A). It therefore appears that low-affinity hapten-specific B cells can also be induced to proliferate by antigens capable of engaging TLR9. Thus stimulation of B cells by BCR-mediated TLR9 ligand delivery is a general mechanism not limited to autoreactive specificities such as RF.

Fragment ICs Stimulate B Cells from 3H9 DNA-Reactive B Cells

The ability of DNA fragments to so effectively promote the activation of AM14 IgG2a B cells as well as NIP-

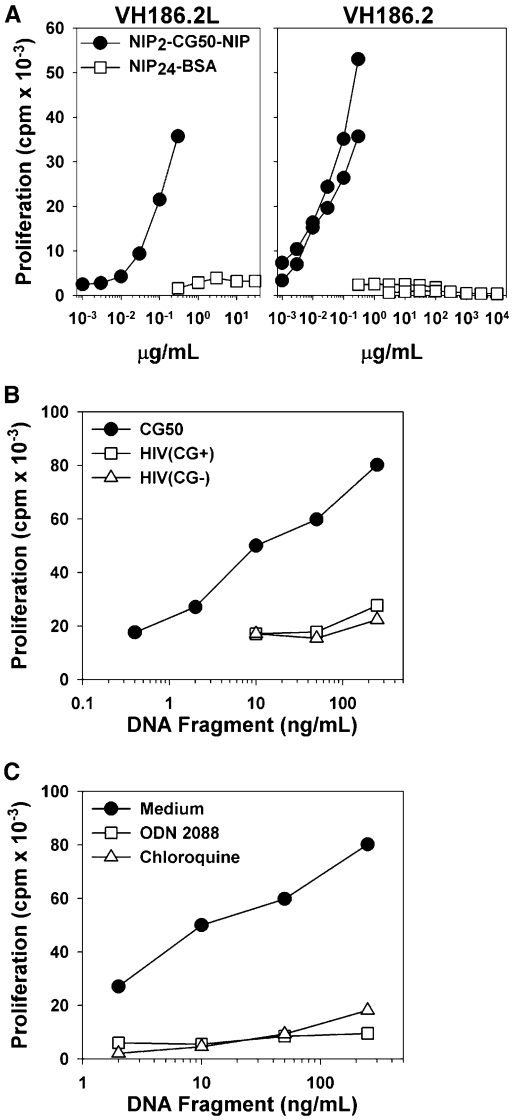

Figure 5. CG50 DNA Fragments Efficiently Stimulate Low-Affinity Hapten-Specific and DNA-Reactive B Cells

(A) Low-affinity (VH186.2L) (1 of 2 comparable experiments) and moderate-affinity (VH186.2) (two of three comparable experiments) hapten-reactive B cells were stimulated with varying concentrations of NIP2-CG50-NIP or NIP-BSA.

(B) 3H9 B cells were stimulated with varying concentrations of the DNA fragments CG50, HIV-(CG+), and HIV-(CG-).

(C) 3H9 B cells were stimulated with varying concentrations of CG50 in the presence or absence of sODN 2088 or chloroquine.

specific B cells raised the possibility that a similar TLR9-dependent process could be involved in the activation of DNA-reactive B cells. To test this possibility, we isolated B cells from anti-DNA 3H9 Tg mice and cultured them with CG50, HIV(CG+), and HIV(CG-) fragments. Proliferation was observed only in the CG50 stimulated cultures (Figure 5B). To determine whether this response was TLR9 dependent, the 3H9 cells were stimulated with CG50 in the presence of either chloroquine or sODN 2088, two inhibitors of the TLR9 signaling pathway. Both chloroquine and sODN blocked the response of the 3H9 cells to CG50 (Figure 5C). Notably, the level of spontaneous proliferation (in the absence of fragments) in the 3H9 cultures was consistently higher than that observed with either the VH186.2 or AM14 B cells; background proliferation was also reduced in the chloroquine and sODN 2088 cultures. This elevated background is presumably due to direct stimulation of chromatin-reactive cells in the population by the chromatin present in the spent culture fluid. These results extend the BCR/TLR9 paradigm to the activation of DNA reactive B cells.

Stimulation of B Cells by DNA-Containing ICs Can Be Blocked by Cyclosporin A

The above studies established a critical role for the BCR in the transport of autoantigen to TLR9. However, they did not address the potential contribution of BCR engagement to the signaling cascade that leads to AM14 or 3H9 proliferation. Cyclosporin A (CsA) has been shown to prevent the BCR crosslinking-induced activation of NFAT required for cellular proliferation (Dongworth and Klaus, 1982; Rui et al., 2003). Consistent with this finding, CsA blocked the AM14 B cell response to anti-IgM $F(ab)'_2$ crosslinking by ~96% and had absolutely no effect on the AM14 B cell response to CpG 1826 (Figure 6A). If the BCR served only as a conduit to TLR9 in the chromatin and/or fragment IC-stimulation of AM14 cells, then that response should be unaffected by CsA. However, CsA consistently blocked the AM14 response to chromatin ICs by 60%–80% and blocked the AM14 response to the fragment ICs by 40%–50%. Chromatin ICs bind more avidly to AM14 B cells than fragment ICs (Figure 3B) and have a lower frequency of CpG motifs than the fragment ICs. Therefore it is not surprising that the AM14 response to chromatin ICs is somewhat more sensitive to CsA than the response to fragment ICs.

A similar pattern was observed with the fragment-reactive cells present in the 3H9 population. CsA completely blocked the response of 3H9 B cells to anti-IgM crosslinking, had little to no effect on the 3H9 response to CpG 1826, or the lipopeptide Pam3Cys, but blocked the 3H9 response to CG50 by approximately 60% (Figure 6B). Together, these data indicate that the AM14 response to ICs containing either mammalian DNA or defined DNA fragments results from signals emanating from both TLR9 and the BCR. However, the contribution of the BCR to signaling events can vary depending on the extent of BCR crosslinking and the density of CpG motifs.

Discussion

We previously reported that ICs formed between IgG2a[a] mAbs and apoptotically released chromatin, present in spent culture supernatants, induced the TLR9-dependent proliferation of AM14 IgG2a-reactive RF B cells. However a number of key issues pertaining to the actual mechanism of chromatin IC-mediated activation of AM14 B cells were left unresolved. The first question was the actual composition of the chromatin portion of the IC ligand. Vertebrate DNA is generally considered a poor activator of TLR9 compared to its putative natural ligand, hypomethylated CpG-containing bacterial DNA. Moreover, naked vertebrate genomic DNA, even when extensively demethylated, does not activate B cells and can actually inhibit activation by bacterial DNA (Krieg

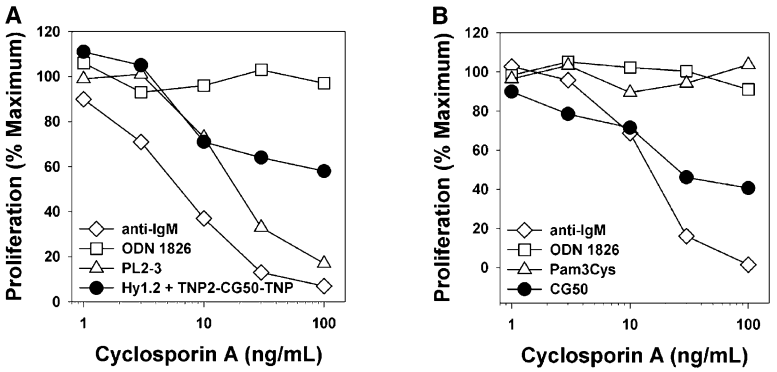

Figure 6. Cyclosporin A Partially Blocks the Proliferation of Autoreactive B Cells Stimulated by Chromatin or DNA Fragment IC

(A) AM14 B cells were stimulated with anti-IgM F(ab')$_2$, sODN 1826, PL2-3, or DNA-fragment ICs in the presence of 1-100 ng/ml CsA. (B) 3H9 B cells were stimulated with anti-IgM F(ab')$_2$, sODN 1826 (1 μg/ml), Pam3Cys (10 μg/ml), or CG50 (250 ng/ml) in the presence of 1–100 ng/ml CsA. Data are representative of 5 (A) and 2 (B) independent experiments and are presented as the percentage of the response to the same stimuli in the absence of CsA. Total ^{3}H-thymidine incorporation (cpm) obtained in the absence of CsA for (a) are anti-IgM F(ab')$_2$ (80,000), sODN 1826 (168,000), PL2-3 (137,000), or DNA-fragment ICs (134,000) and for (b) are anti-IgM F(ab')$_2$ (168,000), sODN 1826 (372,000), Pam3Cys (372,000), or CG50 (111,000).

et al., 1995; Messina et al., 1991). One explanation for the poor stimulatory activity of mammalian DNA is the low frequency of CG dinucleotides. CpG motifs are found in vertebrate genomes at approximately one fourth of the expected random rate and those that are present tend to be methylated and flanked by a 5′ C residue and a 3′ G residue (Karlin et al., 1994), a sequence context that is poorly immunostimulatory (Krieg, 2002). In addition, mammalian DNA may contain regions that specifically block TLR9 activation (Lenart et al., 2001). Therefore, the link between mammalian DNA, TLR9, CpG motifs, and the activation of autoreactive B cells, established by this report, is quite remarkable.

How is it then that mammalian chromatin becomes such an effective ligand in the context of BCR-mediated delivery to TLR9? The data do not support a mechanism involving loss of TLR9 stringency for CpG motifs (Wang and Krieg, 2003). A more likely explanation is that apoptotic chromatin is enriched in hypomethylated stimulatory CpG motifs. The mouse genome is estimated to contain approximately 37,000 CpG islands that include clusters of hypomethylated CpG dinucleotides (Cross et al., 1997). These islands are primarily found in the promoter regions of genes (Cross and Bird, 1995; Gardiner-Garden and Frommer, 1987) and may be preferentially released by the nucleases activated during apoptosis (Krystosek, 1999). The chromatin present in culture fluids may therefore be dramatically enriched in CpG DNA. Notably, anti-DNA/DNA ICs isolated from the serum of SLE patients have been found to have higher GC content than total human DNA (Sano and Morimoto, 1982).

Another possible explanation is that unselected mammalian DNA may have sufficient CpG content to activate B cells as long as it is efficiently delivered to a TLR9-containing compartment. Our data on mammalian genomic DNA fragment ICs (Figure 4D) discount this possibility. However, the importance of delivery was emphasized by the complete inability of either CG50 fragments or relatively low concentrations of *E. coli* DNA fragments to activate AM14 B cells in the absence of a "shuttle" antibody, despite their high content of stimulatory CpG motifs. TLR9 is sequestered away from the plasma membrane in a cytoplasmic compartment (Heit et al., 2003). Although short synthetic sODNs such as CpG 1826 appear to reach TLR9 without an antibody escort, it seems that the BCR is absolutely required to direct conventional dsDNA fragments to the appropriate endosome/lysosome compartment where TLR9 is subsequently engaged (Ahmad-Nejad et al., 2002). Notably, higher concentrations of *E. coli* DNA have been shown to activate B cells in the absence of such an antibody-dependent shuttle mechanism (Messina et al., 1991; Krieg, 2002).

Others have found that the stimulatory activity of mammalian DNA can be markedly enhanced by transfection protocols that deliver DNA to the cytoplasm (Ishii et al., 2001; Zhu et al., 2003). Whether this effect is due to enhancement of the overall level of uptake, selective targeting to a particular cellular location, or artifacts resulting from the transfection procedure is still unclear. Transfection of macrophages or dendritic cells with mammalian DNA or synthetic polydeoxynucleotides can induce the increased expression of both MHC class I and class II molecules as well as various proteins involved in antigen processing and costimulation. For the most part, these affects appear to be independent of both DNA sequence and CpG methylation status (Ishii et al., 2001; Zhu et al., 2003). However, only unmethylated bacterial DNA could induce these same cells to make the cytokines IL-6 and IL-12. By comparison, our findings demonstrate that DNA sequence and CpG methylation are critical factors in the ability of the DNA fragment ICs to activate AM14 B cell proliferation. The most likely explanation for these differences is that the transfected DNAs are at least partially activating the cells through a TLR9-independent pathway. The roles of sequence and methylation status have also been examined in experimental models involving the coactivation of B cells by synthetic ODN and suboptimal concentrations of BCR ligands. In some experiments, only hypomethylated CpG ODN appear to synergize with BCR ligands (Krieg et al., 1995), while in others, methylated CpG or even non-CpG ODN can effectively costimulate B cells (Goeckeritz et al., 1999; Wang and Krieg, 2003). It will be important to determine the role of TLR9 in these particular protocols, by using TLR9 specific inhibitors or appropriately gene-targeted cell populations.

Our findings further indicate that DNA fragment ICs can activate B cell proliferation in the apparent absence of BCR crosslinking. Monohaptenated DNA fragment ICs stimulated AM14 B cells as well as trihaptenated fragment ICs. Moreover, binding of the DNA fragment ICs to AM14 B cells was barely detectable by flow cytometry, in contrast to the enhanced binding observed for multivalent IgG2a protein and chromatin ICs. Studies from Pierce and colleagues have indicated that the BCR continuously cycles from the cell surface to the peptide loading compartment, even in the absence of receptor crosslinking (Song et al., 1995), and it is reasonable to assume that a similar process may mediate the transport of DNA fragments from outside the cell to the TLR9-associated compartment. However, in the case of conventional foreign proteins, this mode of antigen internalization is not sufficient to induce proliferation, as documented in the current report by the failure of monomeric IgG2a to stimulate even the high-affinity 20.8.3 B cells to proliferate.

The inhibitory effect of CsA on the AM14 response to both chromatin and fragment ICs, but not direct TLR ligands, suggests that the BCR serves as more than just a conduit, and in some way contributes to the signaling cascade. It appears that the contribution of the BCR to the activation process can vary depending on the extent of BCR engagement and the density of CpG motifs. Chromatin ICs bind more avidly to AM14 B cells than fragment ICs (Figure 3B), and have a lower frequency of CpG motifs. Therefore it is not surprising that the AM14 response to chromatin ICs is somewhat more sensitive to CsA than the response to fragment ICs. Therefore, we propose that chromatin ICs generate two independent signals when they bind to AM14 B cells: a partial signal through IgG2a engagement of the low-affinity BCR and a suboptimal TLR9 signal mediated by the relatively sparse hypomethylated CpG motifs. The strong proliferative response of the B cells therefore results from the combined signals generated from both the BCR and TLR9, and possibly other receptors such as cellular DNA or nucleosome receptors. This synergy may reflect sequential events that begin at the plasma membrane and then further develop at the endosome/lysosome membrane. Alternatively, the BCR and TLR9 may physically interact at the membrane of the TLR9-associated compartment. The demonstration that hapten-conjugated CG50 DNA fragments can directly stimulate both moderate and low-affinity NP-specific B cells under conditions where NP-BSA does not, further supports our premise that BCR/TLR9 coengagement is a generally effective mechanism for the activation of low affinity B cells. It also raises the possibility that this is a general mechanism for foreign antigen responses in vivo, in situations involving opsinized or intracellular bacteria.

AM14 B cells have served as a useful prototype for low-affinity RF B cells, and have provided a framework for understanding the connection between chromatin-associated autoantigens and TLR9. However, in the context of systemic disease, chromatin-specific autoantibodies are needed before it is possible to form chromatin-containing ICs, and such antibodies, of necessity, come from chromatin-reactive B cells. A major insight from the current report is that the principle established for AM14 cells applies directly to such chromatin-reactive B cells.

The original 3H9 hybridoma cell line was derived from a diseased MRL/lpr mouse as an IgG2a/Vκ4 DNA-reactive antibody (Shlomchik et al., 1987). It was subsequently shown that the 3H9 heavy chain can pair with a variety of endogenous light chains to generate antibodies reactive with both single-stranded and double-stranded DNA (Radic et al., 1991). 3H9+ ssDNA-reactive antibodies can be isolated from hybridoma cell lines derived from nonautoimmune prone 3H9 Tg mice (Roark et al., 1995). Moreover, wild-type mice also contain dsDNA-reactive 3H9λ B cells that can be detected at the T/B interface of the spleen by immunofluorescence (Mandik-Nayak et al., 1997). Thus it is reasonable to assume that low-affinity DNA-reactive B cells are present in the normal repertoire of B6 3H9 heavy chain only mice. It was therefore not surprising that B cells from B6 3H9 heavy chain Tg mice can be stimulated robustly by the same nonhaptenated CG50 fragments that completely failed to stimulate AM14 B cells. 3H9 H Tg mice also have anergic dsDNA-reactive B cells. It is not clear whether these, too, can be stimulated to proliferate by BCR/TLR9 cosignals; if so, this would be another mechanism of breaking tolerance. Studies are in progress to further characterize the 3H9 CG50 reactive cells and to confirm the importance of TLR9 to the in vivo development of systemic disease.

Experimental Procedures

Mice

AM14 RF$^+$ and RF$^-$ littermates were obtained from crosses between MRL or Balb/c AM14 H chain and MRL Vκ8 light chain Tg mice. The high-affinity IgG2a-reactive 20.8.3 mice and 20.8.3 FcγRII$^{-/-}$ were identified by PCR as described previously (Wang and Shlomchik, 1997). The anti-NP Tg line VH186.2 (previously referred to as (m+s)Ig-Tg) has been described previously (Hannum et al., 2000). VH186.2L was made with a similar construct, except that the Vh region was recovered from a GC cell that had 3 mutations in the Vh. One of these resulted in a glycine replacement of histidine at codon 50 that in turn resulted in an $\sim$20-fold reduction in the affinity for NP (Dal Porto et al., 1998, 2002). B6 mice expressing the 3H9 heavy chain transgene were generously provided by Drs. E. Paul and M. Carroll (Harvard Medical School).

mAbs

Monoclonal IgG2a antibodies specific for nucleosome (PL2-3) (Monestier and Novick, 1996), TNP (Hy1.2) (Hannum et al., 2000), or NP/NIP (23) (Wolfowicz et al., 1988) were affinity purified under sterile conditions and determined to be endotoxin free using the Limulus amebocyte lysate assay (BioWhittaker) (less than 0.3 EU/ml).

Plasmids

The plasmid pMCG-50, containing 50 copies of a murine CpG immunostimulatory sequence, was provided by Dr. A. Krieg (Coley Pharmaceuticals). The murine CpG sequences were subcloned into pLITMUS-29 to form pLIT-CG50.1. An HIV-1 sequence containing 25 CpG dinucleotides (nucleotides 112-838 of pNL4-3) predicted to be nonimmunostimulatory was also subcloned into pLITMUS-29 to form pLIT-HIV(CG+). An HIV-1 sequence free of CpG dinucleotides (nucleotides 931-1560 of pNL4-3) was cloned into pUC18 to form pUC-HIV(CG-). The DNA fragments contained in these plasmids were all flanked by an EcoRI site on one end and a BamHI site on the other end. All plasmids were propagated in the dam$^-$/dcm$^-$ *E. coli* strain GM2163.

Hapten-Conjugated DNA Fragments

Plasmids were digested with BamHI and EcoRI and the resultant CG50 fragments were purified and their overhanging 5′ ends were filled with Klenow polymerase in the presence of 5-(3-aminoallyl) dUTP (aa-dUTP) (Molecular Probes, Eugene, OR). The filled in fragments included two primary amine-containing bases at the EcoRI end and one at the BamHI end. Radiolabeled DNA fragments were made by including α-^{32}P-dCTP in the fill-in reaction. All fragments were labeled to comparable specific activities. Some DNA fragments were labeled exclusively at the EcoRI end by excluding dCTP and dGTP from the Klenow reaction. Other fragments were labeled exclusively at the BamHI end by digestion with BamHI and filling in the overhang with dNTPs including aa-dUTP, followed by digestion with EcoRI and filling in the overhang in the absence of aa-dUTP. To label with the haptens TNP, NIP or NP, amine–containing DNA fragments were coupled with succinimidyl ester forms of the haptens (BioSearch Technologies; Novato, CA). Haptenated DNAs were purified using the Qiaquick PCR purification kit. Mouse genomic DNA was isolated from the spleens of MRL mice and *E. coli* genomic DNA was isolated from lyophilized Strain B (Sigma). Endotoxin was removed from the *E. coli* DNA using a Qiagen Endo-free maxi kit. Total genomic DNAs were digested with DdeI. The 5′ overhangs were repaired in the presence of aa-dUTP and hapten groups added as described. The digested DNAs ranged in size from 100–3000 bp. All DNAs were endotoxin-free.

Methylation of Hapten-Labeled DNA Fragments

Hapten-labeled DNA fragments were methylated at their CpG residues using M.Sss I CpG methylase. The degree of methylation was determined by digestion with the methylation sensitive restriction enzyme, HpyCH4 IV.

B Cell Activation

B cells were positively selected from spleen cell suspensions using B220 MicroBeads (Miltenyi Biotech, Auburn, CA). Cells were cultured as described previously (Leadbetter et al., 2002). DNA fragments, mAbs, F(ab′)$_2$ goat anti-mouse IgM (15 μg/ml, Jackson ImmunoResearch Laboratories) or preformed ICs were diluted in RPMI 1640 and added directly to the cultures. DNA fragment ICs and protein ICs were made by incubating the anti-hapten antibodies with haptenated DNA fragments or haptenated-BSA in RPMI 1640 for 2–3 hr at 37°C; complexes were then kept on ice for 1–2 hr until they were added to the B cells. PL2-3 was used at 5 μg/ml and Hy1.2 or 23 were used at 25 μg/ml unless indicated otherwise. Cells were cultured under standard conditions for 30–48 hr, pulsed with ^{3}H-thymidine, and then collected with a 96-sample harvester (Brandel, Gaithersburg, MD); thymidine incorporation during the last 6–16 hr was measured on a 1450 Microbeta Jet Liquid Scintillation Counter (Perkin Elmer, Boston, MA). The sODNs 1826 and 2088 (Krieg et al., 1998) were purchased from Oligos Etc. Data are generally presented as the mean of triplicates with standard deviations $<$10% of total. In the inhibition experiments, chloroquine, cyclosporin A, and ODN 2088 were diluted in RPMI 1640 and preincubated with the splenic B cells for 15–60 min at 37°C before stimuli were added to the cultures.

Detection of IC Binding

Splenic B cells were resuspended in 5 μg/mL mAb 2.4G2 diluted in HBSS and incubated on ice for 15 min to block FcγRII/III binding. Anti-nucleosome ICs were formed by incubating 15 μg/ml PL2-3 (anti-nucleosome/anti-histone Ab) in spent supernatant collected from spleen cell suspensions cultured at 3–5 $\times$ 10^{6} cells/ml for 24–48 hr in RPMI 1640. ICs or IgG2a mAbs were added to the B cells in 50 $\mu\lambda$ of RPMI 1640 and incubated on ice for 45–60 min. Unbound ICs and mAbs were washed away with HBSS. Cell surface-bound ICs and mAbs were detected by staining with 5 μg/ml biotin-conjugated F(ab′)$_2$ goat anti-mouse IgG2a (Southern Biotechnology Associates, Birmingham, AL) followed by 5 μg/ml FITC-conjugated anti-mouse CD45R/B220 (PharMingen, San Diego, CA) and 20 μg/ml phycoprobe R-phycoerythrin streptavidin (Biomeda, Foster City, CA). Samples were analyzed on a FACScan flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA).

To detect binding of ^{32}P-labeled DNA fragments, AM14 RF^{+} or RF^{-} control B cells were incubated with the DNAs either alone or as ICs with the mAb, Hy1.2, at 4°C for 1 hr. Bound DNA was detected by scintillation counting of washed cell pellets.

Acknowledgments

We thank A. Krieg and R. Weeratna for pMCG-50, E. Paul and M. Carroll for 3H9 transgenic mice, A. Haberman, S. Anderson, and J. William for the VH186.2 and VH186.2L transgenic mice, M. Tessel for technical assistance, and R. Corley for helpful discussions and for reviewing the manuscript. This work was supported by grants AR35320 (A.M.R.), PO1-AI36529 (M.J.S.), and AI43603 (M.J.S.) from the National Institutes of Health and the Arthritis Foundation (A.M.R.).

Received: July 16, 2003
Revised: October 16, 2003
Accepted: November 6, 2003
Published: December 9, 2003

References

Ahmad-Nejad, P., Hacker, H., Rutz, M., Bauer, S., Vabulas, R.M., and Wagner, H. (2002). Bacterial CpG-DNA and lipopsaccharides activate Toll-like receptors at distinct compartments. Eur. J. Immunol. *32*, 1958–1968.

Bauer, S., Kirschning, C.J., Hacker, H., Redecke, V., Hausmann, S., Akira, S., Wagner, H., and Lipford, G.B. (2001). Human TLR9 confers responsiveness to bacterial DNA via species-specific CpG motif recogniton. Proc. Natl. Acad. Sci. USA *98*, 9237–9242.

Cohen, P.L., and Eisenberg, R.A. (1991). Lpr and gld: single gene models of systemic autoimmunity and lymphoproliferative disease. Annu. Rev. Immunol. *9*, 243–269.

Cross, S.H., and Bird, A.P. (1995). CpG islands and genes. Curr. Opin. Genet. Dev. *5*, 309–314.

Cross, S.H., Lee, M., Clark, V.H., Craig, J.M., Bird, A.P., and Bockmore, W.A. (1997). The chromosomal distribution of CpG islands in the mouse: evidence for genome scrambling in the rodent lineage. Genomics *40*, 454–461.

Dal Porto, J.M., Haberman, A.M., Kelsoe, G., and Shlomchik, M.J. (2002). Very low affinity B cells form germinal centers, become memory B cells, and participate in secondary immune responses when higher affinity competition is reduced. J. Exp. Med. *195*, 1215–1221.

Dal Porto, J.M., Haberman, A.M., Shlomchik, M.J., and Kelsoe, G. (1998). Antigen drives very low affinity B cells to become plasmacytes and enter germinal centers. J. Immunol. *161*, 5373–5381.

Dongworth, D.W., and Klaus, G.G. (1982). Effects of cyclosporin A on the immune system of the mouse. I. Evidence for a direct selective effect of cyclosporin A on B cells responding to anti-immunoglobulin antibodies. Eur. J. Immunol. *12*, 1018–1022.

Gardiner-Garden, M., and Frommer, M. (1987). CpG islands in vertebrate genomes: measures and values. J. Mol. Biol. *196*, 261–282.

Goeckeritz, B.E., Flora, M., Witherspoon, K., Vos, Q., Lees, A., Dennis, G.J., Pisetsky, D.S.., Klinman, D.M., Snapper, C.M., and Mond, J.J. (1999). Multivalent cross-linking of membrane Ig sensitizes murine B cells to a broader spectrum of CpG-containing oligodeoxynucleotides motifs, including their methylated counterparts, for stimulation of proliferation and Ig secretion. Int. Immunol. *11*, 1693–1700.

Hannum, L.G., Ni, D., Haberman, A.M., Weigert, M.G., and Shlomchik, M.J. (1996). A disease-related rheumatoid factor autoantibody is not tolerized in a normal mouse: implications for the origins of autoantibodies in autoimmune disease. J. Exp. Med. *184*, 1269–1278.

Hannum, L.G., Haberman, A.M., Anderson, S.M., and Shlomchik, M.J. (2000). Germinal center initiation, variable gene region hypermutation, and mutant B cell selection without detectable immune complexes on follicular dendritic cells. J. Exp. Med. *192*, 931–942.

Heit, A., Maurer, T., Hochrein, H., Bauer, S., Huster, K.M., Busch, D.H., and Wagner, H. (2003). Cutting edge: Toll-like receptor 9 expression is not required for CpG DNA-aided cross-presentation of

DNA-conjugated antigens but essential for cross-priming of CD8 T cells. J. Immunol. *170*, 2802–2805.

Hemmi, H., Takeuchi, O., Kawai, T., Kaisho, T., Sato, S., Sanjo, H., Matsumoto, M., Hoshino, K., Wagner, H., Takeda, K., and Akira, S. (2000). A Toll-like receptor recognizes bacterial DNA. Nature *408*, 740–745.

Ishii, K.J., Suzuki, K., Coban, C., Takeshita, F., Itoh, Y., Matoba, H., Kohn, L.D., and Klinman, D.M. (2001). Genomic DNA released by dying cells induces the maturation of APCs. J. Immunol. *167*, 2602–2607.

Jacobson, B.A., Sharon, J., Shan, H., Shlomchik, M., Weigert, M.G., and Marshak-Rothstein, A. (1994). An isotype switched and somatically mutated rheumatoid factor clone isolated from a MRL-lpr/lpr mouse exhibits limited intraclonal affinity maturation. J. Immunol. *152*, 4489–4499.

Karlin, S., Ladunga, I., and Blaisdell, B.E. (1994). Heterogeneity of genomes: measures and values. Proc. Natl. Acad. Sci. USA *91*, 12837–12841.

Kotzin, B.L. (1996). Systemic lupus erythematosus. Cell *85*, 303–306.

Krieg, A.M. (2002). CpG motifs in bacterial DNA and their immune effects. Annu. Rev. Immunol. *20*, 709–760.

Krieg, A.M., Yi, A.-K., Matson, S., Waldschmidt, T.J., Bishop, G.A., Teasdale, R., Koretsky, G.A., and Klinman, D. (1995). CpG motifs in bacterial DNA trigger direct B-cell activation. Nature *374*, 546–549.

Krieg, A.M., Wu, T., Weeratna, R., Efler, S.M., Love-Homan, L., Yang, L., Yi, A.-K., Short, D., and Davis, H.L. (1998). Sequence motifs in adenoviral DNA block immune activation by stimulatory CpG motifs. Proc. Natl. Acad. Sci. USA *95*, 631–636.

Krystosek, A. (1999). Preferential sites of early DNA cleavage in apoptosis and the pathway of nuclear damage. Histochem. Cell Biol. *111*, 265–276.

Leadbetter, E.A., Rifkin, I.R., Hohlbaum, A.H., Beaudette, B., Shlomchik, M.J., and Marshak-Rothstein, A. (2002). Immune complexes activate autoreactive B cells by co-engagement of sIgM and Toll-like receptors. Nature *419*, 603–607.

Lenart, P., Stunz, L., Yi, A.-K., Krieg, A.M., and Ashman, R.F. (2001). CpG stimulation of primary mouse B cells is blocked by inhibitory oligodeoxyribonucleotides at a site proximal to NF-κB activation. Antisense Nucleic Acid Drug Dev. *4*, 247–256.

Mandik-Nayak, L., Bui, A., Noorchashm, H., Eaton, A., and Erikson, J. (1997). Regulation of anti-double-stranded DNA B cells in nonautoimmune mice: localization to the T-B interface of the splenic follicle. J. Exp. Med. *186*, 1–11.

Messina, J.P., Gilkeson, G.S., and Pisetsky, D.S. (1991). Stimulation of in vitro murine lymphocyte proliferation by bacterial DNA. J. Immunol. *147*, 1759–1764.

Monestier, M., and Novick, K.E. (1996). Specificities and genetic characteristics of nucleosome-reactive antibodies from autoimmune mice. Mol. Immunol. *33*, 89–99.

Plotz, P.H. (2003). The autoantibody repertoire: searching for order. Nat. Rev. Immunol. *3*, 73–78.

Radic, M.Z., Mascelli, M.A., Erikson, J., Shan, H., and Weigert, M. (1991). Ig H and L chain contributions to autoimmune specificities. J. Immunol. *146*, 176–182.

Roark, J.H., Kuntz, C.L., Nguyen, K.A., Caton, A.J., and Erikson, J. (1995). Breakdown of B cell tolerance in a mouse model of Systemic Lupus Erythematosus. J. Exp. Med. *181*, 1157–1167.

Rui, L., Vinusa, C.G., Blasioli, J., and Goodnow, C.C. (2003). Resistance to CpG DNA-induced autoimmunity through tolerogenic B cell antigen receptor ERK signaling. Nat. Immunol. *4*, 594–600.

Sano, H., and Morimoto, C. (1982). DNA isolated from DNA/anti-DNA antibody immune complexes in systemic lupus erythematosus is rich in guanine-cytosine content. J. Immunol. *128*, 1341–1345.

Shlomchik, M.J., Aucoin, A.H., Pisetsky, D.S., and Weigert, M.G. (1987). Structure and fundtion of anti-DNA autoantibodies derived from a single autoimmune mouse. Proc. Natl. Acad. Sci. USA *84*, 9150–9154.

Song, W., Cho, H., Cheng, P., and Pierce, S.K. (1995). Entry of B cell antigen receptor and antigen into class II peptide-loading compartment is independent of receptor cross-linking. J. Immunol. *155*, 4255–4263.

Wang, H., and Shlomchik, M. (1997). High affinity rheumatoid factor transgenic B cells are eliminated in normal mice. J. Immunol. *159*, 1125–1134.

Wang, H., and Shlomchik, M.J. (1999). Autoantigen-specific B cell activation in Fas-deficient rheumatoid factor immunoglobulin transgenic mice. J. Exp. Med. *190*, 639–649.

Wang, Y., and Krieg, A.M. (2003). Synergy between CpG- or non-CpG DNA and specific antigen for B cell activation. Int. Immunol. *15*, 223–231.

William, J., Euler, C., Christensen, S., and Shlomchik, M.J. (2002). Evolution of autoantibody responses via somatic hypermutation outside of germinal centers. Science *297*, 2066–2070.

Wolfowicz, C.B., Sakorafas, P., Rothstein, T.L., and Marshak-Rothstein, A. (1988). Oligoclonality of rheumatoid factors arising spontaneously in lpr/lpr mice. Clin. Immunol. Immunopath. *46*, 382–395.

Yamamoto, S., Yamamoto, T., Shimada, S., Kuramoto, E., Yano, O., Kataoka, T., and Tokunaga, T. (1992). DNA from bacteria, but not from vertebrates, induces interferons, activates natural killer cells, and inhibits tumor growth. Microbiol. Immunol. *36*, 983–997.

Zhu, F.-G., Reich, C.F., and Pisetsky, D.S. (2003). Effect of cytofectins on the immune response of murine macrophages to mammalian DNA. Immunol. *109*, 255–262.