In Situ Studies of the Primary Immune Response to (4-Hydroxy-3-Nitrophenyl)Acetyl

IV. Affinity-dependent, Antigen-driven B Cell Apoptosis in Germinal Centers as a Mechanism for Maintaining Self-tolerance

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Summary

Germinal centers (GCs) are the sites of antigen-driven V(D)J gene hypermutation and selection necessary for the generation of high affinity memory B lymphocytes. Despite the antigen-dependence of this reaction, injection of soluble antigen during an established primary immune response induces massive apoptotic death in GC B cells, but not in clonally related populations of nonfollicular B lymphoblasts and plasmacytes. Cell death in GCs occurs predominantly among light zone centrocytes, is antigen specific, and peaks within 4–8 h after injection. Antigen-induced programmed death does not involve cellular interactions mediated by CD40 ligand (CD40L) or Fas; disruption of GCs by antibody specific for CD40L was not driven by apoptosis and C57BL/6 lpr mice, though unable to express the Fas death trigger, remained fully susceptible to soluble antigen. Single injections of antigen did not significantly decrease GC numbers or average size, but repeated injections during an 18-h period resulted in fewer and substantially smaller GCs. As cell loss appeared most extensive in the light zone, decreased GC cellularity after prolonged exposure to soluble antigen implies that the Ig+ centroblasts of the dark zone may require replenishment from light zone cells that have survived antigenic selection. GC cell death is avidity-dependent; oligovalent antigen induced relatively little apoptosis and GC B cells that survived long exposures to multivalent antigen expressed atypical VDJ rearrangements unlikely to encode high affinity antibody. Antigen-induced apoptotic death in GCs may represent a mechanism for the peripheral deletion of autoreactive B cell mutants much as the combinatorial repertoire of immature B lymphocytes is censored in the bone marrow.

Abbreviations used in this paper: AP, alkaline phosphatase; CD40L, CD40-ligand; CGG, chicken ß-globulin; CVF, cobra venom factor; FDC, follicular dendritic cell; GC, germinal center; HRP, horseradish peroxidase; mlg, membrane Ig; NIP, (4-hydroxy-5-iodo-3-nitrophenyl)acetyl; NP, (4-hydroxy-3-nitrophenyl)acetyl; PALS, periaortial lymphoid sheath; PNA, peanut agglutinin; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labeling.

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of the response, and they persist for ~3 wk (2, 23, 24). V(D)J hypermethylation is not active during the early proliferative phase of GC development; Ig mutations are first observed 7–8 d after immunization (11, 24), coincidentally with increased expression of the B7-2 molecule within the GC light zone (25). Before day 8, GC B cell populations are clonally diverse, and lymphocytes expressing V186.2 rearrangements are not dominant (24). In early (day 4–6) λ1+ GCs, the most frequent VDJ rearrangements contain λ1 exons (24). These gene segments share a high degree of sequence similarity with the V186.2 gene, and they can encode λ1+ antibodies that bind NP (26–28). When expressed by transfection, however, these antibodies generally possess lower affinities for NP/NIP than do those encoded by the canonical VDJ (reference 29 and Dal Porto, J., and G. Kelsoe, unpublished data). By day 8 of the response, these atypical cells become infrequent (~5%) in GCs, presumably as a consequence of antigen-driven competition (24).

An undesirable consequence of V(D)J hypermethylation is the generation of autoreactive lymphocytes (30–32). Although the ability to sustain self-reactive mutants is diminished by the reciprocal oligoclonality of GC T- and B cells (33), we and others have developed experimental models to study the fate of GC B cells that acquire specificity for an abundant self antigen present within the splenic compartment. Recently, Pulendran et al. (34) and Shokat and Goodnow (35) demonstrated antigen-specific cell death in normal and transgenic GC B lymphocytes after the injection of soluble antigen. This cell death was characterized by the rapid DNA fragmentation of apoptosis, but was only slightly impeded or completely resistant to constitutive expression of the antiapoptotic protooncogene bcl-2. While neither of these groups could assign a specific mechanism to GC apoptosis, Pulendran and his colleagues concluded that antigen most likely acted directly on GC B cells. Here we show that in C57BL/6 mice immunized with NP conjugated to chicken γ-globulin (NP12-CGG), GCs arise which contain λ1+ B cells that rapidly undergo apoptosis after the injection of soluble proteins bearing the NIP hapten. Apoptosis was confined to GC B cells, and was antigen specific and avidity dependent. Abrogation of cellular interactions mediated through the CD40 ligand (CD40L) resulted in the rapid loss of splenic GCs by migration rather than by cell death, suggesting that free antigen does not act by disrupting lymphocyte collaboration signals. C57BL/6.MRL.Lpr mice remained susceptible to antigen–induced apoptosis, demonstrating the independence of programmed cell death from the Fas molecule. Interestingly, prolonged administration of soluble antigen resulted in the cellular depletion of GCs by sustained apoptosis and selected for a residual population of atypical, presumably low affinity B cell mutants, reversing the usual direction of B lymphocyte evolution.

Materials and Methods

Antigens and Immunizations. The succinyl anhydride esters of NP or NIP (Cambridge Research Biochemicals, Cambridge, UK) were reacted with CGG (Accurate Scientific, Westbury, CT) or BSA (U.S. Biochemical Corp., Cleveland, OH) as described (2); hapten/protein ratios were determined spectrophotometrically. All mice were immunized with a single i.p. injection of 50 μg NP12-CGG precipitated in alum. Cobra venom factor (CVF) (20 U; Quidel, San Diego, CA) was given i.p. 30 min before the injection of soluble NIP–protein to decomplement mice by C3 depletion. Isoproterenol hydrochloride (Sigma Chemical Co., St. Louis, MO) was injected (150 μg/kg body wt) concurrently with NIP–protein to prevent anaphylactoid reactions. Soluble NIP–protein conjugates were administered 9 d after immunization in a single i.p. injection of 4 mg antigen in a 0.5-ml vol of PBS. Some mice received multiple i.p. injections of soluble NIP12-CGG on day 9; 4 mg protein was injected initially and 2 mg was given 6, 12, and 18 h later. These animals were killed 2 h after the final injection of soluble antigen.

Histology. Spleens were prepared for histology as described (2). Splenic cell populations were visualized by immunocytochemical labeling with antibodies or PNA conjugated with biotin, horseradish peroxidase (HRP), or alkaline phosphatase (AP) (2). Bound HRP and AP activities were located by reacting the labeled section with 3-aminon phenyl carbazole and naphthol AS-MX phosphate/Fast Blue BB (Sigma), respectively. Bound biotinylated antibody or lectin was visualized with HRP or AP conjugates of streptavidin (Southern Biotechnology Associates, Birmingham, AL). PNA–HRP and PNA–biotin were obtained from E–Y Laboratories (San Mateo, CA); biotinylated antibodies specific for mouse IgG or B220 were purchased from Southern Biotechnology and Pharmingen (San Diego, CA), respectively; Ls136, GK1.5, and MR1 antibodies, specific for mouse λ1 light chain, mouse CD4, and mouse CD40L, respectively, were produced in vitro and purified by elution from protein A. Terminal deoxynucleotidyl transferase (Tdt)–mediated dUTP–biotin nick-end labeling (TUNEL) was used to identify apoptotic cell nuclei (36) (Apoptag; Oncor, Gaithersburg, MD). Sections with TUNEL+ cells were counterstained either with hematoxylin and eosin, or were labeled with antibodies or PNA.

TUNEL+ cells were counted at a magnification of 200 by systematic scanning of the entire section. Counts were confirmed by blinded recounts; independent duplicate counts varied by ~15%. Amplification and Sequencing of VDJ DNA from GC B Cells. Cellular material representing ~20 TUNEL+ GC B cells was microdissected from single λ1+ GCs as described and submitted to two rounds of PCR amplification (23, 24) driven by the Pfu thermostable polymerase (7). Amplified VDJ fragments were ligated into the plasmid pBSKII to transform bacteria, and inserts obtained from single bacterial colonies were sequenced in both directions (23, 24). The measured error rate of the Pfu polymerase is 1–2 × 10−6 misincorporations/bp per cycle, resulting in two to three artificial mutations per 100 VDJ clones (7). This frequency is sufficiently low to permit the assumption that all recovered mutations represent in vivo events.

Results

Anaphylactoid Reactions Follow the Injection of Soluble Antigen. Though previously unreported (34, 35), our initial experiments were complicated by frequent and severe anaphylactoid reactions triggered by the formation of immune complexes (37). Injection of equivalent amounts of BSA alone or irrelevant hapten conjugates produced no response. Physiologic shock became obvious within 5 min after the
injection of soluble antigen, and it resulted in ~20% mortality. These reactions persisted even after decomplementation with CVF (38), but they were blocked by administering the β-agonist isoproterenol (39, 40) concurrently with soluble NIP-protein. Administration of CVF and/or isoproterenol had no discernable effect on the splenic histology or numbers and distribution of apoptotic cells in immune control mice or in those receiving soluble NIP-protein (not shown). Thus, subsequent experiments were carried out in mice that had received isoproterenol to minimize the anaphylactogenic effect of immune complexes. Pairwise analysis (Student's t test) of induced apoptosis in each group (antigen, antigen + CVF, antigen + isoproterenol, antigen + CVF, + isoproterenol) revealed no statistically significant ($P > 0.05$) differences, thus data from all experiments have been pooled.

**Histology and Kinetics of Antigen-induced Apoptosis in Germinal Centers.** Spleens of control mice that did not receive soluble NIP-protein exhibited typical and vigorous immune responses with large collections of extrafollicular plasmacytes and prominent PNA+ GCs (Fig. 1 A). Low numbers of apoptotic cells (TUNEL+) were identified within normal GCs (Fig. 1 A), and only rarely within the periaortic lymphoid sheaths (PALS) or splenic red pulp. By day 9 of the primary anti-NP response, collections of lymphoblasts and plasmacytes form large foci of NP/NIP-binding cells with abundant cytoplasmic Ig. These foci are located at the periphery of the PALS, and occasional $\lambda^+$ plasmacytes are scattered throughout the red pulp (2). Although these cells are specialized for antibody production and are excluded from the memory compartment (2, 23, 41), they are clonally related to adjacent GC B cell populations (23). Absence of TUNEL+ cells within the PALS and red pulp suggests that GC apoptosis is solely the consequence of that particular microenvironment. Within GCs, the majority of TUNEL+ cells/nuclei were contained within tingible body macrophages as clumps of two to five cells per nuclei (Fig. 1 A). Some of these apoptotic cells could be colabeled by an antibody specific for the B cell marker, B220 (not shown).

In contrast, after injecting NIP15-BSA, the numbers of TUNEL+ cells increased dramatically in GCs, but not elsewhere in the spleen (Fig. 1 B). Apoptotic cells were most frequent in the GC light zone (Fig. 1 B) and were usually clustered in groups of $\geq 10$ cells (Fig. 1 C). Clustering probably represents engulfment by macrophages, although we could not demonstrate the presence of a tingible body macrophage in every section. Despite this large wave of induced cell death, the numbers and cross-sectional area of GCs remained relatively constant during the 18 h of study, presumably because of the continued migration of mIg+ centroblasts from the dark zone (42).

The apoptosis induced by soluble antigen was rapid; by 3 h after injection, the number of TUNEL+ GC cells rose significantly above background levels, reaching a peak of ~35 apoptotic cells per follicle at 5 h, and eventually declining to normal levels by 18 h (Fig. 2). Antigen-induced apoptosis was sensitive to hapten density. Injection of NIP15-BSA resulted in a maximum increase in TUNEL+ GC cells (~28 apoptotic cells per follicle) that was about one-half of that observed with the more heavily substituted NIP15-BSA. Injection of an equal amount of soluble BSA had no effect on the splenic histology or the frequency of TUNEL+ cells (Fig. 2). Thus, GC cell death was antigen specific and proportional to ligand density.

**Soluble Antigen Does Not Initiate Programmed Cell Death by Disrupting T-B Cell Collaboration.** In GCs, both the B and T cell populations are specific for the immunizing antigen (1-3, 5-7). The effects of NIP-BSA could therefore result from interference in cognate T-B interaction; hapten-specific B cells presenting processed BSA fragments would be

![Figure 1](https://example.com/image.png)

**Figure 1.** Antigen-induced apoptosis in splenic GCs. On day 9 of a primary immune response to NP12-CGG/alum, the spleens of control immune C57BL/6 mice (A) contained well-formed GCs (G) within the lymphoid follicles (F) of the white pulp. These GCs contained low numbers of TUNEL+ cells (arrows), ~20 apoptotic nuclei per follicle, which were generally located distally from the PALS (P). In contrast, 5 h after injecting 4 mg of soluble NIP15-BSA (B), the frequency of TUNEL+ cells in splenic GCs increased significantly, to ~60 per follicle. These apoptotic cells were generally clustered (C), probably because of engulfment by tingible body macrophages. Sections were labeled by the TUNEL method (14) and then counter-stained with hematoxylin and eosin. A and B, X100; C, X400.
unable to collaborate with CGG-specific T cells. To test this possibility, we injected soluble NIP$_{12}$-CGG on day 9 and determined the frequency of TUNEL$^+$ cells as before. Fig. 2 illustrates that NIP$_{12}$-CGG induced apoptosis in GCs with kinetics similar to that caused by NIP$_{15}$-BSA. Indeed, the number of apoptotic cells per follicle in mice treated with NIP$_{15}$-CGG was greater (~70 apoptotic cells per follicle) than that found in mice receiving NIP$_{15}$-BSA. This increase represents death of both NIP- and CGG-specific GC B cells (2), but did not result in the elimination of splenic GCs. Thus, a soluble NIP-protein conjugate that could be recognized by collaborating T cells within the GCs induced apoptotic death as effectively as the heterologous NIP–BSA conjugate. This finding suggests that soluble antigen does not block cognate T cell help, but induces GC B cell death directly, or possibly by interfering with B–FDC interaction.

Additional experiments were performed to test the conclusion that antigen-induced apoptosis is not mediated through T–B cell contact. The CD40L expressed on activated T cells is necessary for the GC reaction (43, 44), and injection of the anti–CD40L antibody, MR1, disrupts established GCs within 24 h (25). To establish if this effect is driven by apoptosis and shares the kinetics of antigen-induced cell death, immunized mice were injected with 300 µg of MR1 antibody instead of soluble antigen. To our surprise, MR1 antibody did not abrogate the GC reaction by inducing programmed cell death, but rather, it appeared to initiate GC B cell migration from the lymphoid follicle. Before the administration of anti-CD40L antibody, GCs were abundant and contained normal levels of apoptotic cells (Fig. 3 A). By 6 h after the administration of MR1, the frequency of apoptotic cells rose modestly, reaching a maximum comparable to that seen early (3 h) after injection of NIP–protein (Figs. 2 and 3 B). Higher doses of MR1 antibody (600 µg) elicited similar migrations (not shown). Thus, disruption of T–B interaction through the CD40L is relatively ineffective in causing GC cell death. Injected MR1 antibody, however, did exert a powerful effect on GC B cells, since PNA$^+$ GCs were virtually absent in the spleens of MR1-treated animals at 12–18 h after injection (Fig. 3 C). This loss did not represent downregulation of the PNA-binding phenotype, since staining with anti-IgD antibody revealed no substantial collections of IgD$^-$, B220$^+$ cells remaining within the lymphoid follicles. This role for CD40L in maintaining GC integrity was unexpected and may represent failure to replenish the dark zone by recirculating centrocytes (3, 45). Although our experiments do not permit a detailed accounting of MR1-induced GC cell migration, at least some IgD$^-$, B220$^+$ migrants enter the PALS. Fas-independent Apoptosis in Germinal Center B Cells. T helper cells can cause apoptotic death by cross-linking the Fas molecules that are expressed on the surface of activated B lymphocytes (46–48). Mice homozygous for the lpr mutation poorly express Fas, and their lymphocytes are resistant to this pathway of programmed cell death (49–51). C57BL/6 mice congenic for the lpr locus were fully susceptible to antigen-induced apoptosis, demonstrating that cell death is independent of Fas-L: Fas cross-linking (17). Interestingly, TUNEL$^+$ cells were present at normal levels in control immune C57BL/6/lpr mice (17).

Figure 2. Kinetics of antigen-induced GC apoptosis in C57BL/6 and C57BL/6/lpr mice. On day 9 of a primary response to NIP$_{12}$-CGG, C57BL/6 mice were injected i.p. with 4 mg NIP$_{15}$-BSA (O) or NIP$_{12}$-CGG (■) plus CVF (20 U) and isoproterenol (150 µg/kg). Control mice either received CVF and isoproterenol alone (■) or 4 mg BSA (●). Mice were killed at the times indicated; BSA controls (●) were studied 6 h after injection. Spleens were prepared for histology, and apoptotic nuclei were labeled as for Fig. 1; TUNEL$^+$ cells were enumerated at ×200 in two to three sections per spleen. Results from four independent experiments (n = 2–11) have been averaged (±SEM). Injection of NIP$_{15}$-BSA (O) or NIP$_{12}$-CGG (■) induced large increases in the numbers of TUNEL$^+$ cells/nuclei per follicle. Apoptosis was elevated slightly above background levels (■) by 3 h after injection, and it reached maximum levels at 5–9 h. Antigen treatment did not result in the loss of splenic GCs, and by 18 h after injection, the frequency of follicular apoptotic cells returned to nearly normal levels. Injection of BSA (●) did not result in increased numbers of TUNEL$^+$ cells, and 6 h after the injection of 300 µg of MR1 antibody (●), only a modest increase in the number of apoptotic cells was observed. Fas$^+$ C57BL/6/lpr mice (▲) were fully susceptible to antigen-induced apoptosis, demonstrating that cell death is independent of Fas-L: Fas cross-linking (17). Interestingly, TUNEL$^+$ cells were present at normal levels in control immune C57BL/6/lpr mice (▼).
Figure 3. The anti-CD40L antibody, MR1, disrupts established GCs by cell emigration. 9 d after primary immunization with NP12-CGG (A), splenic GCs consist of large clusters of PNA+ B cells (blue) that contain scattered collections of apoptotic nuclei (red, arrows) usually within tingible body macrophages. 6 h after injecting 300 μg of MR1 antibody (B), the number of TUNEL+ cells within the GC is modestly increased but otherwise the splenic histology appears unchanged. By 12 h after injection (C), however, only occasional collections of PNA+ cells remain in the lymphoid follicles. Sections were labeled by the TUNEL method (14) to identify apoptotic cells, and they were then incubated with PNA-biotin/streptavidin-AP (10) to label GCs (2). X200.

Figure 4. Prolonged administration of soluble antigen depletes GC populations. mIg- centroblasts divide every 7–10 h (2, 3, 42) to produce the Ig+ centrocytes of the GC light zone. Concentrations of circulating NIP12-CGG were maintained for approximately two centroblast doublings (18 h) by repeated i.p. injections. In contrast to a single administration of soluble antigen (see Fig. 1 B), repeated injections of antigen significantly reduced the area of splenic GCs (B) compared with controls given equivalent amounts of BSA (A). The average reduction in GC area was to 20–25% of that seen in controls, indicating a four- to fivefold reduction in cellularity. After 20 h of exposure to soluble antigen, the frequency of TUNEL+ GC cells is reduced to normal levels (C). For comparison to apoptosis in normal GCs and after a single injection of NIP-BSA, see Fig. 1. A and B, X200; C, X100.
from these GCs is unusual; at this time, noncanonical VDJ present in all V186.2 rearrangements at equal frequencies were recovered from all GCs (Fig. 5). Mutations were available from GenBank under accession numbers U34979-U34984.

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B cell precursors to leave for other tissues sites. Interestingly, laremigration (Fig. 3). This activity was unexpected from that this pathway of GC apoptosis is a direct consequence prevent cognate T-B interaction (44) and terminate establishmentsof GC centroblasts (2, 3, 42), and it is consistent with the depletion of dark zone cells take place in this manner, centroblasts themselves must be replenished by returning centrocytes in accordance with the model of Kepler and Perelson (45). Cyclic migration between the two poles of the GC with distinct locales for hypermutation and receptor selection provides a useful model for the stepwise accumulation of large numbers of mutations in functional V(D)J rearrangements and the rapid selection for higher affinity mutants (45).

Prolonged administration of soluble antigen, even if homologous to the eliciting immunogen, does not eliminate GCs, but instead, leaves a small B cell population that appears relatively insensitive to antigen-induced apoptosis (Fig. 4 C). While these persistent cells could represent a population(s) intrinsically resistant to programmed cell death, they express atypical antigen receptors that are likely to bind NP/NIP with low affinity. Sequence analysis of VDJ fragments recovered from splenic GCs that resisted multiple injections of soluble antigen indicated strong selection against the canonical V186.2/DFL16.1 H chain rearrangement (Fig. 5) that is characteristic of NP-reactive GCs after day 7 of the primary response (11, 24). In fact, in 15 VDJ rearrangements recovered from three GCs, only a single V186.2 rearrangement (Fig. 5, SIAB01.1) contained the CDR3 motif YYY(GS), which is typically found in primary anti-NP hybridomas and is important for high affinity binding of the NP and NIP hapteins (22, 28). Significantly, this sequence also contained an unusual Trp->Cys replacement in codon 33 that is likely to reduce the affinity of the antigen-combining site for NIP. Position 33 is an important contact residue within CDR1 that mediates 12-15 van der Waals contacts between the NP-NIP ligand and the third hypervariable loop of the canonical H chain (59). This residue is critical for the heteroclitic behavior of the anti-NP antibodies of IgH mice (59), and a recurrent Trp->Leu mutation in codon 33 increases affinity for NP/NIP ~10-fold (17, 18). Replacement of this critical residue by Cys could reduce the number of interactions between the antigen-combining site and the NIP ligand both directly (a modest effect) or indirectly, by altering the position of another important contact residue, Arg 50 (a very strong effect) (reference 59 and Amzel, M., personal communication). We are in the process of generating transfectomas to measure the affinity of A1 antibodies encoded by the unusual H chain genes that resist antigen-induced apoptosis (29).

Thus, the presence of soluble antigen seems to reverse the usual direction of evolution within GC B cell population. Although resistant cells carry nucleotide substitutions (Fig. 5) typical of Ig hypermutation (55), those that survived 20 h of exposure to free antigen appear selected for decreased antigen affinity. If this pathway of cell death normally serves to eliminate self-reactive lymphocytes arising as a consequence of V(D)J hypermutation (34, 35), then at least two powerful selective forces drive the evolution of memory B cells. Mutants that bind the immunogen with increased affinity exhibit a selective growth advantage within the GC microenvironment; however, should this maturing
affinity introduce self-reactivity, the benefit of this positive selection may be reduced or lost. This developmental boundary could result in evolutionary trajectories that are probable for only a few $V_{H}$ and $V_{L}$ pairings.

Finally, this GC death program is remarkably similar to receptor-driven death in immature/transitional B cells (66–68). In sheep, Peyer’s patch GC B cells undergo antigen-independent $V(D)J$ hypermutation indistinguishable from that observed in unselected murine $\kappa$ transgenes (69). This developmentally regulated post-$V(D)J$ diversification illustrates the potential of this special GC microenvironment to function as a primary lymphoid tissue. It may be that the sharing of many phenotypic characters by mouse bone marrow and GC B cells is not entirely coincidental.

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