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# CpG DNA Is a Potent Enhancer of Specific Immunity in Mice Immunized with Recombinant Hepatitis B Surface Antigen<sup>1</sup>

Heather L. Davis,<sup>2\*†</sup> Risini Weeranta,\* Thomas J. Waldschmidt,<sup>‡</sup> Lorraine Tygrett,<sup>‡</sup> Joachim Schorr,<sup>§</sup> and Arthur M. Krieg<sup>||</sup>

Unmethylated CpG dinucleotides in bacterial DNA or synthetic oligodeoxynucleotides (ODN) cause B cell proliferation and Ig secretion, monocyte cytokine secretion, and activation of NK cell lytic activity and IFN- $\gamma$  secretion in vivo and in vitro. The potent immune activation by CpG ODN suggests possible utility for enhancing immune responses to vaccines. Mice immunized with recombinant hepatitis B virus surface Ag and a CpG ODN as an immune enhancer have titers of Abs against HBsAg (anti-HBs) that are five times higher than those of mice immunized with HBsAg and the standard adjuvant, aluminum hydroxide (alum). Ab titers in mice immunized with HBsAg and both CpG ODN plus alum were 35 times higher than the titers in mice immunized with alum alone, indicating a strong synergistic interaction between the CpG ODN and alum. ODN without CpG motifs had little or no immune-enhancing activity at the doses used herein. Alum induces a Th2 humoral response (mostly IgG1) and no CTL. In contrast, CpG ODN gives a strong Th1 response with predominantly IgG2a Abs and CTL, even when mixed with alum. In vitro studies to determine possible mechanisms of CpG immune-enhancing effects show that CpG ODN induce expression of costimulatory molecules on Ag-presenting cells and drive B cell isotype switching in the appropriate cytokine milieu. These studies demonstrate that CpG ODN are promising new immune enhancers for vaccination applications. *The Journal of Immunology*, 1998, 160: 870–876.

**B**acterial DNA, but not vertebrate DNA, has direct immunostimulatory effects on leukocytes in vitro (1, 2). This lymphocyte activation is due to unmethylated CpG dinucleotides (3), which are present at the expected frequency in bacterial DNA (1/16 bases) but are under-represented (“CpG suppression,” 1/50 to 1/60 bases) and methylated in vertebrate DNA (4). Activation may also be triggered by synthetic oligodeoxynucleotides (ODN<sup>3</sup>) that contain one or more unmethylated CpG dinucleotides in particular sequence contexts (CpG motifs) (3). It appears likely that the rapid immune activation in response to CpG DNA may have evolved as one component of the innate immune defense mechanisms that recognize structural patterns specific to microbial molecules (5).

CpG DNA can induce proliferation of almost all (>95%) B cells and triggers polyclonal Ig secretion. This B cell activation by CpG DNA is T cell independent and Ag nonspecific. However, B cell activation by low concentrations of CpG DNA has strong synergy with signals delivered through the B cell Ag receptor for both B cell proliferation and Ig secretion (3). In theory, this strong synergy between the B cell signaling pathways triggered through the B cell Ag receptor and by CpG DNA may promote Ag-immune responses. In addition to its direct effects on B cells, CpG DNA also directly activates monocytes, macrophages, and dendritic cells to secrete a variety of cytokines, including high levels of IL-12 (6–8). These cytokines stimulate NK cells to secrete IFN- $\gamma$  and have increased lytic activity (6, 8–11). It is noteworthy that although IFN- $\gamma$  inhibits LPS-induced B cell activation, the IFN- $\gamma$  secreted in response to CpG DNA promotes B cell activation and Ig secretion (12).

Overall, CpG DNA induces a Th1-like pattern of cytokine production dominated by IL-12 and IFN- $\gamma$  with little secretion of Th2 cytokines (6). Such a Th1 immune response is associated with the production of predominantly IgG2a Abs, while Th2 responses are associated with the production of IgG1 Abs.

The strong activating effects of CpG DNA on B cells, as well as the induction of cytokines that could have indirect effects on B cells via T-help pathways, suggest utility of CpG ODN as a vaccine enhancer. Here we test a CpG ODN alone, and in combination with alum, for its effects on the humoral and cellular responses against recombinant hepatitis B surface Ag (HBsAg) in mice. We show that a CpG ODN drives the production of higher levels of Ag-specific Abs (predominantly Th1) compared with alum and that there is a strong synergistic response when the ODN is used together with alum. We also demonstrate the ability of CpG ODN to induce costimulatory molecule expression in vitro and in vivo and to drive B cell isotype shifting in vitro, suggesting possible mechanisms for the enhancing effects.

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<sup>3</sup> Abbreviations used in this paper: ODN, oligodeoxynucleotides; alum, aluminum hydroxide (Al<sub>2</sub>O<sub>3</sub>); HBsAg, hepatitis B surface Ag; HBV, hepatitis B virus; MFI, mean fluorescence intensity.

## Materials and Methods

### Oligodeoxynucleotides

ODN used herein were: 1745, TCCATGAGCTTCTGAGTCT; 1911, TC CAGGACTTTCCTCAGGTT; 1982, TCCAGGACTTCTCTCAGGTT; 1983, TTTT TTTT TTTT TTTT TTTT TTTT; 1668, TCCATGACGTTCTCTGAT GCT; and 1826, TCCATGACGTTCTCTGACGTT (CpG dinucleotides underlined for clarity). All ODN were synthesized with a nuclease-resistant phosphorothioate backbone by Oligos Etc. (Wilsonville, OR). The Na<sup>+</sup> salts of the ODN were ethanol precipitated and then resuspended in 10 mM Tris (pH 7.0) 1 mM EDTA for storage at -20°C before dilution into saline for injection. LPS level in ODN was undetectable (less than 1 ng/mg) by *Limulus* assay (Whittaker Bioproducts, Walkersville, MD).

### Immunization of mice against HBsAg

Immunization with HBsAg was conducted on 6- to 8-wk-old female BALB/c mice (Charles River, Montreal, QC, Canada). Each mouse received a single i.m. injection, into the left tibialis anterior muscle, of a solution containing 1  $\mu$ g recombinant HBsAg (*ay* subtype) produced in yeast cells (Medix Biotech, Foster City, CA) in a total volume of 50  $\mu$ l. Control groups ( $n = 10$ ) received HBsAg in saline or with added Al<sub>2</sub>O<sub>3</sub> (Alhydrogel "85," Superfos Biosector, Vedbaek, Denmark; 2.5  $\mu$ l 2% Al<sub>2</sub>O<sub>3</sub> per  $\mu$ g HBsAg to give 25 mg Al<sup>3+</sup>/mg HBsAg). Experimental groups ( $n = 5$  or 10) received HBsAg plus 10, 100, or 500  $\mu$ g of the indicated ODN. These experiments were performed with ODN in which the backbone was nuclease resistant (phosphorothioate) to improve cell uptake and *in vivo* stability (13). Some experimental groups received only CpG ODN while others received both CpG ODN plus alum (as above). All component solutions were added at the same time, mixed with a vortex, and left on ice for about 30 min before injection.

### Evaluation of *in vivo* humoral response to HBsAg

Plasma was recovered from mice at various times after immunization as described previously (14). Abs specific to HBsAg were detected and quantified by end-point dilution ELISA assay (in triplicate) on samples from individual animals (15).

In brief, a solid phase of plasma-derived HBsAg particles (*ay* subtype, 100  $\mu$ l of 1  $\mu$ g/ml per well, overnight at room temperature) was used to capture anti-HBs Abs in the plasma (1 h at 37°C), which were then detected with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG, IgM, IgG1, or IgG2a (1:4000 in PBS-Tween, 10% FBS; 100  $\mu$ l/well; Southern Biotechnology Inc., Birmingham, AL), followed by *o*-phenylenediamine dihydrochloride solution (100  $\mu$ l/well, 30 min at room temperature in the dark; Sigma, St. Louis, MO). End-point titers were defined as the highest plasma dilution that resulted in an absorbance value (OD 450) two times greater than that of nonimmune plasma with a cut-off value of 0.05.

For descriptive purposes, anti-HBs titers were expressed as group means  $\pm$  SEM of individual animal values, which were themselves the average of triplicate assays. The statistical significance of differences between groups was determined from the means and SDs by Student's *t* test (for 2 groups) or 1-factor ANOVA (for three or more groups). All statistical tests were performed using InStat (GraphPad Software, San Diego, CA).

### Evaluation of CTL response

Spleens were recovered under sterile conditions from mice 12 to 16 wk after immunization with HBsAg alone or with CpG ODN and/or alum ( $n = 3$ /group). Single cell suspensions were prepared and suspended in RPMI 1640 (Life Technologies, Grand Island, NY) tissue culture medium supplemented with 10% FBS (Life Technologies) and penicillin-streptomycin solution (final concentrations of 1000 U/ml and 1 mg/ml respectively) (Sigma, Irvine, U.K.). Splenocytes ( $3 \times 10^7$ ) were cocultured with  $1.5 \times 10^6$  syngeneic HBsAg-expressing stimulator cells (P815-preS, generously provided by F.V. Chisari, Scripps Institute, La Jolla, CA), which had been inactivated by irradiation (20,000 rad). The cultures were maintained for 5 days in 10 ml of media in upright 25 cm<sup>2</sup> tissue culture flasks in a humidified atmosphere (5% CO<sub>2</sub>) at 37°C and then were harvested and washed in media. These effector cells were serially diluted and cultured with  $5 \times 10^3$  <sup>51</sup>Cr-labeled HBsAg-expressing targets (P815S) or control target cells (P815) at 37°C in round-bottom 96-well microtiter plates, with each sample in triplicate. After 4 h of incubation, 100  $\mu$ l of supernatant was removed for radiation ( $\gamma$ ) counting. The percent lysis was calculated as [(experimental release - spontaneous release)/(total release - spontaneous release)]  $\times$  100. Spontaneous release was determined by incubating target cells without effector cells, and total release was determined by adding 100  $\mu$ l of 2% Triton X-100 to the target cells. The percent specific lysis was calculated as follows: % lysis with P815S cells - % lysis P815 cells.

### *In vivo* ODN treatment and flow cytometric analysis

BALB/c mice were given a single i.p. dose (500  $\mu$ g) of ODN suspended in saline (0.15 M NaCl). At designated time points after injection, mice were killed and spleen cells analyzed by flow cytometry. Specifically, Ficoll-spun splenocytes were incubated with conjugated Abs for 20 min at 4°C, followed by washing and incubation with the FITC-avidin. In the first incubation, 15  $\mu$ g of the anti-Fc $\gamma$ RII Ab 2.4G2 and 10  $\mu$ l of rat serum were added to minimize nonspecific staining. Cells were stained with Cyanine 5.18-conjugated anti-B220 (6B2) and either biotin-conjugated anti-MHC class II (M5/114), anti-B7.2 (GL1), or anti-Ly-6C (15.1.1). Conjugated purified rat IgG (Jackson ImmunoResearch, West Grove, PA) served as isotype controls. Stained cells were run on a dual laser Becton Dickinson FACS 440 with a minimum of  $3 \times 10^4$  cells collected per sample. The FACS 440 data were analyzed using a VAX station 3200 computer equipped with DESK software (kindly supplied by Wayne Moore, Stanford University, Stanford, CA). Final graphic output was performed with Macintosh Canvas software (Deneba Software, South Miami, FL).

### Preparation and culture of purified B cells for isotype switch studies

Spleens were obtained from 6- to 8-wk-old female BALB/c mice or BALB/c mice genetically lacking the IFN- $\gamma$  gene (The Jackson Laboratory, Bar Harbor ME) that had been maintained under specific pathogen-free conditions in the University of Iowa Animal Care Facility. Splenocyte suspensions were treated with anti-Thy 1 (HO13.4) and baby rabbit complement to eliminate T cells, followed by sedimentation through a discontinuous (50%/60%/70%/75%) Percoll gradient. Dense B cells at the 70 to 75% Percoll interface were collected, washed in balanced salt solution (BSS), and suspended in complete medium consisting of RPMI 1640 with FBS (10%), penicillin (100 unit/ml), streptomycin (100 unit/ml), L-glutamine (2 mM), and 2-ME (0.05 mM). In selected experiments, T cell-depleted splenic B cells were stained with Cyanine 5.18-conjugated anti-B220 (6B2) and FITC-conjugated anti-IgD (11-26) followed by sort purification of the B220<sup>+</sup>IgD<sup>+</sup> cells on a dual laser Coulter Epics 753 (Hialeah, FL).

Percoll dense or sort-purified B cells were cultured in flat-bottom 96-well plates at  $1 \times 10^5$  cells per well in 200  $\mu$ l of complete medium. Cultures were stimulated with 6  $\mu$ g/ml of CpG or non-CpG ODN alone or with either recombinant IL-4 (Peprotech, Rocky Hill, NJ) at 1000 U/ml or IFN- $\gamma$  (kindly provided by Immunex Corp., Seattle, WA) at 20 or 100 U/ml as indicated. LPS (*Escherichia coli* 0111:B4 LPS; Difco, Detroit MI) at 40  $\mu$ g/ml served as positive control. Culture supernatants were harvested after 6 days and assayed for Ig levels.

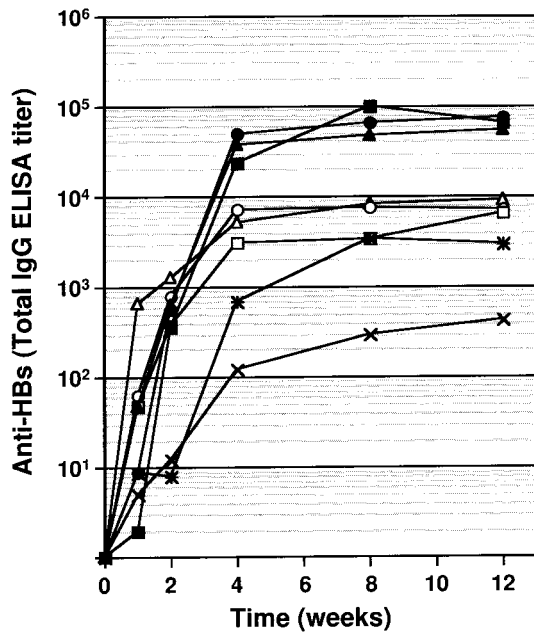
### Isotype-specific Ig ELISA assays

Isotype specific ELISA assays were designed for the measurement of total IgM, IgG1, IgG2a, and IgE from culture supernatants. For each ELISA, 50  $\mu$ l of culture supernatant was assayed and quantified based on a standard curve. These ELISA utilized a biotinylated detection Ab followed by alkaline phosphatase streptavidin (Zymed, San Francisco, CA), and subsequent reaction with phosphatase substrate (Sigma Chemical Co., St. Louis, MO). Capture and detection Abs for the various isotype-specific ELISA were as follows: IgM, goat anti-mouse  $\mu$ -chain-specific Ab (capture) and monoclonal rat anti-mouse IgM b-7-6 (detection); IgG1, goat anti-mouse  $\gamma$ 1 Ab (capture and detection); IgG2a, goat anti-mouse  $\gamma$ 2a Ab (capture and detection); IgE, monoclonal rat anti-mouse  $\epsilon$ -chain B1E3 (capture); and monoclonal rat anti-mouse  $\epsilon$ -chain EM95 (detection). All goat Ab preparations were obtained from Southern Biotechnology Associates (Birmingham, AL). Affinity-purified anti-TNP IgM, IgG1, IgG2a, and IgE mAbs were used as standards.

## Results

### CpG ODN is a potent enhancer of immunization against HBsAg in mice

BALB/c mice immunized on a single occasion by i.m. injection of 1  $\mu$ g of HBsAg without adjuvant attained only low titers of Abs against HBsAg (anti-HBs) by 12 wk (mean endpoint dilution ELISA titer of total IgG about 400). Titers of anti-HBs were nearly seven times higher when the standard adjuvant for humans, aluminum hydroxide (Al<sub>2</sub>O<sub>3</sub>, commonly known as alum), was added at the same ratio (0.5 mg/ml) as used in the commercial hepatitis B vaccines (e.g., Engerix-B, SmithKline Beecham; Recombivax-HB, Merck). In contrast, anti-HBs titers were 20 times higher



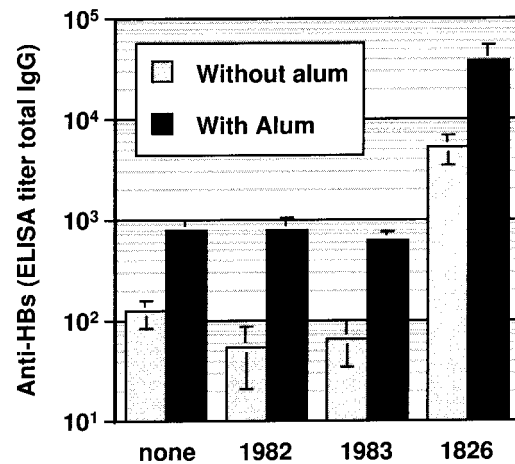
**FIGURE 1.** Kinetics and strength of humoral responses in BALB/c mice immunized against HBsAg by i.m. injection of 1  $\mu$ g of recombinant HBsAg protein, which was given alone ( $\times$ ), with alum (25 mg  $\text{Al}^{3+}$ /mg HBsAg) (\*), or with CpG ODN (1826) in doses of 10 ( $\blacktriangle$ ), 100 ( $\bullet$ ), or 500 (squl)  $\mu$ g. ODN was either given alone (open symbols) or together with alum (closed symbols). Each point represents the group mean ( $n = 8-10$ ) for titers of anti-HBs (total IgG) as determined in triplicate by end-point dilution ELISA assay. End-point titers were defined as the highest plasma dilution that resulted in an absorbance value (OD 450) two times greater than that of control nonimmune plasma with a cut-off value of 0.05.

when HBsAg was mixed with the immune activating CpG ODN 1826 and 180 times higher with this ODN and alum together (Fig. 1). The enhancing effects of ODN 1826 were surprisingly potent, as anti-HBs titers were not significantly different between groups receiving 10-, 100-, or 500- $\mu$ g doses, nor between groups receiving these doses of ODN mixed with alum. In other experiments we found that doses of 1  $\mu$ g or below had reduced effect (not shown). Anti-HBs titers in animals receiving ODN without CpG motifs (#1982 and 1983), alone or with alum, were not significantly different from those in the respective control groups (HBsAg alone or HBsAg plus alum) (Fig. 2). Treatment with CpG DNA was well tolerated by all mice, which exhibited no apparent ruffling of fur, diarrhea, or other signs of toxicity, even with the 500- $\mu$ g dose.

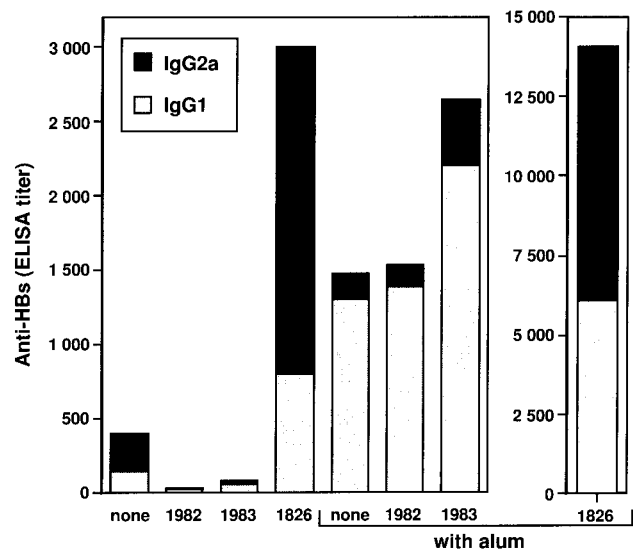
#### *CpG ODN induces a Th1-like response even in the presence of alum*

Immunization with HBsAg alone gave a mixed Th1/Th2 response, but with more anti-HBs Abs of the IgG2a (Th1) than IgG1 (Th2) isotype (Fig. 3). When alum was added as adjuvant, the response became predominantly Th2 with almost all anti-HBs Abs being of the IgG1 isotype. In contrast, in mice injected with HBsAg with CpG ODN (1826), the majority of Abs were IgG2a, indicating a very strong Th1-type response. Remarkably, even when combined with alum, the CpG ODN induced significantly more IgG2a than IgG1, indicating that the effects of CpG ODN dominate over those of alum with respect to T-help. Control ODN without CpG motifs (1982 and 1983) did not affect the isotype profile (Fig. 3).

These observations were further confirmed by the results of the CTL assays. Low levels of CTL were induced by immunization

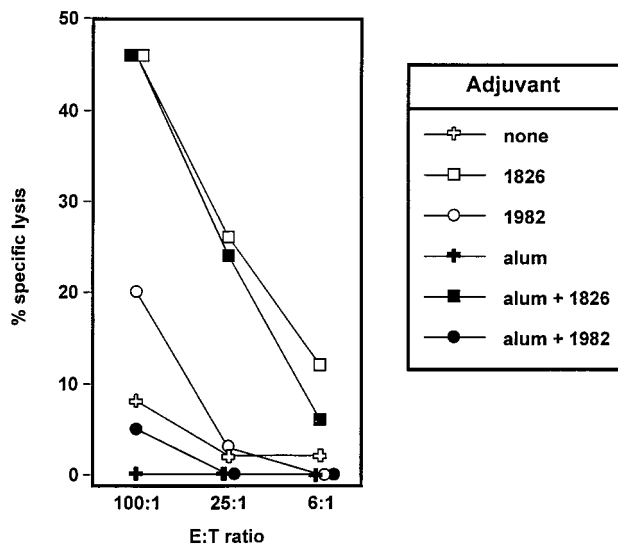


**FIGURE 2.** Strength of humoral response in BALB/c mice immunized i.m. with 1  $\mu$ g recombinant HBsAg without adjuvant or with 10  $\mu$ g of ODN and/or alum. Stippled bars indicate groups without alum and solid bars with alum. Each bar represents the group mean ( $n = 5$  for 1982, 1983;  $n = 10$  for none, 1826) for anti-HBs ELISA titers (total IgG) at 4 wk. Vertical lines represent the SEs of the mean.



**FIGURE 3.** Ab isotypes in BALB/c mice 4 wk after immunization with 1  $\mu$ g recombinant HBsAg protein without adjuvant, or with 10  $\mu$ g ODN and/or alum. Each bar represents the group mean ( $n = 5$  for 1982, 1983;  $n = 10$  for none, 1826) for anti-HBs titers as determined by end-point dilution ELISA assay. Stippled-hatched portions indicate IgG1 isotype (Th2) and solid portions indicate IgG2a isotype (Th1). Note different scale for 1826 with alum.

with HBsAg alone, and no CTL were detected with alum. The CTL response was much stronger with the addition of an immunostimulatory CpG ODN (1826), even in the presence of alum, confirming the ability of CpG ODN to overcome the Th2 bias of alum (Fig. 4). There were no differences between results obtained with 10, 100, or 500  $\mu$ g of the CpG ODN in the absence of alum (results not shown), indicating that complete immunostimulatory effects are realized at low doses. The non-CpG control ODN (1982) augmented the CTL response in the absence of alum, although to a much lesser degree than did 1826, and had very little effect in the presence of alum (Fig. 4).



**FIGURE 4.** CTL activity in splenocytes removed from mice 3 to 6 wk after immunization with 1  $\mu$ g HBsAg, which was given without alum (open symbols) or with alum (solid symbols) with the addition of either no oligo (58), 100  $\mu$ g CpG ODN 1826 (■), or 100  $\mu$ g non-CpG ODN 1982 (●). Each point represents the means of three animals that were each assayed in triplicate. E:T ratios are indicated on the horizontal axis. The vertical axis shows HBsAg-specific lysis as a percentage of the total possible lysis (% specific lysis).

#### *CpG ODN induce costimulatory molecule expression in vitro and in vivo*

Administration of CpG ODN with Ag or with Ag and alum resulted in the production of increased levels of Ab. This effect can be attributed to a number of CpG-mediated activities including the stimulation of monocytic/dendritic cells, the induction of proinflammatory cytokines, and the activation of B cell proliferation and differentiation (5). Collectively, these activities may create an environment highly conducive to Ag presentation and may trigger the expression of costimulatory molecules on Ag-presenting cells, which is required for the induction of T cell differentiation. To test this assumption, the expression of selected surface molecules was monitored on B cells after either *in vitro* or *in vivo* exposure to CpG ODN. In the first set of experiments, T cell-depleted spleen cells (containing predominantly B cells and a minor population of non-B, non-T cells) were cultured with ODN for 48 h and tested for expression of MHC class II, B7.2, and Ly-6C. As shown in Figure 5, B cells treated with CpG ODN demonstrated a marked up-regulation of MHC class II and B7.2, key molecules involved in both the induction of Th cells, and Th cell-B cell cognate interactions. Ly-6C is also up-regulated on B cells, an indicator of IFN- $\alpha$  or - $\gamma$  production in the culture (T. J. Waldschmidt, unpublished observations). The IFN is likely produced by the small number of NK or monocytic cells present in the culture and is seen in all three groups. Of interest, at high doses the non-CpG ODN also induced increases in MHC class II and B7.2, although the level of expression was lower than that observed with the CpG ODN.

To determine whether these *in vitro* activities would also be detectable *in vivo*, additional experiments were performed in which 500  $\mu$ g of CpG or non-CpG ODN was injected into mice *i.p.* and splenic B cell phenotype was assessed 2 and 6 days later by flow cytometry. The results of such an experiment, illustrated in Figure 6, are consistent with the *in vitro* studies in that B cell MHC class II and B7.2 were elevated at day 2 in mice receiving CpG ODN. At 6 days postinjection, class II remained high, whereas

B7.2 dropped to near baseline levels. Expression of Ly-6C was markedly increased at day 2 in response to CpG ODN and was still elevated at day 6. This latter observation suggests that CpG ODN induce high and sustained levels of proinflammatory cytokines, including IFN- $\alpha$  and - $\gamma$ . As in the *in vitro* experiments, the non-CpG ODN exhibited some biologic activity although its effects were generally less potent. Collectively, these data indicate that CpG ODN up-regulate costimulatory molecules on Ag-presenting cells and further enhance the priming environment by inducing the expression of proinflammatory cytokines.

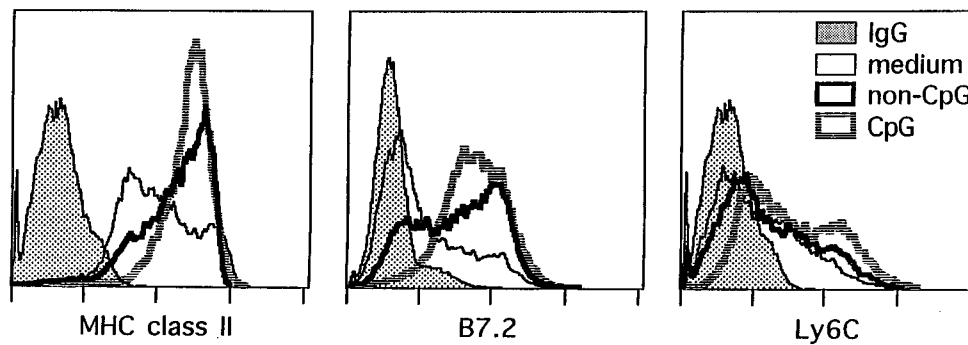
#### *CpG ODN promote in vitro B cell isotype switching*

From the perspective of the B cell, CpG ODN may promote *in vivo* Ag-specific IgG production either directly, by facilitating isotype switching of Ag-selected B cells, or indirectly, by inducing a strong Th cell response. Activation of the switch recombination program in naive B cells is known to require a strong differentiation signal and a specific cytokine that directs which heavy chain locus will be accessible (IFN- $\gamma$  promotes selection of IgG2a; IL-4 promotes the selection of IgE and IgG1) (16). To evaluate the ability of CpG ODN to induce B cell isotype switching, we tested two CpG ODN, 1668 and 1826, which activate B cell differentiation and IFN- $\gamma$  secretion from NK cells, as well as a control non-CpG ODN (1745), which has little effect (8, 10, 17).

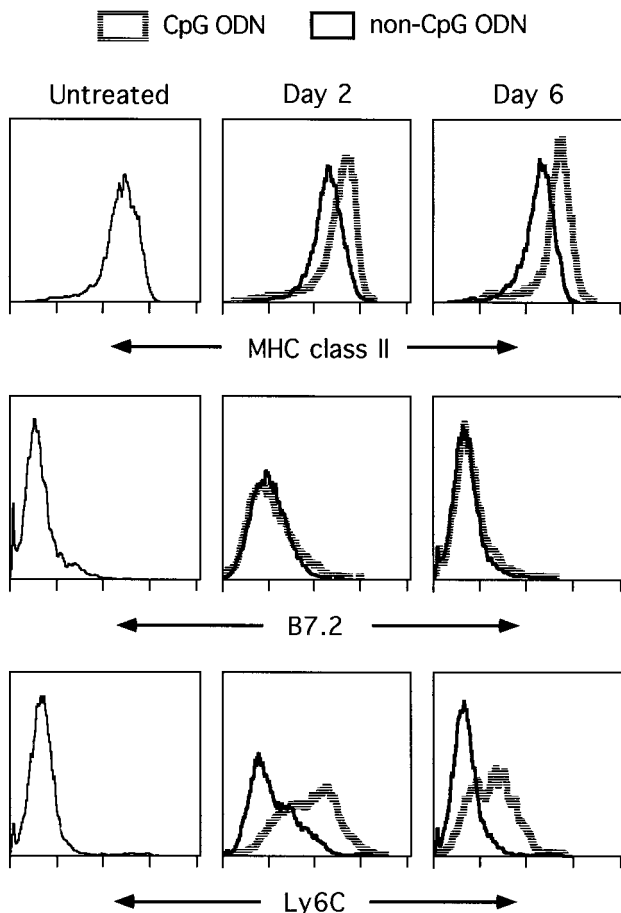
Resting B cells were purified by Percoll sedimentation and then cultured with LPS (as positive control), CpG ODN, or control non-CpG ODN. To mimic the Th1-like cytokine milieu induced *in vivo* by the CpG ODN, recombinant IFN- $\gamma$  was added to the cultures. After 6 days, culture supernatants were assayed for the presence of downstream isotypes. Both LPS and CpG ODN induced high levels of IgM secretion, but in the absence of IFN- $\gamma$  there was minimal secretion of IgG2a (Table I). Upon addition of IFN- $\gamma$  however, both LPS and CpG ODN promoted a marked increase in IgG2a secretion, strongly suggesting the induction of isotype switching. Of interest, the non-CpG ODN allowed for a modest level of IgM and IgG2a secretion, consistent with the low level of biologic activity observed in the other studies (Figs. 5, 6).

In a second set of experiments, the capacity of CpG ODN and IL-4 to promote switching to IgG1 and IgE was examined. In the first experiment, as shown in Table II, Percoll-sedimented B cells were cultured with LPS, CpG ODN, or non-CpG ODN in the presence or absence of IL-4. LPS and CpG ODN again promoted a strong IgM response but, in the absence of IL-4, induced little IgG1 and IgE secretion. In the presence of IL-4, however, both LPS and CpG ODN induced heightened levels of IgG1 and IgE although LPS proved to be a stronger stimulus. Non-CpG ODN induced a modest level of IgM but failed to promote secretion of the downstream isotypes. These results again suggest that CpG ODN directly promotes switch recombination in the presence of cytokines.

The cytokine dependence of IgG1 and IgE secretion is evidence that CpG ODN are not simply promoting Ig secretion from B cells that have already undergone switch recombination. However, to exclude this possibility more rigorously, we repeated the experiment using a highly purified population of naive B cells, which can be distinguished by the fact that they are IgD. IgD<sup>high</sup>B220<sup>+</sup> B cells were sorted purified and stimulated as in experiment 1. The results show that CpG ODN induce IgG1 and IgE secretion only in the presence of IL-4 and confirm the interpretation that the combined action of these signals has induced switch recombination (experiment 2, Table II).



**FIGURE 5.** CpG ODN-mediated induction of costimulatory molecules. T cell-depleted spleen cells were incubated in medium alone, 6  $\mu\text{g}/\text{ml}$  CpG ODN 1826, or 6  $\mu\text{g}/\text{ml}$  non-CpG ODN 1911. After 48 h of culture, cells were harvested, washed, and stained with Abs to MHC class II, B7.2, or Ly-6C followed by flow cytometric analysis. The isotype controls (shaded histograms) are shown for comparison. The derived MFI of the class II MHC stain was 10.84 for cells cultured in medium, 13.47 for non-CpG, and 18.53 for CpG; for the B7.2 stain the MFI was 1.14 for cells cultured in medium, 3.03 for non-CpG, and 5.92 for CpG; for the Ly-6C stain the MFI was 1.16 for cells cultured in medium, 1.60 for non-CpG, and 3.13 for CpG. Thus, although some activation of expression of these markers was observed in the cells treated with the non-CpG ODN, the average levels were approximately twice as high on the cells treated with the CpG ODN. These data are typical of those obtained in three separate experiments. Histograms are derived from the gated viable cells, as determined by forward and orthogonal light scatter.



**FIGURE 6.** In vivo effects of CpG ODN on B cell phenotype. BALB/c mice were injected i.p. with 500  $\mu\text{g}$  of either CpG ODN 1826 or non-CpG ODN 1911. Splenocytes were harvested 2 or 6 days after injection and stained with anti-B220 and either anti-MHC class II, anti-B7.2, or anti-Ly-6C followed by flow cytometric analysis. Spleen cells from an uninjected control mouse are shown for comparison. All histograms are derived from the B220<sup>+</sup> gated B cells.

## Discussion

Alum (e.g.,  $\text{Al}_2\text{O}_3$ ) was developed for therapeutic use about 75 years ago and is still the only adjuvant approved for human use. In

Table 1. Induction of Th-1-like Ab secretion by CpG ODN + IFN- $\gamma$

	Expt 1 <sup>a</sup>		Expt 2 <sup>b</sup>	
	IgM	IgG2a	IgM	IgG2a
LPS	35.5	0.66	43.2	0.1
LPS + IFN- $\gamma$	20	2.2	20.2	0.6
1668	26.3	0.38	17.2	0
1668 + IFN- $\gamma$	15.9	4.2	28.5	1.2
1826	ND	ND	8.5	0
1826 + IFN- $\gamma$	ND	ND	22.9	1.1
1745	1.6	0	1.6	0
1745 + IFN- $\gamma$	1.6	0.97	2.5	0.6

<sup>a</sup> Resting B cells were purified from BALB/c splenocytes (see *Materials and Methods*) and cultured in the presence of IFN- $\gamma$  at 100 U/ml to simulate the cytokine milieu induced by CpG DNA in vivo. Supernatants were then used for ELISA assays to detect IgM and IgG2a isotypes of Ab. Control cultures that were with or without IFN- $\gamma$  but lacking either LPS or ODN showed no IgM or IgG2a secretion (not shown). Results are given in  $\mu\text{g}/\text{ml}$  in both experiments. Less than 50 ng/ml of IgG1 was detected in these cultures.

<sup>b</sup> Resting B cells were purified from splenocytes of IFN- $\gamma$  knockout mice BALB/c genetic background in order to avoid possible CpG-mediated IFN- $\gamma$  secretion by contaminating NK cells. These cells were incubated with IFN- $\gamma$  at 20 U/ml since previous titration studies showed 10 and 100 U/ml to induce equivalent isotype switch. Each value is the mean of four samples, each assayed in triplicate.

the studies presented herein, we show that CpG ODN can induce higher Ab titers than alum, even at low doses. An advantage of the ODN over alum is that it could be used to enhance immune responses to live-attenuated or multivalent vaccines that cannot be mixed with alum. In situations where a particularly potent adjuvant is required, for example with a poorly immunogenic Ag or to overcome non- or hyporesponsiveness, it may be possible to take advantage of the synergistic effect of CpG ODN and alum together. Our studies indicate that the CpG-enhancing effect requires only a single CpG motif (i.e., ODN 1668). In ongoing studies to determine the optimal number and spacing of CpG motifs, we have found that the effect can be somewhat further enhanced with ODN containing two motifs but that additional motifs do not seem to contribute a further increase in the immune response (H. L. Davis et al., unpublished results).

Our studies demonstrate that the in vivo immune-enhancing effect of CpG DNA is associated with the induction of costimulatory molecule expression including class II MHC and B7.2. The induction of class II MHC, a marker for lymphocyte activation and

Table II. Induction of Th-2-like Ab secretion by CpG ODN + IL-4

	Expt 1 <sup>a</sup>			Expt 2 <sup>b</sup>		
	IgM	IgG1	IgE	IgM	IgG1	IgE
LPS	92	0.32	0.05	6.2	0.27	0
LPS + IL-4 <sup>c</sup>	20	1.53	4.0	6.7	3.0	2.25
1668	7.2	0	0	6.9	0	0
1668 + IL-4	8.0	0.18	0.45	11.2	0.57	0.49
1745	0.5	0	0	0	0	0
1745 + IL-4	0.5	0.014	0	0	0.01	0

<sup>a</sup> Resting B cells were obtained by depleting splenocytes of T cells using anti-Thy 1.1 and complement followed by Percoll fractionation (see *Materials and Methods*). Cells were cultured with the indicated additives as described in *Materials and Methods*. Experiments were performed in quadruplicate. Results are given in  $\mu\text{g/ml}$  in both experiments. There was no detectable IgG2A in these cultures. Control cultures that were with or without IL-4 but lacking either LPS or ODN showed no IgM, IgG1, or IgE secretion (not shown).

<sup>b</sup> 6B2<sup>+</sup> IgD<sup>high</sup> B cells were purified from splenocytes by flow cytometry fractionation (see *Materials and Methods*). Experiments were performed in quadruplicate.

<sup>c</sup> IL-4 was used at 1000 U/ml.

Ly-6C, a marker for the presence of IFNs, persisted for at least 6 days following injection of CpG ODN. Since previous studies have shown that the cytokine induction by CpG DNA lasts for less than 24 h (6, 18), this sustained expression demonstrates that the cytokine effect outlasts the elevated cytokine levels. These data suggest that CpG DNA may improve the efficiency with which local Ag-presenting cells present coinjected Ags to T cells and induce T helper cell activity. Indeed, in support of this hypothesis, we have found that the effect of CpG DNA depends on coadministration with the Ag and is substantially reduced if the Ag and ODN are injected in separate sites (G. Weiner et al., H. L. Davis et al., unpublished data). A consistent but less dramatic stimulatory effect of the control ODN on costimulatory molecule expression was also noted. We consistently observed that phosphorothioate backbone ODN without a CpG caused a dose-dependent, non-sequence-specific B cell proliferation and monocyte/macrophage activation, which, while weaker than that seen with phosphorothioate CpG ODN, was more substantial at higher ODN concentrations (not shown). These results are in agreement with the data of Monteith et al., who showed that all phosphorothioate backbone ODN can induce B cell proliferation and splenomegaly *in vivo*, indicating an inherent immune stimulatory effect of the phosphorothioate backbone (19).

In addition to its ability to enhance Ag presentation, and thereby provide T cell help, our previous studies have shown that CpG ODN directly stimulate murine B cells to proliferate and secrete IgM (3). The possibility thus arises that together with appropriate cytokine signals, CpG ODN may directly promote switch recombination at the level of the individual B cell. It is clear from numerous studies that two signals are required to induce the switch recombination program in naive B cells (16). In addition to a specific cytokine that directs which heavy chain locus will be accessible, B cells require a strong differentiation signal. Murine cytokines known to direct isotype-specific switching include IL-4, which promotes switching to IgG1 and IgE (20–23), and IFN- $\gamma$ , which induces switching to IgG2a (24). In the mouse, LPS or reagents that engage CD40 have been demonstrated to supply the necessary differentiation signals for switch recombination. Based on these findings, we tested whether CpG ODN could also induce isotype switching in combination with either IL-4 or IFN- $\gamma$ . Our studies confirmed the ability of CpG ODN to promote isotype switching in either the Th1 or Th2 direction, depending on the cytokine environment. Unlike LPS, CpG DNA induces a high

level of IFN- $\gamma$  secretion from NK cells, which explains the predominance of IgG2a isotypes observed in the *in vivo* studies.

Strong cell-mediated immunity, including CTL activity, is essential for protection against many diseases and is therefore desirable with almost all vaccines. Although not essential for protective immunity against HBV, CTL may be important for avoiding or overcoming the chronic carrier state. Indeed, many previously infected individuals, even years after clinical and serologic recovery, have traces of HBV in their blood and have HBV-specific CTL that express activation markers indicative of recent contact with Ag (25). These results suggest that sterilizing immunity may not occur after HBV infection and that chronic activation of CTL is responsible for keeping the virus under control. There are currently more than 250 million chronic HBV carriers in the world, many of whom will eventually suffer from cirrhosis or hepatocellular carcinoma (26). Repeated doses of an HBsAg subunit vaccine (with alum) reduced viral replication in 50% of vaccinated chronic carriers (27). Addition of CpG ODN would presumably improve these results through its strong Th1 bias and induction of CTL. This might be far more effective than the currently used IFN therapy, which cures only 10 to 20% of treated individuals (28) and would also be much less expensive, an important factor since the highest incidence of HBV chronicity is in less developed areas of the world. Anti-viral drugs (e.g., lamivudine) are also expensive and, although they can reduce the circulating virus to undetectable levels, there is usually a return to pretreatment levels if the drug is stopped.

An important disadvantage of alum is the induction of a Th2-rather than a Th1-type immune response. In the case of recombinant HBsAg, the use of alum as an adjuvant appears to interfere with cell-mediated immunity and blocks activation of CD8<sup>+</sup> CTL (29). In contrast, our studies show that CpG ODN induces strong Th1-type immune responses to HBsAg and can even overcome the Th2 bias of alum for both Ab isotype and CTL response when the two agents are used together. Thus it is possible to induce strong humoral responses, owing to synergistic action of the alum and CpG ODN, while still permitting CTL.

The Th1 effect of CpG DNA may also be relevant to asthma, an immune-mediated inflammatory disease characterized by Th2 immune responses to innocuous, inhaled environmental Ags. The much higher prevalence of asthma in developed nations has been linked to the high hygiene level and rapid treatment of childhood infections (30). We hypothesize that early exposure to bacterial DNA (and its Th1-immunostimulatory CpG motifs) would push the immune system away from Th2- and toward a Th1-type response to environmental Ags. This may account for the lower incidence of asthma in less developed countries, where there is a much higher frequency of childhood upper respiratory infections. Addition of CpG ODN to all pediatric vaccines could re-establish a Th1-type response to environmental Ags. Indeed, we have recently found that CpG ODN are effective at preventing and treating asthma in a mouse model.<sup>4</sup>

Phosphorothioate-modified ODN can be easily produced on a large scale under Good Manufacturing Practices conditions at a cost of approximately \$200/g. In antisense studies, ODN have been found to be safe when administered at doses above 100 mg/kg, which is several orders of magnitude over the dose of 10  $\mu\text{g}$  used herein (0.36 mg/kg). It is noteworthy that human clinical trials have reached continuous daily doses of up to 4 mg/kg without apparent drug-related toxicity (31, 32). Moreover, in other studies

<sup>4</sup> J. N. Kline, T. R. Businga, T. J. Waldschmidt, J. V. Weinstock, and A. M. Krieg. 1997. Modulation of the asthmatic response by CpG oligodeoxynucleotides. Submitted for publication.

we and others have demonstrated that human cells also show immune activation in response to CpG DNA (10, 33). Together, these factors suggest that CpG ODN could be safe, effective, and economical replacements or supplements for alum in human and animal vaccine formulations.

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