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The Molecular Basis for the Lack of Immunostimulatory Activity of Vertebrate DNA[1]

Katryn J. Stacey,[2]*‡§ Greg R. Young,*‡ Francis Clark,¶ David P. Sester,§ Tara L. Roberts,*‡ Shalin Naik,§ Matthew J. Sweet,*‡§ and David A. Hume*†‡§

Macrophages and B cells are activated by unmethylated CpG-containing sequences in bacterial DNA. The lack of activity of self DNA has generally been attributed to CpG suppression and methylation, although the role of methylation is in doubt. The frequency of CpG in the mouse genome is 12.5% of *Escherichia coli*, with unmethylated CpG occurring at ~3% the frequency of *E. coli*. This suppression of CpG alone is insufficient to explain the inactivity of self DNA; vertebrate DNA was inactive at 100 μg/ml, 3000 times the concentration at which *E. coli* DNA activity was observed. We sought to resolve why self DNA does not activate macrophages. Known active CpG motifs occurred in the mouse genome at 18% of random occurrence, similar to general CpG suppression. To examine the contribution of methylation, genomic DNAs were PCR amplified. Removal of methylation from the mouse genome revealed activity that was 23-fold lower than *E. coli* DNA, although there is only a 7-fold lower frequency of known active CpG motifs in the mouse genome. This discrepancy may be explained by G-rich sequences such as GGAGGGG, which potently inhibited activation and are found in greater frequency in the mouse than the *E. coli* genome. In summary, general CpG suppression, CpG methylation, inhibitory motifs, and saturable DNA uptake combined to explain the inactivity of self DNA. The immunostimulatory activity of DNA is determined by the frequency of unmethylated stimulatory sequences within an individual DNA strand and the ratio of stimulatory to inhibitory sequences. *The Journal of Immunology*, 2003, 170: 3614–3620.

Macrophages, dendritic cells, and B cells produce a range of cytokines and up-regulate markers of activation in response to bacterial, but not vertebrate, DNA (1). These responses require the uptake of DNA, and intracellular recognition of unmethylated CG (CpG)-containing sequences. Subsequent signaling requires a member of the Toll-like receptor (TLR)[3] family, TLR-9 (2). Sequences flanking the CpG are important for recognition, as C residues upstream and a G residue downstream greatly reduce activity (1).

Potentially active sequences are present in mammalian genomes (3). The inactivity of vertebrate DNA has been attributed in part to suppression of CpG in vertebrate genomes to ~23% of random frequency (4). In addition, cytosine in vertebrate CpG sequences is 70–80% methylated (5, 6). Methylation reduces the activity of CpG-containing oligodeoxynucleotides (ODN) (7, 8), and treatment of bacterial DNA with CpG methylase renders it inactive (9, 10). A role for methylation in the suppression of activation by self DNA was challenged by the finding that hypomethylated DNA from embryonic stem cells deficient in DNA cytosine methyltransferase 1 (Dnmt1) remains relatively nonstimulatory (11). Dnmt1 is responsible for copying methylation patterns onto the nascent DNA strand in replicated DNA (6). However, Dnmt1-null embryonic stem cells retain methylation of 18% of CpG motifs, probably due to two de novo methylases, Dnmt3a and Dnmt3b (5). Little is known about the specificity of these enzymes, and it is possible that immunostimulatory motifs remain largely methylated in Dnmt1-null cells. Thus, the contribution of CpG methylation to the inactivity of self DNA remains uncertain.

The low activity of hypomethylated mouse DNA led to the proposal that vertebrate DNA contains inhibitory motifs (11). Although a number of studies have shown an inhibitory action of viral (12) and vertebrate (13–15) DNA or specific ODN (12–14, 16–20), there is no consensus in the literature about the nature of such motifs. The suggestions for inhibitory motifs include GC-rich sequences such as CCGG (12), GC-rich sequences when linked closely to CpG (21), methylated CpG motifs (15), certain G-rich sequences such as GGAGGGG (16, 17), and deoxyguanosine (dG) homopolymer (13, 14, 22). In none of these cases is it known how an inhibitory effect is mediated, and competition for CpG DNA uptake has not always been eliminated as a mechanism.

Although a role for recognition of self DNA as an indication of tissue damage has been suggested (23), the effects of self DNA are clearly distinct from those of bacterial DNA. The lack of recognition of self CpG sequences is important in allowing the immune system to distinguish between sterile tissue damage and infectious danger. The roles of both methylation and inhibitory motifs in preventing the activity of self DNA remain unclear. Here we have examined for the first time the activity of fully demethylated vertebrate DNA and assessed the contributions of methylation, inhibitory motifs, and suppression of the frequency of active sequences in preventing inappropriate activation of macrophages by self DNA.

*Cooperative Research Center for Chronic Inflammatory Diseases, †Special Research Center for Functional and Applied Genomics, ‡Institute for Molecular Bioscience, §School of Molecular and Microbial Sciences, and ¶Advanced Computational Modeling Center, University of Queensland, Brisbane, Queensland Australia

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[2] Address correspondence and reprint requests to Dr. Katryn Stacey, Institute for Molecular Bioscience, University of Queensland, Brisbane, Queensland 4072, Australia. E-mail address: k.stacey@imb.uq.edu.au

[3] Abbreviations used in this paper: TLR, Toll-like receptor; dG, deoxyguanosine; Dnmt1, DNA cytosine methyl-transferase 1; GFP, green fluorescent protein; ODN, oligodeoxynucleotide; PO-ODN, phosphodiester ODN; PS-ODN, phosphorothioate ODN; AO, activating ODN; RAO, reverse activating ODN.

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Materials and Methods

Materials

Escherichia coli and calf thymus DNA (Sigma-Aldrich, St. Louis, MO) were purified by two phenol chloroform extractions, ethanol precipitation, followed by four extractions with Triton X-114 to remove LPS (24), a phenol-chloroform extraction, and ethanol precipitation. DNA was isolated from human peripheral blood cells and mouse spleen and purified as described above. Assays of DNA activity after digestion with DNase I showed the lack of activating contaminants. ODN with and without 5′ Cy3 labels were obtained from Geneworks (Adelaide, Australia). The sequences are shown in Table I. All ODN used were phosphodiester (PO-ODN), with the exception of 1668S, which is a phosphorothioate (PS)-modified version of 1668. Stimulatory activating ODN (AO) is similar to 1668 (25). AO was used in double-stranded form by mixing with an equimolar amount of reverse activating ODN (RAO), heating to 65°C, and cooling slowly to room temperature. 2114L and 2088L are based on 2114 and 2088 (16), but lengthened and made as PO-ODN. LPS from *Salmonella minnesota* Re595 (Sigma-Aldrich) was dissolved in 0.1% triethylamine and sonicated.

Cells and cell culture

The murine macrophage-like cell line RAW264 was stably transfected with the human ELAM (E-selectin) promoter (−760 to +60) driving destabilized enhanced green fluorescent protein (GFP; obtained from K. Smith, University of Washington, Seattle, WA). A CpG- and LPS-responsive clone (ELAM9) was selected for use. Cells were cultured in RPMI 1640 plus 10% serum supreme (Invitrogen, San Diego, CA), 20 U/ml penicillin, and 20 μg/ml streptomycin.

PCR amplification of DNA

E. coli DNA was partially digested with *Sau*3A, and calf thymus, mouse, and human DNAs were digested with *Mbo*I. Digested DNA was ligated to double-stranded ODN with complementary ends. The ODN ligated to *E. coli* and calf thymus DNA was Tiny (GAGGTACCTAGAGGGAG) annealed to 5′-phosphorylated Tim (GATCCTCCCTCTAGGTACCTC). ODN ligated to human and mouse DNA were Lil' (TGAGCTGCCTGAT GCTG) and 5′-phosphorylated Ritchie (GATCCAGCATCAGGCAGC). DNA fragments >600 bp were isolated on a 1% low melting temperature agarose gel and used in PCR reactions. *E. coli* and calf thymus DNA were PCR amplified using ODN Huge (GAGGTACCTAGAGGGAGGATC). Human and mouse DNA were amplified using ODN Big (TGAGCTGC CTGATGCTGGATC). ELAM promoter assays on these ODN showed that they were inactive. Amplified DNA was purified by spinning through a 30,000 m.w. cutoff spin column, three extractions with Triton X-114 as described above, phenol-chloroform extraction, and ethanol precipitation.

Assay of ELAM promoter activity

ELAM9 cells were plated at 250,000 cells in 0.5 ml in 24-well plates and incubated overnight. After addition of stimuli, cells were incubated for 4–10 h. Plates were washed once with PBS, and cells were harvested in PBS/1 mM EDTA/0.1% sodium azide. Mean cellular fluorescence (arbitrary units) determined by flow cytometry was used to measure levels of GFP expression.

Assay of ODN uptake

Cells were plated at 250,000 in 1 ml in 24-well plates overnight, and then the volume was reduced to 250 μl for assay. Cy3-labeled ODN were added for 40 min. Cells were washed twice with cold PBS/0.1% BSA/0.1% sodium azide (PBA), incubated on ice for 10 min with 100 μg/ml DNase I in PBA to remove surface-bound ODN, washed once with PBS/0.1% sodium azide, and harvested in PBS/1 mM EDTA/0.1% sodium azide. Relative levels of internalized ODN were assessed by flow cytometry.

Nitrite assay

A nitrite assay was used to indicate NO production. RAW264 cells were plated at 4×10^4 cells/well in 96-well plates in 200 μl of medium and cultured overnight. Cells were then pretreated with 200 pg/ml murine IFN-γ (R&D Systems, Minneapolis, MN) for 2 h, followed by addition of ODN. Supernatants were assayed for nitrite at 24 h (26).

Table I. *ODN sequences*

ODN	Sequence	Ref. No.
AO	GCTCATGACGTTCCTGATGCTG	28
IO-2	GCTCATGCCGGTCCTGATGCTG	
RAO	CAGCATCAGGAACGTCATGAGC	
1668	TCCATGACGTTCCTGATGCT	25
1668GC	TCCATGAGCTTCCTGATGCT	
1896	CCGGCCGGCCGGCCGGCCGG	12
2114L	TTCCTGGAGGGGAAGTTGCT	16
2088L	TTCCTGGCGGGGAAGTTGCT	16
G22	GGGGGGGGGGGGGGGGGGGGGG	
A22	AAAAAAAAAAAAAAAAAAAAAA	
G20	GGGGGGGGGGGGGGGGGGGG	

Results

Demethylation exposes stimulatory motifs in mammalian DNA

Analysis of GenBank sequences (27) shows that the frequency of CpG in mouse DNA is 12.5% that in *E. coli* DNA (Table II). Since 20–30% of the CpG sequences in vertebrate genomes are unmethylated (5), unmethylated CpG occurs in the mouse genome at 2.5–3.8% the frequency of *E. coli*. Given this frequency and a number of reports using CpG ODN that show that methylation of active sequences does not completely prevent their activity (7, 8), vertebrate DNA might be predicted to show activity at an ~40-fold higher concentration than *E. coli* DNA. We examined this using the RAW264 macrophage cell line with the stably integrated NF-κB-dependent ELAM promoter driving GFP reporter expression. In all responses to DNA we have measured, this cell line behaves similarly to bone marrow-derived macrophages. Calf thymus DNA used at concentrations of up to 100 μg/ml had no significant activity, whereas *E. coli* DNA had detectable activity at 0.03 μg/ml (Fig. 1). The lack of activity could be explained by 1) preferential methylation of active sequences over relatively inactive sequences such as CCGG, 2) a much greater suppression of active CpG sequences than inactive, or 3) the presence of inhibitory sequences.

To investigate the role of methylation in the immunological silencing of self DNA, we eliminated all epigenetic modification by PCR amplification. After partial digestion of *E. coli* and vertebrate DNAs with a frequently cutting restriction enzyme, double-stranded primer ODN were ligated on to the DNA, and the genomes were amplified by PCR. Unlike native genomic DNA, unmethylated vertebrate DNA had readily detectable activity (Fig. 2). The response was not linear with concentration and began to plateau above 3 μg/ml (Fig. 2*A*). This response plateau was due to saturation of the DNA uptake process, as a plot of uptake of radiolabeled *E. coli* DNA superimposed well on this activity curve (results not shown). The three vertebrate DNAs tested after PCR amplification showed reproducibly different activities, with mouse having lower activity than human or calf. Given the published differences between optimal DNA sequences for mouse and human cellular responses (1), the lower activity of mouse DNA on mouse cells may reflect a lower concentration of the most potent mouse CpG motifs in the mouse genome.

Table II. *Genome frequencies*[a]

Genome	fC = fG	fCG	fCG/(fC × fG)	fCG/fCG *E. coli*
E. coli	0.254	0.0747	1.16	1
Mouse	0.216	0.00933	0.20	0.125
Human	0.203	0.00951	0.23	0.127

[a] Frequencies (f, number of occurrences/length of genome) analyzed from 2.57×10^9 bases of human phase III sequence, 1.96×10^8 bases of mouse phase III sequence, and the entire *E. coli* genome. fCG/(fC × fG) show CpG suppression, taking into account base usage in the genome, with a value of 1 being the expected random occurrence of CpG.

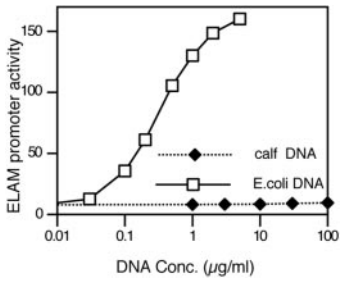

FIGURE 1. Calf thymus DNA fails to activate the ELAM promoter. *E. coli* and calf thymus DNAs were added to ELAM9 cells at the indicated concentrations and incubated for 10 h before measurement of GFP levels by flow cytometry. The results are typical of four experiments.

The activity of demethylated vertebrate DNA was still much less than that of *E. coli* DNA. Using low concentrations of DNA where uptake was not a limiting factor, PCR-amplified mouse DNA at a concentration of 0.5 μg/ml gave a response equivalent to that of amplified *E. coli* DNA at 0.022 μg/ml (Fig. 2*B*). Thus, in the absence of epigenetic modification, mouse DNA had only 4% the activity of *E. coli* DNA.

Can sequence suppression explain the low activity of mouse DNA?

We asked whether the difference in activity between mouse and *E. coli* DNA could be explained by the suppression of active sequences within the mouse genome by examining the frequency of acitve 8-mers in mouse and *E. coli* genomes. These 8-mer sequences would be found in a vast range of sequence contexts in native DNA that may well affect activity. There is currently no comprehensive understanding of the effect of sequence context on activity, so analysis of 8-mers, which is known to be active in ODN, was the best available option. The extent of CpG suppression in the mouse genome is presented graphically in Fig. 3*A*. This shows a strong bimodal distribution of frequency of occurrence of 8-mers, with almost all the rare sequences being CpG-containing 8-mers. Not surprisingly, the rarest sequences contain two or more CpG sequences. The most abundant CpG-containing 8-mers are predominantly CG-rich sequences, which tend to be nonstimulatory. In contrast, CpG-containing 8-mers in the *E. coli* genome show the same distribution as the bulk of sequences (Fig. 3*B*). The average frequency of single CpG-containing mouse 8-mers is 18.9% of the expected random frequency (i.e., $1/4^8$). Eight published active sequences have an average 18.1% of random frequency (Table III), and thus there is no profound suppression of active sequences below the general level of suppression of CpG-containing 8-mers. In Table III we show that active sequences have an average suppression in the mouse genome to 13.6% of that in *E. coli*, a 7-fold difference. This appears insufficient to account for the 23-fold difference in activity between demethylated mouse and *E. coli* DNA. It is possible that this discrepancy could be due to an incomplete state of knowledge on what comprises an active sequence, and sequences outside the 8-mers analyzed may have a profound effect on CpG motif activity in intact vertebrate DNA. The alternative explanation is that vertebrate genomes contain inhibitory sequences that prevent self activation.

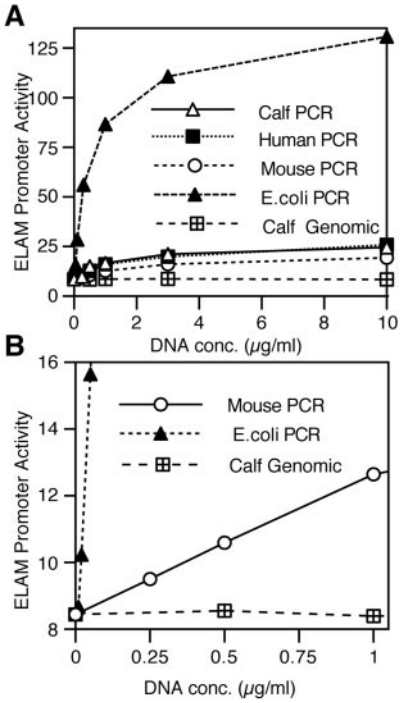

FIGURE 2. PCR amplification of vertebrate DNA reveals a role for methylation in preventing DNA activity. *A*, Calf thymus, human, mouse and *E. coli* DNA annealed to primers were amplified by PCR to remove epigenetic modifications. The ability of various concentrations of these DNAs and calf thymus genomic DNA to activate the ELAM promoter was assessed after 10-h incubation with ELAM9 cells. Results are typical of three experiments. *B*, Data from *A* on an expanded scale to allow estimation of demethylated mouse DNA activity relative to *E. coli*. Error bars showing the values obtained with duplicate samples fall within the symbols.

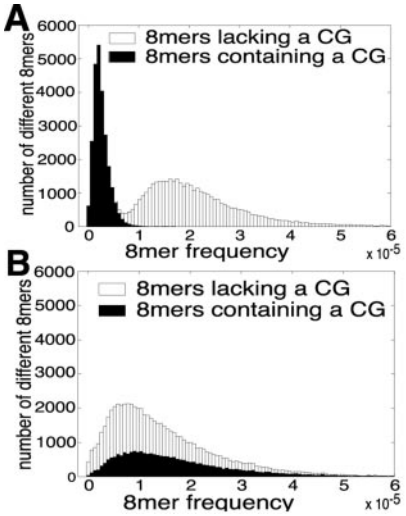

FIGURE 3. Profound suppression of CpG-containing 8-mers in the mouse, but not the *E. coli* genome. Phase 3 sequence of the mouse genome (4.43×10^7 nucleotides) and the entire *E. coli* genome (4.64×10^6 nucleotides) were analyzed for the frequency of occurrence of all possible eight nucleotide sequences. Graphs show the number of different 8-mer sequences falling within particular frequency ranges within the analyzed mouse genome sequence (*A*) and *E. coli* genome (*B*). Results are shown for 8-mer-containing and -lacking CG sequences.

Inhibition by genomic DNA

To look for an inhibitory role for vertebrate DNA, we pretreated cells with various amounts of calf thymus DNA before addition of stimulatory ODN. Calf thymus DNA inhibited the activity of both single- (ss) and double (ds)-stranded CpG ODN (Fig. 4, *A* and *B*). We examined whether this could be due to competition for uptake. Uptake of Cy3-labeled ODN was also greatly inhibited by the

Table III. *Frequency of occurrence of 8-mer sequences*[a]

Sequence	Source (Ref. no.)	Mouse Frequency/ Expected	*E. coli* Frequency/Expected	Mouse Frequency/ *E. coli* Frequency
Active				
TGACGTTC	25, 28	0.214	1.328	0.161
TAACGTTG	38	0.132	2.048	0.064
TGACGTTG	9, 16	0.188	2.289	0.082
AGACGTTA	39	0.177	0.876	0.202
TGACGTTT	40	0.257	1.540	0.167
CAACGTTG	41, 42	0.151	2.020	0.075
TAACGTTC	43	0.137	1.215	0.113
TGTCGTTC	43	0.194	0.862	0.225
Average		0.181	1.522	0.136
Inhibitory				
TGTAGGGG	—[b]	0.896	0.155	5.764
TTTAGGGG	—[b]	1.034	0.057	18.309
TTGAGGGG	—[b]	1.299	0.325	3.998
TGGAGGGG	16	1.791	0.297	6.038
TGGCGGGG	16	0.343	1.427	0.240
AGTGGGGG	18	1.933	0.325	5.948
ATTGGGGG	18	1.306	0.310	4.213
Average		1.229	0.414	5.359
Ratio active: Inhibitory sequences		0.147	3.68	

[a] The frequency of occurrence of the listed activating and inhibitory 8-mer sequences was determined within 4.43×10^7 nucleotides of phase III mouse genomic sequence and in the *E. coli* genome (4.64×10^6 nucleotides). Frequency = number of occurrences/length of genome analyzed. This was divided by the expected frequency of any 8-mer assuming random base composition and usage (i.e., $1/4^8$).

[b] P. Lenert and B. Ashman, unpublished observation.

vertebrate DNA (Fig. 4, *A* and *B*). Fig. 4*C* shows that the concentration of single-stranded stimulatory ODN used, 3 μM, is on a linear part of the dose-response curve for activity. The uptake of ssODN was proportional to concentration between 0 and 5 μM (result not shown). Thus, if uptake of ODN at 3 μM was inhibited by 50%, we would expect an approximate 50% reduction in activity. This was apparent using ssAO in Fig. 4*A*. Thus, the inhibitory activity could apparently be accounted for by inhibition of ODN uptake.

A different picture emerged using dsODN. The dsAO was considerably more active than the ssAO, and this was not due to the independent activity of the reverse strand RAO, which was quite low (Fig. 4*C*). This increase in activity may be due to greater resistance of the double-stranded form to nuclease activity. When cells were incubated with calf thymus DNA before dsAO, there was consistently greater inhibition of activity than inhibition of dsAO uptake (Fig. 4*B*). Since the dose response of dsAO is beginning to plateau at 3 μM (Fig. 4*C*), and, like ssAO, uptake is linear with concentration in this range (result not shown), a 50% drop in uptake should give a <50% drop in activity. The reverse finding implies that the calf thymus DNA has inhibitory activity in addition to direct competitive inhibition of uptake. The apparent lack of inhibition of the activity of the ssODN (Fig. 4*A*) probably relates to opposing effects of calf thymus DNA on ODN stability vs the uptake and activity of ssODN. We present evidence below that the stability of ssODN limits their activity (Fig. 6*A*). Thus, addition of calf thymus DNA may compete for nucleases and stabilize the ODN. Given this evidence of inhibitory activity of genomic DNA, we next looked for inhibition by specific sequences.

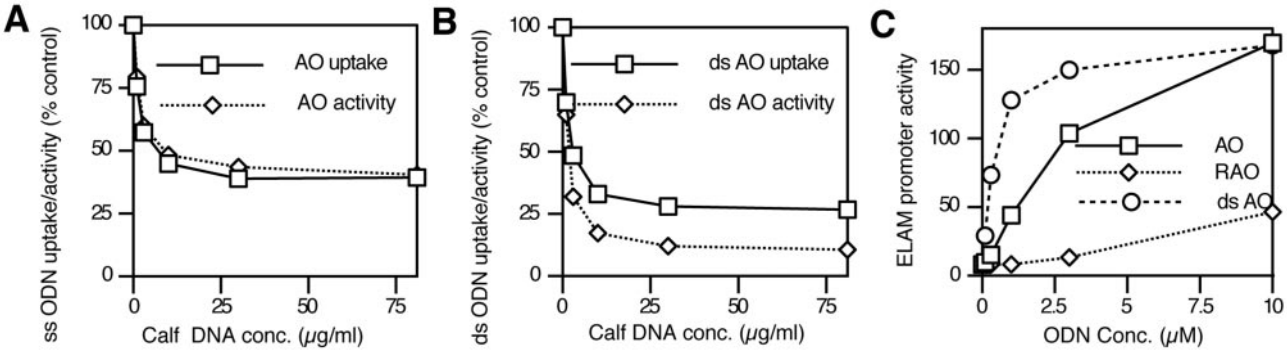

FIGURE 4. Inhibition of uptake and activity of ss- and dsCpG ODN by calf thymus DNA. *A* and *B*, Various amounts of calf thymus DNA were added to ELAM9 cells 20 min before 3 μM ssCpG ODN (AO or Cy3-AO; *A*) or 3 μM dsCpG ODN (AO or Cy3-AO annealed with RAO; *B*). The uptake of Cy3-AO was assessed at 40 min by flow cytometry. The activity of AO was assessed by ELAM promoter activation after 4-h incubation. Results show the percent uptake or activity relative to a control sample with no added calf thymus DNA, and are the mean of two independent experiments. Error bars showing the values obtained in the two experiments are plotted, but fall within the symbol. *C*, Comparison of activity of ss- and dsCpG ODN. Increasing amounts of ssCpG ODN (AO), its complement RAO, and dsCpG ODN (AO annealed to RAO) were incubated with ELAM9 cells for 4 h before determination of promoter activity. The results are the average of duplicate determinations and are typical of three experiments.

Inhibition by specific sequences

Several reports have suggested that ODN of certain sequences can inhibit responses to CpG ODN. Krieg et al. (12) found GC-rich sequences, including CCGG, were inhibitory. When we tested CCGG-containing ODN for inhibitory activity, they were, in fact, active at high concentrations and failed to inhibit the response to a CpG ODN (Fig. 5*A*). The ODN used were either a CCGG repeat sequence (1896) or the AO backbone containing CCGG instead of the normal active CpG motif (IO-2). These ODN were all used in the diester form, as we have previously shown that PS-ODN have a thioate-specific inhibitory function at higher concentrations (28).

Chen et al. (15) showed inhibition of spleen cell responses to *E. coli* DNA by calf thymus DNA and concluded that this effect required methylated CpG sequences. To test whether methylated CpG is important in the inhibitory action observed in Fig. 4*B*, we repeated the experiment using calf thymus DNA amplified by PCR to remove methylation. Fig. 5*B* shows that the PCR product inhibited dsODN activity to a greater extent than it inhibited uptake, similar to native calf genomic DNA. Thus, there is no requirement for methyl cytosine in the inhibitory effect we observed in RAW264 cells.

Another report has shown that certain G-rich sequences have inhibitory properties (16). As these studies were performed with PS-ODN, which have some intrinsic inhibitory activity (28), we remade two of these inhibitory sequences as PO-ODN of the same length as our activating ODN. Both ODN 2114L and 2088L were potent inhibitors of the response to CpG ODN. An equimolar amount of 2114L reduced the response to 1668 by 70% (Fig. 6*A*). The noninhibitory control ODN, 1668GC, and A22 actually increased activity despite being inactive themselves. This may be mediated by the stabilization of 1668 by competition for nucleases, as there was no enhancement of the response to a PS-ODN (Fig. 6*B*), and the effect was less marked if the 1668 concentration was increased to 3 μM (Fig. 6*C*). Since both inhibitory ODN have the sequence GGNGGGG, we examined whether the inhibitory effect could also be seen with oligo-dG. There was a slight enhancement of the response with dG22 (Fig. 6*A*), although less than that seen with 1668GC and dA22. Two possible explanations are that

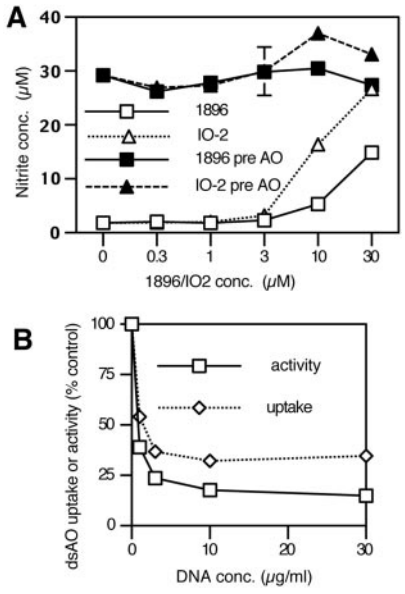

FIGURE 5. Lack of inhibitory effect of CCGG sequences and no requirement for methylated CpG within genomic DNA for inhibitory action. *A*, IFN-γ-primed RAW264 cells were incubated with various concentrations of CCGG-containing ODN (IO-2 and 1896) for 20 min before treatment with or without CpG ODN (AO) for an additional 24 h. Nitrite was assayed as an indication of NO production. The results show the mean and SD of triplicate measurements and are typical of three experiments. *B*, Inhibition of uptake and activity of dsCpG ODN by PCR-amplified calf thymus DNA. Various amounts of calf thymus DNA were added to ELAM9 cells 20 min before 3 μM dsCpG ODN (AO or Cy3-AO annealed with RAO). The uptake of dsCy3-AO was assessed at 40 min by flow cytometry. The activity of dsAO was assessed by ELAM promoter activation after 4-h incubation. Results were corrected for the small amount of activation measured with PCR-amplified calf thymus DNA alone. The data show the percent uptake or activity relative to a control sample with no added calf thymus DNA and are the mean of two independent experiments. Error bars showing the values obtained in the two experiments are plotted, but fall within the symbols.

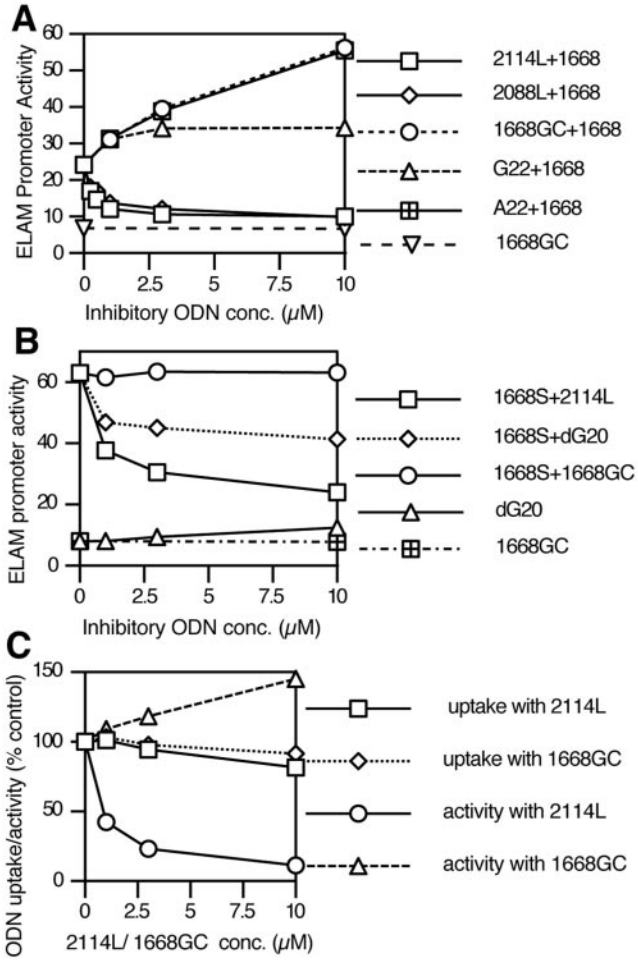

FIGURE 6. Certain G-rich ODN potently inhibit CpG DNA responses. *A*, Various concentrations of ODN (2114L; 2088L, 1668GC, G22, A22) were added to ELAM9 cells at the same time as 1 μM CpG ODN 1668. ELAM promoter activity was determined after a further 4-h incubation. The results are the average of duplicate determinations and are representative of four experiments. Results for 2114L, 2088L, and A22 alone superimpose with those for 1668GC alone and are not shown. *B*, Various concentrations of ODN (2114L, 1668GC, G20) were added to ELAM9 cells 20 min before addition of 0.1 μM CpG PS-ODN 1668S. ELAM promoter activity was determined after a further 4-h incubation. The results are the average of duplicate determinations and are representative of two experiments. *C*, Inhibition by G-rich ODN is not due to inhibition of ODN uptake. ELAM9 cells were incubated with various concentrations of 2114L or 1668GC for 20 min before addition of 3 μM 1668 or Cy3-1668. Uptake of Cy3-1668 was determined after 40 min. ELAM promoter activity in response to 1668 was determined after a further 4-h incubation. The results are the average of duplicate determinations and are typical of three experiments.

oligo-dG is a poor substrate for nucleases, or that the stabilization seen with this ODN is balanced by some inhibitory activity. When we eliminated the stabilization effect by using an active PS-ODN, some inhibitory activity of dG20 was revealed (Fig. 6*B*).

Inhibition by ODN 2114L could not be attributed to inhibition of uptake of DNA, as might be expected from earlier evidence that PO-ODN appear to have low affinity for and hence low saturation of cell surface receptors (28). Fig. 6*C* shows a small effect of both 2114L and the inactive 1668GC on uptake of Cy3-labeled 1668, which clearly does not account for the great inhibition of activity by 2114L plotted on the same graph. The inhibitory ODN inhibited responses to all forms of CpG DNA (ss and dsODN, PS- and PO-ODN, and *E. coli* DNA; results not shown), but there was no inhibition of the LPS response (result not shown), in agreement with previous work (16).

As the inhibitory sequences do not necessarily contain CpG, they are likely to be more prevalent than active sequences. In Table III we see that this is the case for 2114L. However even the CpG-containing 2088L is more highly represented than all the active sequences examined. The situation in the *E. coli* genome is the inverse of that in the mouse genome, with active sequences at 1–2 times the expected random occurrence, and inhibitory sequences being under-represented. With the panel of active and inhibitory sequences used here, the ratio of average occurrence of active to inhibitory sequences is 25-fold different between *E. coli* and mouse.

Discussion

Identification of DNA as foreign requires that there are sufficient structural and sequence differences between host and pathogen genomes. Roles for CpG suppression, methylation, and inhibitory sequences have been proposed, although the contribution of methylation is in doubt (11), and a number of quite different sequences are reported as inhibitory. We sought to resolve these issues. In this study we showed that while suppression of active sequences and methylation both certainly limit the immunostimulatory activity of self DNA, they are insufficient to explain the lack of macrophage response to vertebrate DNA. Demethylated mouse DNA was 23-fold less active than *E. coli* DNA, while the average frequency of known active motifs is only 7-fold lower in mouse than *E. coli*. We therefore examined whether vertebrate genomic DNA contains inhibitory motifs. Responses to CpG ODN were potently inhibited by vertebrate DNA, but a large component of this was due to inhibition of ODN uptake. A proportion of the inhibition of ds CpG ODN activity could not be explained by an effect on uptake (Fig. 4*B*), which provides evidence for inhibitory motifs within vertebrate DNA. We investigated a number of different proposed inhibitory motifs for their effects on CpG responses.

Chen et al. (15) attributed inhibitory action of vertebrate DNA to methylated CpG groups. Given the predominant restriction of methylated CpG motifs to vertebrate genomes, this is an attractive proposal, but we find no evidence for it in our system, as PCR-amplified calf thymus DNA maintained its potency as an inhibitor (Fig. 5*B*). Earlier work showed that DNA from type 2 or 5, but not type 12, adenovirus inhibited responses to *E. coli* DNA (12). The type 2 or 5 adenovirus had much higher representation of GC-rich hexamers, and these sequences, including CCGG-containing sequences, were suggested as inhibitory sequences. We have failed to find any inhibitory action of CCGG-containing PO-ODN, which are stimulatory at high concentrations (Fig. 5*A*). In other published work, GC-rich sequences were found to be inhibitory only when linked on the same molecule within seven bases of the stimulatory motif (21). In that case, the GC-rich sequences may either disrupt the optimal stimulatory motif or bind another factor that sterically hinders interaction with the CpG receptor.

More recent work showed that certain G-rich sequences have a specific effect on B cell activation (16, 17). We found that sequences such as GGCGGGG or GGAGGGG are also potently inhibitory in macrophages, without competing for DNA uptake. These inhibitory sequences are generally more highly represented in mouse than *E. coli* DNA (Table III). The activity of DNA is likely to be determined by the frequency of active sequences and the ratio of active to inhibitory sequences. We found a 25-fold difference in this ratio between mouse and *E. coli* genomes. The challenge remains to determine how these inhibitory sequences are acting; are they direct competitive inhibitors for active sequences, or do they send an independent inhibitory signal? Since they do not inhibit the LPS response (16), which shares many of the same signaling pathways, the latter possibility is less likely.

The G-rich motifs GG(C/A)GGGG were more effective inhibitors than oligo-dG (Fig. 6, *A* and *B*), and they may or may not act on cells in the same manner. Strings of dG residues have the ability to form a four-stranded complex that can bind to the scavenger receptor (29). Stunz et al. (17) suggest that this is not how the G-rich sequence of 2088 is working, as incorporation of deazaguanine, which should disrupt the formation of G base quartets had no effect on the inhibitory action of the ODN. However they did not directly investigate the structures of their ODN. Oligo-dG sequences have complex effects on cells, including formation of macrophage-like colonies from bone marrow (30), inhibition of tumor cell growth (31), and costimulation of T cells (3) in addition to inhibition of macrophage responses to CpG DNA (14, 22). Inclusion of dG runs in ODN may enhance their uptake by scavenger receptor (32), but it would be unwise to assume that all effects of oligo-dG are mediated by this receptor, as other proteins, such as a macrophage cytoplasmic protein, have shown preferential binding to G-rich ODN (30). Inhibition by runs of dG nucleotides probably also explains the inhibition of macrophage activation observed by Häcker et al. (33), and their claim that inhibition was sequence independent may have been due to the confounding effects of PS-specific inhibition at higher concentrations (28).

Saturable uptake of radiolabeled genomic DNA (not shown) and inhibition of ODN uptake by vertebrate DNA (Fig. 4) are consistent with receptor-mediated uptake of DNA (34). The inherent saturability of the uptake process has implications for the inactivity of self DNA. A limitation to the amount of DNA that can be taken up means that DNA with a very low frequency of active CpG motifs may never reach sufficient concentration within the cell to cause cellular activation. A component of ODN uptake remains resistant to inhibition even at high calf thymus DNA concentration (Fig. 4, *A* and *B*). This implicates either a substantial component of uptake being via the fluid phase or use of some different receptors by ODN and *E. coli* DNA, perhaps dependent on recognition of DNA ends.

In summary, using demethylated mouse DNA we have shown for the first time that CpG methylation is essential for the inactivity of self DNA. Analysis of the activity of demethylated DNA and the frequency of active motifs showed that methylation and CpG suppression were insufficient to entirely account for the inactivity of self DNA, implicating a critical role for G-rich inhibitory motifs. In addition, we have recognized the importance of saturable DNA uptake in limiting immunostimulation by DNA with a low frequency of stimulatory motifs. Self DNA is nonstimulatory for inflammatory cytokine production, but recent results suggest that it is not immunologically inert. CpG-independent enhancement of MHC, but not cytokine, expression has been described in response

to transfected dsDNA, but not ssDNA (23, 35). Any immunomodulatory role of self DNA is likely to be specific for the context in which DNA is seen. DNA detected within the cytoplasm could represent either viral infection or leakage of DNA from the nucleus following DNA damage. Extracellular DNA may be found after mechanical tissue damage or chronic inflammatory damage. DNA released in a phagosome after degradation of engulfed apoptotic cells could help prevent macrophage activation and promote ongoing tolerance to self Ags. Each of these situations requires a different immunological response, likely to be controlled by the route of exposure to DNA as well as the presence or the absence of cytokines and microbial molecules.

Recognition of self DNA may have pathological consequences. Chromatin-immune complexes have been reported to stimulate self-reactive human B cells through synergistic activation of Ag receptor and a member of the TLR family (36). Inhibitor studies, including inhibitory ODN, implicated TLR9 in this response. However, it has not been shown that the activation was CpG dependent. We showed that mammalian DNA is immunostimulatory if unmethylated. Recognition of self DNA in autoimmunity could be mediated by hypomethylated DNA as found in lupus (37). Alternatively, there is evidence that B cells, particularly when costimulated through Ag receptor, are able to recognize methylated CpG ODN (7). A role for recognition of self DNA in autoimmunity suggests that inhibitory ODN may have clinical applications, and determination of their mechanism of action is an important challenge.

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