CpG-oligodeoxynucleotides activate HIV replication
in latently infected human T cells

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RUNNING TITLE: CpGs reactivate latent HIV
SUMMARY

CpG oligodeoxynucleotides (CpG ODNs) stimulate immune cells via the toll like receptor 9 (TLR9). In this study we have investigated the effects of CpG ODNs on latent HIV infection in human T cells. Treatment of the latently infected T cell line ACH-2 with CpG ODNs 2006 or 2040 stimulated HIV replication, whereas no effects were evident when ODNs without the CpG motif were used. CpG-induced virus reactivation was blocked by chloroquine, indicating involvement of TLR9. In contrast to the responsiveness of ACH-2 cells, CpG ODNs failed to activate HIV provirus in the latently-infected Jurkat clone J1.1. We also studied the effects of CpG ODNs on productive HIV infection and found enhancement of viral replication in A3.01 T cells, whereas again no stimulating effects were observed Jurkat T cells. CpG ODN treatment activated NF-kB in ACH-2 cells, which was similarly triggered in uninfected A3.01 T cells following exposure to CpG ODNs, indicating that TLR9-induced signal transduction was not dependent on proviral infection. Our study demonstrates that CpG ODNs directly trigger the activation of NF-kB and reactivation of latent HIV in human T cells. Our results point to a novel role for CpG ODNs as stimulators of HIV replication and open new avenues to eradicate the latent viral reservoirs in HIV-infected patients treated with antiretroviral therapy.
INTRODUCTION

The immunostimulatory activity of bacterial DNA was first reported in 1984 (1). This effect is mediated by unmethylated CpG motifs within the DNA and can also be observed with synthetic oligodeoxynucleotides (ODNs) containing these CpG sites (2). The ability of CpG sequences to stimulate the immune system is mainly due to their effects on antigen-presenting cells. (reviewed in (3,4)). CpG ODNs have also been shown to activate T cells via direct (5,6) and indirect mechanisms (7,8).

CpG ODNs act through binding to Toll like receptor 9 (TLR9) (9), one of 10 members of the Toll-like receptor (TLR) family identified in humans (10). TLRs belong to the superfamily of pattern-recognition receptors (PRRs), which are involved in generating innate immune responses. TLR9 is expressed primarily in B cells and plasmacytoid dendritic cells (pDCs) (9,11-13) but a weak TLR9 expression has also been observed in T cells (12).

The immunostimulatory activity of CpG ODNs can be used therapeutically to enhance specific immune responses both in vaccinations and acute infections and has led to promising results for the treatment of various pathogens, including retroviruses (14-19). Stimulating the immune system during HIV infection may not only be useful in enhancing antiviral immune responses but may also resolve the problem of viral latency in antiviral treatment. Highly active antiretroviral therapy (HAART) has had impressive results in the past few years, effectively suppressing virus replication in many patients and reducing virus titers to below clinical detection limits (20-22). However, as soon as antiviral therapy is interrupted the virus load increases again requiring HIV patients to receive life-long maintenance therapy (23-25). Latent reservoirs, such as infected memory T cells, contribute to this viral
reemergence (26) and it is estimated that up to 60 years would be required to eliminate these reservoirs by use of HAART alone (27,28).

It has been proposed that treatment with a combination of cytokines could stimulate the latent reservoirs and thus render them vulnerable to antiviral therapy (29). Clinical studies have been conducted using IL-2 or OKT3 in order to restimulate HIV from its latent reservoirs (30,31).

Here we show that CpG oligodeoxynucleotides directly stimulate HIV replication in latently infected human T cells. The results of our study help to clarify the role of TLR9 in HIV replication and provide a new potential approach to eradicate latent HIV in infected patients treated with antiretroviral therapy.

EXPERIMENTAL PROCEDURES

Cells, viruses, cell culture and reagents.

The latently HIV infected T cell lines ACH-2 (32) and J1.1 (33) and the uninfected T cell lines A3.01 (34) and Jurkat E6-1 (35), were cultured at 37° in 5% CO2 atmosphere in RPMI 1640 (Invitrogen, Karlsruhe, Germany), containing 10% FCS, penicillin, and streptomycin. A3.01 and Jurkat cells were infected with HIV-1 strain IIIB/LAI at an m.o.i. of 1.0 TCID50 per cell. Chloroquine (Calbiochem, Bad Soden, Germany) was dissolved in RPMI, TPA (Phorbol-12-Myristate-13-Acetate (Calbiochem) and ionomycin (Calbiochem)) were dissolved in DMSO. Final DMSO concentration in the experiments was below 0.05%.
**PTO-ODNs**

Phosphothioate-modified (PTO)-oligodeoxynucleotides (MWG-Biotech AG, Ebersberg, Germany) were of HPSF purified quality and dissolved in H$_2$O at a concentration of 1 mg/ml. The following sequences were used (CpG-motifs are underlined):

CpG ODN 2006: TCGTCGTGTTTGTGTTTTGTGTT;
Non-CpG ODN 2006 4xTG: TTGTTGTGTTTTGTTTTTTT;
CpG ODN 2040: CTGTCGTTTGTGTTTTGTCTGG;
non-CpG ODN 2041: CTGCTTTTTGTTTTTTCTGG.

**Flow cytometry**

To determine expression of HIV p24 and TLR9, cells were fixed with 4% formalin in PBS and permeabilised with PBS containing 5% BSA and 0.5% saponin. Expression of HIV-p24 was detected with the mouse anti-HIVp24 mAb 183-H12-5C (AIDS Research and Reference Reagent Program). Expression of TLR9 was detected with the mouse anti-TLR9 mAb 26C593 (BioCarta, Hamburg, Germany). As a second antibody we used a PE-labeled anti-mouse IgG antibody (DAKO, Hamburg, Germany). Cells were analyzed by flow cytometry using a FACScan flow cytometer (Becton Dickinson, Heidelberg, Germany). Markers were set according to staining with an isotype-matched control antibody (DAKO, Hamburg, Germany).

**NF-kB EMSA**

Following stimulation, cells were washed in ice-cold PBS and whole cell extracts prepared by lysing in a high salt, NP-40 buffer on ice for 15 minutes (20mM HEPES pH 7.8, 20% glycerol, 350mM KCl, 1mM MgCl$_2$, 0.5mM EDTA, 0.1mM EGTA, 1% NP-40, 1mM DTT, 1x Complete protease inhibitor (Roche, Basel, Switzerland)).
Extracts were cleared by centrifugation at 15,000g and stored at –80°C. Protein concentration was determined by the Bio-Rad Microassay. 10μg of total protein was incubated with 0.5ng radiolabelled, double-stranded NF-κB consensus oligonucleotide sc-2505 (Santa Cruz Biotechnology, Heidelberg, Germany). The probe was end-labelled with [α-32P] ATP (Amersham Biosciences Europe GmbH, Freiburg, Germany) using T4 polynucleotide kinase (MBI Fermentas GmbH, St. Leon-Rot, Germany) and purified on a G-50 Sephadex quick spin column (Roche). The binding reaction was carried out at room temperature for 20 minutes in a 20μl volume (15mM Tris pH 7.5, 1mM EDTA, 1mM DTT, 5% glycerol, 5μg BSA, 2μg poly dIdC, Complete protease inhibitor). DNA-binding complexes were resolved on a 5% polyacrylamide (29:1) gel containing 0.4X TBE, dried and visualized by autoradiography using Kodak BioMax film. For specificity analysis, 100-fold excess of the unlabelled oligonucleotide or the mutant NF-κB oligonucleotide sc-2511 (Santa Cruz) was added to the binding reaction 10 minutes before the addition of the radiolabelled probe. To confirm the composition of the DNA-binding complexes, antibodies against Rel A (sc-109X, Santa Cruz) and p50 (sc-1190X, Santa Cruz) were preincubated with the extracts for 30 minutes on ice before addition of the radiolabelled probe. For densitometric quantification of NF-κB activation, the intensity of NF-κB bands compared to background was determined with the software Quantity One (BioRad, Munich, Germany).

RESULTS

In this study we have analyzed the effects of CpG oligodeoxynucleotides (ODNs) on latent HIV infection in human T cells. We have exposed latently infected ACH-2 cells...
to different CpG and non-CpG phosphothioate ODNs and measured HIV-p24 production by intracellular staining and flow cytometry. Under normal conditions, ACH-2 cell cultures produce only low amounts of virus with the majority of cells remaining in a latently infected state. Following an appropriate stimulus, transcription of the provirus begins and cells start to produce HIV (32,36).

Treatment of ACH-2 cells for 24 hours with CpG ODN 2006 resulted in a significant increase of cells producing HIV (Fig. 1A). Similar results were obtained with CpG ODN 2040. Non-CpG sequences, such as ODN 2041 or a mutated analog of ODN 2006, in which all cytidine residues are exchanged for thymidine (ODN 2006 4xTG), did not stimulate HIV replication, indicating that the observed effects were sequence specific for CpG sites and not related to phosphothioate modification of the ODNs. CpG-triggered reactivation of HIV was dose dependent as shown for CpG ODN 2006 (Fig. 1B). The kinetics of CpG ODN 2006-mediated HIV expression is depicted in figure 1C. Since CpG ODNs have been reported to act via TLR9 (9), we confirmed expression of TLR9 in ACH-2 cells by RT-PCR (data not shown) and by flow cytometry (Fig. 2).

In contrast to ACH-2 cells, the latently-infected Jurkat clone J1.1 did not show enhancement of virus replication following CpG-treatment (Fig. 3). As a positive control we have used TPA/ionomycin to show that the provirus in J1.1 cells can be activated. Our findings are consistent with previous studies in which it was reported that Jurkat T cells are insensitive towards CpG-signaling, such as activation of NF-κB (11). Since J1.1 cells were positive for TLR9 mRNA (data not shown) and express TLR9 protein (Fig. 2B) we suggest that a signaling defect is either located downstream of the receptor or that additional factors (e.g. costimulatory events) are necessary to observe TLR9 signaling in these cells.
Binding of CpG ODNs to TLR9 occurs after cellular uptake of the DNA and requires endosomal acidification (37). Chloroquine, an inhibitor of endosomal acidification, blocks TLR9 signaling and is therefore being used to study TLR9 activity (37-40). To further confirm involvement of TLR9 in CpG-induced reactivation of HIV, we analyzed the effects of chloroquine on CpG-triggered virus reactivation in ACH-2 cells. As depicted in figure 4, chloroquine completely inhibited CpG-mediated virus reactivation at concentrations to inhibit endosomal acidification. Even at maximum chloroquine concentrations no cell death was observed (according to the FSC/SSC criteria in flow cytometry, data not shown), indicating that inhibition of virus reactivation was not related to any cytotoxic effects.

To analyze whether CpG sequences stimulate HIV replication in cells that are productively infected with HIV, we infected A3.01 T cells with HIV and measured HIV p24-expression by flow cytometry. The CpG ODN 2006 enhanced viral replication compared to untreated cells, whereas no effects could be observed with the non-CpG sequence 2006-4xTG (Fig. 5). Similar to the situation with J1.1 cells, productively-infected Jurkat T cells were insensitive towards CpG-induced enhancement of virus replication (Fig. 5B).

NF-κB is an important transcription factor for replication of HIV (41). Moreover, TLR9 signaling is known to activate NF-κB in antigen presenting cells (42,43). To address the role of NF-κB in TLR9-induced reactivation of HIV, we analyzed cellular extracts of CpG-treated ACH-2 cells by EMSA. Treatment with CpG ODN 2006 activated the p65/p50 NF-κB heterodimer. Increased DNA binding was detectable at 4 hours, reaching a maximum at 8 hours and declining thereafter (Fig. 6B,C). In parallel to the NF-κB studies, we determined HIV expression by flow cytometry. As shown in figure 6A, NF-κB induction preceded HIV expression, suggesting that CpG-induced HIV replication was initiated by NF-κB. It had previously been shown that CpG ODNs do
not activate NF-κB in Jurkat cells, which correlates nicely with the inability of CpG ODNs to reactivate HIV in these cells (11). In contrast, CpG ODNs activated NF-κB in the uninfected A3.01 T cell line, indicating that CpG-triggered signal transduction in these T cells is not dependent on proviral infection but can also be observed in uninfected cells (Fig. 7). A further characterization of the NF-κB complexes showed that both the p50 homodimer and the p50/p65 heterodimer were formed in A3.01 T cells following CpG-treatment (Fig. 7A). The same NF-κB complexes have also been found in CpG-stimulated B cells (44), indicating that the proximal signaling pathways triggered by CpG ODNs in T cells and “classical” CpG-target cells are similar.

**DISCUSSION**

Here we show that CpG ODNs directly activate HIV replication in latently infected human T cells in vitro. The potential effects of CpG ODNs on HIV replication are currently a matter of debate. Equils and colleagues recently observed a stimulatory effect of CpG ODNs on HIV replication in HIV-transgenic mouse spleen cells in vitro (45). It remained unknown, however, whether the increase in virus replication was caused by a direct stimulation of spleen cells by CpG sequences or mediated by indirect effects. Our findings now demonstrate that CpG ODNs can directly trigger signaling events in human T cells that lead to activation of NF-κB and reactivation of latent HIV in vitro. There is evidence that these results have some relevance for HIV reactivation in vivo. It has been reported that purified human T cells (mixed CD4+ and CD8+ T cells) express TLR9 (12). Despite their TLR9 expression effector and memory CD8+ T cells did not respond to CpG ODN treatment (12). However, CD4+ T cells, which are the main target cells for HIV, have not been investigated in this
study. Our current findings together with some anecdotal evidence from HIV patients suggest that CpG ODN might be able to directly stimulate at least certain subpopulations of CD4+ T cells in vivo. For example it was observed that viral loads increase in HIV-infected patients with opportunistic bacterial infections (46,47). Further evidence that the observed effects could be operative in vivo came from Agrawal et al., who recently suggested that the unexpected increase in viral loads observed in HIV-infected patients treated with HIV-gag antisense ODNs could have been due to CpG motifs within the administered sequences (48).

We have recently shown that CpG ODNs are protective in post-exposure treatment of retrovirus-induced disease in the Friend virus mouse model (19). Interestingly, the timing of treatment was a critical factor in treatment efficiency: Only post-exposure treatment was protective, whereas pre-infection treatment resulted in an accelerated development of virus-induced erythroleukemia (49). These observations indicate that CpG-treatment, depending on the time point of inoculation, is able to enhance retroviral replication in vivo and further studies will have to reveal whether mechanisms similar to those described in this manuscript are responsible for the observed effects.

The biological effects of CpG ODNs on the human immune system are currently a matter of great scientific interest and our study contributes to the understanding of the biological activities of these substances. Our data points to a novel role for CpG ODNs as stimulators of HIV replication. This effect might be used in the future to eradicate the latent viral reservoirs in HIV-infected patients treated with antiretroviral therapy. To this end, the ability of CpG ODNs to reactivate the HIV provirus in vivo needs to be urgently explored.
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REFERENCES


**FOOTNOTES**

1 The abbreviations used are: ODN, oligodeoxynucleotide; HIV, human immunodeficiency virus; HAART, highly active antiretroviral therapy, NF-κB, nuclear factor κB; mAb, monoclonal antibody; PTO, phosphothioate.
FIGURE LEGENDS

FIG. 1. CpG ODNs reactivate HIV in ACH-2 cells. A, ACH-2 cells were incubated with the CpG ODNs 2006 and 2040 or with the non-CpG ODNs 2041 and 2006 4xTG at a concentration of 1 µg/ml or with medium alone for 20 hours. B, ACH-2 cells were incubated with the CpG ODN 2006 or the non-CpG ODN 2006 4xTG at the indicated concentrations for 24 hours. C, ACH-2 cells were incubated with the CpG ODN 2006 or the non-CpG ODN 2006 4xTG at a concentration of 1 µg/ml for the indicated times. Expression of HIV-p24 was detected by intracellular staining and flow cytometry. A-C: Values represent means ± S.D. from triplicate analyses.

FIG. 2. Expression of TLR9 on different T cell lines. A-D: Expression of TLR9 was detected by intracellular staining (open line) compared to an isotype matched control antibody (filled line). A: TLR9 expression in latently HIV-infected ACH-2 T cells. B: TLR9 expression in latently HIV-infected J1.1 T cells. C: TLR9 expression in uninfected A3.01 T cells. D: TLR9 expression in uninfected Jurkat E6-1 T cells.

FIG. 3. CpG-induced reactivation of HIV is not observed in latently-infected T cells that are insensitive to TLR9-signaling. ACH-2 cells (A) and J1.1 cells (B) were incubated with medium alone, the CpG ODN 2006 (1 µg/ml), the non-CpG ODN 2006 4xTG (1 µg/ml) or with TPA/lonomycin (10 ng/ml and 0.5 µM, respectively) for 24 hours. Expression of HIV-p24 was detected by intracellular staining and flow cytometry. A-B: Values represent means ± S.D. from triplicate analyses.
FIG. 4. **CpG-induced reactivation of HIV is blocked by chloroquine.** ACH-2 cells were incubated with the CpG ODN 2006 (1 µg/ml) or the non-CpG ODN 2006 4xTG (1 µg/ml) in the presence of the indicated chloroquine concentrations for 24 hours. Expression of HIV-p24 was detected by intracellular staining and flow cytometry. No cytotoxic effects were observed at any chloroquine concentrations. Values represent means ± S.D. from triplicate analyses.

FIG. 5. **CpG sequences enhance productive HIV infection.** A3.01 T cells (A) and Jurkat E6-1 T cells (B) were infected with HIV for 2 hours and then incubated with medium alone, the CpG ODN 2006 (1 µg/ml), or the non-CpG ODN 2006 4xTG (1 µg/ml) for 5 days. Expression of HIV-p24 was detected by intracellular staining and flow cytometry. A,B: Values represent means ± S.D. from triplicate analyses.

FIG. 6. **CpG ODNs activate NF-κB and HIV replication in ACH-2 cells.** A-C: ACH-2 cells were treated with the CpG ODN 2006 or the non-CpG ODN 2006 4xTG at a concentration of 1 µg/ml for the indicated times. A, Expression of HIV-p24 was detected by intracellular staining and flow cytometry. B, C: Cell extracts were analyzed by EMSA to detect NF-κB activation. B: Densitometric analysis of the gel. Relative NF-κB intensity of each band was calculated as the ratio of the intensity of this band divided by the mean intensity of all samples treated with ODN 2006 4xTG. C: Autoradiography of the EMSA quantified in B. The cells analyzed in A and B, C were from the same experiment.
FIG. 7. **CpG ODNs activate NF-κB in uninfected A3.01 T cells.** A, B: A3.01 cells were treated with CpG- and non-CpG ODNs for 24 hours and cell extracts were analyzed by EMSA to detect NF-κB activation. A: Lane 1: untreated cells; lane 2: CpG ODN 2006 treated cells; lane 3: non-CpG ODN 2006 4xTG treated cells; lanes 4-7: extracts from ODN 2006-treated cells; lane 4: 100 fold excess of cold NF-κB probe; lane 5: 100 fold excess of cold mutated NF-κB probe (sc-2511); lane 6: p65 supershift with mAb sc-109X; lane 7: p50 supershift with mAb sc-1190X. B: Densitometric analysis of NF-κB intensity of lanes 1-3. Relative NF-κB intensity of a given band was calculated as the ratio of the intensity of each band divided by the intensity of the medium-treated sample.
FIGURES

figure 1

A

HIV-p24 expressing cells (%)

ODN 2006

ODN 2006 4xTG

ODN 2040

ODN 2041

B

HIV-p24 expressing cells (%)

ODN 2006

ODN 2006-4xTG

ODN concentration (μg/ml)

C

HIV-p24 expressing cells (%)

ODN 2006

ODN 2006 4x-TG

Treatment (h)
**Figure 2**

A. ACH-2

B. J1.1

C. A3.01

D. Jurkat E6-1
figure 3

**A**

![Graph ACH-2 showing HIV-p24 expressing cells (%) for Medium, ODN 2006, ODN 2006 4xTG, and TPA/Ionomycin.]

**B**

![Graph J1.1 showing HIV-p24 expressing cells (%) for Medium, ODN 2006, ODN 2006 4xTG, and TPA/Ionomycin.]

CpGs reactivate latent HIV
**figure 6**

**A**

![HIV-p24 expressing cells (%) vs Treatment (h)]

**B**

![Relative NF-kB activation (%) vs Treatment (h)]

**C**

![Western blot for p65/p50 vs Treatment (h)]
figure 7

A

p65 supershift
p50 supershift
p65/p50
p50/p50

1 2 3 4 5 6 7

B

relative NF-κB activation (%)

Medium ODN 2006 ODN 2006 4xTG
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