Differences in Macrophage Activation by Bacterial DNA and CpG-Containing Oligonucleotides


*J Immunol* 2005; 175:3569-3576; doi: 10.4049/jimmunol.175.6.3569

http://www.jimmunol.org/content/175/6/3569

**References**

This article cites 35 articles, 16 of which you can access for free at:
http://www.jimmunol.org/content/175/6/3569.full#ref-list-1

**Subscription**

Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

**Permissions**

Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**

Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts

*The Journal of Immunology* is published twice each month by The American Association of Immunologists, Inc., 1451 Rockville Pike, Suite 650, Rockville, MD 20852

Copyright © 2005 by The American Association of Immunologists All rights reserved.

Print ISSN: 0022-1767 Online ISSN: 1550-6606.
Differences in Macrophage Activation by Bacterial DNA and CpG-Containing Oligonucleotides

Tara L. Roberts,*† Jasmin A. Dunn,*† Tamsin D. Terry,‡ Michael P. Jennings,‡ David A. Hume,*‡§ Matthew J. Sweet,*‡‡ and Katryn J. Stacey2*†‡

Bacterial DNA activates mouse macrophages, B cells, and dendritic cells in a TLR9-dependent manner. Although short ssCpG-containing phosphodiester oligonucleotides (PO-ODN) can mimic the action of bacterial DNA on macrophages, they are much less immunostimulatory than Escherichia coli DNA. In this study we have assessed the structural differences between E. coli DNA and PO-ODN, which may explain the high activity of bacterial DNA on macrophages. DNA length was found to be the most important variable. Double-strandedness was not responsible for the increased activity of long DNA. DNA adenine methyltransferase (Dam) and DNA cytosine methyltransferase (Dcm) methylation of E. coli DNA did not enhance macrophage NO production. The presence of two CpG motifs on one molecule only marginally improved activity at low concentration, suggesting that ligand-mediated TLR9 cross-linking was not involved. The major contribution was from DNA length. Synthetic ODN of two CpG motifs on one molecule only marginally improved activity at low concentration, suggesting that ligand-mediated TLR9 transport system was absent from B cells and fibroblasts.

CpG ODN response was found to correlate with the presence in macrophages of a length-dependent uptake process for DNA. This activity as bacterial DNA. The response of macrophages to CpG DNA requires endocytic uptake. The length dependence of the CpG DNA action now use phosphorothioate-stabilized ODN (PS-ODN), which have sulfur substituted for a nonbridging oxygen in the DNA backbone. PS-ODN have an activity level similar to that of bacterial DNA in macrophages in vitro (10) and are potent immunomodulators in vivo. Although the high activity of PS-ODN is generally assumed to be due to their stability, the PS modification also greatly enhanced ODN uptake in macrophages (11), and this is likely to contribute to the potency of PS-ODN. In macrophages, short CpG phosphodiester ODN (PO-ODN) are much less potent activators, on a weight basis, than Escherichia coli DNA despite the high molarity of active motifs per microgram of ODN. The increased potency of E. coli DNA might be due to double-strandedness, length, extra uncharacterized sequences, epigenetic factors, or a combination of these. These factors may affect the rate of uptake, intra- or extracellular stability, or CpG recognition and consequent cellular activation. In this work we investigated the physical characteristics of E. coli DNA that make it such a potent immunostimulatory molecule in macrophages and find that the length of DNA is critically important for efficient uptake of DNA and immunostimulatory activity.

Materials and Methods

Cells

The murine macrophage cell line RAW264.7, the human B cell lymphoma RPMI8226, and the murine fibroblast line L929 were purchased from American Type Culture Collection. WEHI231 cells were a gift from D. Tarlinton (Walter and Eliza Hall Institute, Melbourne, Australia). Medium for cell culture was RPMI 1640 with 10% FCS, 2 mM l-glutamine, 20 U/ml penicillin, and 20 μg/ml streptomycin. For culture of WEHI231 and RPMI8226 cells, medium also contained 10 mM HEPES buffer, 1 mM sodium pyruvate, and 0.05 mM 2-ME. FCS was screened for low basal activity and optimal CpG DNA responses in the ELAM promoter assay. Bone marrow-derived macrophages (BMM) were differentiated from bone marrow cells of adult male BALB/C mice as described previously (12). Bone marrow-derived dendritic cells (BMDC) were differentiated from bone marrow cells of male BALB/C mice. Femurs and tibias were flushed with complete culture medium and plated out on tissue culture plastic

Copyright © 2005 by The American Association of Immunologists, Inc.

0022-1767/05/S02.00
DNA and ODN

ODN were purchased from Generexx. Sequences are shown in Table I. E. coli DNA (Sigma-Aldrich) and calf thymus DNA (Sigma-Aldrich) were purified using phenol chloroform extraction and ethanol precipitation, followed by four extractions using Triton X-114 to remove LPS, phenol chloroform extraction, and ethanol precipitation as described previously (13). The purity of the DNA was confirmed by the lack of activity of DNase I-digested DNA. Plasmid pBluescript-SK+ was grown in either the DNA adenine methyltransferase (Dam) or DNA cytosine methyltransferase (Dcm) strains. To examine the Dam status of the Dam and Dcm loci. Plasmids were isolated using the Qiagen Plasmid Midi kit and were purified to remove LPS as described for Dam and Dcm loci. Plasmids were isolated using the Qiagen Plasmid Midi kit and were purified to remove LPS as described for Dam and Dcm loci. Plasmids were isolated using the Qiagen Plasmid Midi kit and were purified to remove LPS as described for Dam and Dcm loci.

Table I. ODN sequencesa

<table>
<thead>
<tr>
<th>ODN</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>AO-1</td>
<td>GCCTAGACGTTTCCTGATGCTG</td>
</tr>
<tr>
<td>RAO</td>
<td>CAGCATTGAGAACCTCTGAGGC</td>
</tr>
<tr>
<td>LO</td>
<td>GCCATTAGGACACTATGCTATAAGTTTCCTGATGCTGATGAGTAGG</td>
</tr>
<tr>
<td>LOR</td>
<td>CCTACTCTACAGCTACGAGGAAAGTTATGACCTGTCCTCCATAAGGC</td>
</tr>
<tr>
<td>LIO</td>
<td>GCCATTAGGACACTATGCTATAGGTTCTCTGATGCTGATGAGTAGG</td>
</tr>
<tr>
<td>CpGA14</td>
<td>AAAATGAGCTTAAAAA</td>
</tr>
<tr>
<td>CpGA18</td>
<td>AAAATGAGCTTAAAAAAA</td>
</tr>
<tr>
<td>CpGA22</td>
<td>AAAATGAGCTTAAAAAAA</td>
</tr>
<tr>
<td>CpGA32</td>
<td>AAAATGAGCTTAAAAAAA</td>
</tr>
<tr>
<td>CpGA44</td>
<td>AAAATGAGCTTAAAAAAA</td>
</tr>
<tr>
<td>5′ CG</td>
<td>TCCATGACGTTTCCTGATGCTGCTGATCTATCCCAGAGCTTCTCTGAT</td>
</tr>
<tr>
<td>3′ CG</td>
<td>TCCATGACGTTTCCTGATGCTGCTGATCTATCCCAGAGCTTCTCTGAT</td>
</tr>
<tr>
<td>Double CG</td>
<td>TCCATGACGTTTCCTGATGCTGCTGATCTATCCCAGAGCTTCTCTGAT</td>
</tr>
</tbody>
</table>

a All ODN have a phosphodiester backbone. Active CpG dinucleotides are shown in bold.

ELAM promoter assay

The RAW264.7 cell line stably transfected with the human ELAM promoter driving expression of GFP has been described previously (13); these transgenes) for 10–12 days.

Nitrite assay

RAW264.7 cells (100,000 cells/well) were plated in a 96-well plate overnight in 200 μl of complete medium. Cells were then pruned with 375 pg/ml IFN-γ (R&D Systems) for 2 h, followed by addition of stimulatory DNA. After 24-h incubation, supernatants were collected and assayed for the presence of nitrite with Griess reagent as previously described (14).

Results

E. coli DNA is more active than ODN in macrophages

We have previously shown that treatment of IFN-γ-primed RAW264.7 macrophage-like cells with purified plasmid DNA up-regulated inducible NO synthase mRNA and NO production (4, 14). To compare the potencies of ODN and E. coli DNA, we measured nitrite as an indicator of NO production by RAW264.7 cells (Fig. 1). E. coli DNA induced NO production at a 10-fold lower concentration than a 22-base CpG ODN (AO-1) on a per weight basis. A similar effect was seen in all other assays of macrophage activation that we performed in RAW264.7 and BMM (data not shown).

Activities of dsDNA and ssDNA

We investigated whether the relative potency of E. coli DNA compared with that of CpG PO-ODN was due to its double-stranded nature. E. coli DNA was denatured by boiling for 10 min, followed by cooling on ice and rapid addition to the medium. E. coli DNA was 30–100% more active in single strand than double strand form in the induction of NO production, depending on the concentration of DNA used (Fig. 2A). This agrees with Krieg et al. (3), who noted that single-stranded E. coli DNA was 10–30% more active than dsDNA in B cell activation, and is consistent with the lack of interaction of TLR9 with dsDNA (15). Thus, the increased potency absorption at 550 nm. Control incubations of cells with Cy-labeled ODN on ice showed that dextran sulfate reduced ODN cell surface binding to negligible levels.

FIGURE 1. E. coli DNA is more active than the PO-ODN AO-1. RAW264.7 cells were primed with 375 pg/ml IFN-γ for 2 h, then treated with various concentrations of DNA for 24 h. Supernatants were assayed for the presence of nitrite. Results show the average and SD of triplicate assays and are representative of three experiments.
of *E. coli* DNA compared with CpG-containing PO-ODN was not due to its double-stranded nature.

This result appeared inconsistent with our own previous work, which showed that a dsPO-ODN was more efficient than an ssPO-ODN in activating the expression of an NF-κB-dependent reporter gene in RAW264.7 cells (13). We determined whether there were differences between activities of ss- and dsPO-ODN in the induction of NO production from RAW264.7 cells. The active CpG-containing ODN AO-1 was made double strand by annealing to its complementary strand, RAO. RAO itself was almost inactive, and the dsAO-1:RAO was significantly more active than AO-1 alone (Fig. 2B). The higher activity could be due to enhanced uptake of the double strand form. To test this hypothesis, AO-1 was labeled with Cy3 dye at the 5′ end, and uptakes of the single strand and double strand forms were compared by FACS. There was some increased uptake of the ds form, although this may not account for all the difference in activities of ss- and dsPO-ODN (Fig. 2C). To validate the use of Cy-labeled ODN for estimating uptake, the activity of Cy3- AO-1 was compared with that of its unlabeled counterpart. Addition of the Cy3 label to 22-mer PO-ODN did not change their ability to activate either an NF-κB-dependent reporter assay (result not shown) or NO production (Fig. 2D), so it is likely to give a true representation of the rate of uptake of unlabeled ODN.

In summary, for long DNA such as *E. coli* DNA, ssDNA was moderately more active than dsDNA, presumably because of the necessity for strand separation before recognition by TLR9. For short DNA such as a 22-mer, double strandedness dramatically improved activity at low concentration. This was partially due to improved uptake, but could also be due to increased nuclease resistance of the ds form. For ODN 44 bases in length, there was little difference between the activities of double strand and single strand forms (see Fig. 6). Our interpretation is that as ODN length increases, the probability that the CpG motif will be destroyed by nuclease digestion decreases. Hence, increased stability of dsODN is only likely to be relevant to short ODN (e.g., 22-mer). Thus, strandedness does not explain the high activity of *E. coli* DNA, but length-dependent resistance to nuclease may be a partial explanation.

**Effect of epigenetic modifications on activity of *E. coli* DNA**

Bacterial genomes contain methylation patterns not found in mammalian DNA. Epigenetic differences could be detected by immune cells, enhancing immunostimulation by *E. coli* DNA. The two major modifications to *E. coli* DNA are mediated by Dam and Dcm (16). Other workers have recently reported an effect of the N6-methyladenine found in Dam methylation sites on responses to CpG DNA (17). However, we found plasmid grown in Dam−/Dcm− *E. coli* and plasmid grown in *E. coli* wild type for these two loci resulted in identical induction of NO production from RAW264.7 cells (Fig. 3) and IL-12 production from BMM (data not shown). Although it is possible that other epigenetic modifications could enhance responses to bacterial CpG motifs in our assays, we have not found an effect of the most frequent methylation patterns.

**DNA length is critical for high response**

A remaining major difference between *E. coli* DNA and ODN is the length of the DNA being presented to the cell. To examine whether length affects activity, ODNs were synthesized containing the archetypal CpG motif active on murine cells (TGACGTT) flanked by oligo(deoxyadenine) (oligo(dA)) regions of varying length (see Fig. 6). Our interpretation is that as ODN length increases, the probability that the CpG motif will be destroyed by nuclease digestion decreases. Hence, increased stability of dsODN is only likely to be relevant to short ODN (e.g., 22-mer). Thus, strandedness does not explain the high activity of *E. coli* DNA, but length-dependent resistance to nuclease may be a partial explanation.
lengths. These ODN were chosen to eliminate any effects on activity by specific sequences or secondary structure in flanking regions that may variably be present in ODN of different lengths. The abilities of these ODN to induce NO production were then assayed. Fig. 4A shows that increasing the length of ODN between 14 and 44 bases gave stepwise increases in activity. A 22-nt oligo(dA) ODN had no stimulatory activity (result not shown), showing that the increased content of oligo(dA) was not responsible for increasing activity with length.

**Length-dependent uptake**

We investigated whether increasing length increased endocytic uptake of the ODN (Fig. 4B). Indeed, at 1 μM, the 44-mer had 8-fold higher uptake than the 22-mer. The 22-mer gave much more potent activation than the 14-mer at 1 and 3 μM (Fig. 4A), but there was only a modest difference in uptake (Fig. 4B). This suggests that between 14 and 22 bases, the length of ODN affects processes other than uptake. It is likely that there is a minimal ODN length for efficient recognition by the CpG receptor. In addition, there may be some contribution of stability to the increased potency of long ODN (i.e., an endonuclease cutting a longer ODN is less likely to destroy the CpG motif).

Apparent differences in uptake of ODN of different lengths could be explained by more rapid degradation of short ODN with subsequent release of Cy3 from the cell. To assess whether this was likely, ODN uptake was analyzed over a 2-h period. Length-dependent uptake was apparent from the earliest time point of 10 min, before degradation and loss of label from the cell could be at all significant (Fig. 4C). In addition, uptake of all ODN was close to linear during the first hour. As long as the measured uptake is linear with time, we may assume that the loss of Cy3 from the cell is negligible, and Cy3 accumulation therefore gives an accurate measure of ODN uptake. Thus, the length-dependent uptake observed at 1 h (Fig. 4B) is not an artifact of ODN degradation.

Length-dependent uptake could be due to the enhanced stability of a receptor-DNA complex when two or more receptors can bind to the one molecule. We next considered whether the 44-mer ODN was actually sufficient to generate maximal uptake. The uptake of longer pieces of DNA was assessed by preparing PCR products of

---

**FIGURE 3.** Dam/Dcm methylation found in *E. coli* DNA has no effect on NO production. Plasmid pBS-SK<sup>−</sup> was grown in *E. coli* strain DH5a (wild type (wt)) or the Dam<sup>−/−</sup>/Dcm<sup>−/−</sup> strain GM2163 (Dam<sup>−/−</sup>/Dcm<sup>−/−</sup>). The nitrite assay was performed as described in Fig. 1. The indicated concentrations of plasmid or Dnase I-digested plasmid were incubated with IFN-γ-primed RAW264.7 cells for 24 h. Results show the average of triplicate assays, and error bars, which fall largely within the size of the symbol, show the SD. Results are representative of two experiments.

**FIGURE 4.** Increasing the length of the ODN increases its stimulatory activity. A. Activity was determined by nitrite assay with RAW264.7 cells as described in Fig. 1. All ODN contained the central core TGACGTT flanked by a varying number of A residues. The number in the ODN refers to the total length of the ODN. Results show the mean and SD of triplicate assays and are representative of two experiments. B. Flow cytometric analysis of uptake of Cy3-labeled ODN of different lengths by RAW264.7 cells. Results show the average of duplicate assays, and error bars show the values obtained. Results are representative of two assays. C. ODN uptake is linear with time up to 125 min. RAW264.7 cells were incubated with 1 μM Cy3-labeled ODN for various times before analysis of uptake by flow cytometry. Results are representative of two experiments. D. Maximal DNA uptake is reached at a length of 250 bp. A Cy3-labeled PCR primer was used to synthesize β-actin DNA fragments of varying length. DNA was incubated with RAW264.7 cells for 2 h before DNA uptake was measured by flow cytometry. Results are representative of three experiments.
100, 250, and 500 bases from the mouse β-actin gene using a Cy3-labeled primer. Fig. 4D shows that uptake on a molar basis did not reach a maximum until DNA was 250 bp.

To confirm that the pattern of uptake and cellular activation observed in the RAW264.7 cell line was relevant to primary cells, experiments were repeated using BMM and BMDC. Like RAW264.7 cells, they also displayed length-dependent DNA uptake (Fig. 5) and consequently had a much enhanced response to longer ODN (data not shown).

A long PO-ODN has activity equal to E. coli DNA

The oligo(dA) flanking sequences in the CpGA44 ODN do not give optimal stimulatory activity (result not shown). To assess whether an increase in ODN length could give activity similar to that of E. coli DNA, we used a 44-mer ODN based on the sequence of AO-1. This ODN (LO) had similar activity to E. coli DNA (Fig. 6). Given the finding that uptake of dsDNA is not maximal until 250 bp (Fig. 4D), it is likely that the 44-mer has not reached the maximal potential activity, and increasing the length of the ODN further may correspondingly increase its activity. Given the range of CpG motifs of varying potency in E. coli DNA, no comparison of activity between E. coli DNA and ODN based on molar concentration of CpG motifs can be made. However, it is clear that the difference in activity between E. coli DNA and 22-mer PO-ODN is largely explained by length-dependent uptake.

Effect of physical linkage of CpG motifs

Published work has suggested that TLR9 clustering caused by ODN aggregation is necessary for signaling (18). Cross-linking of TLR9 molecules by DNA ligands containing more than one CpG motif could be an additional factor contributing to the high immunostimulatory activity of E. coli DNA. To address this possibility, we created a family of 44-mer ODN containing a 5′, a 3′, or both 5′ and 3′ CpG motifs. Fig. 7 shows that at concentrations of ≤0.05 μM, the double-CG ODN activated ELAM promoter activity slightly more than the sum of activation by the two single-CpG motif-containing ODN. At higher concentrations, the double-CG ODN had activity less than the sum of the 5′ and 3′ CG ODNs. By 3 μM, all ODN had similar activity. This suggests that at low ODN concentration, interaction of an ODN with two TLR9 molecules may provide slightly enhanced signaling, but the effect is not dramatic. It is possible that the two CpG motifs are not optimally spaced to allow interaction with two receptors. It should be noted that ligand-induced cross-linking of receptors is not essential for cellular activation, because LO, an ODN containing only one CpG motif, is as active as the double-CG ODN at all concentrations tested.

Length-dependent DNA uptake is cell type specific

The conclusion of this work to this point is that E. coli DNA is a far more potent stimulus than a 22-mer PO-ODN for macrophages, because uptake of short pieces of DNA is inefficient. The identity of the DNA uptake receptor is unknown. Because macrophages are specialized endocytic cells with a wide diversity of receptors that might recognize DNA, including the macrophage scavenger receptor, we examined whether the uptake process was macrophage specific. Comparison of macrophage DNA uptake with other cell types is shown in Fig. 8.
types showed that length-dependent uptake is not a universal phenomenon (Fig. 8). Mouse L929 fibroblasts, the mouse immature B cell line WEHI231, and the human B cell line RPMI8226 showed low DNA uptake with minimal effect of length. Thus, there appears to be a macrophage-specific length-dependent DNA uptake receptor. Because both the B cell lines tested in this study respond to CpG DNA (data not shown), the length-dependent uptake is clearly not absolutely linked to CpG recognition.

Discussion

DNA length is critical for macrophage CpG responses

*E. coli* DNA is much more immunostimulatory to macrophages than short (22-base) CpG-containing PO-ODN (Fig. 1). We considered five characteristics of *E. coli* DNA that could explain its superior activity: 1) double-stranded form; 2) epigenetic factors, i.e., adenine and cytosine methylation; 3) additional uncharacterized stimulatory sequences; 4) multiple linked CpG motifs; and 5) length. The length of DNA proved to be the critical factor, as increasing the CpG ODN length to 44 bases gave an activity equivalent to that of *E. coli* DNA (Fig. 6). The ability to cross-link several TLR9 molecules may play some role in the activity of *E. coli* DNA at low concentration.

Strandedness affects long and short DNA differently

Although double-stranded did not explain the high activity of *E. coli* DNA, it did affect the immunostimulatory potential of both *E. coli* DNA and short ODN. *E. coli* DNA was more active when boiled to result in ssDNA (Fig. 2A). This is consistent with the finding that TLR9 only interacts with ssDNA (15). How strand separation normally occurs before DNA interaction with TLR9 is unknown. In contrast, for short ODN, double-strandedness resulted in increased activity. The increased activity of dsODN appears to be partially mediated by increased uptake (Fig. 2C), but there may also be a contribution of improved stability to nucleosome attack.

Contribution of epigenetic modification of *E. coli* DNA

*E. coli* strain K-12, from which derive all strains for recombinant DNA work, has four characterized methylases (see http://tools.neb.com/~/vincze/genomes/view.php?enzname=EcoKMr)). The two major methylases are Dam, which methylates the N6 position of adenine in GATC sequences, and Dcm, which methylates the C5 position of the second cytosine in CCA/TG sequences (16). Two other methylases generating N6-methyladenine are EcoKI and EcoKII DNA methyltransferases, but they would have a lesser genome-wide effect because they have 6- and 7-bp recognition motifs. A recent publication shows a small induction of cytokines in vivo in response to PS-ODN containing N6-methyladenine as found in the Dam methylation site, and a significant enhancement of CpG responses by coadministration of N6-methyladenine ODN (17). They also found that Dam modification of plasmid enhanced IL-12 production in vivo when plasmid was administered as a lipofectin complex. N6-methyladenine is not found in the mammalian genome (19), so its detection could provide an additional immune stimulus. However, we did not detect an effect of the adenine or cytosine methylation present in the Dam or Dcm modifications of *E. coli* DNA on induction of NO production by RAW264.7 (Fig. 3) or IL-12 production by BMM (data not shown). Differences between our results and theirs regarding a role for Dam modification could result from recognition of Dam-modified DNA in vivo by nonmacrophage cells or an alteration in the response to N6-methyladenine by either PS modification or complex formation with lipofection reagent. However, our results show that Dam/Dcm methylation does not contribute to the superior activity of *E. coli* DNA as measured in our macrophage activation assays. Other characterized methylases are unlikely to play a role because they generate N4-methyladenine at infrequent sites, the sequence of which is strain and species specific.

Receptor cross-linking may enhance activity at low ligand concentration

Individual molecules of *E. coli* DNA contain multiple CpG motifs, and even with nuclease digestion in the endosome, it is likely that DNA fragments will retain the ability to bind to more than one TLR9 molecule. Previous workers have suggested that cross-linking of TLR9 is essential for cellular activation by CpG ODN (18). Data were presented showing that among a number of CpG-containing 40 mer, only PO-ODN that aggregated due to the presence of G-rich tails could activate BMM (18). The nonaggregating ODN they used seemed to be peculiarly inactive, perhaps due to the CpG motif being too close to the 5' end. LO or AO-1, used in this study, do not self-aggregate, as assessed by HPLC (result not shown). Despite this, LO is clearly quite a potent TLR9 agonist (Fig. 6). However, at low ODN concentrations, the presence of two CpG motifs in one molecule conferred a slight advantage (i.e., activation is more than the sum of activation for the two analogous single-CpG ODN; Fig. 7). Thus, ligand-induced TLR9 dimerization may enhance responses at low DNA concentration, but is certainly not essential for signaling in macrophages. Kerkmann et al. (20) have shown that IFN-α production by plasmacytoid dendritic cells requires ODN to be part of a large complex. This requirement was fulfilled by either type A CpG ODN, which associate to form nanoparticles due to oligo(deoxyguanine) (oligo(dG)) flanking sequences forming four-stranded structures, or by binding nonaggregating ODN to polystyrene particles. The requirement for large ODN complexes seems to be a particular requirement for IFN-α, but not TNF-α, induction by plasmacytoid cells. One possible explanation would be a requirement for TLR9 cross-linking, but this does not appear to be required for all responses and not in all cell types.

Although Wu et al. (18) attribute the superior activity of the aggregating ODN to TLR9 cross-linking, they also presented data suggesting that aggregating ODN are more efficiently taken up. The latter effect is consistent with our conclusion that DNA length is a limiting factor for uptake and subsequent activity, and the work of others showing that oligo(dG) regions improve ODN uptake (21).

DNA length affects uptake and activity

Increasing ODN length from 22 to 44 bases produced a large increase in both ODN uptake and activity. Although there may be some contribution of stability to the increased potency of long ODN, much of the effect can be attributed to length-dependent uptake. Given that pinocytic uptake should be independent of length, this indicates the involvement of an uptake receptor. In addition, Fig. 4B shows some degree of saturation of uptake with increasing concentration of the 44-mer, consistent with receptor-mediated uptake. Thus length-dependent uptake is the major contributing factor to the increased activity of *E. coli* DNA over a CpG-containing, 22-mer PO-ODN.

We previously showed that uptake by RAW264.7 of the mixed base, 22-mer PO-ODN AO-1 was linear with concentration up to 5 μM (33 μg/ml) (K. J. Stacey, unpublished observation) and was not inhibited by large amounts of unlabeled PO-ODN (11), but could be inhibited by vertebrate genomic DNA (13). These data indicated that at the concentrations normally used, 22-mer PO-ODN is of low affinity and does not saturate receptor binding. Conversely, uptake of high m.w. DNA is a much higher affinity...
process, with saturation of uptake of $^{32}$P-labeled E. coli DNA occurring at 5–10 μg/ml in RAW264.7 and BMM (K. J. Stacey, unpublished observation). Thus, increased length greatly increases the affinity and/or avidity of DNA for cell surface proteins. There is no doubt a minimum length for binding to a cell surface receptor monomer, but uptake is likely to be greatly enhanced if the DNA reaches a length able to interact with two receptors. This type of receptor clustering would stabilize an interaction that is of inherently low affinity to an individual receptor molecule, resulting in an increased rate of uptake. Surprisingly, enhancement of uptake by increased length is not seen in all cell types (Fig. 8). Thus, the length-dependent DNA uptake receptor may be macrophage and DC restricted. Consequently, in contrast to macrophages, B cells do not show increased activation with long PO-ODN or E. coli DNA (our manuscript in preparation).

**A macrophage-specific uptake receptor?**

The mechanism of DNA uptake has been a topic of interest to the fields of antisense therapy, gene therapy, and DNA vaccination as well as immune stimulation by DNA. Studies of DNA uptake have used a variety of different types of DNA and cell types, only some of which are relevant to the work in this study. Many studies have used PS-ODN, which are known to have much higher binding to a range of cellular proteins than PO-ODN (22). We have previously shown that uptake of PS-ODN by mouse macrophages is 10-fold higher than PO-ODN (11), and this is probably at least as important as stability to the increased potency of PS-ODN. PO-ODN have a higher affinity for the cell surface, and PS-ODN uptake cannot be appreciably inhibited by PO-ODN (11). Whether this reflects a higher affinity for the same receptor(s) involved in PO-ODN uptake or binding to additional receptors is not known.

The macrophage/granulocyte integrin Mac-1 (CD11b/CD18) is reported to be an uptake receptor for PS-ODN on polymorphonuclear leukocytes (23). If this is the case, a drop in surface expression of Mac-1 would be expected shortly after exposure to DNA, but we have previously found no effect of DNA treatment on the cell surface expression of CD11b as measured by flow cytometry (24). Others have cited unpublished data that Mac-1 knockout mice respond and take up DNA normally (25), although it is not stated whether this work was performed with PS-ODN, which may bind more promiscuously to the cell surface. Takakura et al. (26) note that the profile of inhibitors of plasmid DNA uptake into macrophages is similar to that of those binding to Mac-1. We re-examined a role for Mac-1 by looking at the localization of Mac-1 in cells treated with DNA using confocal microscopy. In untreated RAW264.7 cells, CD11b was localized to the cell surface, and after treatment with calf thymus DNA, E. coli DNA, or CpGAA4 ODN for 10 or 20 min, no change in localization of Mac-1 was detected (data not shown), suggesting that Mac-1 is not involved in DNA uptake into endosomes.

Siess et al. (27) attempted expression cloning of a cell surface DNA receptor and obtained a membrane-associated nucleic acid binding protein (MNAB), a 150-kDa protein. The full-length cDNA was obtained from a T cell line, although T cells are not known for efficient DNA uptake. This protein appears to be expressed in a wide range of cell types, but is found predominantly in the perinuclear region. Abs directed against MNAB were not able to block DNA binding to the cell surface, and no definitive role in DNA uptake has been established for MNAB.

A number of groups have speculated that a scavenger receptor (SR) is involved in DNA uptake. SRs are a broad group of molecules involved in the uptake of polyanionic ligands, with the best characterized being the macrophage receptors SR-A I and II (28). The defining ligands for SRs are modified low density lipoproteins (LDL) such as acetylated and oxidized LDL. Different SR molecules, including SR-A, bind well to polynucleotides that form four-stranded complexes such as poly(I) and oligo(dG), but early experiments showed that nucleic acids such as M13 or plasmid were not good ligands for SR-A (29). Macrophage uptake of ODN and longer DNA is inhibited by SR ligands such as oligo(dG) and poly[I] (30) (results not shown). However, using SR-A-null cells, it is evident that SR-A does not play a nonredundant role in the uptake of plasmid into peritoneal macrophages or in vivo clearance of injected DNA (26) and is not required for responses of BMM to E. coli DNA (31). Other molecules defined as SRs are not likely to play a role, because DNA uptake was not inhibited by acetylated or oxidized LDL, the defining SR ligands (26).

**Conclusion**

In macrophages, length-dependent uptake was the major reason for the increased potency of E. coli DNA over short ODN. Longer molecules may promote uptake receptor clustering and internalization by cross-linking several receptors. The identity of the putative macrophage-specific receptor involved in length-enhanced DNA uptake remains to be established.

Current studies of therapeutic ODN have focused on short PS-ODN, which are known to have non-CpG-mediated side effects, such as induction of B cell proliferation, splenomegaly, and tissue infiltration by mononuclear cells (32–35). This work has shown that the limiting factor in macrophage responses to PO-ODN is uptake into the cell. This limit can be overcome by increasing the length of the PO-ODN. In therapeutic situations where the side effects of PS-ODN are unacceptable, long PO-ODN may be used as an alternative.

**Disclosures**

The authors have no financial conflict of interest.

**References**


