Brief Definitive Report

DNA as an Adjuvant: Capacity of Insect DNA and Synthetic Oligodeoxynucleotides to Augment T Cell Responses to Specific Antigen

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Summary

How strong adjuvants such as complete Freund's adjuvant (CFA) promote T cell priming to protein antigens in vivo is still unclear. Since the unmethylated CpG motifs in DNA of bacteria and other nonvertebrates are stimulatory for B cells and antigen-presenting cells, the strong adjuvanticity of CFA could be attributed, at least in part, to the presence of dead bacteria, i.e., a source of stimulatory DNA. In support of this possibility, evidence is presented that insect DNA in mineral oil has even stronger adjuvant activity than CFA by a number of parameters. Synthetic oligodeoxynucleotides (ODNs) containing unmethylated CpG motifs mimic the effects of insect DNA and, even in soluble form, ODNs markedly potentiate clonal expansion of T cell receptor transgenic T cells responding to specific peptide.

It is now well established that unmethylated CpG dinucleotide motifs of bacterial DNA have the capacity to cause polyclonal activation of B cells and stimulation of APCs (1–8). The immunostimulatory property of unmethylated CpG motifs is not unique to bacteria and applies to a wide spectrum of nonvertebrates including insects, nematodes, mollusks, and yeast (3, 9, 10); by contrast, DNA from various vertebrates, e.g., frogs and fish, is nonstimulatory. The capacity of nonvertebrate DNA to stimulate B cells and APCs is shared by synthetic oligodeoxynucleotides (ODNs) containing unmethylated CpG motifs (5, 11). When coinjected with antigen, these agents also enhance the generation of cytotoxic T cell activity and production of specific antibody and IFN-γ (12–15).

Stimulation of APCs via unmethylated CpG motifs could explain the remarkable efficacy of "naked" DNA vaccines (16). In this respect, the induction of antigen-specific responses after DNA vaccination is reported to be much more efficient when the plasmid vector for mammalian DNA contains unmethylated CpG motifs (17, 18). In light of this finding, DNA vaccines may operate not only by providing a source of specific antigen (peptide) but by acting as an adjuvant, i.e., by enhancing the immunogenicity of APCs. According to this notion, the poor immunogenicity of proteins or peptides given in solution could be overcome simply by coinjecting any source of DNA containing stimulatory CpG motifs. In support of this prediction we show here that, when suspended in mineral oil, insect DNA and ODNs containing unmethylated CpG motifs act as powerful adjuvants in mice when coinjected with foreign peptides or proteins. ODNs also have adjuvant activity in soluble form and markedly amplify clonal expansion of TCR transgenic T cells responding to specific peptide.

Materials and Methods

Mice. C57BL/6J (B6) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). 2C TCR transgenic mice (9) were bred and maintained at The Scripps Research Institute (La Jolla, CA).

Proteins and Peptides. Fowl γ-globulin (FγG) was purchased from Pel-Freez Biologicals (Rogers, AR). A synthetic peptide, SIYRYYGL (19), recognized by 2C TCR transgenic cells in the context of H-2K^b was provided by Z. Cai (R.W. Johnson Pharmaceutical Research Institute, San Diego, CA). This peptide was synthesized on a synthesizer (431 A; Applied Biosystems, Foster City, CA) and purified with C18 reverse-phase high performance liquid chromatography.

DNA and ODNs. DNA from the *Drosophila melanogaster* cell line, SC2, was prepared as described (20). For injection, DNA was used without denaturation. CpG (GCATGACGTTGAGCT) and ZpG (GCATGAZGTTGAGCT, Z = 5′-methyl-C) phosphorothioated ODNs were designed using published sequences (5). The ODNs were synthesized and purified using HPLC by Research Genetics, Inc. (Huntsville, AL). Residual LPS in DNA preparations was measured (Limulus Amebocyte Lysate QCL-1000 kit; BioWhittaker, Walkersville, MD). *D. melanogaster* DNA preparations contained 0–10 pg of LPS/mg of DNA.

Immunization with FγG and antibody production. Mice were injected subcutaneously with FγG ± adjuvant in the lower portion

 J. Exp. Med. © The Rockefeller University Press • 0022-1007/98/04/1145/06 $2.00
Volume 187, Number 7, April 6, 1998 1145–1150
http://www.jem.org

of both hind limbs in a volume of 50 μl/limb. For injection, FγG was either (*a*) suspended in saline with soluble insect DNA or ODNs, or (*b*) mixed with CFA or IFA (mineral oil) plus either insect DNA or ODNs; in each situation, the dose of FγG injected was the same. Specific antibody production was determined by coating 96-well flat-bottomed plates with 2 μg FγG/well. Serially diluted antiserum were allowed to bind to the plates, washed, and then detected with isotype-specific biotinylated antibodies and streptavidin–horseradish peroxidase (PharMingen, San Diego, CA).

In Vitro T Proliferation Assay and IFN-γ Production. To measure proliferative responses to FγG, draining (popliteal) LN cells from FγG-primed mice were removed at day 9 after priming and then cultured with the indicated concentration of FγG in 200 μl volumes in 96-well plates using standard tissue culture medium plus FCS (21); in some experiments, B cell–depleted LN cells or purified LN $CD4^+$ cells (21) were used as responders. To measure proliferation, cultures were harvested on day 4 after overnight addition of 1 μCi [^{3}H] thymidine (TdR). To measure IFN-γ production, aliquots of culture supernatants were collected on day 2 or 3; IFN-γ production was measured with an ELISA assay using anti–IFN-γ mAbs from PharMingen.

Adoptive Transfer, Immunization, and In Vivo Proliferation of TCR Transgenic T Cells. For adoptive transfer, doses of 2×10^7 unseparated spleen plus LN cells from 2C mice on a B6 background were injected intravenously into normal B6 mice. The recipients were then injected subcutaneously with peptide ± soluble ODNs in both hind limbs. To measure proliferation in vivo, groups of the recipients were given a single injection of 1 mg bromodeoxyuridine (BrdU) intraperitoneally at 3, 4, or 5 d after immunization; BrdU incorporation was measured 4 h after BrdU injection.

Cell Surface Staining and Flow Cytometry. As described elsewhere (22), cell suspensions were first surface stained for expression of CD8 and the TCR clonotype of 2C cells, detected by 1B2 mAb (23). After fixation, the cells were then stained internally for BrdU incorporation using an anti-BrdU mAb (Becton Dickinson, San Jose, CA). Stained cells were analyzed on a FACScan® flow cytometer.

Results

In previous studies, the effects of SssI methylase treatment indicated that the capacity of insect (*D. melanogaster*) DNA to cause polyclonal activation of B cells was controlled, at least in part, by unmethylated CpG motifs (9). To test whether insect DNA could act as an adjuvant for antigen-specific T cell responses, mice were injected subcutaneously with FγG mixed with insect DNA (100 μg/mouse); since DNA is highly unstable when injected in vivo, some mice received FγG plus insect DNA suspended in IFA. Control mice received FγG in saline or suspended in CFA or in IFA without DNA.

Adjuvant Effect of Insect DNA on Normal $CD4^+$ T Cells. Priming of mice injected with FγG ± adjuvant was measured by removing the draining LN (DrLN) at 9 d after immunization and culturing LN cell suspensions with or without FγG in vitro. The results of culturing either unseparated LN cells or purified $CD4^+$ cells with FγG are shown in Fig. 1 *A*. As expected, for both cell populations, in vitro T proliferative responses to FγG were substantial with in vivo priming to FγG in CFA and somewhat lower with priming in IFA. When mice were primed with FγG in saline alone (not shown) or with FγG plus soluble insect DNA (Fig. 1 *A*), secondary responses to FγG in vitro were virtually undetectable, indicative of minimal immunization. In marked contrast, priming with FγG plus insect DNA suspended in IFA led to strong secondary responses to FγG in vitro. Significantly, these responses were appreciably

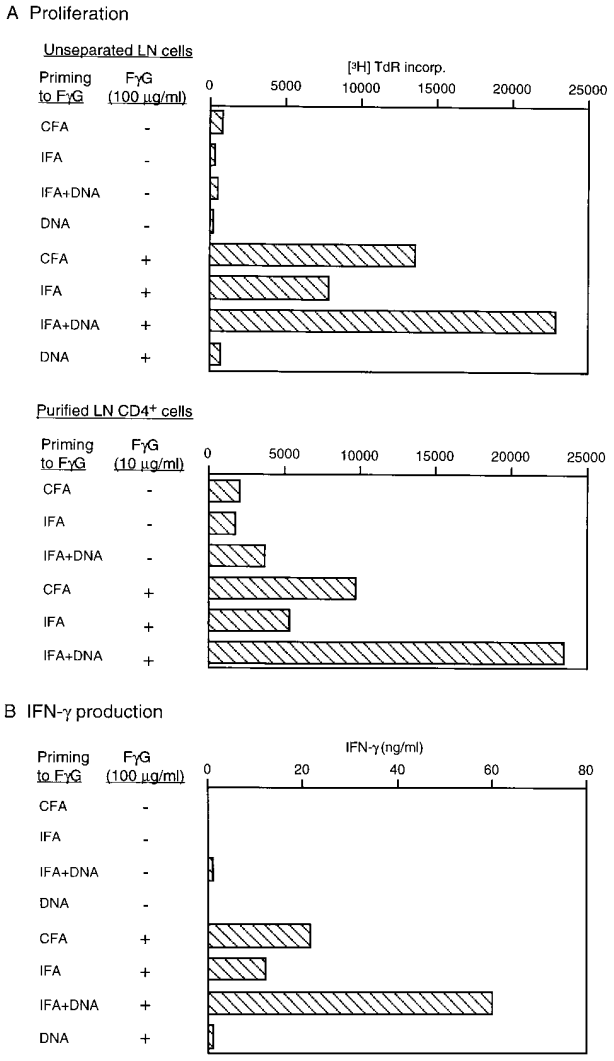

Figure 1. Adjuvant function of insect DNA for T cell responses to FγG. Normal B6 mice were immunized in the hind limbs with FγG (25 μg/limb for *A*, *top* and *B*, 5 μg/limb for *A*, *bottom*) mixed with CFA, IFA, DNA, or IFA and DNA; for DNA, insect DNA was injected at 50 μg/limb. Cell suspensions were prepared from the DrLN 9 d later. For *A*, *top* and *B*, unseparated LN cells were cultured at 2.5×10^5 cells/well ± FγG (20 μg/well, 100 μg/ml). For *A*, *bottom*, purified $CD4^+$ cells were prepared from DrLN cells and cultured at 10^5/well in the presence of T-depleted spleen cells (4×10^5/well) ± FγG (10 μg/ml). To measure [^{3}H]TdR incorporation (*A*), cultures were pulsed with 1 μCi/well of [^{3}H]TdR on day 3 and harvested for radioactive counting on day 4. For IFN-γ production (*B*), supernatants were tested on day 3. The data show means of triplicate cultures; SDs were within 10–20% of the means.

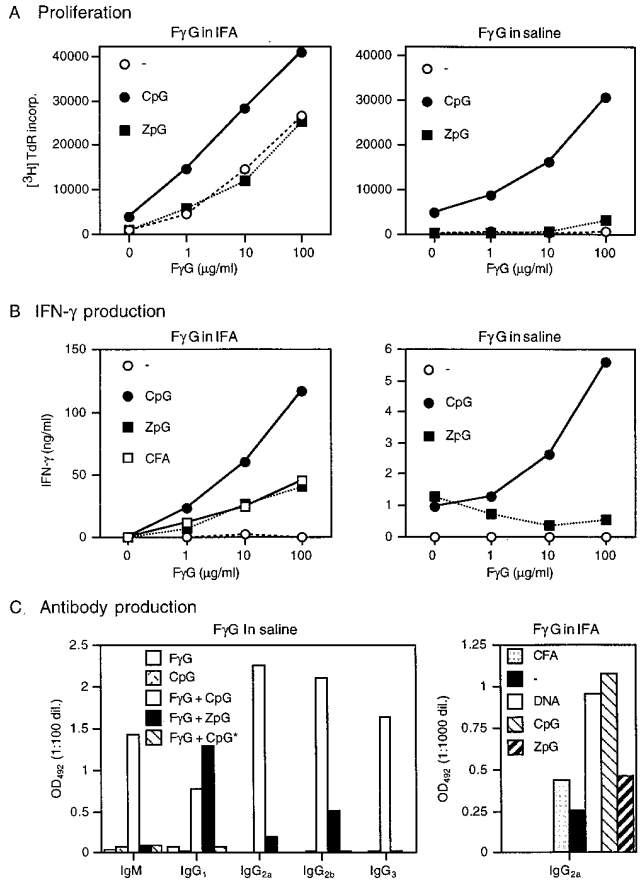

Figure 2. Adjuvant function of ODNs for T cell responses to FγG. Mice were immunized to FγG (5 μg/limb) as described for Fig. 1, using CpG and ZpG ODNs (25 μg/limb) instead of DNA; ODNs were either mixed with IFA (*A*; *B*, *left*; and *C*, *right*) or suspended in saline (*A*; *B*, *right*; and *C*, *left*). Antiserum was collected by tail bleeding on day 9 and tested at 1:100 or 1:1,000 dilution (*C*). For Ab production, some mice received CpG ODNs in the front limbs and FγG in the hind limbs (*FγG + CpG**). For in vitro responses (*A* and *B*), DrLN cells were removed on day 9, depleted of B cells, and cultured with graded doses of FγG for 3 d (IFN-γ production) or 4 d ([^{3}H]TdR incorporation). The data show means of triplicate cultures. Two other experiments gave similar findings.

higher than with priming to FγG in CFA. Similar results occurred for production of IFN-γ in vitro (Fig. 1 *B*) and also for production of specific Ab (see below). As a negative control for insect DNA, we used DNA from nonvertebrates (salmon testes; reference 10). In contrast to insect DNA, salmon DNA in IFA plus FγG was no more immunogenic than IFA plus FγG alone (data not shown).

Adjuvant Effect of Synthetic ODNs on Normal T Cells. The above data indicate that insect DNA acts as a powerful adjuvant, though only when suspended in IFA. To assess whether the adjuvanticity of DNA is controlled by unmethylated CpG motifs, we prepared two synthetic 15-mer ODNs containing a single CG dinucleotide pair. The only difference between the two ODNs was that, for the CG pair, C was unmethylated for one ODN (CpG ODN) but methylated (ZpG ODN) for the other; to retard degradation in vivo, both ODNs contained a phosphorothioated backbone.

To assess adjuvanticity, mice were primed with FγG plus 50 μg/mouse of CpG or ZpG ODNs suspended either in IFA or saline. As measured by secondary T proliferative responses in vitro to graded concentrations of FγG (Fig. 2 *A*, *left*), priming with FγG in IFA was considerably augmented by addition of CpG ODNs; by contrast, addition of ZpG ODNs to IFA had no effect. Thus, for ODNs in IFA, only CpG and not ZpG ODNs had demonstrable adjuvant activity (relative to priming in IFA alone). The results were quite similar for IFN-γ production, except that, for this assay, FγG priming with IFA plus ZpG was clearly higher than with IFA alone (Fig. 2 *B*, *left*). In general, FγG priming with IFA and CpG ODNs was substantially more effective than priming with CFA, especially for IFN-γ production (Fig. 2 *B*, *left*, and data not shown).

The above data refer to ODNs suspended in IFA. Significantly, in contrast to soluble insect DNA, CpG ODNs in saline displayed quite strong adjuvant activity for T proliferative responses (Fig. 2 *A*, *right*); by contrast, ZpG ODNs in saline were ineffective. For IFN-γ production, priming with CpG ODNs in saline led to much lower responses than with CpG ODNs in IFA (Fig. 2 *B*). Nevertheless, IFN-γ production elicited by ODNs in saline was clearly demonstrable with CpG ODNs, but undetectable with ZpG ODNs (Fig. 2 *B*, *right*).

Confirming the results of others (12–15), CpG ODNs acted as a powerful adjuvant for specific Ab production (Fig. 2 *C*). In saline (Fig. 2 *C*, *left*), CpG ODNs augmented both IgM and IgG Ab to FγG; except for IgG_1 Ab, ZpG ODNs were much less effective. Addition of IFA considerably augmented the adjuvant activity of CpG (but not ZpG) ODNs, especially for IgG_{2a} (Fig. 2 *C*, *right*), IgG_{2b}, and IgG_3 (data not shown) Ab. For these isotypes, Ab production elicited by CpG ODNs in IFA was substantially higher than with CFA immunization (Fig. 2 *C*, *right*); similar findings occurred with insect DNA in IFA (Fig. 2 *C*, *right*). By contrast, CpG ODNs or insect DNA in IFA inhibited the production of IgG_1 Ab, relative to immunization with CFA or IFA alone (data not shown). Significantly, the adjuvant activity of CpG ODNs required coinjection with FγG in the same site. Thus, injection of CpG ODNs in the front limbs and FγG in the hind limbs failed to elicit Ab production (Fig. 2 *C*, *left*).

Adjuvant Effects of ODNs on TCR Transgenic CD8$^+$ Cells. Since adjuvants presumably act largely by augmenting the clonal expansion of antigen-specific T cells, we sought direct evidence on this issue by studying the capacity of ODNs to augment proliferation of TCR transgenic T cells to specific peptide in vivo. For these studies we used 2C TCR transgenic mice. For this line, CD8$^+$ T cells have strong reactivity for a synthetic peptide, SIYRYYGL (19), presented by self (K^b) class I molecules. Using 2C mice on a B6 background, the approach taken (24) was to transfer doses of 2×10^7 2C lymphoid cells (pooled from spleen and LN) intravenously into normal B6 mice and then inject the mice subcutaneously with specific peptide ± CpG or ZpG ODNs in saline. To measure T cell proliferation in vivo, groups of the recipients were injected with the DNA

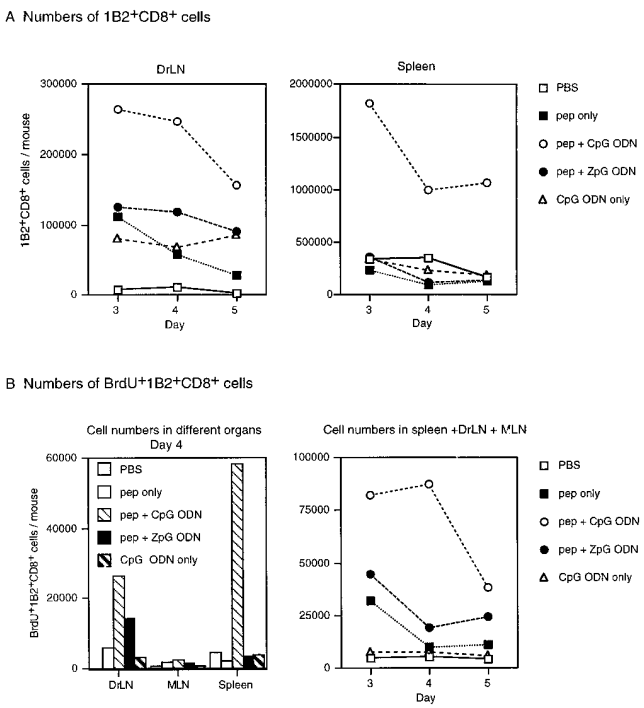

Figure 3. Capacity of ODNs to augment clonal expansion of 2C transgenic $CD8^+$ cells responding to specific peptide. Groups of B6 mice were injected intravenously with 2×10^7 2C lymphoid cells and then injected subcutaneously in the hind limbs with specific peptide (25 μg/limb) ± soluble CpG or ZpG ODNs (25 μg/limb). At 3, 4, or 5 d after immunization, the mice received a single intraperitoneal injection of 1 mg BrdU. Lymphoid organs were removed from the mice 4 h later and cell suspensions were stained for CD8 and 1B2 expression and then for BrdU incorporation followed by FACS® analysis. The data show mean values (2–3 mice/group) for total numbers of $1B2^+$ $CD8^+$ cells (*A*) and $BrdU^+$ $1B2^+$ $CD8^+$ cells (*B*) in the DrLN, spleen, and MLN. Essentially identical results were seen in a second experiment.

precursor, BrdU, at 3, 4, or 5 d after immunization; 4 h after BrdU injection, cell suspensions were stained for surface markers and then, after fixation, for BrdU incorporation. This 4-h pulse approach (22) thus provided an indication of the extent of donor cell proliferation at daily intervals from days 3 through 5. Donor $CD8^+$ cells were detected by staining for expression of CD8 and the 2C TCR clonotype, 1B2.

As shown in Fig. 3 *A*, *left*, total numbers of donor $CD8^+$ cells ($1B2^+$ $CD8^+$ cells) in the DrLN were substantially higher after injection of peptide and CpG ODNs than with injection of peptide alone; ZpG ODNs were much less effective. Similar findings applied to donor cell proliferation, i.e., to total numbers of $BrdU^+$ $1B2^+$ $CD8^+$ cells in the DrLN (Fig. 3 *B*, *left*).

These data refer to the response of donor $CD8^+$ cells in the DrLN. Surprisingly, injecting peptide plus CpG ODNs caused a marked increase in total numbers of both $1B2^+$ $CD8^+$ cells (Fig. 3 *A*, *right*) and $BrdU^+$ $1B2^+$ $CD8^+$ cells (Fig. 3 *B*, *left*) in the spleen, though not in mesenteric LN (MLN). By contrast, injection of peptide alone or peptide plus ZpG ODNs caused little, if any, evidence of proliferation in the spleen.

A rough estimate of the extent of donor T cell proliferation in the whole animal was obtained by calculating total numbers of $BrdU^+$ $1B2^+$ $CD8^+$ cells in DrLN + MLN + spleen at days 3 through 5. By this parameter, priming with peptide plus CpG ODNs was far more effective than priming with peptide alone (Fig. 3 *B*, *right*); priming with peptide plus ZpG ODNs was only slightly better than with peptide alone.

Discussion

Since CFA has long been the "gold standard" for adjuvant function, it is of interest that the adjuvant activity of insect DNA in mineral oil (IFA) surpassed the activity of CFA by three different parameters, namely T proliferative responses, IFN-γ synthesis, and production of specific Ab. This finding supports the view that the adjuvant activity of CFA is due, at least in part, to the presence of dead bacteria, a source of immunostimulatory DNA (3).

It should be emphasized that insect DNA only displayed adjuvant activity when suspended in mineral oil; in soluble form, insect DNA was ineffective, presumably reflecting rapid degradation by enzymes. In view of this problem, we resorted to the use of phosphorothioate-modified synthetic ODNs, which are comparably resistant to degradation in vivo (5). Except for CpG methylation, the two ODNs studied were identical. Confirming the findings of others (5), preliminary data established that CpG ODNs were highly effective in stimulating B cell proliferation in vitro, whereas ZpG ODNs had minimal activity (our unpublished data). Significantly, this marked difference between CpG and ZpG ODNs also applied to adjuvant activity. Thus, unlike ZpG ODNs, CpG ODNs acted as a strong adjuvant when used to prime mice for T proliferative responses, IFN-γ synthesis, and production of specific Ab to FγG. Although the adjuvant activity of CpG ODNs was clearly much higher when suspended in IFA, priming in the presence of soluble CpG ODNs led to quite strong T proliferative responses and low but significant production of specific Ab and IFN-γ. Confirming previous findings (12–15), the adjuvant activity of CpG ODNs for Ab production was much more prominent for certain Ig isotypes, e.g., IgG_{2a}, than for others, notably IgG_1; similar findings applied to insect DNA. Thus, for synthetic ODNs and DNA, the adjuvant function of CpG motifs appears to be skewed to Th1 function (12–15).

Examining the influence of adjuvants during the early primary response is difficult in normal mice, but relatively easy in TCR transgenic mice. When TCR transgenic T cells are exposed to specific peptide on adoptive transfer, it is well established that a mixture of peptide in CFA leads to a prolonged proliferative response (24); by contrast, injection of peptide alone elicits only transient proliferation followed by rapid elimination of the responding T cells. In line with these findings, the response of 2C $CD8^+$ cells to specific peptide alone was very brief and declined abruptly after day 3. By contrast, supplementing peptide with solu-

ble CpG ODNs augmented and considerably prolonged the proliferative response, indicative of an adjuvant effect.

Rather surprisingly, the proliferative response elicited by peptides plus CpG ODNs involved not only the DrLN but also the spleen. Yet proliferation in MLNs was undetectable. How can this distribution be explained? The simplest possibility in our view is that, in contrast to peptide alone, exposure to peptide plus ODNs in the DrLN signaled the responding T cells to survive and make their way into the circulation, thus reaching the spleen. The failure of the cells to reach MLNs may have reflected that antigen activation of T cells often leads to downregulation of the LN homing receptor, CD62L (25), thus preventing migration to LN but not to spleen. In fact, in more recent studies, a high proportion (50%) of the $1B2^+$ $CD8^+$ cells in the spleen on day 4 were $CD62L^{lo}$ (data not shown); such downregulation of CD62L did not apply to MLNs and, in spleen, was only seen with injection of peptide plus CpG ODNs.

How DNA and ODNs potentiate clonal expansion of antigen-specific T cells in vivo is still unclear. Others have postulated that ODNs act directly on T cells and provide a second signal for cells subjected to TCR ligation (12). The alternative possibility is that ODNs function largely by potentiating APC function, e.g., by inducing synthesis of cytokines such as IL-1, TNF-α, and IL-6 (6, 7, 26, 27), thus causing migration of APCs to DrLN (28), and perhaps also by stimulating upregulation of costimulatory molecules on APC precursors, e.g., by IFN-I (15, 29, and our unpublished data). However, direct evidence on the mechanism of action of ODNs under in vivo conditions is still unavailable.

We thank Ms. Barbara Marchand (The Scripps Research Institute, La Jolla, CA) for typing the manuscript.

This work was supported by grants CA38355, CA25803, AI21487, AI32068, AI07244, and AG01743 from the United States Public Health Service. H. Kishimoto is the recipient of a fellowship from the Cancer Research Institute. This work has publication No. 11253-IMM from the Scripps Research Institute.

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Received for publication 1 December 1997 and in revised form 29 January 1998.

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