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Immunology

FAS is highly expressed in the germinal center but is not required for regulation of the B-cell response to antigen

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ABSTRACT **In establishing the memory B-cell population and maintaining self-tolerance during an immune response, apoptosis mediates the removal of early, low-affinity antibody-forming cells, unselected germinal center (GC) cells, and, potentially, self-reactive B cells. To address the role of the apoptosis-signaling cell surface molecule FAS in the B-cell response to antigen, we have examined the T-cell-dependent B-cell response to the carrier-conjugated hapten (4-hydroxy-3-nitrophenyl)acetyl (NP) in *lpr* mice in which the *fas* gene is mutated. High levels of FAS were expressed on normal GC B cells but the absence of FAS did not perturb the progressive decline in numbers of either GC B cells or extrafollicular antibody-forming cells. Furthermore, the rate of formation and eventual size of the NP-specific memory B-cell population in *lpr* mice were normal. The accumulation of cells with affinity-enhancing mutations and the appearance of high-affinity anti-NP IgG1 antibody in the serum were also normal in *lpr* mice. Thus, although high levels of FAS are expressed on GC B cells, FAS is not required for GC selection or for regulation of the major antigen-specific B-cell compartments. The results suggest that the size and composition of B-cell compartments in the humoral immune response are regulated by mechanisms that do not require FAS.**

Apoptosis, or programed cell death, is critical to the regulation of immune responses (1). It is thought to be necessary not only for the removal of effector lymphocytes once an antigen has been cleared but also for maintaining the efficiency of the germinal center (GC) reaction. Somatic hypermutation of immunoglobulin V genes occurring in the GC generates changes that may either increase, decrease, or not change affinity for antigen or that may destroy the capacity of an antibody to bind antigen at all. The ability to select those B cells with enhanced affinity for antigen is clearly paramount to the functioning of the GC. Furthermore, somatic mutation of immunoglobulin variable region (V) genes could alter the antigen specificity of a B cell allowing it to become self-reactive. The ability to recognize and remove such B cells is important in maintaining immunological tolerance (2).

It has been proposed that two routes to apoptosis are of particular importance in lymphocytes (reviewed in refs. 1 and 3). One of these is triggered through the cell surface glycoprotein FAS (APO-1 or CD95), a member of the tumor necrosis factor superfamily (reviewed in ref. 4). The other, which may be activated by a number of stimuli, is defined by its ability to be blocked by expression of BCL-2 (5). *In vitro* evidence suggests that BCL-2 and FAS operate relatively independently in controlling lymphocyte apoptosis (reviewed in ref. 3). While there is ample evidence documenting the role of BCL-2 in immune responses (reviewed in ref. 5), the exact role played by FAS is more uncertain. FAS-mediated apoptosis has been shown to be important in T-cell regulation, allowing the removal of activated T cells in the periphery (6, 7) and mediating some cytotoxic T lymphocyte killing (8). FAS is also thought to provide a mechanism for clearance of lymphocytes activated in the course of the immune response to antigen (1).

There is increasing evidence that FAS may also be involved in regulating normal B-cell immune responses. *In vitro* activation of B cells has been shown to induce FAS expression (9) and to induce sensitivity to apoptosis through FAS (10). Liu *et al.* (11) have shown that human tonsillar GC B cells express FAS and that they can be killed with anti-FAS antibody *in vitro*. GC B cells can be rescued from apoptosis *in vitro* by stimulation through either the antigen receptor or CD40 (12). Stimulation through CD40, and not the antigen receptor, increases FAS expression and renders the cells sensitive to the induction of apoptosis through FAS. This has led to the proposal that the role of FAS is to prevent the nonspecific rescue from apoptosis of "bystander" low-affinity or autoreactive B cells stimulated through CD40 but not through the antigen receptor, thus maintaining GC selection and affinity maturation (12).

Additional evidence supporting a role for FAS in B-cell responses comes from study of the *lpr* mouse (13), which bears mutated *fas* genes (14). Although the most noticeable abnormality in these mice is a progressive accumulation of a population of $B220^+$ $CD4^-$ $CD8^-$ T cells, an accumulation of B cells also occurs. Indeed, B cells with the *lpr* mutation are required for full development of the *lpr* autoimmune phenotype (15), suggesting a direct role for FAS in either establishing or maintaining B-cell tolerance. Polyclonal antibody-forming cell (AFC) activation has been reported in *lpr* mice (13) as has a diminished serological response to haptenic antigens in older mice (16).

Despite the evidence that B-cell function is abnormal in *lpr* mice and that FAS is up-regulated on human GC B cells, its role in primary T-cell-dependent humoral immune responses *in vivo* is unclear. The ability to isolate and fully phenotype single (4-hydroxy-3-nitrophenyl)acetyl (NP)-specific isotype-switched B cells has allowed us to analyze GC function and the development of B-cell memory in normal C57BL/6 mice (17–19) and in mice transgenic for *bcl-2* (20). We have now used similar methodology to perform a detailed assessment of the response to NP in *lpr* mice, in the expectation that this might provide insight into the role of FAS in humoral immunity.*

MATERIALS AND METHODS

Mice and Immunization. C57BL/6 MRL/*lpr* (originally obtained from the Jackson Laboratory) and C57BL/6 control mice were immunized by intraperitoneal injection of 100 μg of alum-precipitated NP conjugated to keyhole limpet hemocyanin (NP:KLH conjugation ratio, 17:1), prepared as described (18). *lpr* mice bearing the *bcl-2* transgene were generated by crossing C57BL/6*lpr* mice with mice of the Bcl-2-36 strain (21)

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Abbreviations: AFC, antibody-forming cell; CDR, complementarity-determining region; FR, framework region; GC, germinal center; KLH, keyhole limpet hemocyanin; NP, (4-hydroxy-3-nitrophenyl)acetyl; PNA, peanut agglutinin; V, variable region.

*The sequences reported in this paper have been deposited in the GenBank data base (accession nos. U32231–U32246).

backcrossed onto the C57BL/6 background for 10 generations (kindly provided by A. W. Harris). Progeny were typed for presence of the *bcl-2* transgene as described (22) and for presence of the *lpr* mutation using a polymerase chain reaction (PCR) strategy (6).

ELISA and ELIspot Assays. NP-specific antibody of high and low affinity was detected by ELISA with NP_2-bovine serum albumin (BSA) and NP_{17}-BSA plate coats, respectively, as described (20). ELIspot assays for NP-specific IgG1-secreting cells were performed exactly as described (18).

Immunofluorescence and Flow Cytometry. For identification of NP-specific GC and memory cells, spleen cells were prepared (23) and stained to reveal a population of cells defined as $B220^+$ $IgG1^+$ IgM^- IgD^- PI^- and NP^+ cells (20). Cells were analyzed and sorted using a FACStar Plus (Becton Dickinson) equipped with an automated cell deposition unit. To detect FAS expression on antigen-specific cells 7 days after immunization, cells were prepared as described above, except that anti-IgG1 was omitted and replaced by biotin conjugates of RA3-6B2 (anti CD45R/B220; ref. 20) or 281.2 (rat anti-mouse syndecan, kindly provided by Ralph Sanderson; ref. 20), which were revealed with avidin-Texas red (Caltag, South San Francisco, CA). FAS expression was then determined using purified hamster anti-mouse FAS (PharMingen) revealed with a mixture of fluorescein isothiocyanate-conjugated monoclonal mouse anti-hamster IgG antibodies (PharMingen). Thus the level of anti-FAS staining was determined on cells that were IgM^- IgD^- PI^- NP^+ and either $B220^+$ (GC cells) or $Syndecan^+$ (AFCs). To determine FAS expression on total GC cells and B cells, spleen cells from three unimmunized mice were stained with phycoerythrin-conjugated RA3-6B2, biotinylated peanut agglutinin (PNA, Vector Laboratories) revealed with avidin-Texas red, and hamster anti-mouse FAS revealed as before. Hamster anti-human BCL-2 antibody (PharMingen) revealed with the same second step was used as a control for FAS staining in all experiments.

V_H Gene Amplification and Sequencing from Single Cells. Forty-two days after immunization, spleen cells from pools of three mice were prepared, stained, and analyzed as described above. Single $IgG1^+$ NP^+ memory B cells were sorted into tubes containing 4 μl of buffer (0.8 μl of RNase inhibitor, 2 μl of buffered saline solution, 0.4 μl of 10 mM dithiothreitol, and 0.8 μl of water), and lysed by the addition of 7 μl of lysis mix (300 ng of random hexamer, 1 μl of 10% Nonidet P-40, 0.25 μl of RNase inhibitor, and 5.65 μl of water). cDNA was made using Superscript II (GIBCO/BRL), incubation being for 30 min at 37°C. The reverse transcriptase was inactivated by incubation at 90°C for 6 min; 2.5 μl of this cDNA solution was added to the first round PCR. Two rounds of PCR using nested primers were performed as described (17). Products with bands of the expected size were purified over a Qiaquick column (Qiagen, Chatsworth, CA). Eight microliters of the eluant from the column was used as a template for sequencing using the PRISM reaction system (Applied Biosystems). Sequences were run on a 373 DNA sequencer (Applied Biosystems) and analyzed using the SEQED program (Applied Biosystems).

RESULTS

High Levels of FAS Are Expressed on GC B Cells. Spleen cells from C57BL/6 mice, stained with polyclonal hamster anti-murine FAS antibody, were counterstained with B220 and PNA to allow identification of GC B cells (Fig. 1*A*). High levels of FAS antigen were detected on GC B cells compared to that observed on resting B cells and *lpr* GC cells (Fig. 1*B*).

FAS expression could be specifically high on GC B cells or, alternatively, could be due to cell activation and be present on B cells of the intra- and extrafollicular pathways. To examine this we stained splenic cells from mice 7 days after immunization with NP_{15}-KLH and compared FAS expression on antigen-specific AFCs (NP^+ IgM^- IgD^- $Syndecan^+$), almost all of which are extrafollicular at this time (18), and on antigen-specific GC cells (NP^+ IgM^- IgD^- $B220^+$). FAS was expressed on both B cell types, but levels on AFCs were clearly lower (Fig. 1*C*). This result suggests that the high level of FAS is confined to GCs and is consistent with a functional role for FAS on GC B cells.

The Kinetics of Memory B-Cell and AFC Generation Are Normal in *lpr* Mice. The high levels of expression of FAS on GC B cells along with the observation of prominent apoptosis in the GCs of normal mice raise the possibility that FAS-mediated apoptosis may be involved in involution of the GC response. To test this hypothesis antigen-specific GC and memory B cells were enumerated by flow cytometry in control and *lpr* mice at regular intervals after immunization. The reduction of antigen-specific GC B cells after their peak at day 14 and the establishment of a stable memory cell population numbering ≈1 in 10,000 spleen cells were not influenced by the absence of FAS (Fig. 2*A*). Antigen-specific AFCs of the extrafollicular foci, which rapidly die by apoptosis *in situ* after day 7 of the NP response (K.G.C.S., T. D. Hewitson, G.J.V.N., and D.M.T., unpublished data), decline to a similar number in *lpr* and control mice by day 21, though the rate of decline in *lpr* mice may be somewhat slower than in controls (Fig. 2*B*). These

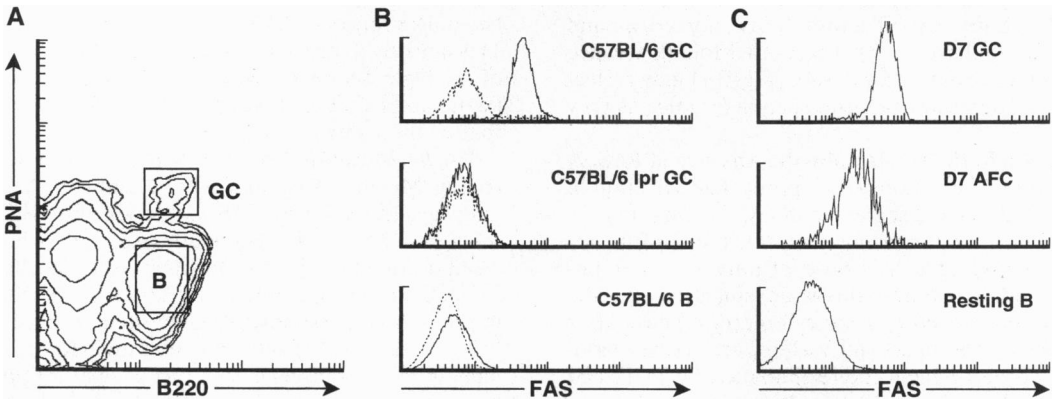

FIG. 1. FAS expression on GC B cells. (*A*) Germinal center B cells and resting B cells (indicated as GC and B, respectively) were identified by their characteristic staining patterns with PNA and anti-B220. (*B*) The expression of FAS was determined on cells of each type in control and *lpr* mice. Staining for FAS is high on GC cells (top) compared to resting B cells (bottom). No significant staining was seen with a control antibody (dashed line) nor on *lpr* GC cells (middle). (*C*) Seven days after immunization with NP-KLH, FAS was expressed at higher levels on GC cells (IgM^- IgD^- NP^+ $B220^+$; top) than on AFCs (IgM^- IgD^- NP^+ $Syndecan^+$; middle) or resting B cells (bottom).

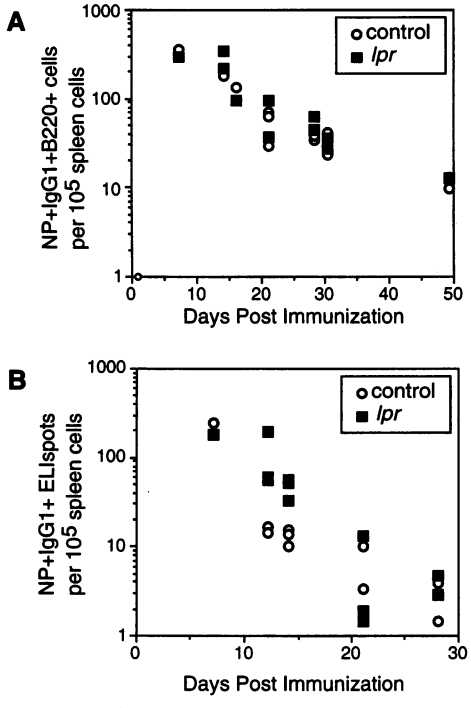

FIG. 2. (*A*) Splenic antigen-specific cells of the GC/memory pathway were enumerated by flow cytometry at various times after immunization. Each point represents an individual mouse, except at days 7 and 49 when pools of three mice were used. (*B*) Splenic antigen-specific IgG1 AFCs were enumerated by ELIspot assays. Each point represents the mean of 10 wells containing either 10^5 or 10^6 spleen cells of an individual mouse, except at day 7 when a pool of three mice was used.

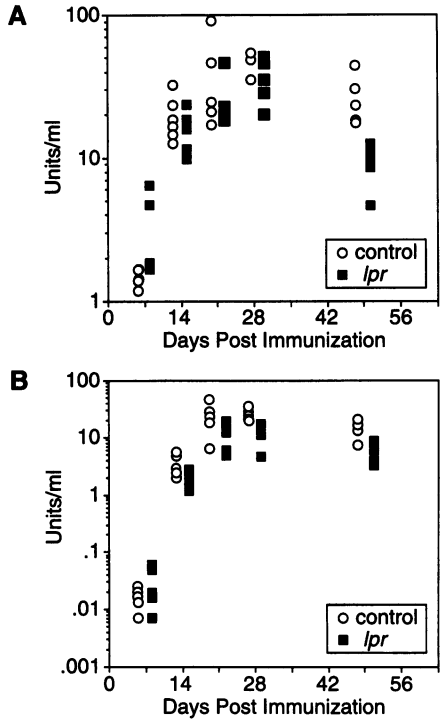

FIG. 3. Affinity maturation of anti-NP IgG1 is normal in immunized *lpr* mice. Five mice per group were immunized with 100 μg of NP-KLH and serum was collected at 7-day intervals. Total NP-specific IgG1 (*A*) and high-affinity anti-NP IgG1 (*B*) were determined by ELISA.

results are in contrast to those seen in *bcl-2* transgenic mice (20, 22, 24).

The Appearance and Titer of High-Affinity Anti-NP Antibody Are Not Influenced by the *lpr* Mutation. Affinity maturation of the humoral immune response, resulting in the generation of high-affinity antibody, is largely a consequence of B-cell somatic hypermutation and selection within the GC. Since the appearance of high-affinity antibody in the serum coincides with the detection of somatically mutated, antigen-specific long-lived AFCs in the bone marrow (K.G.C.S., T. D. Hewitson, G.J.V.N., and D.M.T., unpublished data), its detection is one reflection of GC function. Titers of high-affinity (anti-NP_2) and low-affinity (anti-NP_{17}) NP-specific IgG1 antibodies were thus determined in serum samples from *lpr* and control mice (Fig. 3). The initial appearance and accumulation of total and high-affinity anti-NP antibody in cohorts of *lpr* and control mice were similar (Fig. 3). The 2- to 3-fold difference in titer that becomes apparent by day 49 (Fig. 3*A*) may reflect the reduction in serological responses seen in *lpr* mice as they age (16).

Normal Selection in the GC Despite the Absence of FAS. A related outcome of GC function is the appropriate selection of somatically mutated V gene segments in GC B cells—that is, cells containing affinity-enhancing mutations should be retained at the expense of cells whose affinity is either unchanged or reduced. To address this issue, single NP-specific memory B cells were sorted by flow cytometry 42 days after immunization, cDNA was made, and V_H186.2 genes (the canonical anti-NP V_H gene segment) were amplified by PCR and sequenced (Fig. 4). The number (9.0 per V_H gene) and distribution of mutations in the 16 sequences analyzed from *lpr* mice were similar to those observed in the NP-specific memory B-cell population from control animals (Table 1). In particular, the tryptophan to leucine exchange at amino acid position 33, which alone is sufficient to raise the binding affinity 10-fold to a level typically seen in antibodies of the secondary response (26), was present in 10 of 16 (63%) sequenced genes from the *lpr* mice. This frequency is similar to that seen in memory and secondary response B cells in this and other studies of the NP response (Table 1; refs. 17, 27, and 28).

R:S ratios in CDRs and FRs were relatively normal in *lpr* mice. In CDRs, selection for replacement mutations, which may increase affinity for antigen, results in an increase in the R:S ratio compared to predictions from models of random mutagenesis (28). Conversely, the R:S ratio observed in FRs is typically less than that predicted by these models, as framework replacements are more likely to destroy antibody conformation than to increase affinity for antigen. In *lpr* mice, the R:S ratios in FRs of memory cell V_H186.2 genes are similar to values seen in control mice rather than to those predicted from random mutagenesis (Table 1). The difference observed between R:S ratios in CDRs between *lpr* and control mice (Table 1) is unlikely to be significant, as the removal from the analysis of a single sequence with four silent mutations in CDR2 (sequence 139, Fig. 4) returns this ratio to a value similar to that of the control group.

The *lpr* Mutation Does Not Synergize with BCL-2 in Influencing Memory Cell and AFC Numbers. As the apoptotic pathways triggered by FAS and blocked by BCL-2 appear to function relatively independently (e.g., ref. 3), it is possible that a cell destined to die may do so by either mechanism and that its death may be prevented only if both are blocked. To test this in the humoral response we generated *lpr* mice transgenic for *bcl-2* (*bcl-2*$^+$ *lpr*$^{-/-}$) and compared them to their littermates—namely, *bcl-2* transgene only (*bcl-2*$^+$ *lpr*$^{+/-}$), *lpr* only (*bcl-2*$^-$ *lpr*$^{-/-}$), and control mice (*bcl-2*$^-$ *lpr*$^{+/-}$). As has been demonstrated previously (20), transgenic bcl-2 expression increased memory B-cell recruitment and prolonged the life of AFCs (Fig. 5) compared to *lpr* and wild-type mice. The addition of the *bcl-2* transgene to *lpr* mice resulted in a reduction in the peak size of the immune response compared

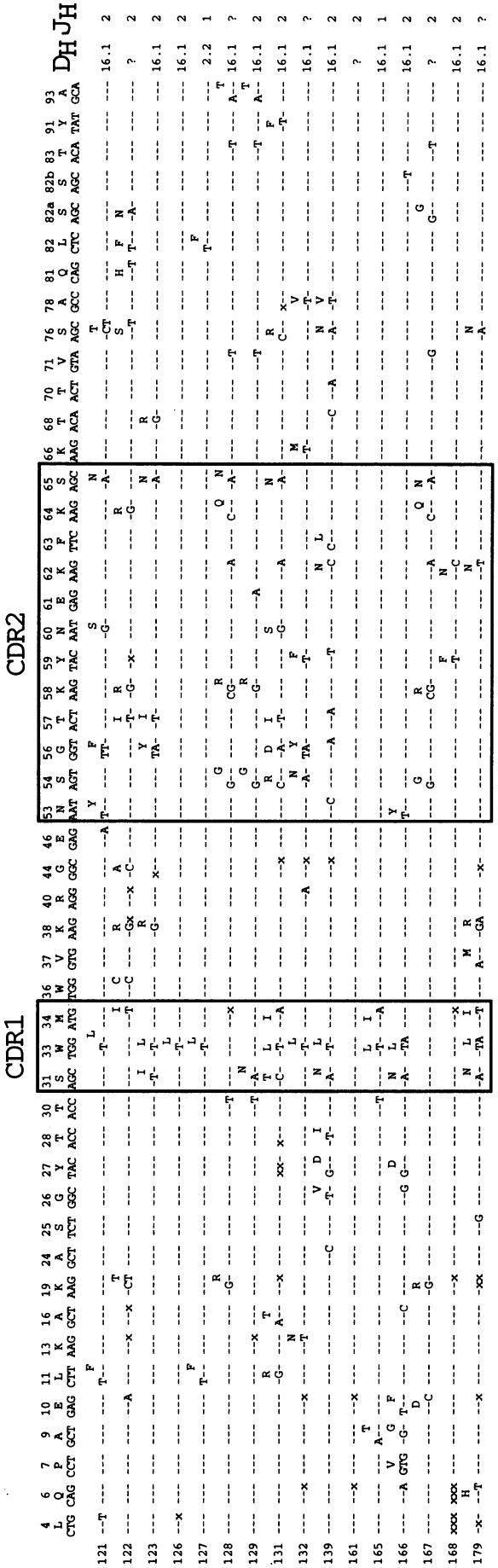

Table 1. Selection of somatic mutations in *lpr* and control mice

Parameter	Random	Control	*lpr*
No. of V_H186.2 genes sequenced	—	20	16
Total mutations (range)	—	8.3 (0–20)	9.0 (0–16)
Position 33 Trp → Leu (%)	—	55	63
R:S ratio			
CDRs 1 and 2*	6.2:1	11:1	6.8:1
FRs 1–3	3.1:1	1.1:1	2.0:1

*See Fig. 4.

to mice transgenic for *bcl-2* alone, and no alteration in the duration of the response could be seen. Thus, the BCL-2 and FAS pathways do not seem to offer alternative routes to death in establishing the memory B-cell pool or in clearance of the extrafollicular AFCs, but rather the pathway that can be inhibited by BCL-2 seems to be of primary importance.

DISCUSSION

Apoptosis of B cells can be achieved through ligation of the plasma membrane receptor FAS or by pathways that do not involve FAS but can be efficiently blocked by BCL-2 (1, 3, 29). BCL-2 blocks killing through FAS quite poorly (30), even though both molecules are thought to regulate apoptosis mediated by CED-3-like proteases (31), which implies that BCL-2 and FAS operate largely independently in lymphocytes (3). Thus, a comparison of previous studies examining the effect of transgenic *bcl-2* expression on the humoral immune response (20, 22, 24, 32) with this analysis of the effect of the *lpr* mutation allows us to assess the relative contributions of each of these apoptotic pathways to the regulation of B-cell responses to antigen.

We have previously shown that transgenic expression of *bcl-2* prevents clearance of extrafollicular AFCs and of excess GC cells, resulting in increased recruitment (20), and perhaps survival (24), of memory B cells. We have now shown that both of these processes are normal in *lpr* mice, indicating that FAS is not required. Thus, apoptosis signaling pathways susceptible to blocking by BCL-2 are of predominant importance in determining the size of antigen-specific B-cell compartments after encounter with antigen.

Selection of high-affinity somatic mutants in the GC occurs normally in the absence of FAS, just as it does in mice expressing transgenic *bcl-2* (20). These observations have implications for how selection operates in the GC. The increasing representation of cells with higher affinity for antigen could presumably occur either by the preferential expansion of these cells (positive selection) or by the removal of cells whose affinity is either unchanged or decreased. If positive selection was of paramount importance in GC selection, then blocking

FIG. 4. Nucleotide sequences of V_H186.2 genes from single NP-specific memory cells of *lpr* mice 42 days after primary immunization. The sequences are compared with the germ-line V_H186.2 gene. Nucleotides identical to the reference sequence are indicated by dashes and uncertainties are indicated by x. Amino acid substitutions relative to the reference sequence are marked above the nucleotide sequence. Only those codons in which a mutation was observed in one of the sequences are shown and codons are numbered according to Kabat *et al.* (25). Complementarity-determining region 3 (CDR3) is not shown, but the D_H and J_H elements used are listed except where too few nucleotides existed to allow accurate designation (indicated by "?"). All sequences were clonally unrelated as defined by unique CDR3s. Replacement to silent mutation ratios (R:S ratios) and mean mutation numbers given in the text are calculated for CDRs 1 and 2 and for framework regions (FRs) 1–3.

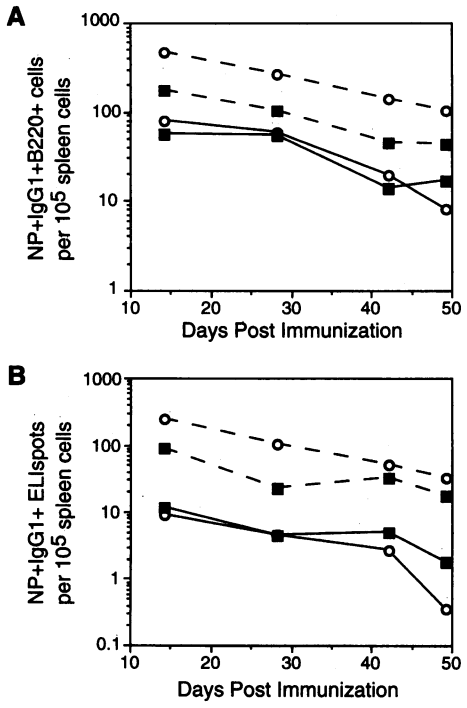

FIG. 5. Cellular kinetics of the NP response in *lpr* mice transgenic for *bcl-2*. (*A*) NP-specific GC/memory cells in the spleen were enumerated by flow cytometry. Results in *lpr* (—■—) and control (—○—) mice were similar, confirming the results shown in Fig. 2. The rate of decline in GC cells after the peak of the response was similar in *bcl-2/lpr* (– ■ –) and *bcl-2* (– ○ –) mice, though the latter mounted a larger response. (*B*) Antigen-specific AFC number, determined by ELIspot, showed similar results to those in *A*. The addition of the *lpr* mutation to the *bcl-2* transgene did not alter the kinetics of decline but did reduce the magnitude of the response.

apoptosis of B cells would not greatly affect the outcome. Thus, our observation that blocking apoptosis through either the FAS or BCL-2 pathway does not affect GC selection is consistent with the notion that the expansion of cells with increased affinity is more important in affinity maturation within the GC than removal of lower-affinity cells by apoptosis.

Our contention that appropriate selection of GC B cells is not critically dependent on the apoptotic death of unselected cells is further supported by our analysis of the immune response in *lpr* mice transgenic for *bcl-2*. It could be argued that more than one clearance mechanism operates in the GC and that in order to observe interference with selection, it may be necessary to block some or all of them. Rather than observing an uncontrolled expansion of antigen-specific B cells, the immune response in the *bcl-2*$^+$ *lpr*$^{-/-}$ mice was reduced in magnitude relative to that of *bcl-2* transgenic mice (Fig. 5). This may well be due to the accelerated accumulation of abnormal T cells and other cells and the resultant splenomegaly and lymphadenopathy seen in these mice (A. Strasser, A. W. Harris, P. H. Krammer, and S. Cory, personal communication), which might reasonably be expected to worsen the reduction in immune responsiveness observed in *lpr* mice as they age (16). We did not observe synergy between BCL-2 and *lpr* in influence on AFC clearance or memory cell recruitment. While these data do not exclude a role for apoptosis in GC selection, they suggest that other clearance mechanisms may operate in the GC. For example, lymphocytes earmarked for clearance may express "engulfment receptors" and be phagocytosed despite not undergoing apoptosis, in a manner analogous to neutrophils expressing a *bcl-2* transgene that undergo phagocytosis and normal clearance despite suppression of apoptosis (33).

There has been increasing speculation that FAS-mediated apoptosis could play an important role in regulation of the B-cell response to antigen. Despite the fact that GC cells express high levels of FAS, we have found no evidence for the involvement of FAS in the recruitment of memory B cells or in clearance of extrafollicular AFCs, processes that therefore appear to be primarily mediated by apoptotic mechanisms that can be inhibited by BCL-2. In addition, we could find no defects in affinity maturation or selection in GCs in mice lacking FAS, supporting the importance of positive selection in the GC and raising the possibility that other clearance mechanisms, in addition to those controlled by BCL-2 and FAS, operate there.

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