CD5-Mediated Negative Regulation of Antigen Receptor-Induced Growth Signals in B-1 B Cells

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A subset of B lymphocytes present primarily in the peritoneal and pleural cavities is defined by the expression of CD5 and is elevated in autoimmune diseases. Upon signaling through membrane immunoglobulin M (mIgM), splenic B lymphocytes (B-2) proliferate, whereas peritoneal B cells (B-1) undergo apoptosis. However, in CD5-deficient mice, B-1 cells responded to mIgM crosslinking by developing a resistance to apoptosis and entering the cell cycle. In wild-type B-1 cells, prevention of association between CD5 and mIgM rescued their growth response to mIgM crosslinking. Thus the B cell receptor–mediated signaling is negatively regulated by CD5 in normal B-1 cells.

Peritoneal B lymphocytes (B-1 cells) differ from conventional B lymphocytes (B-2 cells) in their function and expression of surface markers; B-1 cells are subdivided into "sister cells," B-1a (Mac-1$^+$CD5$^+$IgM$^+$) and B-1b (Mac-1$^+$CD5$^-$IgM$^+$) populations, whereas B-2 cells are Mac-1$^-$CD5$^-$IgM$^+$ (*1*). B-1 cells have a limited repertoire that is dominated by antibodies that crossreact with self antigens in mice and humans and are increased in autoimmune diseases (*1*, *2*). Mechanisms that limit the expansion of these self-reactive B cells have not yet been elucidated. The ligation of mIgM on splenic B cells (B-2) with antibodies to IgM (anti-IgM) triggers a cascade of events leading to the entry of these cells into the S phase of the cell cycle. In contrast, ligation of mIgM by autoantigen or anti-IgM on B-1 cells induces apoptosis (*3*–*5*). Activation mediated by mIgM crosslinking is blocked in B-1 cells early in signal transduction, possibly at the stage of protein kinase C (PKC) activation, whereas B-1 and B-2 cells proliferate equally when stimulated with activated T cells or lipopolysaccharide (LPS) (*4*). Although CD5 appears to have a role in T cell–B cell interactions (*6*), its function in B-1 cells remains unknown. CD5 associates with the B cell antigen receptor (BCR) after mIgM ligation in human tonsillar B cells (*7*), raising the possibility of a functional role for CD5 in mIgM-induced signals. Here we show that CD5 acts as a negative regulator of mIgM-mediated signaling in B-1a cells.

To examine the importance of CD5 in mIgM signaling, we compared the anti-IgM induced growth response in B-1 and B-2 cells from either wild-type or CD5 deficient (−/−) mice (*8*). Purified splenic B-2 and peritoneal B-1 cells were stimulated in vitro with LPS, F(ab′)$_2$ goat anti-mouse IgM (GαmIgM) or anti-CD40 (Fig. 1). Splenic B cells from both wild-type and mutant mice responded equally to all the stimuli employed (Fig. 1, A and C). Control and CD5$^{-/-}$ B-1 cells also responded equally to LPS or anti-CD40, two stimuli known to bypass mIgM dependent signal transduction (Fig. 1, B and D) (*9*). However, in contrast to normal B-1 cells, CD5$^{-/-}$ mutant B-1 cells were induced to proliferate by GαmIgM (Fig. 1D), indicating that in the absence of CD5, mIgM-induced proliferation is restored in peritoneal B-1 cells. The negative regulatory function of CD5 appeared to affect only mIgM signaling, because LPS- and CD40-mediated signaling remained unaltered in CD5$^{-/-}$ B-1 cells.

BCR-mediated proliferation, but not apoptosis-inducing signals, are impaired in B-1

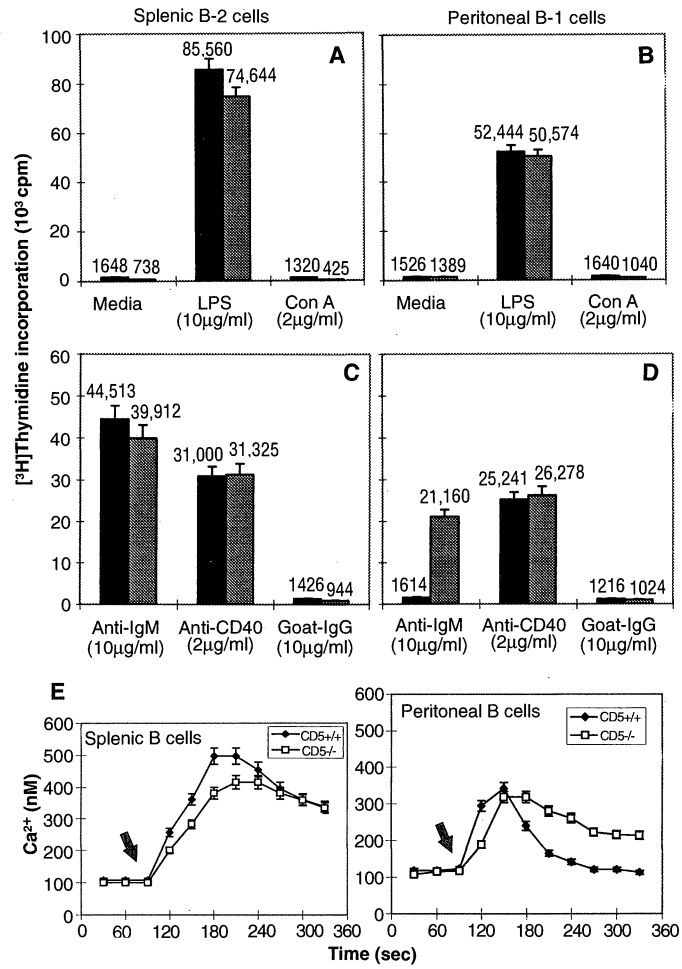

Fig. 1. Mitogen and mIgM-mediated responses of splenic and peritoneal B cells from control (+/+) and CD5$^{-/-}$ (−/−) mice. (**A** and **B**) Proliferative responses of B-2 and B-1 cells to LPS (Difco Lab) and concanavalin A (Con A) (Sigma). (**C** and **D**) B-2 and B-1 cell responses to F(ab′)$_2$ GαmIgM (Cappel) and monoclonal antibodies to mouse CD40 (*9*). Black bars, CD5$^{+/+}$; gray bars, CD5$^{-/-}$. B-1 and B-2 cells were obtained as described (*20*). The bars represent mean and standard error of triplicate cultures. The results represent one of eight independent experiments showing mIgM-mediated response of CD5$^{-/-}$ B-1 cells. The observed differences between the +/+ and −/− mice are not due to strain variation, because the B-1 cells from five different strains (DBA/2, C57BL/6, BALB/c, 129, and CB17) did not respond to anti-IgM. (**E**) mIgM-induced Ca^{2+} mobilization in CD5$^{-/-}$ and control (129) B cells. Intracellular free Ca^2 was evaluated (*21*). Briefly, splenic B cells and peritoneal B-1 cells were purified by depleting T cells and adherent cells as described (*20*). Two color immunofluorescence was performed on cells loaded with indo-1 (Molecular Probes) at 37°C, and stained with anti-B220 for splenic B cells or anti–Mac-1 for peritoneal B cells. The cells were stimulated with F(ab′)$_2$ GαmIgM (Cappel). Arrows indicate the time of addition of anti-IgM. The results represent mean and standard error of calcium responses from three independent experiments. In every experiment three samples were analyzed for each time point. Both B-1 and B-2 cells had similar intracellular Ca^{2+} concentrations (1200 nM) when treated with ionomycin.

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cells (5). The role of CD5 in mIgM-induced programmed cell death of B-1 cells was investigated by inoculating normal or CD5$^{-/-}$ mice with GαmIgM intraperitoneally and assessing DNA content as a measure of cell cycle (*10*) (Fig. 2). Peritoneal B cells from control mice had a substantial decrease in the number of cycling B cells and a concomitant increase in cells undergoing apoptosis when exposed to GαmIgM for 24 or 48 hours (Fig. 2, A, C, E, and G). In contrast, the peritoneal B cells from the CD5$^{-/-}$ mice did not undergo apoptosis after injection with GαmIgM but progressed into S phase of the cell cycle at 24 and 48 hours after injection (Fig. 2, B, D, F, and H). Using Mac-1 to distinguish B-1 and B-2 cells in the peritoneum, we found the apoptotic response in the wild-type mice to be mostly in the B-1 population. Thus 48 hours after injection with GαmIgM, 56 ± 8% of B-1 (Mac-1^{+}) but only 10 ± 2% of the B-2 (Mac-1^{-}) cells were apoptotic. Moreover, fluorescence-activated cell sorting (FACS)–purified B-1a, as well as B-1b cells from 129 mice, did not proliferate in response to anti-IgM, whereas the B-2 cells proliferated (*11*). Thus, the absence of CD5 redirects B-1 cells from an apoptotic to a proliferative pathway in response to mIgM ligation. In agreement with Tarakhovsky *et al.* (8), there was no change in the ratios of B-1 to B-2 cells as judged by Mac-1 and B220 expression. Thus B-1 and B-2 cells in the peritoneum were 87% and 13%, respectively, in the wild-type and 84% and 16% in the CD5$^{-/-}$ mice. Although the numbers of B-1a and B-1b B cells in CD5$^{-/-}$ mice cannot be rigorously determined, IgD staining is consistent with them being present in normal proportions. Thus, a shift in peritoneal B cell phenotype from B-1 to B-2, due to the absence of CD5 in mutant mice, did not account for these results.

The B-1 cells respond to anti-Ig by an elevation of calcium that is of lower magnitude than in splenic B-2 cells (*12*). Intracellular Ca^{2+} concentrations in control B-1 cells treated with GαmIgM were modest and transient in comparison to splenic B-2 cells (Fig. 1E), whereas Ca^{2+} mobilization in CD5$^{-/-}$ B-1 cells was more sustained than in normal B-1 cells. Also, the percentage of responding cells decreased more rapidly for wild-type B-1 cells than for mutant cells (*11*). Thus CD5 may have a role in regulating mIgM-mediated Ca^{2+} release from intracellular stores or its influx through ion channels. These results are consistent with the increased Ca^{2+} mobilization reported for anti-CD3–stimulated T cells from CD5$^{-/-}$ mice (*13*).

B cell activation through mIgM leads to activation of PKC and the subsequent induction of target genes by activated transcription factors (*14, 15*). One transcription factor that is regulated by PKC is nuclear factor κB (NF-κB) (*16*), which translocates into the nucleus within the first 2 hours of mIgM ligation in B-2 but not in B-1 cells (*14, 16, 17*). Because the data suggested that the mIgM-induced proliferative response was restored in CD5$^{-/-}$ peritoneal B-1 cells, we investigated the nuclear translocation of NF-κB in these cells (Fig. 3). Confocal laser microscopy of B-1 cells stained with anti–NF-κB (p65) revealed that in contrast to control cells, NF-κB was found in the nuclei of mIgM-stimulated CD5$^{-/-}$ B-1 cells (Fig. 3, C and D). Ligation of mIgM caused a fivefold increase of nuclear NF-κB fluorescence in CD5$^{-/-}$ B-1 cells (Fig. 3E); about 62% of the cells (on average, 37 cells out of 60) from CD5$^{-/-}$ mice showed nuclear expression of NF-κB. In comparison, less than 2% (1 cell out of 60, on average) of wild-type B-1 cells showed nuclear localization of NF-κB. In the mIgM-stimulated B-1 cells from CD5$^{-/-}$, but not control mice, nuclear NF-κB bound specifically to oligonucleotides containing an NF-κB binding consensus sequence as revealed by electrophoretic mobility shift assay (Fig. 3F), suggesting that nuclear NF-κB could be involved in the transcriptional activation of genes required for cell proliferation.

To confirm the regulatory role of CD5 in mIgM-mediated B-1 cell signaling, the proliferative responses of normal B-1 cells to the crosslinking of mIgM and CD5 molecules were examined (Fig. 4). Crosslinking of either mIgM or CD5 alone did not induce a proliferative response in normal B-1 cells. In contrast, when CD5 molecules were first crosslinked such that they could not interact with mIgM, the subsequent addition of anti-IgM initiated a significant proliferative response in B-1 cells from wild-type mice (Fig. 4). Primary crosslinking of mIgM molecules

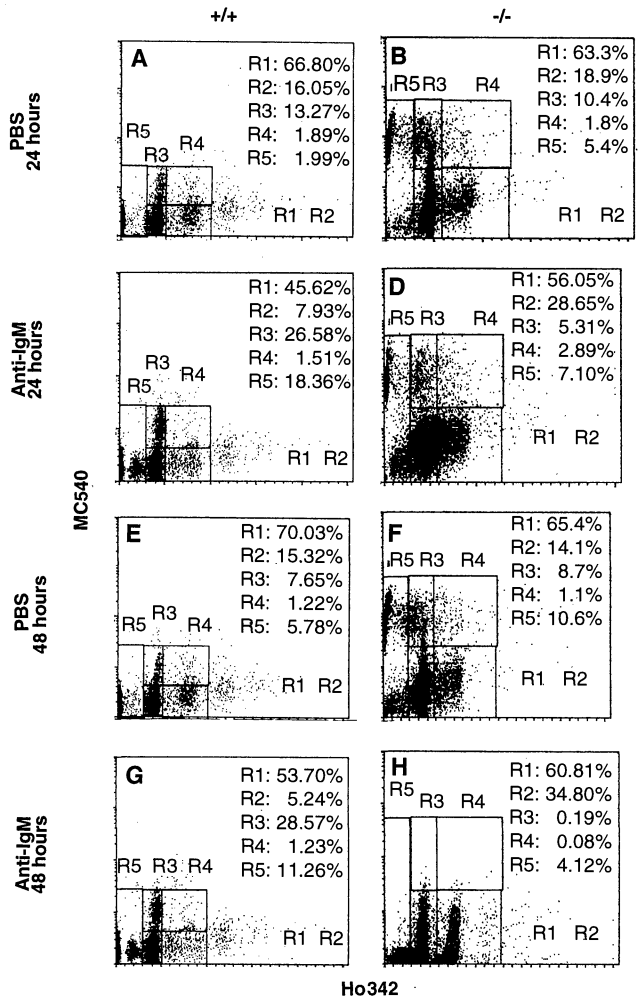

Fig. 2. Apoptosis and cell cycle analysis of peritoneal B cells stimulated with goat anti-mIgM. Control and CD5$^{-/-}$ mice were injected intraperitoneally with F(ab′)$_2$ GαmIgM (100 μg/300 μl) or 300 μl of phosphate buffered saline (PBS). Cells were extracted by peritoneal lavage 24 and 48 hours after injections and stained with Hoechst 33342 (Ho342), merocyanin 540 (MC540), and anti-B220-FITC (*10, 22*). The gates were set for B220^{+} cells, in order to analyze B cell cycle and apoptosis stages. Ho342 is a DNA specific dye that measures the amount of DNA. MC540 adheres to inner membrane phospholipids made available for binding during the process of apoptosis (*10*). Together, these two dyes allow for the separation of five distinct populations of B cells: viable resting cells, 2*n* and MC540^{dull} (R1); viable cycling cells, >2*n* and MC540^{dull} (R2); resting cells undergoing apoptosis, 2*n* and MC540^{bright} (R3); cycling cells undergoing apoptosis, >2*n* and MC540^{bright} (R4); R5 represents cells that have moved into the later stages of apoptosis as they become Ho342^{dull}, an indication of DNA fragmentation. The results presented are representative of one of three independent experiments. For each experiment, three mice were similarly injected with GαmIgM or PBS. Each peritoneal B cell content was analyzed independently. Results from one mouse are shown and are nearly identical to those from the other two mice. The mean per cent ± SD values (from three experiments) for the numbers of apoptotic B cells in mice treated for 48 hours with anti-IgM were 41 ± 2.8 and 4 ± 0.7 for the +/+ and −/− mice, respectively.

followed by the addition of anti-CD5 did not induce B-1 cell proliferation. Crosslinking of another B-1 cell surface molecule, the major histocompatibility complex (MHC) class II, followed by GαmIgM stimulus also did not induce a proliferative response, establishing the specificity of the rescue of mIgM signaling to the initial crosslinking of CD5 molecules. Thus, the primary ligation of CD5 allows for mIgM-mediated proliferative signaling to be restored in B-1 cells, suggesting that surface CD5 regulates, directly or indirectly, mIgM-mediated activation signals. Because CD5 has been reported to be associated with mIgM on $CD5^+$ B cells (*7*), and the data indicate that CD5 negatively regulates mIgM signaling, the crosslinking of CD5 on B-1 cells could sequester these molecules away from mIgM, consequently preventing the blockade of mIgM-mediated signals. These results are consistent with the suggestion that CD5 might similarly inhibit positive selection as well as anti-CD3–mediated T cell proliferation (*13*).

Presently, we do not know if the B-1b response to anti-IgM is altered in the $CD5^{-/-}$ mice, because the two populations (B-1a and B-1b) cannot be easily distinguished in the absence of CD5 (*1*, *2*). The purified B-1b population ($CD5^-$ Mac-1^+) in wild-type mice is also unresponsive to anti-IgM and may be regulated by another mechanism. Although the mechanism by which CD5 regulates mIgM-mediated signaling remains to be elucidated, preliminary studies suggest that CD5 may directly regulate Ig mediated signaling, because it is associated with the cytosolic protein tyrosine phosphatase (SHP-1) (*18*) in resting but not activated T cells and in BKS-2 B lymphoma cells (*11*). Thus, CD5 could negatively regulate the BCR-mediated signaling in B-1 cells in a manner analogous to the inhibition of the BCR signaling by CD22, or that of the T cell receptor signaling by CTLA-4, by recruiting the cytoplasmic tyrosine phosphatases SHP-1 and SHP-2, respectively (*19*).

The B-1 cell repertoire is primarily directed against microbial and self-antigens; thus regulation of the BCR signaling by CD5 may have evolved as a mechanism to check the uncontrolled expansion of B-1 cells. The CD5-mediated negative regulation can be overcome, however, with strong growth signals provided by mitogenic moieties of microbial antigens or by T cell help. Thus, these data suggest that $CD5^{-/-}$ mice may have an increased incidence of autoantibodies, which accumulate with age in wild-type mice, and that certain autoimmune states may be due to defects in CD5-mediated negative regulation of mIgM signaling.

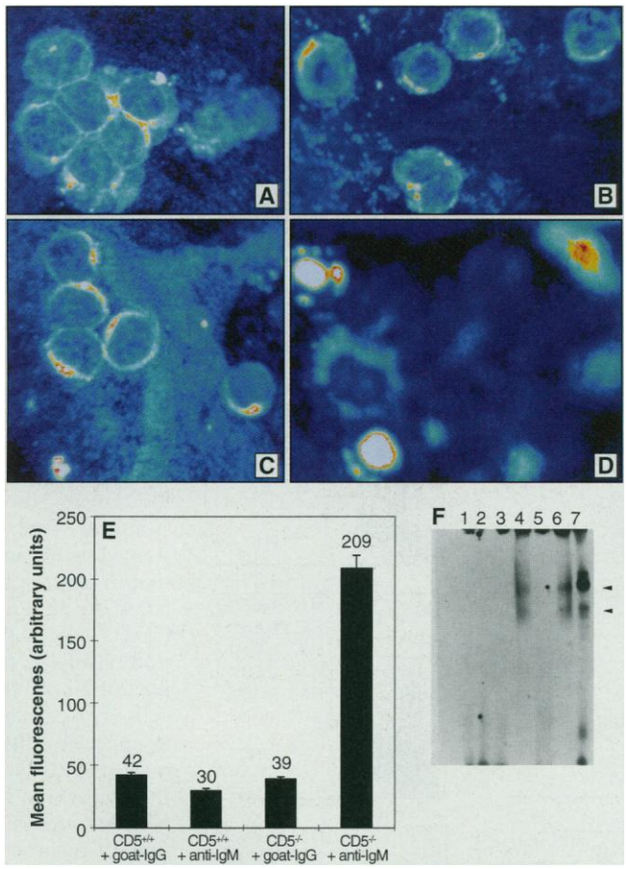

Fig. 3. Induced nuclear localization of the transcription factor NF-κB by mIgM crosslinking. Photomicrographs of NF-κB fluorescence in control (**A** and **C**) or $CD5^{-/-}$ (**B** and **D**) B-1 cells. (A and B) Cells treated with normal goat-IgG. (C and D) Cells treated with $F(ab')_2$ GαmIgM (*23*). Images are optical sections of cells obtained with a confocal laser scanning microscope and are representative of three different experiments. Fluorescence intensity is represented on a color scale with blue being the lowest, yellow medium, red high, and white being the maximum intensity. Each treatment of cells was done in triplicate; therefore for every treatment nine slides were examined. On each slide, 60 cells were counted, the mean fluorescence and the percentage of cells showing nuclear staining were derived from these 60 cells (**E**). The values for average intensity of nuclear staining are represented by the numbers above the bars. (**F**) NF-κB binding activity in response to mIgM stimulus measured by electrophoretic mobility shift assay (*24*). Nuclear proteins from $CD5^{+/+}$ B-1 cells stimulated with goat-IgG (lane 1) or with $F(ab')_2$ GαmIgM (lane 2). Nuclear proteins from $CD5^{-/-}$ B-1 cells stimulated with goat-IgG (lane 3) or with $F(ab')_2$ GαmIgM (lane 4). Lane 5, same as in lane 4, plus addition of 1 μg of cold probe (competitor); lane 6, same as in lane 4, plus addition of 1 μg of a nonspecific competitor, activator protein 2 oligo; lane 7, positive control from HeLa cell extract. Arrowheads indicate the position of NF-κB; the two bands are consistent with other studies on B lymphocytes (*13*–*15*). The results shown are representative of three different experiments.

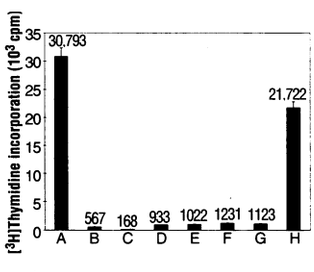

Fig. 4. Proliferative response of normal peritoneal B cells to mIgM and CD5 crosslinking. Purified B-1 cells from normal mice (strain 129) were stimulated with LPS (10 μg/ml) (**A**), avidin (10 μg/ml) (**B**), $F(ab')_2$ GαmIgM (10 μg/ml) (**C**), biotinylated anti-MHC class II (clone AF6-120.1) (50 μg/ml) plus avidin (**D**), or biotinylated anti-CD5 (clone 53.7) (50 μg/ml) plus avidin (**E**). B-1 cells were treated for 1 hour with biotinylated anti-MHC class II plus avidin (**F**), $F(ab')_2$ GαmIgM (**G**), or biotinylated anti-CD5 plus avidin (**H**) at 37°C in 5% CO_2 before the addition of biotinylated anti-CD5 plus avidin (G) or $F(ab')_2$ GαmIgM (F and H). The cells were cultured for 48 hours, pulsed with [^{3}H]thymidine for the last 4 hours and proliferation was measured as a function of thymidine uptake. The values represent mean ± standard error of responses of triplicate samples. Similar results were obtained in another experiment with 129 mice and two experiments with B-1 cells from BALB/c and CB17 mice.

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20. Control mice were 129. The mutant CD5$^{-/-}$ mouse strain was derived by intercrossing and subsequent inbreeding of heterozygous littermates born to C57BL/6 females, mated with the male chimeras, obtained by injecting CD5 gene targeted embryonic stem (ES) cells into C57BL/6 blastocysts (*8*). Splenic and peritoneal washout cells were obtained from 7- to 12-week old mice, and depleted of T cells by treatment with a cocktail of antibodies (anti-Thy 1.2, anti-CD8, and anti-CD4) followed by rabbit complement. Splenic and peritoneal B cells were depleted of macrophages by plastic adherence overnight at 37°C in 5% CO_2. B-1 cells were further purified by dual staining with Mac-1 and B220 and sorting of the double positive cells with a FACStar (Becton Dickinson) flow cytometer. Cells (2.5×10^5) in 0.2 ml of Iscove F-12 media, 5% fetal calf serum were cultured in flat bottom microtiter wells.
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22. Peritoneal B cells were pelleted and resuspended in 100 μl of Ho342 (5 μg/ml) (Molecular Probes). After a 30 min incubation at 37°C in the dark, the cells were pelleted and resuspended in 100 μl of a 0.4 μg/ml stock solution of MC540 (Molecular Probes). After a 20-min incubation at room temperature (in the dark), 1 μg of anti-B220 conjugated to fluorescein isothiocyanate (FITC) (Sigma) was added, and incubated on ice for 30 min. The cells were then pelleted, resuspended in 1 ml of PBS and 10,000 cells were analyzed immediately on a FACStar flow cytometer (Becton Dickinson). The gates to distinguish MC540 dull (R1, R2) and bright cells (R3, R4) were set by looking for a natural break in the staining profile of the resting or cycling B cells. The CD5$^{-/-}$ mice required different gates, presumably due to higher background staining of the untreated cells with MC540, which occurs whenever the responding population is activated (*10*). Data with peritoneal B cells from wild-type 129 mice were shown in Fig. 2 and similar results were obtained with peritoneal B cells from BALB/c mice.
23. Control and CD5$^{-/-}$ B-1 cells were purified as described (*20*). Cells (2×10^5) were incubated with F(ab')$_2$ GαmIgM (Cappel) or normal goat IgG (Sigma) for 60 min at 37°C in 5% CO_2. The cells were transferred onto microscope slides by cytocentrifugation, fixed with 4% paraformaldehyde, and permeabilized in 0.05% Triton X-100 (Bio-Rad). Cells were subsequently incubated with anti-NF-κB p65 (Santa Cruz Biotech) for 2 hours, then with biotinylated anti-rabbit-IgG (Vector) for 1 hour. Cells were finally incubated with avidin-FITC (Vector) for 30 min, and examined on a confocal laser scanning microscope (Molecular Dynamics, Sarastro 2000). To allow for quantitative comparisons of the relative fluorescence between the cells, the intensity of the laser beam and the sensitivity of the photodetector were held constant. The "Imagespace" software supplied by the manufacturer was used to obtain the values for average intensity of nuclear staining. Several fields of cells were examined on each slide.
24. B-1 cells (10^7) from wild-type or CD5$^{-/-}$ mice were stimulated with F(ab')$_2$ GαmIgM or goat-IgG for 1 hour at 37°C in 5% CO_2. Nuclear proteins were obtained as described (*25*). Protein concentrations were standardized (BCA, Pierce), and the electrophoretic mobility shift assay was done with a NF-κB binding protein assay kit (Gibco-BRL), following the procedure of the manufacturer.
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26. All animal procedures were conducted in accordance with the University of Kentucky "Guide for the Care and Use of Laboratory Animals."

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Quantal Duration of Auditory Memories

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Neuronal responses in the caudomedial neostriatum (NCM) of adult zebra finches (*Taeniopygia guttata*) decreased upon repeated, unreinforced presentations of conspecific song, calls, or other complex sounds. This "stimulus-specific habituation" is a form of learning, and its spontaneous loss, a form of "forgetting." Spontaneous forgetting occurred only at narrowly defined times (2 to 3, 6 to 7, 14 to 15, 17 to 18.5, 46 to 48, or 85 to 89 hours after first exposure to a stimulus), determined by stimulus class, number of presentations, and interval between presentations. The first five forgetting times coincided with periods when gene expression and protein synthesis in NCM were required for maintenance of the longer lasting (85 to 89 hours) habituation. The number of successive episodes of gene expression induced by a stimulus, but occurring long after stimulus presentation, appears to determine the quantal duration of auditory memories.

The songs and calls of songbirds have characteristics that can be used for species identification and individual recognition (*1*, *2*). In previous experiments, we used multi-unit activity (MUA) data to show (i) that auditory responses in populations of neurons in the zebra finch NCM habituate specifically to individual song stimuli; (ii) that this habituation can be long-lasting; and (iii) that the duration of habituation is longer for conspecific songs than for white noise, pure tones, or some exemplars of heterospecific sounds (*3*). We showed, too, that this habituation was anatomically circumscribed: It occurred in caudal, but not in rostral, NCM (*3*). NCM is one of the highest stations of the ascending auditory pathway (*4*). Here we describe habituation at the single-unit level and its relation to MUA habituation. We then use recordings of MUA to determine in a systematic manner how stimulus class, interstimulus interval (ISI), number of stimulus presentations, and manner of presentation affect the duration of stimulus-specific habituation in neurons of caudal NCM. Finally, we examine the relation between the duration of habituation and RNA and protein synthesis.

The firing rates of single neurons in caudal NCM were initially high and then decreased upon repeated presentations of the same conspecific song (Fig. 1) (*5*). After 100 presentations there was little if any further decrement in responsiveness. The reduction in firing rate was not selective for any one subset of the song's components but affected the whole song (Fig. 1C). The stimulus-specific habituation seen in multi-unit recordings does not appear to result from individual neurons "tuning in" to particular features of a stimulus and ceasing to respond to the rest of that stimulus, but rather from a general reduction in the responsiveness elicited by that stimulus.

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