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*J Immunol* 2003; 171:2320-2325; doi: 10.4049/jimmunol.171.5.2320

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CpG DNA Induces a Class II Transactivator-Independent Increase in Class II MHC by Stabilizing Class II MHC mRNA in B Lymphocytes

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Microbial products, such as CpG DNA and LPS, enhance class II MHC (MHC-II) expression and Ag presentation by dendritic cells, but this effect does not occur with macrophages and is largely unexplored in B cells. Although MHC-II expression is influenced by transcriptional regulation, which is governed by class II transactivator (CIITA) in all cells, microbial products enhance MHC-II expression by dendritic cells in part by increasing MHC-II protein stability. In this study, we show that the CpG-induced increase in MHC-II expression by B lymphocytes is not due to protein stabilization or changes in CIITA expression or activity, but instead is due to increased stability of MHC-II mRNA. This CIITA-independent mechanism adds a new layer of complexity to regulation of MHC-II and may increase T cell help for B cell Ab responses to microbial or vaccine Ags. The Journal of Immunology, 2003, 171: 2320–2325.
of MHC-II mRNA. The enhancement of B cell MHC-II expression and Ag presentation resulting from MHC-II mRNA stabilization may increase T cell help for B cell Ab responses during vaccination or infection.

Materials and Methods

Cells

Naive conventional B cells were isolated from spleens of 8- to 12-wk-old female FVB/H mice (The Jackson Laboratory, Bar Harbor, ME). After disruption of spleens by passage through a 70-µm filter and depletion of erythrocytes, CD34+ cells were removed using S7 anti-CD34-conjugated magnetic microbeads (Miltenyi Biotec, Auburn, CA), according to the manufacturer’s instructions. After incubation with anti-CD34 microbeads, cells were passed over a magnetic column; CD43− cells were passed over a magnetic column; CD43+ cells were collected in the flow-through. More than 95% of CD43− cells expressed high levels of B220, but little or no TCR β-chain or Mac-1, as detected by flow cytometry. These B cells were cultured at 2-× 10^6 cells/ml in standard medium (RPMI 1640 supplemented with 10% FCS, 50 µM 2-ME, 100 U/ml penicillin, and 100 µg/ml streptomycin).

Ag processing and presentation assays

Nonmethylated, phosphorothioate-modified CpG ODN 1826 (TCCAT GACGTTCCTAGACGTT) and non-CpG ODN 1982 (TCCATGACGTTC TCTAGACGTT) were generously provided by Coley Pharmaceutical Group (Ottawa, Ontario, Canada). B cells were cultured ± 1 µg/ml CpG ODN 1826 or 1 µg/ml non-CpG control ODN 1982 for the indicated times. After incubation with ODN, cells in each sample were counted, plated in U-bottom 96-well plates at 2 × 10^5 cells/well, and incubated for various periods. Hen egg lysozyme (HEL, Sigma-Aldrich, St. Louis, MO) was added during or after the initial incubation with CpG ODN. Cells were washed, fixed, and incubated with 3A9 T hybridoma cells (10^5 /well) (18), previously described (12), to detect HEL(48–61):I-A^k complexes. IL-2 secretion by 3A9 cells was determined using a colorimetric bioassay with Alamar Blue to measure IL-2 secretion (19). Results are presented as OD_{590} -OD_{550}. Each data point is the mean ± SD of triplicate wells.

Flow cytometry

B cells were isolated as above, rested overnight, and incubated ± CpG ODN 1826 (1 µg/ml) for various times. All subsequent incubations for flow cytometry staining were done on ice in PBS with 2% BSA. Fc receptors were blocked with anti-FcγRIIIA/CD16/CD32 Ab (BD Biosciences, San Dieg o, CA), and cells were incubated for 30 min with biotinylated I-A^k- and I-A^k-α-chain, or anti-I-αβ-chain, 209 anti-I-E^k (BD Biosciences), or biotinylated IgG2a isotype control (BD Biosciences). Alternatively, to assess the stability of peptide:MHC-II complexes, B cells were cultured overnight ± 100 µM HEL(48–61) peptide +/− 1 µg/ml CpG ODN 1826, washed, cultured for various periods, and incubated with biotinylated C4H3 mAb (20) to specifically label HEL(48–61):I-A^k complexes. Cells were washed, incubated for 15 min with streptavidin-Cy-Chrome (BD Biosciences), washed, and fixed in PBS with 2% paraformaldehyde. A FACScan flow cytometer and CellQuest software (BD Biosciences) were used for analysis. Cells were gated by optical scatter parameters to exclude cells that were nonviable before fixation. To identify gates containing nonviable cells, separate samples of cells were incubated with 5 µg/ml ethidium monoxide bromide (EMAB) (Molecular Probes, Eugene, OR) for 1 min in the dark. As with propidium iodide, EMA passively diffuses into dead cells and binds DNA. Cells were exposed to light from an ordinary fluorescent lamp to photo-cross-link EMA to DNA before fixation of the cells.

Quantification of total MHC-II and CIITA mRNA

B cells were isolated, rested overnight, incubated ± CpG ODN 1826 (1 µg/ml) for various periods, harvested, and counted. Cells (6–10 × 10^6) were pelleted, and total RNA was isolated using an RNeasy kit (Qiagen, Valencia, CA). RNA was suspended in 40 µl RNAse-free water and stored at −80°C. A total of 8 µl of RNA was reverse transcribed to DNA using Superscript First-Strand Synthesis kit (Invitrogen, Carlsbad, CA) or oligo(dT) primers. Real-time quantitative PCR was performed with 5% of the reverse-transcription product using premixed PCR buffer with hot start Taq polymerase and SYBR green (iQ SYBR Green Supermix; Bio-Rad, Hercules, CA). Triplicate PCR were performed on a thermocycler equipped with optics to monitor SYBR green fluorescence (iCycler; Bio-Rad). Primer pairs to detect I-αβ- β-chain, total CIITA (all isoforms detected), or individual CIITA isoforms (types I, III, and IV) have been described (12, 16). Gel-purified cDNA standards for each amplicon were produced by PCR, quantified by spectrophotometry, and used to generate a standard curve for each real-time PCR run to convert threshold cycles to number of molecules. Normalization to a housekeeping gene, e.g., GAPDH, was not possible, as CpG rapidly increases GAPDH mRNA expression. Therefore, data are presented as number of molecules/cell.

Quantification of nascent RNA transcripts

B cells were purified, rested overnight, and then stimulated for indicated times ± 1 µg/ml ODN 1826. All subsequent steps were done at 4°C using RNAse-free chemicals and water. To isolate nuclei, B cells were lysed for 5 min in 140 mM NaCl, 1.5 mM MgCl_2, 0.5% Nonidet P-40, 1,000 U/ml RNase inhibitor, 1 mM DTI, and 50 mM Tris (pH 8). Nuclei were pelleted by centrifugation at 300 × g, washed three times to remove cytoplasmic RNA, and resuspended by incubation for 10 min in 300 mM NaCl, 1 M urea, 1% NP-40, 7.5 mM MgCl_2, 0.5 mM EDTA, 1 mM DTI, and 20 mM HEPES (pH 7.6) (21). Histone-bound chromatin was pelleted by centrifugation at 15,000 × g for 10 min. RNA was isolated using a RNeasy kit with DNase treatment. A random hexamer-primed reverse-transcription reaction was conducted using Superscript First-Strand Synthesis kit. Transcripts for I-A^k α- and β-chain were quantified by quantitative real-time PCR, as described above.

Results

CpG ODN enhance Ag processing and presentation by B cells

CpG ODN induce proliferation and increase surface expression of MHC-II by human or murine B cells (17). We investigated the effect of CpG ODN on Ag processing and presentation by murine B cells. Because CD43 is expressed on all murine splenocytes except resting conventional (B-2) B cells (22, 23), B cells were isolated from mouse spleen by negative selection using anti-CD43 mAb conjugated to magnetic microbeads. B cells were incubated for 18 h ± CpG ODN 1826 and then for various periods with HEL before fixation and 3A9 T hybridoma assay for HEL(48–61):I-A^k complexes. Maximum presentation of these complexes was only achieved by 8 h or longer (Fig. 1), indicating a slower rate of processing than observed with macrophages, which achieve maximal presentation efficiency within 2 h of Ag addition (24). CpG ODN 1826 greatly enhanced the efficiency of HEL processing (in terms of both rate and dose response). This effect was CpG specific, because non-CpG ODN 1982 had no effect (Fig. 1D). CpG ODN 1826 also enhanced cell surface expression of MHC-II protein by 2- to 3-fold by 24 h (Fig. 2A), providing a potential mechanism for the enhanced Ag-processing efficiency.

CpG ODN increase surface expression of MHC-II, but does not stabilize peptide:MHC-II complexes

We investigated whether the CpG-induced enhancement of MHC-II expression involves the freeze-frame mechanism seen with dendritic cells, i.e., stabilization of peptide:MHC-II complexes accompanied by reduction in MHC-II mRNA and protein synthesis. To assess the stability of peptide:MHC-II complexes, B cells were incubated overnight ± CpG ODN 1826 with HEL(48–61) peptide to generate a cohort of HEL(48–61):I-A^k complexes. The cells were washed, incubated for various chase intervals, and assessed for expression of HEL(48–61):I-A^k complexes by flow cytometry after staining with C4H3 mAb. The t1/2 of HEL(48–61):I-A^k complexes was 18 h without CpG ODN 1826 and 16 h with CpG ODN (Fig. 2B). Thus, in contrast to its effect on dendritic cells (12), CpG ODN 1826 did not stabilize peptide:MHC-II complexes on B cells, indicating that the freeze-frame mechanism is not induced in B cells by CpG ODN.

CpG ODN increase MHC-II mRNA without an increase in CIITA mRNA

Given the absence of posttranslational regulation of MHC-II by the freeze-frame mechanism, we tested whether the CpG-induced increase in MHC-II protein expression was caused by an increase in
CpG DNA STABILIZES MHC-II mRNA IN B CELLS

FIGURE 1. CpG ODN enhance Ag processing and presentation by naive murine B cells. Resting B cells were isolated from mouse splenocytes by negative selection using anti-CD43 Abs conjugated to magnetic beads. A–C, B cells were incubated for 18 h with () or without (○) 1 μg/ml CpG ODN 1826; plated (2 × 10^5 cells/well) in 96-well plates; incubated with HEL for 2, 4, or 8 h; fixed; washed; and incubated with 3A9 T hybridoma cells to assess presentation of HEL(48–61):I-A^b complexes (using a colormetric IL-2 bioassay; see Materials and Methods). D, B cells were incubated 48 h with HEL and 1 μg/ml CpG ODN 1826 (○), 1 μg/ml non-CpG ODN, 1982 (△), or no ODN (○). The cells were then counted and plated as above for T hybridoma assay to assess Ag presentation. Results are expressed as the mean of triplicate wells ± SD. When error bars are not visible, they are smaller than the symbol width. These results are representative of six independent experiments. Values of p resulting from a two-tailed t test comparing results with and without CpG ODN are shown (#, p < 0.05; +, p < 0.01; *, p < 0.001).

MHC-II mRNA. Upon stimulation of B cells with CpG ODN 1826, MHC-II message increased rapidly (within 1–3 h) by ~2-fold (Fig. 3A), similar to the magnitude of increase in MHC-II protein expression at the cell surface (Fig. 2A). These data indicate that CpG ODN 1826 regulates MHC-II expression by regulating MHC-II mRNA expression.

We next investigated whether CpG ODN regulation of MHC-II mRNA occurred by transcriptional or posttranscriptional mechanisms. Surprisingly, CIITA mRNA levels did not increase upon stimulation of B cells with CpG ODN 1826, but rather declined by ~25–50% over 24 h (Fig. 3B). Three different forms of CIITA (I, III, and IV) arise from alternate transcription initiation sites, with type III CIITA the most abundant in B cells (16, 25). Because an increase in a low abundance isoform might be masked by a decrease in the dominant isoform, we quantified transcripts from each isoform. Type III CIITA was the dominant isoform in B cells and was decreased by 50% by treatment with CpG ODN 1826; there was no significant change in type IV CIITA, and type I was barely detectable (Fig. 3C). We conclude that CpG ODN 1826 does not increase CIITA mRNA, suggesting that the CpG-induced increase in MHC-II mRNA does not result from increased expression of CIITA.

CpG ODN do not increase transcription of MHC-II mRNA

It is possible that MHC-II mRNA could be increased by posttranscriptional enhancement of CIITA activity, e.g., by phosphorylation of CIITA (8). If so, CpG ODN could increase the rate of transcription of MHC-II mRNA. To investigate the rate of transcription, we isolated nascent RNA transcripts and quantified nascent MHC-II transcripts with real-time PCR, as previously described (15, 21, 25). Nascent RNA transcripts were isolated by lysis of B cell nuclei in a buffer that included 300 mM NaCl, 1 M urea, and 1% Nonidet P-40. This lysis condition preserves interactions between nascent RNA transcripts, RNA polymerase, and DNA, and retains chromatin-bound histones, allowing for isolation of chromatin-associated nascent RNA transcripts by low-speed centrifugation (15, 21, 25). After DNase digestion of genomic DNA and purification of RNA, the RNA was reverse transcribed using random hexamer primers. Nascent MHC-II RNA was quantified by real-time PCR using primers located near the 5’ end of the gene for either I-A^a α-chain or I-A^b β-chain. Despite the CpG-induced increase in total MHC-II mRNA, treatment of B cells with CpG ODN 1826 did not increase nascent MHC-II mRNA transcripts (Fig. 4). This result suggests that the CpG-induced increase in MHC-II mRNA is not caused by an increase in the rate of transcription of MHC-II mRNA resulting from increased CIITA activity. Furthermore, this finding suggests the hypothesis that the CpG-induced enhancement of MHC-II mRNA is caused by posttranscriptional regulation, i.e., enhanced stability of MHC-II mRNA.
CpG ODN stabilize MHC-II mRNA

Regulation of mRNA stability is another way to control mRNA expression. To test this possibility, we measured the $t_{1/2}$ of I-A$^b$ mRNA in B cells prior to treatment with CpG ODN 1826 by measuring the decline in MHC-II mRNA after addition of 5,6-dichlorobenzimidazole riboside (DRB) to inhibit RNA synthesis. CpG ODN 1826 stabilized MHC-II mRNA, increasing its apparent $t_{1/2}$ from 1.7 to 4.5 h (Fig. 5). To determine whether this increase in $t_{1/2}$ is sufficient magnitude to explain the observed change in MHC-II mRNA expression, it is possible to perform an analysis similar to the secular equilibrium analysis for production and decay of a radiouclide (26). If mRNA molecules are synthesized at a rate of $R$ and undergo stochastic decay with a decay constant of $\lambda$ (fraction of molecules decaying per unit time), the number of mRNA molecules at equilibrium (N) will be given by the following equation: $n = R/\lambda$. Thus, $n = R/T_{1/2}$, which reveals that the expression of mRNA at equilibrium is proportional to the $t_{1/2}$ of the mRNA. If $R$ is unchanged (Fig. 4), a change in $t_{1/2}$ from 1.7 to 4.5 h (as observed after CpG stimulation; Fig. 5) is predicted to increase mRNA expression by a factor of (4.5/1.7) = 2.6. Therefore, the observed 2- to 3-fold increase in MHC-II mRNA over the 22 h following CpG stimulation (Fig. 3) can be explained by the observed stabilization of MHC-II mRNA without any change in MHC-II mRNA synthesis, consistent with the lack of increase in CIITA mRNA.

Discussion

We have shown that CpG ODN increase Ag processing and presentation capability of CD43$^-$ conventional naive B cells from the spleen (Fig. 1), consistent with the finding that CpG ODN increase MHC-II expression on B cells (Fig. 2) (17). This mechanism may provide a period of increased MHC-II expression, even as B cell differentiation is initiated, which may later result in decreased expression of MHC-II if plasma cell differentiation proceeds. CpG ODN 1826 has been shown to maintain increased MHC-II expression on B220$^-$ spleen cells in vivo for a period of 6 days (27), and we have seen elevated MHC-II on CD43$^-$ splenocytes for up to 2323The Journal of Immunology

![FIGURE 3. Treatment of B cells with CpG ODN increases MHC-II mRNA expression without a corresponding increase in CIITA mRNA. B cells were isolated and rested overnight. Cells were incubated with (filled symbols) or without (open symbols) CpG ODN 1826 (1 µg/ml) for the indicated time periods. The cells were then counted, RNA was extracted, and individual mRNA species were determined by real-time RT-PCR. A, Quantitation of mRNA for MHC-II (I-A$^b$-chain). B, Quantitation of mRNA for total CIITA (using primers that amplify sequence common to types I, III, and IV CIITA). The results in A and B are representative of three independent experiments. C, Quantitation of types I, III, and IV CIITA after 22 h ± CpG ODN using primers specific for each isoform. Results of statistical analysis by two-tailed student’s t test are shown to compare results with and without CpG ODN (#, p < 0.05; +, p < 0.01; *, p < 0.001). The results in C are representative of two independent experiments. Data are presented as number of mRNA copies/cell.](http://www.jimmunol.org/)

![FIGURE 4. Rate of MHC-II mRNA transcription is not increased by treatment of B cells with CpG ODN. B cells were rested overnight and then incubated ± CpG ODN 1826 (1 µg/ml) for 0, 1, or 2 h. Nascent transcripts were isolated, and the amount of nascent MHC-II mRNA was determined by quantitative real-time PCR using primers for MHC-II I-A$^b$ α-chain (A) and I-A$^b$ β-chain (B). For each time point, nascent transcripts were isolated from three separate cultures (○, []), or △). Each data point from a triplicate PCR is shown as a separate open symbol; x-axis values may be displaced slightly to make individual symbols visible. The mean of all nine points is shown as a horizontal line. There is no statistically significant difference between results with untreated cells (time = 0) and CpG-treated cells (time = 1 or 2 h). The p values for two-tailed t tests, using the mean value for triplicate PCR, were 0.37 for time = 0 vs time = 1 and 0.39 for time = 0 vs time = 2 in A, and 0.9 for time = 0 vs time = 1 and 0.15 for time = 0 vs time = 2 in B. These results are representative of three independent experiments.](http://www.jimmunol.org/)

![FIGURE 5. MHC-II mRNA is stabilized by treatment of B cells with CpG ODN. A, MHC-II mRNA was quantified by real-time RT-PCR at various times after addition of the transcriptional inhibitor, DRB. Cells were incubated for 16 h with (filled symbols) or without (open symbols) CpG ODN 1826 (1 µg/ml) before the addition of DRB. Log$_2$ of mRNA copies/cell is expressed as a function of time. The apparent $t_{1/2}$ of MHC-II mRNA, determined as the negative inverse of the slope (m) of the best fit line, y = mx + b, was 1.7 h for cells without exposure to CpG ODN and 4.5 h after incubation with CpG ODN for 16 h. These results are representative of three independent experiments.](http://www.jimmunol.org/)
72 h (Fig. 2A and data not shown). The enhancement of B cell MHC-II expression and Ag presentation may increase T cell help for B cell Ab responses to microbial or vaccine Ags, contributing to Ig isotype switching observed in mice vaccinated with CpG ODN as an adjuvant (28).

For other APCs, CpG ODN generally depress MHC-II expression by transcriptional control, with decreased expression of CIITA leading to decreased expression of MHC-II mRNA and protein. In macrophages, induction of MHC-II by IFN-γ is inhibited by CpG ODN, and this is due to a rapid silencing of MHC-II mRNA transcription due to a corresponding decrease in CIITA mRNA (data not shown) (29). In dendritic cells, PAMPs cause a transient increase in MHC-II mRNA (the mechanism for this is unknown, but it is interesting to speculate that it may be caused by increased mRNA stability), but the progression of dendritic cell maturation involves declining levels of CIITA mRNA and consequent reduction in MHC-II mRNA and MHC-II protein synthesis (15, 16). Despite the decrease in MHC-II mRNA, a stable enhancement of MHC-II protein expression occurs because of increased stability of peptide:MHC-II complexes (12), which we refer to as the freeze-frame mechanism.

We expected a similar freeze-frame mechanism to explain the CpG-induced increase in MHC-II expression by B cells, but our investigations revealed that CpG did not enhance MHC-II protein stability (Fig. 2B). Instead, we found that the 2- to 3-fold increase in MHC-II protein expression was preceded by a similar increase in MHC-II mRNA, which is maintained for at least 24 h (Fig. 3A). We found that CIITA mRNA did not increase, but rather decreased slightly. Although different in magnitude, this decrease in CIITA is consistent with the effect of CpG ODN and other PAMPs on macrophages (data not shown) (29–31) and dendritic cells (15, 16). Although increased transcriptional activity of CIITA due to phosphorylation could theoretically account for the increase in MHC-II mRNA, we found no increase in nascent MHC-II transcripts preceding the increase in MHC-II mRNA (Fig. 4). This result suggested mRNA stabilization as the most likely cause of the increase in MHC-II mRNA, and this was confirmed by the finding that CpG ODN increased the t1/2 of MHC-II mRNA from 1.5 to 4.5 h (Fig. 5), a change of sufficient magnitude to account for the observed increase in MHC-II mRNA expression.

Previous studies showed that MHC-II mRNA stability depends on active protein synthesis, suggesting involvement of trans-activating RNA-binding proteins in determining the message t1/2 (32, 33). Expression of many other genes, including cytokines, is regulated by changing mRNA stability, which results from a change in mRNA degradation rate of destruction of the mRNA by RNA nucleases (regulated by changing mRNA stability, which results from a change in mRNA transcription due to a corresponding decrease in CIITA mRNA). A common mRNA degradation pathway involves endonuclease cleavage, which can be regulated by altering activity of a specific endonuclease or by RNA-binding proteins that protect against endonuclease activity. Analysis of the mouse I-Aβ α-chain sequence for sites to which such proteins bind (34), however, revealed no such sites. Thus, at this point, the mechanism of MHC-II mRNA stabilization is unknown.

With some exceptions (32, 33, 36), regulation of MHC-II expression has been attributed primarily to transcriptional regulation mediated by CIITA. Our results show that physiological stimuli (e.g., signaling induced by CpG DNA) can regulate MHC-II expression by stabilization of MHC-II mRNA, which is a mechanism that is predicted to be independent of CIITA. Mathematical analysis indicates that the observed mRNA stabilization can completely account for the CpG-induced increase in MHC-II mRNA, therefore explaining the regulation of MHC-II expression independent of transcriptional regulation by CIITA.

In summary, MHC-II expression is regulated by a combination of mechanisms that are used to varying degrees in different cell types. Transcriptional regulation by CIITA is often the major mechanism for regulating MHC-II expression, but MHC-II can be regulated by other mechanisms. Posttranslational stabilization of MHC-II protein contributes to the increase in MHC-II expression during dendritic cell maturation. Another CIITA-independent mechanism is induced in macrophages by type I IFN, which inhibits CIITA-driven transcription of MHC-II (36). Stabilization of MHC-II mRNA by CpG ODN, as demonstrated in this study, adds another layer of complexity to the regulation of MHC-II expression.

Acknowledgments

We thank Art Krieg and Coley Pharmaceutical Group for generously providing helpful advice and the CpG and non-CpG ODN.

References


