CpG DNA increases primary malignant B cell expression of costimulatory molecules and target antigens

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Abstract: Multiple factors, including expression of costimulatory molecules, antigen-presenting molecules, and target antigens, likely impact the efficacy of antibody therapy and other approaches to the immunotherapy of B cell malignancy. Unmethylated CpG-dimemotides in select base contexts (“CpG motifs”) that resemble sequences found in bacterial DNA are potent immunostimulatory agents capable of inducing a complex immune response, including a strong B cell stimulus. We examined the effect of a potent human CpG oligonucleotide (CpG ODN 2006) on different types of primary human malignant B cells and reactive follicular hyperplasia. CpG oligodeoxynucleotide (CpG ODN), but not control (non-CpG ODN), increased the expression of costimulatory molecules (CD40, CD80, CD86, CD54) on malignant B cells without altering the phenotype of B cells obtained from reactive follicular hyperplasia. CpG ODN also enhanced expression of class I and class II MHC in most samples. CD20 expression was increased in response to CpG ODN, most notably in B-CLL and marginal zone lymphoma. An inverse correlation was found between baseline expression of CD20 and CD40 and their expression after exposure to CpG ODN, thus the most significant increase in expression of these molecules was found in those samples that had the lowest baseline levels. In conclusion, CpG ODN can lead to increasing expression of molecules involved in costimulation, antigen presentation, and as targets for antibody-based therapy and deserve further evaluation as potential immunotherapeutic agents for B cell malignancy. J. Leukoc. Biol. 69: 81–88; 2001.

Key Words: ODN · follicular hyperplasia · B cell activation · non-Hodgkin lymphoma · CLL · monoclonal antibodies

INTRODUCTION

Extensive cross-talk exists between healthy B cells and T cells. There is evidence that malignant B cells also communicate with T cells. However, malignant cells appear to differ from their normal counterparts in a number of ways, including a decreased tendency to undergo apoptosis in response to normal signals [1–3], altered expression of a variety of surface markers, and altered ability to function as effective antigen-presenting cells [4]. Immunotherapeutic approaches have become part of our therapy of some B cell malignancy subtypes recently. Improvements in the immunotherapy of B cell malignancy will be based on the growing understanding of the cellular immunology of this disease [5].

A variety of cellular receptors and antigens are involved in growth, differentiation, and apoptosis of B cell malignancies. Antibodies or ligands against a variety of antigens, including CD20, surface immunoglobulins, major histocompatibility complex (MHC) II, CD80, CD86, and CD40, can cause growth inhibition by inducing cell-cycle arrest or apoptosis [6–17]. The relative contribution of antibody-dependent cellular cytotoxicity (ADCC) versus trans-membrane signaling mediated by anti-B cell antibodies remains unclear. Nevertheless, there is reason to predict that upregulation of the target antigens would improve elimination of the malignant cell by either mechanism.

Previously, we found that unmethylated CG-dinucleotides within certain sequence contexts (CpG DNA) are recognized by the vertebrate immune system as foreign DNA (bacterial or viral). CpG DNA activates a coordinated set of immune responses that include innate immunity [macrophages, dendritic cells, and natural killer (NK) cells], humoral immunity, and cellular immunity [18–20]. As a vaccine adjuvant, CpG DNA is at least as effective as the gold standard complete Freund’s adjuvant (CFA) but induces higher Th1 activity and demonstrates less toxicity [21–25]. Recently, we identified a human CpG motif that triggers proliferation and activation of primary human B cells [26]. Synthetic oligodeoxynucleotides (ODN) containing the CpG motif have been shown to alter the phenotype of B-CLL cells [27]. In the present study, we examined how CpG DNA impacts primary human malignant B cells from a variety of histologies with a particular focus on how such changes might impact the efficacy of monoclonal antibody (mAb) therapy.
MATERIALS AND METHODS

Cell culture

Fresh lymph node samples were obtained from the operating site and were minced with a scalpel under aseptic conditions. The resulting suspension was passed sequentially through a sterilized sieve-tissue grinder containing a nylon mesh screen, a 150 μm mesh screen, and a 60 μm mesh screen. Alternatively, mononuclear cells were obtained from peripheral blood or pleural fluid as described [28]. Red blood cells were removed by resuspending the cells in 5 ml red cell lysis buffer, according to standard procedures. Cells were frozen slowly and stored in liquid nitrogen. For analysis, cells were thawed and resuspended in 10% (v/v) heat-inactivated (56°C, 30 min) fetal calf serum (FCS; HyClone, Logan, UT), 1.5 mM L-glutamine (all from Gibco BRL, Grand Island, NY), and incubated on a 96-well plate (1×10⁶ cells/ml) in the presence of ODN as indicated below. Not all assays were performed for all samples because of the limited number of cells available for some samples.

ODN

Nucleoside-resistant phosphorothioate-modified ODN were provided by Coley Pharmaceutical Group Inc. (Wellesley, MA). Endotoxin levels in all ODN were <0.075 enzyme unit (EU)/ml by Limulus amoebocyte lysate (LAL). Control ODN included an ODN in which unmethylated cytosines from a CpG ODN were replaced by methylated cytosines and a poly C ODN. Specific sequences were as follows: CpG ODN 2006, 5'-TCG TCG TTT TGT CGT TTT GTCG TT-3'; control ODN 2017: 5'-CCC CCC CCC CCC CCC CCC CC-3'. ODN were diluted in TE [10 mM Tris-HCl, 1 mM ethylenediaminetetraacetate (EDTA), pH 8] using pyrogen-free reagents. ODN were added at a final concentration of 5 μg/ml.

Flow cytometry

Cells were washed and resuspended in ice-cold phosphate-buffered saline (PBS) or Annexin V binding buffer (10 mM HEPES/NaOH, 140 mM NaCl, 2.5

TABLE 1. Percentage of CD19+ Cells in Samples Tested

<table>
<thead>
<tr>
<th>Histology</th>
<th>Source</th>
<th>%CD19+ cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chronic lymphocytic leukemia 1</td>
<td>Peripheral blood</td>
<td>&gt;98%</td>
</tr>
<tr>
<td>Chronic lymphocytic leukemia 2</td>
<td>Peripheral blood</td>
<td>70%</td>
</tr>
<tr>
<td>Large-cell lymphoma 1</td>
<td>Pleural fluid</td>
<td>55%</td>
</tr>
<tr>
<td>Large-cell lymphoma 2</td>
<td>Lymph node</td>
<td>75%</td>
</tr>
<tr>
<td>Mantle-cell lymphoma</td>
<td>Lymph node</td>
<td>98%</td>
</tr>
<tr>
<td>Diffuse mixed small- and large-cell lymphoma</td>
<td>Lymph node</td>
<td>50%</td>
</tr>
<tr>
<td>Marginal zone lymphoma 1</td>
<td>Lymph node</td>
<td>80%</td>
</tr>
<tr>
<td>Marginal zone lymphoma 2</td>
<td>Peripheral blood</td>
<td>&gt;94%</td>
</tr>
<tr>
<td>Reactive follicular hyperplasia</td>
<td>Lymph node</td>
<td>35%</td>
</tr>
</tbody>
</table>

Fig. 1. Impact of ODN on morphology of marginal zone lymphoma cells. Malignant B cells from a patient with marginal zone lymphoma were stimulated with 5 μg/ml of no ODN (A and D), control ODN (B and E), or CpG ODN (C and F) for 72 h and analyzed by flow cytometry. A, B, and C illustrate FSC (x-axis) versus SSC (y-axis). D, E, and F illustrate CD19 expression (x-axis) against FSC (y-axis), allowing for separation of B cells from other leukocyte subpopulations. Upon stimulation with CpG ODN, B cells shifted up and to the right, indicating an increase in granularity and size. No changes could be detected without stimulation or on stimulation with the non-CpG ODN. Similar changes were found in other samples tested (G).
mM CaCl₂, pH 7.4). Murine or human serum was added (final concentration, 1% by v) to block nonspecific binding of antibodies. Surface antigen staining was performed as described [28]. In brief, 1×10⁶ cells per sample were stained with CyChrome-labeled anti-CD19 and fluorescein isothiocyanate (FITC)-or phycoerythrin (PE)-labeled antibodies as indicated for 20 min on ice. Then, they were washed and analyzed by flow cytometry. mAbs to CD40 (FITC)- or phycoerythrin (PE)-labeled antibodies as indicated for 20 min on stained with CyChrome-labeled anti-CD19 and fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-labeled antibodies as indicated for 20 min on ice. Then, they were washed and analyzed by flow cytometry. mAbs to CD40 (FITC)- or phycoerythrin (PE)-labeled antibodies as indicated for 20 min on stained with CyChrome-labeled anti-CD19 and fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-labeled antibodies as indicated for 20 min on ice. Then, they were washed and analyzed by flow cytometry. mAbs to CD40 (FITC)- or phycoerythrin (PE)-labeled antibodies as indicated for 20 min on

Carboxyfluorescein diacetate succinimidyl ester (CFSE) staining

CFSE (5- and 6-carboxyfluorescein diacetate [CFDA], Molecular Probes, Junction City, OR) is a fluorescein-derived intracellular fluorescent label, which is divided equally between daughter cells upon cell division. Staining of cells with CFSE allows quantification and immunophenotyping of proliferating cells in a mixed-cell suspension. Interference between oligonucleotide degradation products and thymidine uptake (standard proliferation assay) is avoided by using this method. The technique is described in detail by Lyons and Parish [29]. Briefly, cells were washed twice in PBS, resuspended in PBS (1×10⁶ cells/ml) containing CFSE at a final concentration of 1 μM, and incubated at 37°C for 10 min. Cells were washed three times with PBS.

RESULTS

Morphologic changes induced by CpG-ODN

Our prior studies demonstrated that activation of naive human B cells by CpG ODN results in increased cell size (FSC) and granularity (SSC) [26]. Therefore, we determined first whether such changes also occur in malignant B cells. Primary malignant B cells were obtained from lymph node biopsies, peripheral blood, or pleural fluid of patients with various types of B cell malignancy. In addition, cells from the lymph node of a patient with benign, reactive, follicular hyperplasia were studied. Nine samples in total were evaluated (see Table 1). Cells were incubated for 72 h in media containing CpG ODN 2006 (5 μg/ml) or control ODN 2017. FSC and SSC were examined with gating on CD19+ viable cells (Fig. 1). Varying degrees of change in FSC were noted in response to CpG ODN 2006 when compared with control ODN 2017 or medium alone. Similar changes were not seen in the cells from the patient with benign, reactive, follicular hyperplasia. The changes in SSC for each case were comparable with those in FSC.

Phenotypic changes induced by CpG-ODN

Expression of CD20, CD40, CD69, CD80, CD86, surface Ig, CD54, MHC I, MHC II, and an HLA-DR variant antigen (recognized by moAb 1D10) was examined on viable CD19+ cells after incubation of cells with CpG ODN for 72 h. Each of these markers was upregulated on cells obtained from the malignant samples—to varying extents in response to the CpG ODN 2006 compared with the control ODN 2017 (Figs. 2–4). CpG ODN 2006 and control ODN 2017 had no detectable effect on binding of isotype-matched control antibodies, demonstrating enhanced binding was not a result of upregulation of Fc receptors.

CD20 was expressed to varying degrees in all samples tested. As is well-known, baseline CD20 expression was lower in the B-CLL samples when compared with the B cell malignancies of other histologies. CpG ODN 2006, but not the control ODN 2017, increased CD20 expression in both B-CLL samples and both marginal zone lymphomas. Limited upregulation was seen in the other lymphoma samples. Nonmalignant CD19+ cells derived from the reactive follicular hyperplasia actually decreased expression of CD20 in response to CpG ODN (Fig. 3). These data demonstrated a reverse correlation between baseline expression of CD20 and CD40 and expression of these markers after incubation with CpG ODN. Those with lower baseline levels of CD20 and CD40 (r: −0.6; −0.4; Fig. 4) were the most responsive samples. This correlation was less clear for the other markers. The effect of CpG DNA on expression of CD20 and 1D10 in nine additional CLL samples was also studied. As indicated in Figure 5, CpG DNA increased expression of both CD20 and 1D10 in these samples.

Fig. 2. Impact of ODN on expression of surface antigens on marginal zone lymphoma cells. Flow cytometric analysis of surface-antigen expression on malignant B cells from a patient with marginal zone lymphoma was performed 72 h after stimulation with 5 μg/ml CpG ODN or non-CpG ODN. On stimulation with CpG ODN, median fluorescence intensity for all markers tested shifted to the right, indicating an increase in surface expression. Thin curves indicate incubation with medium alone; dotted curves, incubation with control ODN; and bold curves, incubation with CpG ODN.

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Proliferation of malignant B cells in response to CpG-ODN

CpG ODN induce a strong proliferative response of primary, benign, human B cells [26]. Before assessing whether CpG ODN induce proliferation of primary malignant B cells in vitro, we assessed whether CpG ODN impact the spontaneous apoptosis that results when primary malignant B cells are cultured in vitro. CpG ODN had no clear effect on the percent of dead cells seen after in vitro culture as determined by morphological criteria or the presence of DNA breaks (unpublished results) [30]. We then evaluated whether CpG ODN impact the proliferation of malignant B cells. This was done only on select samples because of the high rate of cell death seen with many samples after 4 days in vitro. Cells were stained with CFSE (see Materials and Methods) and incubated for 4 days with and without ODN. Proliferation of cells was demonstrated by a loss of CFSE stain with every cell division. In B-CLL, malignant cells were identified by their expression of CD5 and CD19. Proliferation in response to CpG ODN was noted in a subpopulation of malignant B cells (CD5+ and CD19+) and benign B cells (CD5− and CD19+), although the proliferating cells in the benign population appeared to have undergone more cell divisions (Fig. 6). CpG ODN also appeared to induce proliferation of the CD19+ cell population in the marginal zone lymphoma (Fig. 6). No proliferation was seen in response to the control ODN (unpublished results). Proliferation studies were not done on the other samples because of the limited number of cells available.

**DISCUSSION**

Bacterial DNA and select synthetic ODN containing the CpG motif can induce activation and proliferation of benign, human,
primary B cells [26, 31]. In addition, we have demonstrated in a mouse model that CpG ODN can enhance the efficacy of mAb therapy of lymphoma [32]. Understanding the direct effect of CpG ODN on malignant B cells will be important as we consider how this potent class of agents might be used in the immunotherapy of lymphoma.

In the present study, we evaluated samples of various primary, malignant, human B cells to assess how they respond to CpG ODN. All B cell lymphoma cells tested increased in size and granularity, upregulated activation markers (CD80, CD86, CD40, CD54, CD69), and upregulated antigen-presentation molecules (MHC I, MHC II) in response to CpG ODN. A control poly C ODN showed only minor effects, confirming that the specific sequence of the CpG ODN, and not the phosphorothioate backbone, is responsible for the change in malignant, B cell phenotype.

The extent of phenotypic change induced by CpG ODN differed from sample to sample. Interestingly, there was an inverse correlation for CD20 and CD40 between the baseline level of antigen expression and the extent of the response to CpG ODN, which had little effect on nonmalignant B cells obtained from benign, reactive, follicular hyperplasia. The phenotype from these B cells suggested they were already highly activated. The number of samples studied was too small for us to assess rigorously as to whether there is a correlation between response to CpG ODN and histology. Nevertheless, these data suggest CpG ODN may reverse low expression of costimulatory molecules on malignant B cells that correspond to a low level of activation, although its effects on cells already in an activated state are less profound.

An important question relates to whether the observed phenotypic responses are a result of direct effects of the CpG ODN on the malignant B cells or whether CpG ODN induced production of cytokines from benign cells within the samples that secondarily induced the phenotypic changes in the malignant B cells. Measurement of the induction of cytokine production was not part of the experimental design of the present study. However, indirect evidence suggests cytokines were not responsible for the observed effects. We observed no correlation between the purity of the malignant B cell populations and the degree of antigen upregulation. For example, the marginal zone lymphoma and CLL samples highlighted in Figure 3 demonstrated a high degree of upregulation of CD20 and other antigens, although these samples consisted almost exclusively of malignant B cells (see Table 1). Nevertheless, the question of whether the observed effects are a result of direct effects of CpG ODN, or indirect effects mediated by cytokines, is an important one that is currently under investigation.

A growing body of data indicate the intensity of target-antigen expression impacts on the efficacy of therapy with
mAbs directed at that particular target. CpG ODN could enhance the efficacy of therapy by increasing expression of target antigen. The anti-CD20 mAb Rituximab has been shown to be effective clinically in several trials [6, 33–37] and has recently been approved for the therapy of follicular B cell lymphoma. Expression of CD20 is relatively low on B-CLL cells, which may provide a partial explanation as to why Rituximab is less effective for CLL than for some other B cell malignancies [38]. Also, there are now studies showing that a small minority of tumors that re-emerge following Rituximab therapy can lack CD20 expression [39, 40]. The humanized mAb 1D10 recognizes an HLA-DR variant antigen [16]. We are evaluating this antibody currently in a phase I clinical trial in patients with lymphoma. One limitation to the use of this antibody is that the target antigen is expressed only by approximately 60% of B cell lymphomas. Interestingly, the expression of the 1D10 antigen was upregulated by CpG ODN in all lymphoma samples tested. In addition, CpG ODN have been shown to increase NK cell activity [31, 41] and the activity of macrophages [42]. We have found synergy between CpG ODN and mAb in a murine lymphoma model [32]. Thus, CpG ODN could impact the success of antibody therapy by both enhancing expression of the antigen expression and effector cell-mediated killing.

The data outlined above suggest CpG ODN have the potential to enhance development of an immune response against malignant B cells even in the absence of anti-tumor antibody.

Fig. 6. Comparison of CpG ODN-induced proliferation of malignant and normal B cells. Peripheral blood mononuclear cells from two patients, one with B-CLL and one with marginal zone lymphoma with circulating malignant cells, were incubated for 72 h with CpG ODN or medium alone and evaluated by two-color flow cytometry. CFSE fluorescence (x-axis) and expression of CD5 (CLL) or CD19 (marginal zone lymphoma; y-axis) were evaluated.
CpG ODN alter the phenotype of malignant B cells (increased expression of CD80, CD86, class I and class II MHC) and thus could render malignant B cells more immunogenic. A number of studies by us and others have shown that CpG ODN can activate antigen-presenting cells [26, 43–46]. Indeed, in our prior studies, CpG ODN were as effective adjuvants as CFA in a murine lymphoma tumor vaccine model [22]. Therefore, CpG ODN could alter the target cell and antigen-presenting cells in a manner that could induce development of a more potent active anti-tumor response.

In conclusion, the studies outlined above suggest CpG ODN have potential as an effective immunotherapeutic agent in a variety of B cell malignancies. Possible beneficial effects include: 1) increased expression of antigens targeted by therapeutic antibodies; 2) activation of effector cells responsible for ADCC; 3) improved immunogenicity of malignant B cells; and 4) enhanced development of an antigen-specific immune response by the adjuvant activity of CpG ODN. To explore these possibilities further, continued investigation of the effects of CpG ODN at the molecular level and on tumor-cell phenotype, proliferation, and apoptosis is needed. Clinical-grade ODN are relatively easy to synthesize and have been evaluated extensively in anti-sense clinical trials. Based on the data outlined above, we have begun a clinical trial recently with CpG ODN, exploring the immunologic and therapeutic effects of CpG ODN in patients with B cell malignancy.

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REFERENCES


