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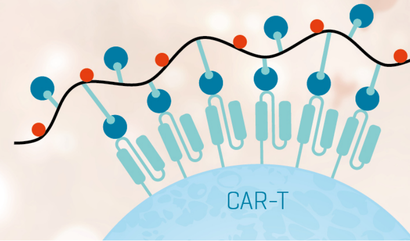

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Macrophages Ingest and Are Activated by Bacterial DNA[1]

Katryn J. Stacey,[2] Matthew J. Sweet, and David A. Hume[3]

Recent evidence suggests that bacterial DNA activates immune responses. Here we showed that TNF-α mRNA was induced in bone marrow-derived macrophages and the macrophage cell line RAW 264 by plasmid DNA, but not by DNaseI-digested plasmid, plasmid methylated on CpG dinucleotides, or by vertebrate genomic DNA, which is naturally largely methylated on these sequences. Synthetic polynucleotides poly d(I-C) and poly I·poly C also induced TNF-α. IL-1β and plasminogen activator inhibitor-2 mRNAs were induced by plasmid DNA, and IFN-γ-pretreated macrophages responded to DNA with induction of inducible nitric oxide synthase. The HIV-1 long terminal repeat was activated by exogenous DNA in a manner similar to TNF-α, and was also activated by a CpG-containing oligonucleotide. Transcription factor nuclear factor-κB (NF-κB) is involved in regulation of the HIV-1 long terminal repeat and many inflammatory response genes. NF-κB binding activity was increased by plasmid DNA. An important question is whether these effects involve DNA binding to a cell surface receptor that signals to the interior, or whether internalization is necessary. Here we found that plasmid was taken up by RAW 264 cells and remained sufficiently intact to code for luciferase protein. Results suggest that DNA is taken up by macrophages and characteristic bacterial DNA sequences, which include an unmethylated CpG sequence, activate a signaling cascade leading to activation of NF-κB and inflammatory gene induction. Relevance to DNA vaccination, gene therapy, antisense, and transfection studies is discussed. ***The Journal of Immunology,* 1996, 157: 2116–2122.**

A number of bacterial products such as LPS, lipoarabinomannan, peptidoglycan, and formyl methionine are well characterized as activators of immune function. There is increasing evidence that mammalian immune systems can distinguish between bacterial and vertebrate DNA, with bacterial DNA directly activating immune cells (1). B cell mitogenesis and total Ab production were activated by treatment with bacterial DNA but not mammalian DNA in a T cell-independent manner (2). This work suggested that structures or sequences in bacterial DNA that do not exist in mammalian DNA are being recognized by the immune system. One major difference between bacterial and vertebrate DNA is that vertebrate DNA contains a relatively low frequency of CpG dinucleotides, and those that occur tend to be methylated on the cytosine residue (3). In a study on B cells, activation by bacterial DNA required unmethylated CpG motifs, and was mimicked by some CpG-containing oligonucleotides (4). The most active oligonucleotides contained CpG flanked by two 5′ purines and two 3′ pyrimidines.

B cells are not the only cell type to respond to foreign DNA. Using murine spleen cells, Yamamoto et al. (5) showed that viral, bacterial, and invertebrate DNA increased NK cell activity and induced IFNs, whereas plant and vertebrate DNA did not. This activation correlates well with the DNA methylation pattern, only vertebrate and plant DNAs are largely methylated on CpG motifs (3, 6, 7). CpG-containing palindromic sequences from bacterial DNA were identified that induced NK cell activity (8). Some of these sequences are similar to those found by Krieg et al. (4) for B cell activation. Injection of these CpG-containing oligonucleotides or bacterial DNA into tumors has been found to lead to immune cell infiltration and tumor regression (9).

Published evidence suggests that entry of DNA into the cell is required for activation. Immobilized oligonucleotide was unable to stimulate B cells (4), and lipofection of active oligonucleotides to increase cellular uptake greatly enhanced their effect in activating NK activity (10). Also, no difference has been detected in the binding of activating and nonactivating oligonucleotides to the cell surface (4, 10). This suggests that there is no discrimination at the level of cell surface binding, and determination of whether DNA is of foreign origin may take place intracellularly. Uptake of DNA by monocytes has been observed, but it was largely degraded within an endosomal compartment (11). In this paper we show that some DNA taken up into a macrophage cell line remains in an intact form and can be expressed, and that bacterial DNA activates transcription factor nuclear factor-κB (NF-κB),[4] and expression of a number of genes in macrophages. Cellular responses to DNA have experimental implications for transfection and antisense oligonucleotide studies, and therapeutic implications in gene therapy, DNA immunization, and antisense therapy.

Materials and Methods

Materials

The synthetic dsDNA, poly d(I-C), was purchased from Boehringer Mannheim. Salmon sperm DNA, poly I·poly C (dsRNA), poly dC (ssDNA), and poly I and poly C (ssRNAs) were purchased from Sigma Chemical Co. (St. Louis, MO). LPS from *Salmonella minnesota* RE595 and *Escherichia coli* serotype 0111:B4 were purchased from Sigma Chemical Co. Murine IFN-γ was purchased from Genzyme (Cambridge, MA).

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[4] Abbreviations used in this paper: NF-κB, nuclear factor-κB; BMM, bone marrow-derived macrophages; PAI-2, plasminogen activator inhibitor-2; LTR, long terminal repeat; EMSA, electromobility shift analysis.

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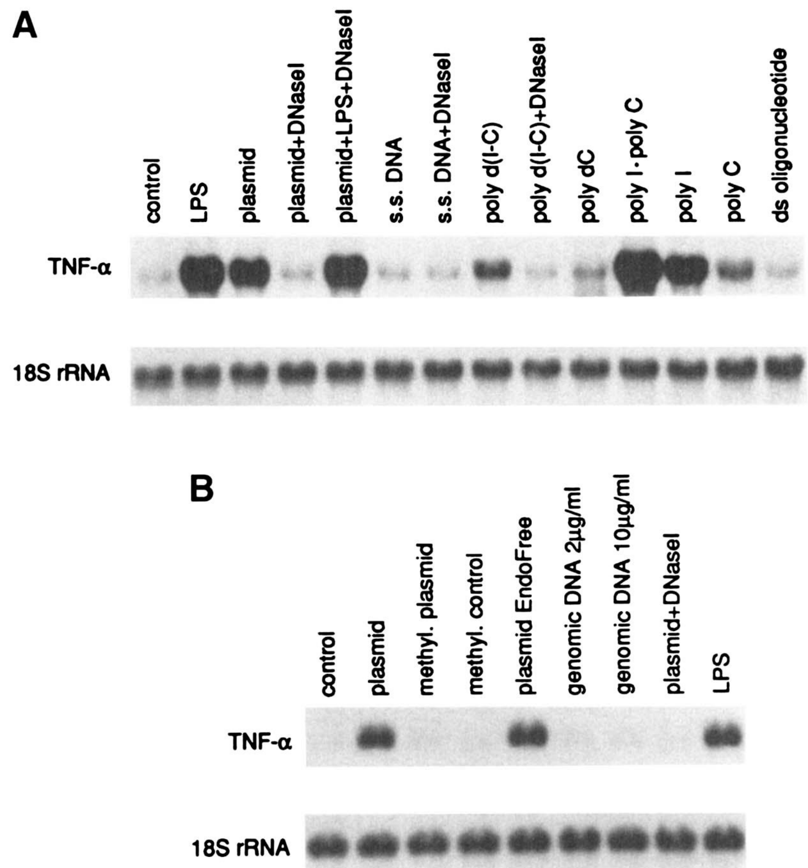

FIGURE 1. Northern blot analysis of induction of TNF-α mRNA by nucleic acids. *A*, BMM were incubated in 10 ml medium for 1 h with LPS or various nucleic acids. Treatments were: control, no additions; LPS, 10 ng *Salmonella minnesota* LPS; plasmid, 10 μg pBluescript; plasmid + DNaseI, 10 μg pBluescript DNA digested with DNaseI; plasmid + LPS + DNaseI, 10 μg pBluescript DNA and 10 ng *S. minnesota* LPS treated with DNaseI; s.s. DNA, 10 μg salmon sperm DNA; s.s. DNA + DNaseI, 10 μg salmon sperm DNA digested with DNaseI; 10 μg poly d(I-C); 10 μg poly d(I-C) digested with DNaseI; 10 μg poly dC; 10 μg poly I·poly C; 10 μg poly I; 10 μg poly C; ds oligonucleotide, 10 μg ds oligonucleotide (5′-GGCCAGGACCAAATG AGGAGATCTT-3′). Hybridization to 18S rRNA is shown as a loading control. *B*, RAW 264 cells were incubated in 5 ml medium for 1 h with LPS or various nucleic acids. Cell treatments were: control, no additions; plasmid, 10 μg pBluescript; methyl. plasmid, 10 μg pBluescript treated with CpG methylase; methyl. control, CpG methylase in buffer for methylation; plasmid EndoFree, 10 μg pBluescript prepared with Qiagen EndoFree kit; genomic DNA 2 μg/ml, 10 μg mouse genomic DNA; genomic DNA 10 μg/ml, 50 μg mouse genomic DNA; plasmid + DNaseI, 10 μg pBluescript digested with DNaseI; LPS, 10 ng/ml *E. coli* LPS. Hybridization to 18S rRNA is shown as a loading control.

Cell culture

The murine macrophage-like cell line RAW 264 (12) was obtained from American Type Culture Collection (Rockville, MD) and cultured in RPMI 1640 with 10% FCS, 20 U/ml penicillin, and 20 μg/ml streptomycin. Bone marrow-derived macrophages (BMM) were obtained from BALB/c mice as described (13) and used after 7 days in culture.

Northern Blot analysis

Cells were plated on tissue culture plastic approximately 16 h before commencement of the experiment. Nucleic acids or LPS were added to the cells for various times as indicated in the figure legends. RNA was prepared by the method of Chomczynski and Sacchi (14). Ten micrograms of each sample was run on denaturing MOPS formaldehyde gels, blotted, and probed according to Hybond N protocols (Amersham, Arlington Heights, IL). cDNA probes were labeled with [α-^{32}P]dCTP by random priming (Amersham). An oligonucleotide probe complementary to mouse 18S rRNA (5′-CATGGTAGGCACGGCGACTACCAT-3′) was used as a loading control, and was end-labeled using [γ-^{32}P]ATP and T4 polynucleotide kinase.

DNA preparation

pBluescriptSK (Stratagene, La Jolla, CA) and pGL3C (Promega, Madison, WI) were prepared by alkaline lysis followed by CsCl gradient centrifugation. Ethidium bromide was then removed by repeated extraction with isopropanol, plasmid was diluted threefold, and precipitated with 2 vol of ethanol. The precipitation was repeated and the pellet rinsed with 70% ethanol before resuspension in water. Mouse genomic DNA prepared by the method of Laird et al. (15) was obtained from Dr. R. Passey (Heart Research Institute, Sydney, Australia). This DNA was then treated with RNaseA, followed by phenol/chloroform extraction, ether extraction, and ethanol precipitation. For digestion of DNA with DNaseI, 20 to 50 μg of DNA was incubated with 10 U of RNase-free DNaseI (Boehringer Mannheim, Mannheim, Germany) in 20 mM Tris-HCl, pH 7.6, and 20 mM $MgCl_2$ for 6 to 8 h. Complete digestion was confirmed by agarose gel electrophoresis. For methylation of plasmid DNA, 30 μg of pBluescriptSK was incubated with 15 U CpG methylase (New England Biolabs, Beverly, MA) and 160 μM *S*-adenosylmethionine in 10 mM Tris-HCl, pH 7.9, 10 mM $MgCl_2$, 50 mM NaCl, and 1 mM DTT. Methylation was complete, as assessed by inability of the methylation-sensitive enzyme *Hpa*II to digest the methylated plasmid DNA. Single stranded oligonucleotides used were AAC-22 (5′-ACCGATAACGTTGCCGGTGACG-3′) and ACC-22 (5′-ACCGATACCGGTGCCGGTGACG-3′) (10).

Activation of A4 cells

A4 cells, which are RAW 264 cells stably transfected to express the luciferase gene under the control of the HIV-1 long terminal repeat (LTR), have been described previously (16). Cells were plated out 16 h before each experiment (10^6 cells in 5 ml medium). Medium was reduced to 1 ml and then LPS or nucleic acid was added to the medium for 2 h. Cells were harvested for luciferase analysis per Promega protocols.

Electromobility shift analysis (EMSA) of NF-κB activity

BMM were plated out overnight in medium with 10^4 U/ml CSF-1, and then DNA or LPS was added for 1 h before harvest of cells. Nuclear extracts were prepared as described previously (17), except that 0.5% Nonidet P-40 was used in the cell lysis. Two micrograms of nuclear protein extract was incubated with 0.02 pmol of end-labeled double stranded oligonucleotide probe in a total volume of 10 μl, containing 20 mM HEPES (pH 7.9), 0.5 mM DTT, 12% glycerol, 40 mM KCl, 0.5 μg poly d(I-C), and 1 mM EDTA. Samples were incubated at room temperature for 30 min, and then run on 5% acrylamide gel (acrylamide:bis ratio, 29:1) at 100 V in 1× Tris-borate-EDTA buffer. Gels were dried and exposed to x-ray film. The double stranded oligonucleotide probe used is a NF-κB binding site from the TNF-α promoter (κB site 3 (18) - 5′-CAA ACA GGG GGC TTT CCC TCC TC-3′).

Results

Treatment of both BMM cells and the mouse macrophage cell line RAW 264 with pBluescript plasmid DNA (1 or 2 μg/ml) for 1 h led to induction of TNF-α mRNA (Fig. 1, *A* and *B*). Since TNF-α is well characterized as an LPS-inducible gene, it was necessary to

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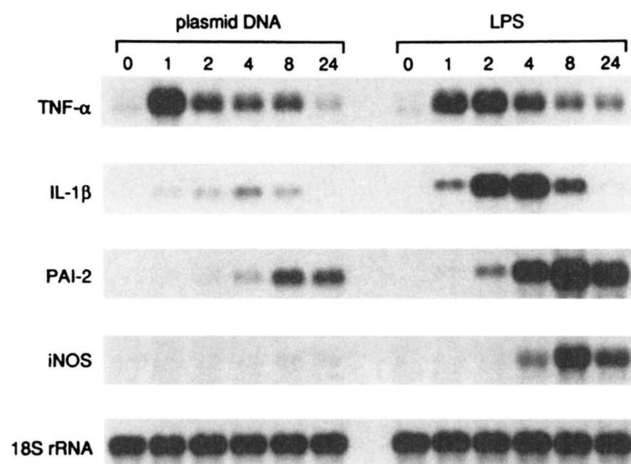

FIGURE 2. Northern blot analysis of the timecourse of induction of various genes by LPS and plasmid DNA. RAW 264 cells were incubated from 1 to 24 h with either 100 ng/ml *Salmonella minnesota* LPS or 2 μg/ml pBluescript. Expression of TNF-α, IL-1β, PAI-2, and iNOS mRNA was assessed by hybridization with labeled cDNA probes. Hybridization to 18S rRNA is shown as a loading control.

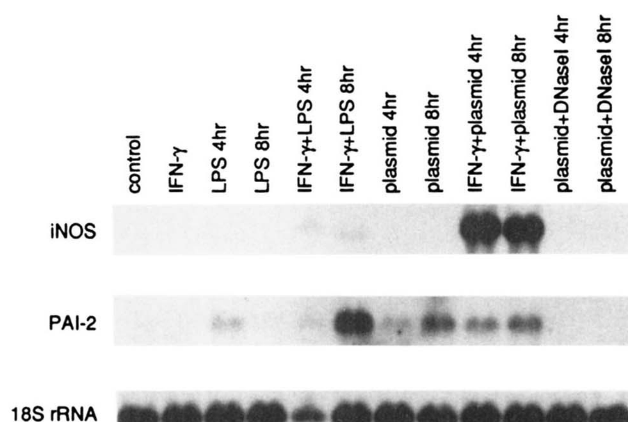

FIGURE 3. Northern blot analysis of the effect of IFN-γ pretreatment on induction of PAI-2 and iNOS mRNA. RAW 264 cells were plated for 14 h with or without 20 U/ml murine IFN-γ. Control and IFN-γ samples were harvested at this time, and other samples were treated for a further 4 or 8 h with either 0.1 ng/ml *Salmonella minnesota* LPS, 5 μg/ml pBluescript, or DNaseI-treated plasmid. The concentration of LPS used was suboptimal, to best see the priming effect of IFN-γ. Expression of PAI-2 and iNOS mRNA was assessed by hybridization with labeled cDNA probes. Hybridization to 18S rRNA is shown as a loading control.

determine whether this induction was due to contaminating LPS. Digestion of the plasmid DNA with DNaseI prevented any response (Fig. 1, *A* and *B*), showing that the DNA itself was responsible for the induction of TNF-α. If plasmid DNA was spiked with LPS before DNaseI treatment, the response to LPS was unchanged (Fig. 1*A*), showing that the DNaseI treatment in no way diminished the response to LPS. In addition, plasmid prepared by a method designed to remove endotoxin (EndoFree; Qiagen, Hilden, Germany) gave the same level of TNF-α induction (Fig. 1*B*). Induction of TNF-α by plasmid DNA led to the question of what other forms of DNA could produce a similar response. Figure 1*A* shows that the synthetic dsDNA poly d(I-C) also induced TNF-α, and this effect was prevented by DNaseI digestion. In other experiments (data not shown), the level of induction by poly d(I-C) was similar to that by plasmid. Single stranded poly dC did not induce TNF-α message (Fig. 1*A*). Some background hybridization in this lane was due to probe hybridization to poly dC taken up by or bound by the cells at the time of harvest. (The TNF-α cDNA used as probe contains poly dG sequences introduced during cloning.) Although not necessarily acting via the same pathway as DNA, synthetic RNAs also induced TNF-α mRNA. Synthetic dsRNA (poly I·poly C) was the most active, followed by poly I and poly C (Fig. 1*A*). However, salmon sperm DNA (Fig. 1*A*) and mouse genomic DNA (Fig. 1*B*) were without effect on TNF-α mRNA levels. This lack of effect is likely to be due to the methylation of vertebrate DNA, as TNF-α was no longer induced when plasmid was methylated on CpG motifs (Fig. 1*B*). A double stranded oligonucleotide which contained no CpG sequence was also nonstimulatory (Fig. 1*A*).

Since DNA is able to mimic the effect of LPS in induction of TNF-α, its effect on the induction of a number of other LPS-inducible genes was investigated. Figure 2 shows timecourses of treatment of RAW 264 cells with either LPS or plasmid DNA. TNF-α was strongly induced by a 1-h treatment with DNA, and induction was a little less sustained than in response to LPS. IL-1β, an important inflammatory mediator in response to LPS (19), was induced with similar kinetics by LPS and DNA, but the DNA response was lower. Plasminogen activator inhibitor type 2 (PAI-2) is another LPS-responsive macrophage gene (20), and was induced by DNA. Treatment of plasmid with DNaseI prevented the induction of PAI-2 (Fig. 3), once again confirming that responses observed are not due to a contaminant of the plasmid. iNOS, a gene involved in the tumoricidal response of macrophages (21), was not responsive to DNA alone (Figs. 2 and 3), but was dramatically induced by DNA if the cells were pretreated with IFN-γ (Fig. 3). In contrast, IFN-γ pretreatment enhanced the induction of PAI-2 mRNA by LPS, but not by DNA (Fig. 3). Thus, despite similarities between LPS and DNA responses, the pathways of activation are not entirely convergent.

The HIV-1 LTR is another LPS-responsive promoter, and experiments were performed to assess whether it is responsive to DNA. In earlier work we had stably integrated an HIV-1 LTR-luciferase construct into RAW 264 cells, and shown the LTR to be readily activated by LPS (16). Treatment of these cells with various types of DNA and RNA showed that the HIV-1 LTR was activated by plasmid, poly d(I-C) (Fig. 4*A*), and poly I·poly C (Fig. 4*B*). No response was seen to DNaseI-digested or methylated plasmid, salmon sperm DNA, or mouse genomic DNA (Fig. 4*A*). Thus the HIV-1 LTR responded in a similar manner to the TNF-α gene. Dose-response curves for activation of the HIV-1 LTR by plasmid DNA, poly d(I-C), and poly I·poly C showed a sensitive response to concentrations of 2 μg/ml and less, with only a moderate further increase in transcription when concentration was increased from 2 to 20 μg/ml (Fig. 4*B*). Responses to plasmid concentrations as low as 0.1 μg/ml have been detected (result not shown). DNaseI-digested plasmid (Fig. 4*B*) and salmon sperm DNA (not shown) did not activate the HIV-1 LTR at concentrations of up to 20 μg/ml. Hence induction by higher concentrations of plasmid was not boosted by LPS contaminating the plasmid preparation.

Two palindrome-containing oligonucleotides that have been characterized as either activating (AAC-22) or not activating (ACC-22) NK cell activity when added as naked DNA to spleen cells (10), were also tested for ability to activate the HIV-1 LTR. AAC-22, containing the palindrome AACGTT, activated the HIV-1 LTR, whereas ACC-22, containing the palindrome ACCGGT, did not (Fig. 4*B*). No response was seen to oligonucleotide below a concentration of 2 to 5 μg/ml, so it is a less potent activator of the HIV-1 promoter than plasmid.

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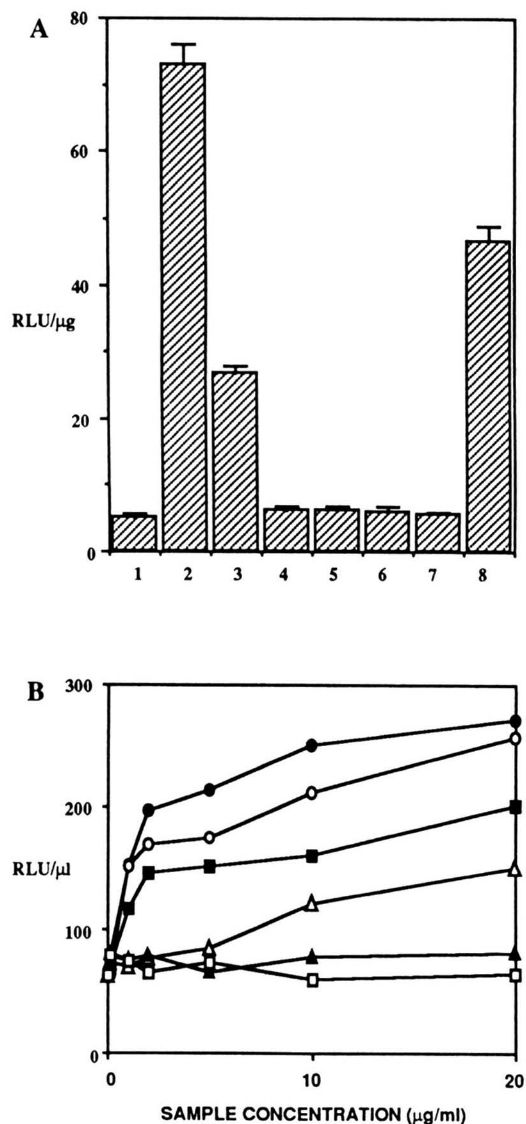

FIGURE 4. Activation of the integrated HIV-1 LTR in A4 cells by nucleic acids. *A*, A4 cells in 1 ml medium were incubated for 2 h with 1) no additions; 2) 100 ng *Salmonella minnesota* LPS; 3) 2 μg pBluescript; 4) 2 μg pBluescript digested with DNaseI; 5) 2 μg pBluescript treated with CpG methylase; 6) 2 μg salmon sperm DNA; 7) 2 μg murine genomic DNA; and 8) 2 μg poly d(I-C). Cells were then lysed and assayed for luciferase. Results are expressed as relative light units (RLU) per μg cellular protein. Error bars show the SEM (n = 5). *B*, A4 cells were incubated for 2 h with various concentrations of nucleic acids. Treatments were: poly d(I-C) (●); poly I·poly C (○); pBluescript (■); pBluescript digested with DNaseI (□); oligonucleotide AAC-22 (△); and oligonucleotide ACC-22 (▲). Cells were harvested in 100 μl lysis buffer, and results are presented as RLU/μl.

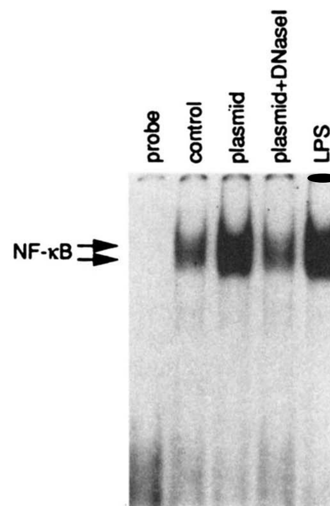

FIGURE 5. Induction of NF-κB binding activity by plasmid DNA. BMM in 10 ml medium were incubated for 1 h with the following treatments: control, no additions; plasmid, 10 μg pBluescript; plasmid + DNaseI, 10 μg pBluescript digested with DNaseI; and LPS, 100 ng/ml *Salmonella minnesota* LPS. EMSA of nuclear NF-κB was performed using an NF-κB binding site from the TNF-α promoter.

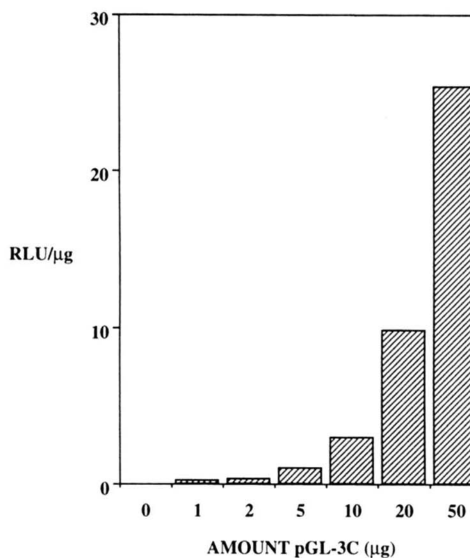

FIGURE 6. Expression of luciferase from plasmid taken up from the medium. RAW 264 cells (1.5 million) were incubated with various amounts of the luciferase reporter construct pGL3C (Promega) in 100 μl of medium for 1 h. Cells were then plated in 5 ml of medium for 23 h before harvest for analysis of luciferase activity.

The transcription factor NF-κB is activated by LPS treatment of macrophages and is implicated in induction of TNF-α, IL-1β, and HIV transcription (18, 22, 23). The possibility that NF-κB mediates some of the effects of DNA was investigated. Figure 5 shows an EMSA of BMM nuclear extracts using an NF-κB-binding oligonucleotide derived from the TNF-α promoter. In three separate experiments, treatment of cells with plasmid DNA was found to increase binding of NF-κB (Fig. 5 and unpublished observations). This increase in NF-κB was prevented by digestion of the plasmid with DNaseI (Fig. 5). CpG-methylated plasmid and salmon sperm DNA were unable to induce NF-κB binding (result not shown). Thus, the induction of NF-κB was well correlated with factors that induced TNF-α and activated the HIV-1 LTR.

An important issue in the activation of cells by exogenous DNA is whether the DNA binds a cell surface receptor and signals from outside the cell, or whether internalization is necessary. To examine whether DNA could be taken up by cells and escape from endosomes in an intact form, RAW 264 cells were incubated with various concentrations of the luciferase reporter construct pGL3C. Results in Figure 6 show that plasmid merely incubated with cells was indeed taken up and expressed in a dose-dependent manner. Cells were incubated for 1 h in a small volume with a high concentration of plasmid (10 to 500 μg/ml) and then diluted out to a

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final plasmid concentration of 0.2 to 10 μg/ml for a 23-h incubation. The initial high concentration incubation was necessary to get good levels of expression, as samples incubated for 24 h with 5 μg/ml plasmid gave detectable but very low expression (result not shown).

Discussion

Work on cellular responses to DNA in our laboratory was initiated by the observation that transient transfection with DNA had an LPS-like effect on macrophages. Subsequent work showed that this cellular activation was due to exposure to bacterial DNA, and did not require electroporation. Results presented here show that treatment of macrophages with plasmid DNA induced expression of the TNF-α, IL-1β, and PAI-2 genes; induced iNOS mRNA in IFN-γ-primed cells; and activated an integrated HIV-1 LTR. Some or all of these effects may be mediated by the activation of transcription factor NF-κB, which occurred in cells exposed to plasmid DNA. NF-κB sites are found in many genes involved in the inflammatory response and NF-κB is believed to regulate TNF-α (18), IL-1β (23), and HIV-1 (22) transcription. Induction of TNF-α, NF-κB, and the HIV-1 LTR was mediated by plasmid DNA, but not salmon sperm or murine genomic DNA. This result is complementary to studies showing activation of B cells (2) and NK activity (5) by bacterial DNA, but not vertebrate genomic DNA. Thus it seems that DNA can be added to the list of bacterial products with immune-stimulating activity.

Genes or proteins reported as being induced in response to bacterial DNA are IL-6 by B cells and $CD4^+$ T cells (24, 25), IL-12 by B cells and an adherent population of spleen cells (25, 26), IFN-α/-β by spleen cell cultures (5), and TNF-α, IL-1β, PAI-2, and iNOS by macrophages (this report). Induction of TNF-α by DNA in splenocytes has previously been inferred by the finding that neutralizing anti-TNF-α Abs inhibited the induction of IFN-γ in response to DNA, although TNF-α could not be detected by ELISA (26). Our work confirms that TNF-α production in splenocyte cultures is probably due to macrophage activation. Using ELISA we could readily detect TNF-α in macrophage culture medium after addition of DNA, and in BMM, expression was enhanced by IFN-γ pretreatment (result not shown). Induction of IFN-γ by DNA has been found in NK cells and $CD4^+$ T cells (25), but this may be a secondary response to induced IL-12 and TNF-α rather than a direct effect of the DNA (25, 26). It is clear that DNA acts directly on several cell types to induce cytokines, and its action on a mixed population of immune cells is likely to result in induction of a wide range of cytokines. Here we found that treatment of macrophages with IFN-γ primed them for production of iNOS mRNA in response to DNA. Similarly, Yi et al. (24) found that IFN-γ increased B cell activation by DNA. Thus, bacterial DNA may induce cytokines such as IL-12 and TNF-α in vivo, leading to increased production of IFN-γ, which then enhances macrophage and B cell activation by DNA.

Obvious questions arising from this work are what features of bacterial DNA are recognized as foreign, and how is the recognition mediated. Krieg et al. (4) found that bacterial DNA induced proliferation and IgM production by B cells, and that this was dependent on unmethylated CpG motifs in the bacterial DNA. We have confirmed this in macrophages by showing that CpG methylation of plasmid DNA prevented it from inducing NF-κB, TNF-α, and the HIV-1 LTR. Extensive work has shown that some 6-bp palindromic sequences containing a CpG motif could induce IFN and NK cell activity in spleen cells (8, 27), and we have found that a CpG-containing palindromic oligonucleotide also activated macrophages (Fig. 4*B*). Oligonucleotide sequences mediating activation of B cells were not all palindromic, but had a preference for two 5′ purines and two 3′ pyrimidines surrounding the CpG motif (4, 25). In mammalian systems, CpG methylation and a low frequency of activating sequences may combine to prevent general immune activation by self-DNA. "CpG suppression" in vertebrates means that CpG sequences occur at approximately 25% of the level predicted from genome base composition (3). In addition to CpG suppression, Krieg et al. (4) found that B cell stimulatory hexamer sequences are found in human coding DNA sequences at less than one-third the frequency of CpG-containing hexamers with little stimulatory activity. In about 98% of the mammalian genome, approximately 70% of the CpG motifs are methylated (3). The other 2% of genomic DNA consists of unmethylated "CpG islands" associated with the 5′ ends of many genes (3). This level of CpG suppression and methylation is apparently sufficient to prevent unwanted activation of immune function. Whether there is any preferential methylation of stimulatory sequences, or a further lowered frequency of stimulatory sequences in CpG islands, has not been determined.

A number of synthetic DNA molecules such as a random copolymer, poly (dG, dC) (28), $(dG)_{18}$ (29), and in our study poly d(I-C) but not poly dC, have been shown to activate immune cells. These polynucleotides clearly do not all contain the activating sequences identified by Krieg et al. (4) and Kuramoto et al. (27), or even CpG dinucleotides. Whether they are being detected by the same system that mediates responses to bacterial DNA and active oligonucleotides is not known. It is possible that cellular activation is mediated through recognition of a DNA conformation rather than specific sequence or methylation status.

Despite the high molar concentration of stimulatory sequences when oligonucleotides are used to activate cells, we and others (26) found that bacterial DNA was a more effective activating agent than oligonucleotide. In our work with macrophages, and in spleen cell cultures (10), response to naked oligonucleotide was not detected at less than 2 μg/ml, whereas we have found responses to plasmid DNA as low as 0.1 μg/ml. We considered that this difference may be due to more rapid degradation of oligonucleotides than plasmid DNA. However, phosphorothioate-linked oligonucleotides, which are not readily degraded, were no more effective at activating macrophages, nor did using double stranded oligonucleotides have much effect (result not shown). It may be either that the combination of different stimulatory sequences found in plasmid DNA is more effective, or that longer molecules are more active. One group has found cytokine production by spleen cells in response to a 20-bp phosphorothioate-linked oligonucleotide at concentrations as low as 0.03 μg/ml (25). They also found responses to oligonucleotides of only 8 bp (4, 25), which were nonstimulatory to macrophages at even high concentrations (result not shown). Thus there may be differences in DNA detection systems between cell types, or the various responses measured may have different thresholds for activation.

Synthetic dsRNA has a well-established role in induction of IFNs and an antiviral state (30), activities that are not generally attributed to DNA (31). In this study, synthetic dsRNA and DNA had similar effects in inducing TNF-α mRNA and HIV-1 LTR activity. A candidate for mediating these responses to dsRNA is the RNA-dependent protein kinase (PKR), which is known to activate NF-κB by phosphorylating IκB (32), and may participate in tumoricidal activation of macrophages in response to LPS (33). Whether the pathways of activation by RNA and DNA converge remains to be determined. RNA certainly had additional effects on macrophages, as treatment with high concentrations of poly I·poly C was toxic, while DNA had no such effect (result not shown).

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Macrophage scavenger receptors, which bind a number of polyanions, are a candidate for mediating some of the effects of nucleic acids. Poly I, and to an extent poly I·poly C, are ligands for the scavenger receptor (34, 35), and they induced TNF-α more effectively than did poly C, which does not bind to scavenger receptor. Although some of the effects seen here may be mediated through binding to scavenger receptor, it is unlikely to account for the action of all the nucleic acids tested, as phage and plasmid DNA were not found to be good ligands (35).

Most evidence suggests that determination of whether DNA is of bacterial or self-origin occurs within the cell (see introduction). A number of groups have provided evidence for specific binding of DNA to cell surface receptors (reviewed in Ref. 36). It appears that DNA uptake is receptor mediated and DNA is largely broken down within an endosomal compartment (11). While it is possible that recognition of bacterial DNA occurs within endosomes, we have shown in this paper that intact plasmid can reach the nucleus. So a protein detecting foreign DNA could also be located in the nucleus or cytoplasm. This would enable detection of viral DNA. Infection with HIV has been shown to activate NF-κB (22), and although several systems are likely to play a role in this, it could be mediated in part by detection of HIV DNA. We have previously observed cell death of primary macrophages in response to transfected DNA, and proposed that this may be a defense against viral infection (13). However, that effect and the immune activation by DNA observed here seem to be differently mediated, as transfected salmon sperm DNA was also toxic to the cells.

Cellular uptake of DNA is of interest to the fields of gene therapy and DNA immunization. For some Ags, injection of DNA encoding the Ag can lead to a protective immune response (37). It was initially shown that muscle cells at the site of injection could express β-galactosidase from injected plasmid (38), but which cell types are critical in the expression and presentation of Ag leading to an immune response has not been determined. Macrophages are a possible site of uptake and expression of the foreign DNA, and results here show that a macrophage cell line can internalize and express exogenous DNA. However, mechanisms leading to presentation of Ag in association with MHC class II molecules, which is presumably necessary for the immune response seen in DNA immunization, remain unknown. Another aspect of this work that is relevant to DNA immunization is the general activation of immune function in response to foreign DNA. The adjuvant-like activity of the DNA itself may be required for the immune response in DNA immunization.

Cellular activation by DNA has many experimental and therapeutic implications. Obvious caution should be used in interpretation of transfection experiments using cells that respond to bacterial DNA. Nonspecific immune activation is also documented in experiments involving antisense oligonucleotides (4). While immune activation may be desirable in DNA immunization, it may be a complicating factor in gene therapy and antisense therapy. Immune stimulation by DNA containing unmethylated CpG may explain a number of biologic observations. CpG suppression is thought to be caused by the deamination of 5-methylcytosine and a failure in DNA repair, leaving TpG dinucleotides (7). The fact that mammals have not developed a more efficient mechanism to prevent this potentially harmful mutagenesis suggests that CpG suppression may be desirable. Evolutionary pressure for lowering of CpG levels may have been driven by the development of a means to distinguish between mammalian and foreign DNA (4). Pathogenic organisms may then also be subject to a selective pressure to lower CpG and evade immune responses, and indeed, CpG suppression is observed in a large number of viruses (7).

In the normal in vivo situation, immune activation by DNA could occur either by extracellular DNA from dead organisms, by bacterial DNA released by cell killing in phagolysosomes, or by invading viral DNA. A recognition system for the DNA, and a mechanism for subsequent activation of transcription factors such as NF-κB, remain to be established.

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