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Activation of Cutaneous Dendritic Cells by CpG-Containing Oligodeoxynucleotides: A Role for Dendritic Cells in the Augmentation of Th1 Responses by Immunostimulatory DNA

Thilo Jakob, Patricia S. Walker, Arthur M. Krieg, Mark C. Udey, and Jonathan C. Vogel

Genetic vaccination depends at least in part on the adjuvant properties of plasmids, properties that have been ascribed to unmethylated CpG dinucleotides in bacterial DNA. Because dendritic cells (DC) participate in the T cell priming that occurs during genetic vaccination, we reasoned that CpG-containing DNA might activate DC. Thus, we assessed the effects of CpG oligodeoxynucleotides (CpG ODN) on Langerhans cell (LC)-like murine fetal skin-derived DC (FSDDC) in vitro and on LC in vivo. Treatment with CpG ODN as well as LPS induced FSDDC maturation, manifested by decreased E-cadherin-mediated adhesion, up-regulation of MHC class II and costimulator molecule expression, and acquisition of enhanced accessory cell activity. In contrast to LPS, CpG ODN stimulated FSDDC to produce large amounts of IL-12 but only small amounts of IL-6 and TNF-α. Injection of CpG ODN into murine dermis also led to enhanced expression of MHC class II and CD86 Ag by LC in overlying epidermis and intracytoplasmic IL-12 accumulation in a subpopulation of activated LC. We conclude that immunostimulatory CpG ODN stimulate DC in vitro and in vivo. Bacterial DNA-based vaccines may preferentially elicit Th1-predominant immune responses because they activate and mobilize DC and induce them to produce large amounts of IL-12. The Journal of Immunology, 1998, 161: 3042–3049.

D

NA-based vaccines may be the first gene therapies to be successfully employed for the treatment or prevention of human diseases. The mechanisms responsible for elicitation of humoral and cellular immune responses following genetic immunization remain incompletely characterized. Intracutaneous or intramuscular injection of bacterial plasmids carrying eukaryotic expression vectors results in synthesis of the encoded proteins in multiple cell types, including dendritic cells (DC)4 (1–6). It is now apparent that successful genetic vaccination depends not only on the ability of injected cDNAs to be transcribed and translated, but also on the adjuvant properties of bacterial DNA (7–11).

Recent studies demonstrated that the adjuvant properties of bacterial DNA are related to the high frequency of unmethylated CpG dinucleotides in bacterial compared with eukaryotic DNA (reviewed in Ref. 12). Synthetic phosphorothioate oligodeoxynucleotides (ODN) containing a consensus immunostimulatory motif (5′-purine-purine-CpG-pyrimidine-pyrimidine-3′; CpG ODN) can also act as adjuvants (9). We became interested in the adjuvant properties of bacterial DNA and CpG ODN while studying the efficacy of genetic vaccines in a murine model of leishmaniasis. We determined that a plasmid encoding gp63, a Leishmania major cell surface protein, induced a gp63-specific Th1 response and protected some BALB/c mice from infection (~30%), while gp63 protein-based vaccines were ineffective (13). Gurunathan et al. recently demonstrated that plasmids encoding another L. major protein, the LACK Ag, induce considerably more protection (14). In a subsequent study, we observed that an ODN containing two CpG immunostimulatory motifs in a tandem array (CpG ODN 1826) converted an ineffective parasite lysate vaccine into one with considerable efficacy (i.e., one that afforded ~40% protection).5 Co-administration of CpG ODN 1826 and live parasites to susceptible mice also abrogated infection in most animals (~65% of those inoculated; see Footnote 5). Similar results have recently been reported by Lipford et al. (15).

Because DC are thought to be essential for the initiation of primary immune responses in T cells in vivo (16), we hypothesized that CpG ODN might be potent activators of DC. We focused on immature (nonlymphoid) DC because they are more likely to be involved in the acquisition of Ag (including Leishmania Ag) in peripheral tissues than mature interdigitating DC in lymphoid organs (17, 18), and made use of an in vitro model of murine epidermal Langerhans cells (LC), i.e. fetal skin-derived DC (FSDDC), that has recently been developed in our laboratory (19). FSDDC are expanded in primary cultures of C57BL/6 fetal skin and are isolated as tight aggregates comprised of cells (immature FSDDC-I) with an LC-like phenotype. Like LC, FSDDC-I spontaneously give rise to cells that resemble mature

4 Abbreviations used in this paper: DC, dendritic cell; CpG ODN, oligodeoxynucleotide containing the CpG motif; LC, Langerhans cell; FSDDC-I (-M), fetal skin-derived dendritic cells-immature (-mature); GM-CSF, granulocyte-macrophage CSF; PE, phycoerythrin.
interdigitating DC (mature FSDDC (FSDDC-M)) during a several-day subculture period (19) and can be triggered to differentiate into FSDDC-M by inflammatory mediators (IL-1, TNF-α, and LPS) (20). Because FSDDC model the transition of LC into interdigitating DC with considerable fidelity, we assessed the responses of these cells to various ODN, including CpG ODN 1826. We also assayed ODN for LC-activating activity in vivo. Our results indicate that CpG ODN that function as adjuvants in vivo (21–23) are assayed ODN for LC-activating activity in vivo. Our results indicate that CpG ODN that function as adjuvants in vivo (21–23) are potent activators of immature cutaneous DC in vitro and in vivo. In addition, the ability of CpG ODN 1826 to induce the production of large amounts of IL-12 by DC may explain its propensity to augment the development of Th1 immune responses in vivo (22, 23).

Materials and Methods

Animals

Eight- to twelve-week-old female C57BL/6, timed pregnant C57BL/6, and female BALB/c mice were obtained from the National Cancer Institute Animal Production Program (Frederick, MD) and were housed and used in accordance with institutional guidelines. Gestational day 0 represents the day of conception.

Propagation and stimulation of FSDDC

FSDDC were propagated from day 16 C57BL/6 fetal skin in FCS-containing medium supplemented with murine recombinant GM-CSF (10 ng/ml; R&D Systems, Minneapolis, MN) and CSF-1 (10 ng/ml; PeproTech, Rocky Hill, NJ) and were isolated as aggregates as recently described (19). When necessary, FSDDC-I were dissociated with trypsin in EDTA (0.025% trypsin in calcium- and magnesium-free HBSS containing 1 mM EDTA for 30 min at 37°C) to allow enumeration of cells. FSDDC aggregates were subcultured in cytokine-supplemented complete medium at 5 × 10^5 cells/ml in T-25 flasks or 24-well plates in the presence or the absence of ODN and LPS as indicated.

Oligodeoxynucleotides and LPS

ODN with a nucleoside-resistant phosphorothioate backbone and ODN with a phosphodiester backbone (see Table I) were synthesized by Oligos, Etc. (Wilsonville, OR), a GMP facility. Sodium salts of ODN were ethanol precipitated, stored at −20°C, and diluted in PBS immediately before use. Escherichia coli and calf thymus DNA were purchased from Sigma (St. Louis, MO) and prepared for use as previously described (24). LPS levels in phosphorothioate ODN were undetectable (<1 pg/ml) by Limulus amebocyte lysate assay (Whittaker Bioproducts, Walkersville, MD). Amounts of LPS in phosphodiester ODN, calf thymus DNA, and E. coli DNA were detectable, but were <10% of the threshold amounts required to activate FSDDC in vitro (≥10 pg/ml final concentration) (20) (T.J. and M.C.U., unpublished observations). LPS (E. coli K235-derived LPS; <0.008% protein) was provided by Dr. Stephanie Vogel (Uniformed Services University of the Health Services, Bethesda, MD).

Abs and flow cytometry

Anti-I-A^b (Y3P, mouse IgG2a), anti-I-A^a (MKD6, mouse IgG2a), and anti-I-A^b, I-E^b (M5/114, rat IgG2a)-producing hybridomas were obtained from American Type Culture Collection (Manassas, VA), and an anti-E-cadherin mAb-producing hybridoma (ECCD-2, rat IgG2a) was provided by Dr. Masatoshi Takeichi (Kyoto University, Kyoto, Japan). Mouse mAb were purified from hybridoma supernatants using immobilized protein A (Pierce, Rockford, IL), and rat mAb were prepared using immobilized protein G (Pierce). Y3P, MKD6, and M5/114 were conjugated with FITC (Sigma) as previously described (19). Anti-CD40 (HM40–3, hamster IgM), anti-CD45 (30F11.1, rat IgG2b), anti-CD80 (1G10, rat IgG2a), anti-CD86 (GL-1, rat IgG2a), anti-mlL-12 p40 (C15.6, rat IgG1), and isotype controls were purchased as purified biotin-, PE-, or FITC-modified mAb from PharMingen (San Diego, CA). PE-streptavidin was obtained from Tago (Burlingame, CA).

FSDDC aggregates were subcultured in GM-CSF- and CSF-1-supplemented media for 18 h in 24-well plates (5 × 10^5 cell equivalents/1 ml well) in the presence or the absence of LPS or ODN. Before staining, FSDDC aggregates were dissociated in HBSS containing 1 mM EDTA (30 min at 37°C) in 10% Chelex-100 treated FCS (Sigma, St. Louis, MO) (25). For multicolor flow cytometry, cells were suspended in cold PBS containing 5% FCS and 0.02% NaN3, preincubated with saturating concentrations of anti-FeRIV/III (2.4G2, supplied by Julie Tius, National Cancer Institute), and then serially incubated with saturating concentrations of FITC-mAb, biotin-mAb, and PE-streptavidin. Stained cells were analyzed using a FACScan flow cytometer equipped with CellQuest software (Becton Dickinson, Mountain View, CA). Propidium iodide-permeable (nonscorable) cells were excluded from analyses.

Flow cytometric assessment of DC-associated IL-12 in FSDDC-I incubated with LPS or ODN was conducted as described above, except that brefeldin A (1 μg/ml; PharMingen) was added for the last 4 h of the 18-h incubation period (26). FSDDC were subsequently harvested, dissociated with EDTA, preincubated with anti-FeRIV/III mAb, stained with FITC-I-A^a or control mAb, fixed and permeabilized (4% paraformaldehyde and 0.1% saponin) for 20 min at 4°C, washed, and then stained with PE-conjugated anti-IL-12 p40 (C15.6) or isotype control. Propidium iodide was omitted from the staining protocol.

FSDDC cytokine production

Aggregates of FSDDC were suspended (0.5 × 10^6 cell equivalents/ml) in GM-CSF- and CSF-1-containing DC media and incubated for 18 h in the presence or the absence of LPS or ODN. At the end of the incubation, supernatants were decanted, nonadherent cells were removed by centrifugation, and the cell-free supernatants were stored at −70°C. Concentrations of IL-1β (19), IFN-γ and TNF-α (27), and IL-6 and IL-12 p40 (11) in culture supernatants were determined as recently described.

Mixed leukocyte reactions

EDTA-dissociated FSDDC were exposed to 20 Gy, suspended in RPMI 1640 supplemented with 10% FCS, and added to 2 × 10^6 accessory cell-depleted BALB/c lymph node T cells in 96-well, flat-bottom microtiter plates as previously described (27). After 72 h, [3H]Thymidine (New England Nuclear, Boston, MA) was added at 1 Ci/well, and the incubation was continued for an additional 18 h. Cell-associated radioactivity was determined by direct beta counting.

Assessment of LC activation in vivo

Mice were anesthetized via i.p. injection of ketamine (2.5 mg/animal) and xylazine (0.05 mg/animal). The dorsum of each ear was injected with recombinant mouse IL-1β (50 ng in 50 μl; Genzyme Diagnostics, Cambridge, MA) or ODN (50 μg) in PBS (four mice per group). After 12 h, epidermal cell suspensions were obtained from the skin overlying the injection site by limited trypsinization as previously described (28), and the surface phenotype of LC was characterized using flow cytometry. Intraepidermal IL-12 p40 accumulation in LC activated by in vivo administration of LPS (200 ng) or CpG ODN 1826 (50 μg) was evaluated in epidermal cell suspensions obtained 12 h after injection and an additional 5-h culture in complete medium (1 × 10^5 cells/ml) (28) supplemented with brefeldin A (1 μg/ml) using a procedure analogous to that used to quantitate intraepidermal IL-12 in FSDDC (see above).

Statistical analysis

The statistical significance of differences in cytokine production of FSDDC and LC surface Ag expression was calculated using the paired Student’s t test.

Results

Induction of DC maturation by CpG ODN

We previously demonstrated that immature DC expanded in primary cultures of murine fetal skin cells (FSDDC-I) and isolated as

Table I. Oligodeoxynucleotide sequences*
concentrations
CpG ODN 1826 was dose dependent, with no effects observed at
expression (data not shown) (20). The disaggregation observed with
hesion was accompanied by a reduction in E-cadherin surface ex-
1836 were without effect (see Fig. 1). Loss of homotypic cell ad-
ning (CpG ODN 1758 and 1835) or tandem repeats (CpG ODN
used are presented in Table I. Each ODN, except ODN 1911 and
FSDDC cell surface Ag expression were assessed using mul-
Cytokine- and LPS-induced disaggregation of FSDDC-I is also
associated with DC maturation, manifested by increased expres-
sion of MHC Ag and costimulator molecules (20). Effects of ODN
on FSDDC cell surface Ag expression were assessed using multi-
color flow cytometry. Single cell suspensions of EDTA-dissoci-
ated FSDDC-I were stained with anti-CD45 mAb and mAb reac-
tive with MHC Ag and costimulator molecules, and viable CD45+ cells (>95% of all cells present) were selected for analysis. Treat-
ment of FSDDC-I with LPS (100 ng/ml) or CpG ODN 1826 (6 
µg/ml) for 18 h induced dramatically increased expression of

trypsin-resistant aggregates resemble epidermal LC (19). Adhesion
within aggregates is E-cadherin mediated (19) and serves as an in
vitro correlate of E-cadherin-mediated adhesion of LC and kera-
tinocytes in the epidermis (29). We have also determined that the
ability of inflammatory mediators to induce loss of adhesion in
FSDDC aggregates and initiate maturation correlates with their
ability to activate and mobilize LC in vivo (20). Thus, we initially
screened phosphorothioate ODN for FSDDC-activating activity
using a simple disaggregation assay. The sequences of the ODN
used are presented in Table I. Each ODN, except ODN 1911 and
2067, contained two immunostimulatory CpG motifs in overlap-
ing (CpG ODN 1758 and 1835) or tandem repeats (CpG ODN
1826 and 2061). FSDDC-I were incubated in ODN or LPS for 18 h
and observed at various times. CpG ODN 1826 (6 µg/ml) and LPS
(100 ng/ml) caused almost complete loss of E-cadherin-mediated
adhesion in FSDDC aggregates within 18 h, whereas the base com-
position-matched ODN 1911 (6 µg/ml) and CpG ODN 1758 or
1836 were without effect (see Fig. 1). Loss of homotypic cell ad-
hesion was accompanied by a reduction in E-cadherin surface ex-
pression (data not shown) (20). The disaggregation observed with
CpG ODN 1826 was dose dependent, with no effects observed at
concentrations <1.5 µg/ml, and, like that due to LPS, began after
a lag period of approximately 6 h (data not shown).

Cytokine- and LPS-induced disaggregation of FSDDC-I is also
associated with DC maturation, manifested by increased expres-
sion of MHC Ag and costimulator molecules (20). Effects of ODN
on FSDDC cell surface Ag expression were assessed using multi-
color flow cytometry. Single cell suspensions of EDTA-dissoci-
ated FSDDC-I were stained with anti-CD45 mAb and mAb reac-
tive with MHC Ag and costimulator molecules, and viable CD45+ cells (>95% of all cells present) were selected for analysis. Treat-
ment of FSDDC-I with LPS (100 ng/ml) or CpG ODN 1826 (6 
µg/ml) for 18 h induced dramatically increased expression of

MHC class II Ag, CD40, and CD86 (see Fig. 2). Cell surface levels
of CD80 were also somewhat increased. Identical concentrations
(6 µg/ml) of CpG ODN 1758 and 1835 and control ODN 1911 did
not result in significant changes in cell surface phenotype (Fig. 2).
To determine whether the failure of ODN other than 1826 to ac-
tivate FSDDC-I was relative or absolute, we conducted dose-re-
response studies. FSDDC-I were exposed to concentrations of ODN
ranging from 0.6 to 200 µg/ml for 18 h, and MHC class II Ag
levels were evaluated. Concentrations of CpG ODN 1826 that
were >2 µg/ml resulted in FSDDC activation (see Fig. 3). In the
experiment depicted, CpG ODN 1758 (20 µg/ml) also induced
almost complete FSDDC-I activation, and CpG ODN 1835 had
similar effects at 60 µg/ml. Indeed, even ODN 1911, an ODN with
the same base content as CpG ODN 1826 but lacking the immu-
nostimulatory CpG motifs (see Table I), activated FSDDC-I to
some extent at high concentrations (≥60 µg/ml).

Stimulation of FSDDC IL-12 production by CpG ODN
The accessory cell activity of APC and the type of response that
ensues after interactions of T cells with APC and Ag depend on the
cytokines produced by APC as well as the costimulator molecules
that they express on their cell surfaces. To characterize the cyto-
kines produced by FSDDC following CpG ODN stimulation,
FSDDC aggregates were cultured for 18 h in control medium (sup-
plemented with GM-CSF and CSF-1) or in medium also contain-
ing LPS (100 ng/ml) or ODN (6 µg/ml), and immunoreactive IL-
1β, IFN-γ, TNF-α, IL-6, and IL-12 p40 were assayed in cell-free
supernatants by ELISA. IL-1β and IFN-γ were not detected in
FSDDC supernatants under any of these conditions (data not

FIGURE 2. Enhanced expression of MHC and costimulator molecules
by FSDDC-I treated with CpG ODN 1826. FSDDC-I were incubated in
medium containing GM-CSF and CSF-1 alone (control) or in medium also
containing LPS (100 ng/ml) or the indicated ODN (6 µg/ml) for 18 h, cultures
were treated with EDTA to obtain single cells, and FSDDC were
analyzed for expression of the indicated Ag using flow cytometry. Shaded
areas indicate the Ab of interest; the solid line shows the isotype control.
Representative data from one of three (n = 3) experiments are shown.

FIGURE 1. Activation of LC-like FSDDC-I by CpG ODN 1826.
FSDDC aggregates were harvested from 14-day cultures of day 16
C57BL/6 fetal skin and incubated in GM-CSF- and CSF-1-supplemented
with medium alone (A) or in medium containing LPS (100 ng/ml; B), CpG
ODN 1826 (6 µg/ml; C), ODN 1911 (6 µg/ml; D), CpG ODN 1758 (6 
µg/ml; E), or CpG ODN 1835 (6 µg/ml F). Morphologic changes were
documented after 18 h using a phase contrast photomicroscope (×40).
Control (None); †

Stimulation of cytokine release from skin-derived DC by CpG

The relationship between the immunostimulatory activity of CpG ODN 1826 and its phosphorothioate backbone was explored by incubating FSDDC-I with phosphodiester ODN that were sequence-identical with CpG ODN 1826 and ODN 1911 (CpG ODN 2061 and ODN 2067, respectively). Like CpG ODN 1826, CpG ODN 2061 triggered enhanced MHC class II expression and induced a dramatic dose-dependent up-regulation of intracellular IL-12 p40 in 5 to 36% of the FSDDC treated, whereas ODN 2067 (and ODN 1911) activated FSDDC to a limited extent and only at the highest concentration tested (60 µg/ml). ODN 2067 was somewhat less active in this regard than ODN 1911 in two experiments. To compare the DC-activating potential of bacterial and mammalian DNA, FSDDC-I were also incubated with E. coli DNA and calf thymus DNA. E. coli DNA (30 µg/ml) clearly up-regulated FSDDC MHC class II expression and IL-12 production, whereas calf thymus DNA (30 µg/ml) was without effect (Fig. 4).

Augmentation of FSDDC accessory cell activity by CpG ODN

To determine whether increases in cell surface MHC Ag and co-stimulator expression and cytokine production were accompanied by an increase in the ability of FSDDC to stimulate unprimed T cells, C57BL/6 FSDDC-I were incubated for 18 h in LPS (10 ng/ml) or ODN (6 µg/ml), washed, and then tested for allostimulatory activity in a mixed leukocyte reaction using accessory cell-depleted BALB/c T cells as responders. Treatment of FSDDC with CpG ODN 1826 resulted in a severalfold increase in the potency of FSDDC as an allostimulator (see Fig. 5). This was considerably less than the approximately 10-fold increase in accessory cell activity induced by LPS, but was clearly increased relative to that due to other ODN. ODN 1911, CpG ODN 1758, and CpG ODN 1835 had no accessory cell-augmenting activity at 6 µg/ml.

Activation of epidermal LC by CpG ODN

Although FSDDC accurately model various aspects of LC biology, we sought to determine whether immunostimulatory ODN also had the capacity to activate immature nonlymphoid DC in vivo. Subpopulations of LC become activated by exposure to contact allergens, IL-1, TNF-α, and LPS and are subsequently induced to emigrate from epidermis (30–33). Before they exit the epidermis, activated LC express increased levels of cell surface MHC class II and CD86 Ag and can be differentiated from unstimulated LC using multicolor flow cytometry. To test the ability of CpG ODN

![FIGURE 3.](http://www.jimmunol.org/) Concentration dependence of ODN-induced enhancement of FSDDC MHC class II Ag expression. FSDDC aggregates were incubated for 18 h in various concentrations of the indicated ODN or LPS (100 ng/ml), and MHC class II Ag expression was evaluated by flow cytometry (n = 2).

Table II. Stimulation of cytokine release from skin-derived DC by CpG ODN

<table>
<thead>
<tr>
<th>Agonist</th>
<th>TNF-α</th>
<th>IL-6</th>
<th>IL-12</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>ND</td>
<td>46 ± 36</td>
<td>80 ± 80</td>
</tr>
<tr>
<td>LPS</td>
<td>757 ± 228</td>
<td>948 ± 453</td>
<td>1,643 ± 520</td>
</tr>
<tr>
<td>CpG ODN 1826</td>
<td>155 ± 114</td>
<td>133 ± 16</td>
<td>16,582 ± 5,742</td>
</tr>
<tr>
<td>ODN 1911</td>
<td>ND</td>
<td>62 ± 28</td>
<td>1,266 ± 1,215</td>
</tr>
<tr>
<td>CpG ODN 1758</td>
<td>ND</td>
<td>47 ± 35</td>
<td>392 ± 235</td>
</tr>
<tr>
<td>CpG ODN 1835</td>
<td>ND</td>
<td>52 ± 39</td>
<td>152 ± 131</td>
</tr>
</tbody>
</table>

*Numerical values represent pg/5 × 10⁶ cells/18 h. FSDDC aggregates were incubated for 18 h in medium containing GM-CSF and CSF-1 alone (None) or in medium additionally supplemented with LPS (100 ng/ml) or ODN (6 µg/ml); supernatants were harvested and cytokines were quantitated by ELISA. The data presented are from four experiments and represent the mean ± SD (p < 0.05 as compared to control (None); † p < 0.05 as compared to CpG ODN 1826).

* ND = not detectable; < 40 pg.
1826 and ODN 1911 to stimulate LC in situ, we injected IL-1β (50 ng in 50 μl of PBS), CpG ODN 1826 (50 μg/50 μl), ODN 1911 (50 μg/50 μl), or PBS into the dorsal ear skin of BALB/c mice, prepared epidermal cell suspensions from the overlying epidermis 12 h later, and stained cells for simultaneous expression of MHC class II and CD86 Ag. Introduction of IL-1β and CpG ODN 1826 into murine skin led to increased expression of MHC class II and CD86 Ag by significant subpopulations of LC in three experiments (see Fig. 6 and Table III). The proportion of LC activated by CpG ODN 1826 in vivo was always less than that activated by IL-1β, but was significantly greater than that due to injection of control ODN 1911 or PBS. Similar results were obtained in an identical experiment conducted with C57BL/6 mice.

Intracellular IL-12 accumulation was assessed in LC from the ears of BALB/c mice injected with LPS (200 ng) or CpG ODN 1826 (50 μg) using methodology analogous to that used to evaluate IL-12 production by FSDDC. IL-12 levels in LC from sham-injected or ODN 1911-injected skin could not be assessed because LC with baseline levels of MHC class II Ag could not definitively be resolved from fixed, permeabilized, MHC class II-negative keratinocytes. Activated LC, however, expressed increased levels of MHC class II Ag (see also Fig. 6) and could be clearly resolved from MHC class II-negative epidermal cells. Therefore, we compared intracellular levels of IL-12 in LC activated by CpG ODN 1826 with those in LC stimulated with CpG ODN 1826. We found that in three experiments a small subpopulation of LC activated by CpG ODN 1826 in vivo (4.3 ± 1.6%) contained detectable levels of intracellular IL-12 p40, whereas LPS-activated LC did not (Fig. 7). Thus, CpG ODN 1826 selectively induced IL-12 production by cutaneous DC in vivo as well as in vitro.

Discussion

Genetic vaccines that rely on injection of naked DNA are efficacious because plasmid expression vectors are taken up by cells in skin and muscle and expressed (1–6) and also because bacterial
DNA has immunostimulatory properties (7–12). Much of the immunostimulatory activity of bacterial DNA resides in unmethylated CpG dinucleotide sequences and can be mimicked by synthetic ODN that carry a consensus 5'-purine-purine-CpG-pyrimidine-pyrimidine-3' motif (9). Previous studies demonstrated that CpG ODN can activate B cells (9, 11), NK cells (24, 27), and macrophages (15, 34–37). The present studies extend the immunostimulatory effects of CpG ODN and bacterial DNA to cells of the DC lineage.

Exposure of LC-like FSDDC-I to low concentrations of CpG ODN (especially CpG ODN 1826) in vitro resulted in loss of E-cadherin-mediated adhesion within DC aggregates, pronounced up-regulation of MHC class II Ag and costimulatory molecule (CD40, CD80, and CD86) expression, and acquisition of enhanced accessory cell activity. In addition, CpG ODN resulted in a large increase in IL-12 production by FSDDC. Significantly, intradermal injection of CpG ODN also led to increased expression of MHC class II Ag and costimulatory molecules as well as induction of IL-12 synthesis by LC in overlying epidermis. CpG ODN had no effect on cytokine production or cell surface Ag expression by primary murine keratinocytes (data not shown), suggesting that, at least in murine skin, immunostimulatory DNA acts primarily on DC.

The CpG ODN-induced enhancement of accessory cell activity that we observed was less marked than the changes in cell surface phenotype or cytokine (especially IL-12) production that were detected. We attribute this difference to the characteristics of the cells that we studied and the assays that we used. We have previously shown that FSDDC-I differentiate into cells that resemble mature interdigitating dendritic cells over a several-day period in isolation without additional stimulation (19). Presumably FSDDC also mature at a similar (or faster) rate when cocultured for several days with allogenic T cells. Thus, we are detecting enhanced function in an in vitro assay in which accessory cell activity is already being rapidly up-regulated. Assays of the effects of CpG ODN on cell surface Ag expression and cytokine production involve incubations of hours rather than days, such that backgrounds attributable to spontaneous maturation are considerably lower.

A variety of CpG ODN have immunostimulatory activity in vivo (10, 15, 21–23, 38), and it has been suggested that different

**Table III. Activation of LC by CpG-containing ODN in situ**

<table>
<thead>
<tr>
<th>LC Phenotype</th>
<th>None</th>
<th>PBS</th>
<th>IL-1β</th>
<th>CpG ODN 1826</th>
<th>ODN 1911</th>
</tr>
</thead>
<tbody>
<tr>
<td>MHC class II&lt;sup&gt;high&lt;/sup&gt;</td>
<td>2.9 ± 0.9</td>
<td>9.0 ± 5.2</td>
<td>71.6 ± 13.2&lt;sup&gt;†&lt;/sup&gt;</td>
<td>29.6 ± 13.5</td>
<td>10.4 ± 4.7</td>
</tr>
<tr>
<td>CD86&lt;sup&gt;high&lt;/sup&gt;</td>
<td>1.2 ± 0.5</td>
<td>4.4 ± 2.1</td>
<td>64.6 ± 7.0&lt;sup&gt;†&lt;/sup&gt;</td>
<td>28.6 ± 2.6&lt;sup&gt;†&lt;/sup&gt;</td>
<td>5.1 ± 1.6</td>
</tr>
<tr>
<td>MHC II&lt;sup&gt;high&lt;/sup&gt;/CD86&lt;sup&gt;high&lt;/sup&gt;</td>
<td>0.5 ± 0.2</td>
<td>2.6 ± 1.1</td>
<td>56.9 ± 8.6&lt;sup&gt;†&lt;/sup&gt;</td>
<td>19.4 ± 7.6&lt;sup&gt;†&lt;/sup&gt;</td>
<td>3.2 ± 0.3</td>
</tr>
</tbody>
</table>

* Epidermal cell suspensions were prepared from BALB/c dorsal ear skin overlying dermis that had been injected with IL-1β (50 ng), ODN (50 μg), or PBS 12 h earlier, stained for simultaneous expression of MHC class II and CD86 Ag, and analyzed by flow cytometry. Analysis gates were adjusted to include >95% of LC from uninjected mice (gate A). Numerical values are percentages of all LC (MHC class II<sup>high</sup> cells) analyzed (≥2000/sample) with fluorescence intensities that exceeded the upper limit of gate A and represent the mean ± SD from three separate experiments (<sup>†</sup> p < 0.05; <sup>‡</sup> p < 0.01 as compared with PBS-injected animals).
CpG ODN may act preferentially on different leukocyte subpopulations and/or induce different kinds of responses in distinct subpopulation of cells. The immature DC used in the present study (FSDDC and LC) preferentially responded to CpG ODN 1826, an ODN with tandem nonoverlapping immunostimulatory motifs, compared with other CpG ODN (1758 and 1835). Interestingly, an ODN that is base composition matched to CpG ODN 1826 but lacks CpG sequences (ODN 1911) also activated FSDDC at high concentrations. It is difficult to explain this observation because we do not fully understand how CpG ODN activate immune cells. Nonsequence-specific effects of ODN have been attributed to phosphorothioate modification of the backbone (39–43). In the present study, however, phosphodiester ODN and sequence-matched phosphorothioate ODN had comparable DC-activating potentials (Fig. 4). Inasmuch as immunostimulatory properties of other anionic polymers, including single- and double-stranded polynucleotides (34), dextran sulfate (44), and heparan sulfate (45), are well known, additional physicochemical properties of ODN may also be relevant.

The propensity of bacterial plasmid-based vaccines to induce Th1-predominant responses has been previously ascribed to their ability to induce IL-12 production by macrophages in a CpG dinucleotide-dependent fashion (15, 35–37). This interpretation is difficult to reconcile with data indicating that DC, rather than macrophages, are primarily responsible for T cell priming in vivo (16). Reis e Sousa and co-workers have recently demonstrated that Toxoplasma gondii extracts that activate innate immunity selectively induce rapid and dramatic IFN-γ-independent up-regulation of IL-12 production in splenic DC in vivo and also cause DC redistribution within central lymphoid tissue (46). These authors have also implicated IL-12-producing DC as important regulators of the initial phases of Th1 differentiation/expansion. Our results indicate that CpG ODN have similar dramatic effects on DC IL-12 production, and that they can also activate immature nonlymphoid DC. To our knowledge, these results represent the first direct demonstration that LC can be stimulated to produce IL-12.

We suggest that intracutaneous genetic vaccines induce Th1 responses because CpG dinucleotide sequences in bacterial DNA activate DC in skin and induce them to produce large amounts of IL-12. Similar scenarios may occur in the setting of chronic infections that probably result in the release of immunostimulatory DNA into skin or in instances where CpG-containing DNA is co-administered with simple (22, 23, 38) or complex Ag, or even live organisms (see Footnote 5) (14). We propose that introduction (or accumulation) of immunostimulatory CpG-containing DNA in skin leads to LC activation manifested by attenuation of E-cadherin-mediated adhesion of LC to keratinocytes (29, 47) and enhanced expression of MHC Ag and costimulator molecules. LC MHC Ag could be loaded with antigenic peptides synthesized endogenously (e.g., during the course of immunization with naked DNA) (4, 6), peptides synthesized by other skin cells (e.g., keratinocytes or fibroblasts) (3), or nominal Ag derived from infectious organisms themselves. Ultimately, epidermal LC (and perhaps DC in the dermis) are mobilized, such that DC bearing Ag acquired in skin migrate from skin to regional lymph nodes, where they localize in T cell-dependent areas as mature interdigitating DC (4, 6, 31) capable of initiating Th1 responses in naive T cells.

Other immunostimulatory activities of CpG ODN may also be important. The ability of CpG ODN to induce regional lymphadenopathy after s.c. injection (A. M. Krieg, unpublished observation) suggests that CpG ODN distribute to the lymph node and could activate DC in lymphoid tissues. In addition, CpG ODN are known to act directly on B cells (inducing decreased apoptosis (48), proliferation, enhanced Ig synthesis, and IL-6 release (11)) and on macrophages (resulting in increased IL-12 production) (15, 34–37). Indirect actions of CpG ODN on NK cells (IL-12-dependent IFN-γ production) (37) may also be relevant, especially in chronic infections. Although the relative importance of the effects of CpG ODN on immature DC vis-a-vis effects on immune responses in vivo remains to be conclusively demonstrated, we propose that the effects of CpG ODN as well as immunostimulatory DNA derived from pathogens on T cell priming are probably mediated via DC. Thus, results of in vitro and in vivo studies of the DC-activating potential of CpG ODN such as we have reported here may allow rapid screening of ODN for possible Th1 response-promoting activity and may facilitate identification of CpG ODN that show promise as preventive or therapeutic agents for infectious and inflammatory diseases or cancer.

Note added in proof. In subsequent studies, small amounts of immunoreactive IL-12 p70 (10 pg/10⁶ cells) have been detected in supernatants of FSDDC incubated with CpG ODN 1826 for 72 h. IL-12 p70 was not released by FSDDC treated with the control ODN 1911. A similar report, coauthored by T. Sparwasser et al., appeared while this manuscript was in press (Sparwasser, T., E. S. Koch, R. M. Vabulas, K. Heeg, G. B. Lipford, J. W. Ellwart, and H. Wagner. 1998. Bacterial DNA and immunostimulatory CpG oligonucleotides trigger maturation and activation of murine dendritic cells. Eur. J. Immunol. 28:2045).

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