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A Proviral Role for CpG in Cytomegalovirus Infection¹

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TLR9-dependent signaling in plasmacytoid dendritic cells is a key contributor to innate immune defense to mouse CMV infection. We aimed to study the expression and potential contribution of TLR9 signaling in human CMV (HCMV) infection of primary fibroblasts. HCMV infection strongly induced TLR9 expression in two of three fibroblast types tested. Furthermore, the TLR9 ligand CpG-B induced a strong proviral effect when added shortly after HCMV infection, enhancing virus production and cell viability. However, not all CpG classes displayed proviral activity, and this correlated with their IFN- β -inducing ability. The proviral effect of CpG-B correlated completely with concurrent viral up-regulation of TLR9 in fibroblasts. Importantly, the timing of CpG addition was a critical parameter; in striking contrast to the proviral effect, CpG addition at the time of infection blocked viral uptake and nearly abolished HCMV production. The contrasting and time-dependent effects of CpG on HCMV infectivity reveal a complex interplay between CpG, TLR9, and HCMV infection. Additionally, the data suggest a potentially harmful role for CpG in the promotion of HCMV infection. *The Journal of Immunology*, 2009, 182: 5672–5681.

Human CMV (HCMV)³ (the prototypic β -herpesvirus) causes little to no pathogenicity in immunocompetent individuals, and following acute infection establishes lifelong persistence with latency. However, in cases of immunosuppression (e.g., transplant recipients and AIDS patients) or congenital infection, uncontrolled HCMV replication can result in serious disease (1). HCMV uses many immunomodulatory strategies to influence both the cell-intrinsic and cell-extrinsic response to infection, such as down-regulation of host MHC class I expression, production of inhibitory molecules and decoy receptors, and inhibition of apoptosis. These effects likely contribute to establishment of lifelong HCMV infection (2, 3).

In fibroblasts, HCMV binds cell surface heparan sulfate (4) and cellular entry involves fusion between the viral envelope and the plasma membrane (5). Viral nucleocapsids are then transported to the nucleus for release of the viral genome (6). HCMV infection of primary fibroblasts rapidly activates cell-intrinsic innate response pathways, resulting in the translocation of factors like IFN regu-

latory factor 3 and NF- κ B to the nucleus (within 15–30 min), ultimately leading to transcription of host inflammatory and IFN-dependent gene products (7, 8). The canonical (I κ B α - and RelA-dependent) NF- κ B pathway is not required for HCMV replication in fibroblasts (9), but is important for HCMV-induced IFN- β production (10, 11). Signaling by lymphotoxin (LT)- β R, TNFR1, IL-1R, and by overexpression of the adapter kinase RICK, all induce NF- κ B-dependent production of IFN- β at early times after infection (likely overcoming an HCMV immediate-early 2 (IE2)-mediated block to IFN- β transcription (12)), which inhibits HCMV spread in human fibroblasts via autocrine/paracrine signaling (10, 13, 14). Importantly, primary human NK cells use a similar LT-dependent mechanism to restrict the spread of HCMV in fibroblasts (11).

The TLRs comprise a family of pathogen recognition receptors essential for innate immunity to infection. Host immune control of CMV infection in mice involves TLR9-dependent recognition of mouse CMV (MCMV) by plasmacytoid dendritic cells (DCs) and macrophages, resulting in high levels of type I IFN production at later times of infection (15–17). In contrast, the initial type I IFN response to MCMV infection in the spleen is independent of TLR signaling, and is derived from stromal cell cross-talk with B cells expressing LT- $\alpha\beta$ (18). Type I IFN contributes to NK cell activation and control of the acute MCMV infection. Mice with mutations in TLR9 are hypersusceptible to acute MCMV infection, and TLR3-deficient mice also show modest increases in MCMV replication, highlighting the potential involvement of viral dsDNA and dsRNA in host immune recognition of CMV (16, 19). Both bacterial and viral DNA contain unmethylated CpG DNA sequences (15, 20, 21), the specific ligand for TLR9 (22). In DCs, uptake of synthetic CpG oligodeoxynucleotides (ODNs) is mediated by clathrin-dependent endocytosis into early endosomes, where it interacts with TLR9 that is recruited from the endoplasmic reticulum (23). TLR9 then interacts with MyD88, leading to the activation of the transcription factors NF- κ B, AP-1, and IFN regulatory factor 7 and resulting in production of type I IFN and inflammatory cytokines (24). Depending on cell type, CpG can induce IFN- γ , IL-6, IL-8, IL-12, and TNF, increase proliferation and cell survival (25–27), and even sensitize cells to apoptosis

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³ Abbreviations used in this paper: HCMV, human CMV; DC, dendritic cell; IE2, immediate-early 2; LT, lymphotoxin; MCMV, mouse CMV; MOI, multiplicity of infection; NHDF, neonatal human dermal fibroblast; ODN, oligodeoxynucleotide; poly(I:C), polyinosine-polycytidylic acid; PTO, phosphorothioate; HEK, human embryonic kidney.

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(28). The cell type-specific activity of CpG is highlighted by three unique classes of CpG with variation in DNA sequence and secondary structure (29, 30). Class A CpG is the most potent activator of type I IFN production in plasmacytoid DCs (31), whereas CpG-B activates TLR9 in B cells (29–31). Class C CpG combines the structure and immunologic function of class A and class B CpG, and activates B cell responses, type I IFN secretion, and NK cell cytolytic activity (29, 30, 32).

Synthetic CpG ODNs are potent inducers of Th1 immune responses, and are currently in clinical testing as vaccine adjuvants, immunoprotective agents or anti-allergens for the treatment of various diseases, including cancers and viral infections (33). Ongoing studies indicate that CpG shows little toxicity in humans, both administered alone or in combination with tumor Ags, mAbs, or DCs (33).

Fibroblasts participate in immune defense through their secretion of inflammatory mediators like ILs and IFNs, and express many of the TLRs at the mRNA level (34, 35). However, whether fibroblasts express TLR9 is still somewhat controversial (34–39). In this study, we examined the expression and potential contribution of TLR9 signaling in HCMV infection of several primary fibroblast types. We found that HCMV infection induced potent up-regulation of TLR9 expression, highlighting a potential novel role for this TLR in human fibroblasts. Surprisingly, CpG had dichotomous effects on the outcome of HCMV infection depending on the time of its administration, promoting viral replication when added after entry. These results suggest that CpG treatment may play a harmful role in potentiating HCMV infection.

Materials and Methods

Cells and virus

Neonatal human dermal fibroblasts (NHDF; Clonetics) were cultured in DMEM supplemented with penicillin, streptomycin, L-glutamine, 5 μ g/ml insulin (Sigma-Aldrich), 1 ng/ml basic fibroblast growth factor (Sigma-Aldrich), and 10% FBS (Euroclone). Patient-matched primary fibroblasts derived from skin and oral mucosa were generated at Cardiff University (Cardiff, U.K.). Human embryonic kidney (HEK)293 cells stably expressing TLR9 and the luciferase reporter (HEK293-TLR9) was generously provided by S. Ishizaka (Eisai Research Institute, Andover, MA). All cells tested negative for mycoplasma infection by MycoAlert Assay (Cambrex Bioscience). HCMV strain AD169 (American Type Culture Collection) and strain Toledo, a gift from S. E. Starr (Children's Hospital of Philadelphia, Philadelphia, PA), were prepared and quantified by limiting dilution plaque formation assay on NHDF as described (9).

TLR ligands

CpG 2006 ODN with phosphorothioate (PTO) backbone (class B CpG (29)) used for all experiments shown were from TIB Molbiol, but CpG-B produced by MWG-Biotech AG was also tested with comparable results. Class A CpG 2216 (29), class C CpG M362 (30), and control GpC were from TIB Molbiol and synthetic dsRNA polyinosine-polycytidylic acid (poly(I:C)) from Amersham Biosciences. LPS from *Escherichia coli* (O111:B4; InvivoGen) was sonicated before use.

Virus infection of fibroblasts

As described (10, 11), NHDF, skin or oral mucosa fibroblasts were cultured to 80% confluency and infected with HCMV at low multiplicity of infection (MOI 0.001–0.5). Virus was allowed to adsorb for 2 h at 37°C before the cells were washed with medium and cultured in fresh medium for an additional 2 h. For postinfection exposure, CpG, poly(I:C), LPS, or medium only was added and left in the culture medium for a 1- to 7-day incubation period. In cytokine-neutralizing experiments, blocking polyclonal Ab against human IFN- β (Research Diagnostics) was added together with CpG, poly(I:C), LPS, or medium only. For addition at the time of infection, CpG, poly(I:C), or LPS were added to the cells during the 2 h of virus exposure and then removed when washing the cells. The cells were then incubated with culture medium for the remaining 1–7 days. HCMV-induced cytopathic effects on the infected fibroblasts were observed by light microscopy during the incubation time.

For quantification of HCMV PFU, culture supernatants were collected at day 7, and PFU was determined on NHDF in 96-well plates after 7 days by counting of infected foci observed by light microscopy.

RT-PCR

Low MOI HCMV-infected and uninfected fibroblasts were incubated for 24 h in culture medium. Total RNA was extracted by High Pure RNA Isolation kit (Roche) and cDNA transcribed using Superscript III Reverse Transcriptase (Invitrogen). PCR was performed with AmpliTaq Gold DNA polymerase (Applied Biosystems) and the specific primers for TLR9 5'-CTGCGGCATCTCAACCTCAA-3' and 5'-CCAGCCACGGAACCAACT-3' and β -actin (Clontech). PCR products were separated by 1.5% agarose gel electrophoresis, and visualized by ethidium bromide staining and UV detection in a GelDoc 1000 analyzer (Bio-Rad). PCR products were verified as TLR9-specific using BigDye Terminator version 1.1 Cycle Sequencing kit (Applied Biosystems).

For quantitative real-time RT-PCR, uninfected or HCMV-infected NHDF, HEK293 cells or HEK293-TLR9 cells were incubated with or without addition of ODNs 2 h postinfection, and harvested after 1–5 days in culture. RNA was extracted and cDNA synthesized as described, and quantitative real-time PCR was performed by the StepOnePlus real-time PCR System (Applied Biosystems). TLR9 was detected using the primers (sense) 5'-TCCTGATGCTAGACTCTGCCAG-3' and (antisense) 5'-CGTCCATGAATAGGAAGCGC-3'. TATA-binding protein primers were 5'-TTGCTGCGGTAATCATGAGG-3' and 5'-GCCAGTCTGACTGTTCCTTC-3', HCMV IE2 primers were 5'-CCTGGTTGGTGAGAAGATG-3' and 5'-TTGTAACGAAGGCGTCAAGG-3', and HCMV pp65 was detected by 5'-TCGCCGAGGATGCTGATTTG-3' and 5'-TCCGACGAAGAAGCTCGTAAC-3'.

Flow cytometry analysis

Uninfected and HCMV-infected NHDF were stained with PE-conjugated anti-human TLR9 mAb (eBioscience) and for isotype control, rat IgG2a-PE (eBioscience). Intracellular staining was performed as described (40, 41). Briefly, NHDF were fixed and permeabilized by 2% formaldehyde and 0.1% saponin buffer (with 20% FCS), stained with mAbs in saponin staining buffer (with 2% FCS), and analyzed by flow cytometry (Beckman Coulter).

For FACS analysis of viral and CpG effect on fibroblast cell size and granularity, uninfected or HCMV-infected NHDF \pm 10 μ M CpG-B were harvested after 3 days. Cells were treated identically to those analyzed for TLR9 expression, and were analyzed for forward and side scatter by flow cytometry (Beckman Coulter).

Detection of cell death (apoptosis and necrosis) in uninfected and HCMV-infected NHDF was performed using the APOTEST-FITC kit (Nexins Research) according to the manufacturer's instructions.

Western blot

Uninfected and HCMV-infected NHDF in 85-mm petri dishes were incubated for 3 days before lysis in 250 μ l of lysis buffer (10% SDS and 10 mM Tris-HCl (pH 6.8)) at 50°C using a Hamilton injector. The samples were denatured in NuPAGE LDS sample buffer (Invitrogen), and electrophoresis of 50–70 μ g of lysate performed using NuPAGE novex 7% Tris-acetate polyacrylamide gels (Invitrogen). The samples were blotted onto 0.45- μ m nitrocellulose filters (Bio-Rad). Following blocking in 50 mM Tris-buffered saline (pH 7.5) supplemented with 0.05% Tween 20 and 5% nonfat dried milk (Nestlé), membranes were incubated with Abs. TLR9 expression was detected by rabbit anti-human TLR9 polyclonal Ab (InvivoGen) and HRP-conjugated polyclonal anti-rabbit Ig (DakoCytomation) and loading confirmed by anti-human Lamin B mAb (Calbiochem Immunochemicals) and goat anti-mouse Ig-HRP (DakoCytomation). The proteins were visualized by ECL Western blotting detection reagents (Amersham Biosciences).

MTT assay

Uninfected and HCMV-infected NHDF in 96-well plates were treated with CpG, poly(I:C), LPS, or medium only. After 7 days in culture, MTT assay was performed as originally described by Mosmann (42). In brief, 10 μ l of 5 mg/ml MTT (Sigma-Aldrich) was added per well and incubated at 37°C for 4 h before addition of 100 μ l of 0.01 M HCl with 10% SDS and incubation overnight at 37°C. The plates were read at 570 nm on a Wallac 1420 Victor3 reader (PerkinElmer) and cell viability was calculated as OD values \pm SD of triplicates.

Confocal microscopy

HCMV was labeled with the PKH26 Fluorescent Cell Linker kit (Sigma-Aldrich). Equal volumes of PKH26 (4×10^{-6} M) and HCMV AD169

(3.6×10^6 PFU/ml) were incubated for 20 min on ice, and 1% BSA in PBS was added to terminate the reaction. The 80% confluent NHDF on 35-mm glass bottom gamma-irradiated tissue cell dishes (MatTek) were added 10 μ M CpG-B or medium only before PKH26-labeled HCMV AD169 infection at MOI of 1.6. After 1 h at 37°C, cells were washed, fixed with 4% paraformaldehyde, and observed by confocal microscopy using an Axiovert 100-M inverted microscope (Zeiss) with an LSM 510 laser scanning unit and a 63X 1.4-NA plan Apochromat oil immersion objective (Zeiss) and appropriate filters.

Results

CpG-mediated enhancement of HCMV infection

NHDF were infected with HCMV for 2 h at a low MOI. The cells were subsequently washed and incubated for an additional 2 h before addition of class B CpG, LPS, or medium only. Cells were then incubated for 7 days, allowing completion of at least two replication cycles, before analyzing virus production (Fig. 1, A and B). LPS was found to reduce the level of HCMV production in a dose-dependent manner (Fig. 1A). In contrast, the addition of CpG-B to infected NHDF significantly enhanced virus production (Fig. 1A), suggesting a proviral effect of this TLR9 ligand. Consistent with their effects on virus production, LPS and CpG-B reduced and enhanced, respectively, the virus-induced cytopathicity in the fibroblast cultures (data not shown). The CpG-mediated viral enhancement was dose-dependent (Fig. 1A) and apparent at various low MOIs (0.002–0.014) (Fig. 1B). DNase-degraded CpG and control ODN poly(A) did not enhance HCMV titers (data not shown), and LT- α potently inhibited HCMV spread (>99%) as expected from previous work (10, 11). The CpG-B-mediated enhancement of viral production was confirmed by quantitative real-time RT-PCR analysis of HCMV gene expression (IE2 and pp65) showing elevated viral gene expression at day 3 postinfection, i.e., within the first round of viral replication in NHDF (Fig. 1C). For comparison, control ODN GpC had no significant effects on HCMV titers and viral gene expression (data not shown).

To determine whether the proviral CpG effect was restricted to NHDF isolated from foreskin, primary adult fibroblasts derived from skin and oral mucosa isolated from the same individual were infected and exposed to CpG-B (Fig. 2). The proviral effect of CpG-B observed in NHDF was also readily apparent in primary skin fibroblasts (Fig. 2A). In contrast, HCMV production was not enhanced by CpG-B in oral mucosa fibroblasts, and instead a weak inhibition was observed at several low MOIs (Fig. 2B), suggesting that not all fibroblast types respond to CpG in a similar fashion.

To determine whether the proviral activity of CpG-B was TLR9-dependent, control HEK293 cells and HEK293 cells stably expressing TLR9 (HEK293-TLR9) were HCMV-infected and analyzed for HCMV pp65 gene expression at day 3 postinfection. CpG-B clearly up-regulated HCMV gene expression in TLR9-expressing cells only, indicating a TLR9-dependent proviral activity of CpG-B (Fig. 3). Control ODN GpC did not influence viral gene expression in either HEK293 cell type (Fig. 3).

TLR9 expression in human fibroblasts

The differential effect of class B CpG in HCMV-infected NHDF and skin fibroblasts compared with oral mucosa fibroblasts (Figs. 1 and 2), prompted the examination of TLR9 expression in the three fibroblast types. TLR9 mRNA levels were detectable in all fibroblast types, albeit at low levels (Fig. 4A). Strikingly, at 24 h postinfection, HCMV strongly up-regulated TLR9 mRNA levels in both NHDF and skin fibroblasts, but not in oral mucosa fibroblasts (Fig. 4A). The HCMV-induced increase in TLR9 mRNA levels persisted and was further increased throughout the first 5 days postinfection (Fig. 4B), and TLR9 protein levels were also

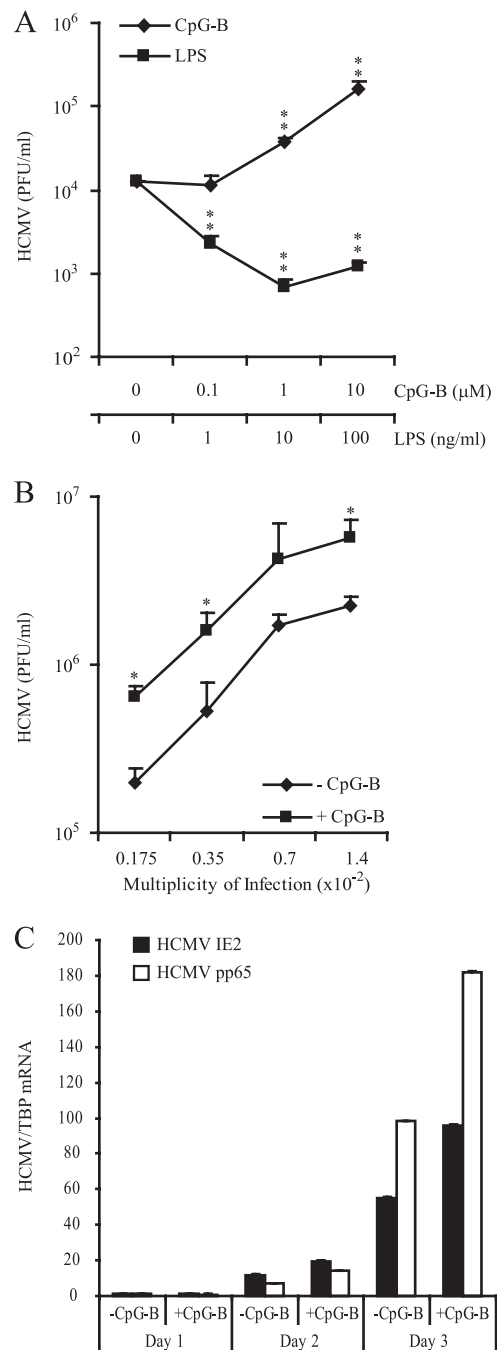


FIGURE 1. CpG enhances HCMV production in NHDF postinfection. **A**, NHDF were infected with HCMV AD169 at MOI 0.004 for 2 h, rested in culture medium for additional 2 h and added CpG-B (10 μ M), LPS (100 ng/ml) or medium only in 10-fold dilutions as indicated. After culture for 7 days the supernatants were collected, analyzed for PFU on fresh NHDF and presented as mean \pm SD of PFU/ml in quadruplicates. **, $p < 0.001$ using Student's t test for two-tailed test. **B**, NHDF were infected with HCMV AD169 at various MOIs as indicated. Four hours after viral addition CpG-B (10 μ M) or medium only was added and left in culture for 7 days before HCMV PFU analysis on the supernatants. Data are presented as mean \pm SD of PFU/ml in quadruplicates. *, $p < 0.01$, using Student's t test for two-tailed test. **C**, NHDF were infected with HCMV AD169 at an MOI of 0.01 for 2 h and incubated with or without CpG-B (10 μ M) 2 h postinfection. Cells were collected for RNA-isolation at 1, 2, and 3 days postinfection, and HCMV IE2 and pp65 mRNA levels (shown normalized to the level of TATA-binding protein mRNA) were quantified by quantitative real-time RT-PCR. Data are presented as mean \pm SD of triplicates. Similar results were obtained in five (A), four (B), and two (C) experiments.

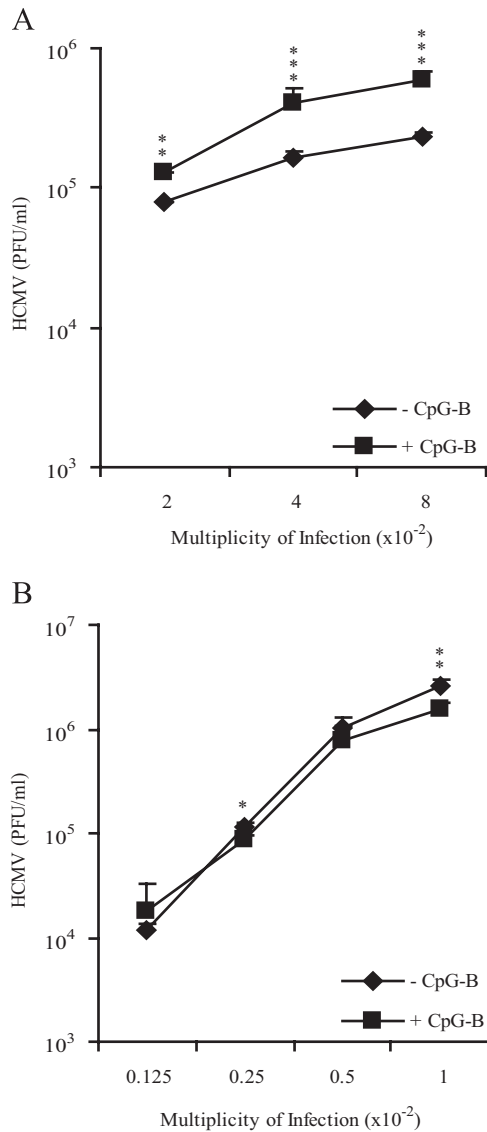


FIGURE 2. CpG effect on HCMV production in skin and oral mucosa fibroblasts. Patient matched skin (A) or oral mucosa (B) fibroblasts were infected with HCMV AD169 at various MOIs as indicated. Four hours after viral addition CpG-B ($10 \mu\text{M}$) or medium only was added and left in culture for 7 days before release of virus to the supernatant was determined by HCMV PFU analysis. Data are presented as mean \pm SD of PFU/ml in quadruplicates. The experiment shown is representative for two independent experiments. *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$ using Student's *t* test for two-tailed test.

modestly increased during this time period (Fig. 4C) with a 1.7-fold increase in relative mean fluorescence intensity (adjusted to respective isotype controls) between uninfected and HCMV-infected NHDF. The TLR9 expression in NHDF was intracellular only, as no surface expression of TLR9 was detected by FACS analysis of nonpermeabilized cells (data not shown). The enhanced TLR9 expression observed using the "high-passage" HCMV strain AD169, was also observed with the more virulent Toledo strain (Fig. 4D). Toledo-infected NHDF showed a 5-fold increase in TLR9 mRNA (data not shown), and a corresponding increase in TLR9 protein expression (Fig. 4D). Interestingly, the induction of TLR9 required live virus because UV-inactivated virus had no effect on TLR9 levels (data not shown). Importantly, TLR9 expression in uninfected NHDF was low, but detectable, as confirmed by all assays.

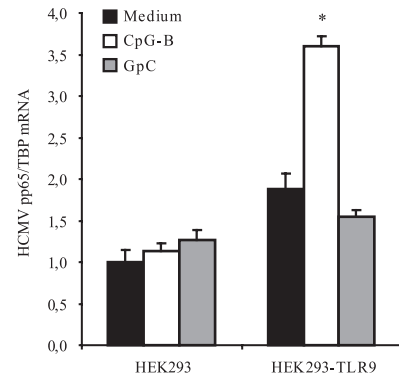


FIGURE 3. TLR9-dependence of proviral CpG-B activity in HEK293 cells. Control HEK293 cells and HEK293-TLR9 cells were infected with HCMV AD169 at an MOI of 0.04 for 2 h and incubated with or without CpG-B or GpC (both $10 \mu\text{M}$) 2 h postinfection. Cells were collected for RNA-isolation at 72 h postinfection, and HCMV pp65 mRNA levels (shown normalized to the level of TATA-binding protein mRNA) were quantified by quantitative real-time RT-PCR. Data are presented as mean \pm SD of triplicates and results are representative for two experiments. *, $p < 0.05$ for statistical significance using Student's *t* test for two-tailed test.

CpG-mediated inhibition of HCMV infection

In light of the enhancement of HCMV replication observed when adding CpG at 2 h postinfection, we were interested in examining whether class B CpG might exhibit even greater activity if added concurrently upon infection. Surprisingly, addition of CpG-B during the initial 2-h HCMV adsorption period had the complete opposite effect, with a greater than 99.9% reduction in HCMV PFU and inhibition of cytopathic effects (Fig. 5A and data not shown). The amount of CpG-B needed for this potent inhibition was considerably lower than that required for enhancement of HCMV production when added 2 h postinfection (Fig. 5A).

CpG sequence specificity

Three different classes of CpG with unique immunostimulatory activities have been characterized (29, 30). We used class B CpG for the initial experiments because the immunostimulatory functions of this TLR9 ligand are well documented. Treatment with CpG-A and CpG-C revealed that the proviral HCMV activity was indeed ODN sequence specific (Fig. 5B). CpG-A did not significantly enhance HCMV production from NHDF cultures, whereas CpG-C only modestly enhanced viral production, compared with CpG-B (Fig. 5B). In contrast, all three CpG classes potently inhibited HCMV infection when added during initial HCMV exposure to the cells (>99.9% inhibition) (Fig. 5B).

Because TLR3 also contributes to innate host defense against CMV in vivo (16, 19), we tested whether the TLR3 ligand poly(I:C) altered the spread of HCMV in fibroblast cultures. When poly(I:C) was added to NHDF cells 2 h postinfection with HCMV, viral production was reduced by >97%, whereas addition at the time of infection showed an even more potent antiviral activity (>99.99% inhibition) (Fig. 5B). When adding LPS during HCMV infection in a similar regimen, >90% reduction in HCMV production was observed, independent of whether LPS was added at the time of infection or shortly after (Fig. 5B). Thus, activation of TLR3 or TLR4 shortly after HCMV entry into fibroblasts did not mediate a proviral effect as seen with CpG-B. Instead, TLR3 and TLR4 ligands inhibited HCMV replication, correlating to recent findings by Harwani et al. (39) on HCMV-infected fibroblasts

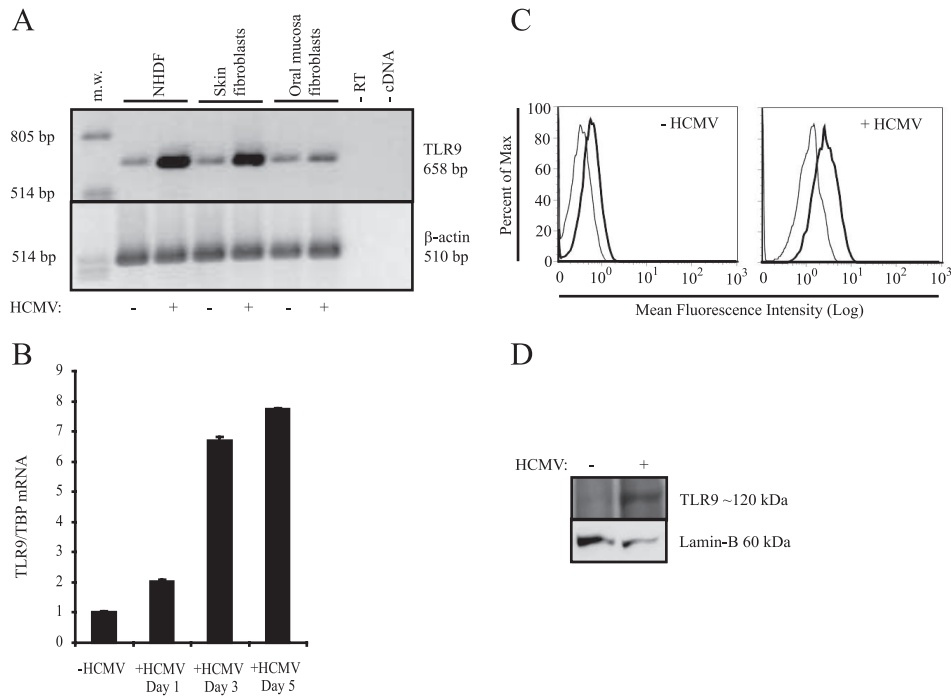


FIGURE 4. TLR9 expression in primary fibroblasts. Fibroblasts were infected with HCMV strain AD169 at MOI 0.04 (A, B, and C) or Toledo at MOI 0.5 (D). A, NHDF, skin and oral mucosa fibroblasts were infected and analyzed by RT-PCR for TLR9 and β -actin gene expression at 24 h postinfection. The m.w. standard (*Pst*I-fragmented λ -DNA) and RT-PCR controls (without RT or cDNA) were included. Data shown are representative of three (NHDF) and two (skin and oral mucosa fibroblasts) experiments. B, RNA was extracted from uninfected (first time point only) and HCMV-infected NHDF at 1, 3, and 5 days postinfection and TLR9 mRNA levels (shown normalized to the level of TATA-binding protein mRNA) were quantified by quantitative real-time RT-PCR. Data are presented as mean \pm SD of triplicates and similar results were obtained in three experiments. C, Uninfected and infected NHDF were incubated for 4 days before permeabilizing the cells and analyzing total cellular TLR9 expression (thick line histogram) by flow cytometry (isotype Ab control staining shown as a fine line). Similar results were obtained in three experiments. D, Uninfected and infected NHDF were incubated for 3 days before Western blot detection of TLR9 expression in total cell extracts. Lamin B expression levels are included as a loading control. Results are representative for three experiments.

treated with poly(I:C) or LPS prior to infection, and similar to what we have previously observed for LT- β R and TNFR signaling in this system (10, 11).

Involvement of IFN- β in CpG effects postinfection

The three classes of CpG possess unique, cell type-dependent activities, one being their varied ability to induce type I IFNs (29–31). IFN- β potently inhibits HCMV replication (12), and we and others have reported that cytokine-induced secretion of endogenous IFN- β from NHDF represent a major cell-intrinsic, antiviral response in this system (10, 11, 13). Therefore, we tested whether the three CpG classes differed in their ability to induce IFN- β . Addition of a neutralizing IFN- β Ab to the cultures increased virus production in the case of CpG-C, indicating that autocrine production of IFN- β contributed to the antiviral HCMV effects of this ODN (Fig. 5C). The proviral effect of CpG-B was, however, unaltered by anti-IFN- β (Fig. 5C), and CpG-A showed only a modest proviral activity when commensurately neutralizing IFN- β (only statistically significant in two of three experiments). Interestingly, CpG-C enhanced HCMV production to levels comparable to those induced by CpG-B when IFN- β was blocked, indicating that simultaneous proviral and antiviral action was mediated by this ODN. Poly(I:C) and LPS induce TLR-dependent IFN- β production in fibroblasts (43, 44). In accordance with this effect, inhibition of HCMV production by poly(I:C) and LPS was abolished when neutralizing IFN- β in the cultures (Fig. 5C).

CpG enhances viability of infected NHDF and blocks HCMV uptake

CpG has been demonstrated to enhance the survival of some cells (27), and consequently we examined whether CpG-B might enhance the growth or survival of NHDF during HCMV infection. HCMV infection alone at low MOI resulted in an increase in cellular metabolism (as assessed by hydrolyzation of the tetrazolium salt MTT) (Fig. 6, A and B), a process shown to reflect viral transcriptional regulation of cellular metabolic pathways (45). At higher HCMV concentrations (above MOI 0.01), the MTT signal was reduced compared with uninfected NHDF, consistent with the observed cytopathic effects and reduced viability in these cultures (Fig. 6, A and B, and data not shown). HCMV enhanced MTT signals at low viral infectious doses (Fig. 6, A and B) correlated with an increase in NHDF cell size and granularity as analyzed by flow cytometry (Fig. 6C).

Addition of CpG-B 2 h postinfection shifted the HCMV dose-response curve to the right, indicating that the fibroblasts “tolerated” \sim 2–4 times greater amounts of virus when CpG-B was added after HCMV infection (Fig. 6A). In combination with the FACS analysis, it is likely that CpG-B addition increases viability of the infected cells (Fig. 6, A and C), consistent with the CpG-B enhanced HCMV production from NHDF when added postinfection (Fig. 1). Addition of LPS and poly(I:C) postinfection prevented the HCMV-induced increase in viability/metabolic activity at MOI below 0.01 (Fig. 6A). None of the TLR ligands tested (CpG-B, LPS, and poly(I:C)) altered MTT

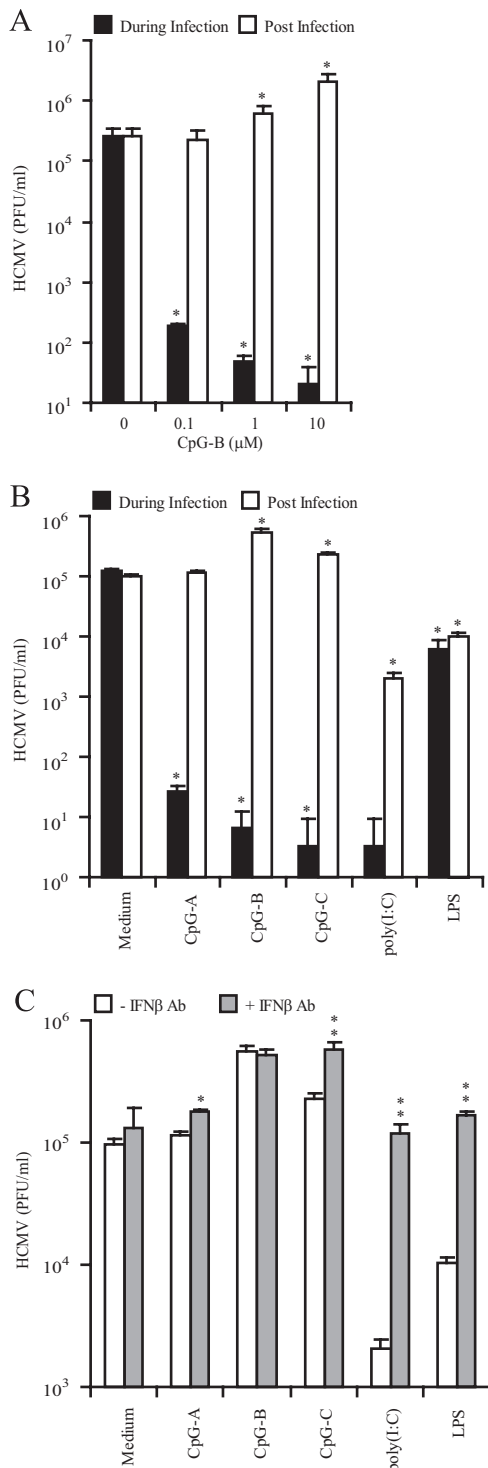


FIGURE 5. CpG class specific and time-dependent influence on HCMV production. NHDF were infected with HCMV AD169 at MOI 0.01. Cells were incubated with or without (A) CpG-B (0.1–10 μM) or (B) CpG-A, CpG-B, or CpG-C (all 10 μM), poly(I:C) (50 μg/ml) and LPS (100 ng/ml) either during the 2 h HCMV infection only (■) or 2 h postinfection, and kept for 7 days in culture (□). HCMV PFU analysis on cell supernatants is presented as mean ± SD in quadruplicates. *, $p < 0.001$, statistical significance from Student's *t* test for two-tailed test shown for comparison to medium control. C, The same experiment as in B, shown with addition of reagents added postinfection (□), and simultaneous addition of blocking IFN-β polyclonal Ab (500 U/ml) (▨). *, $p < 0.05$ and **, $p < 0.001$ for statistical significance comparing each treatment with or without IFN-β polyclonal Ab. Similar results were obtained in five (A and B) and three (C) experiments.

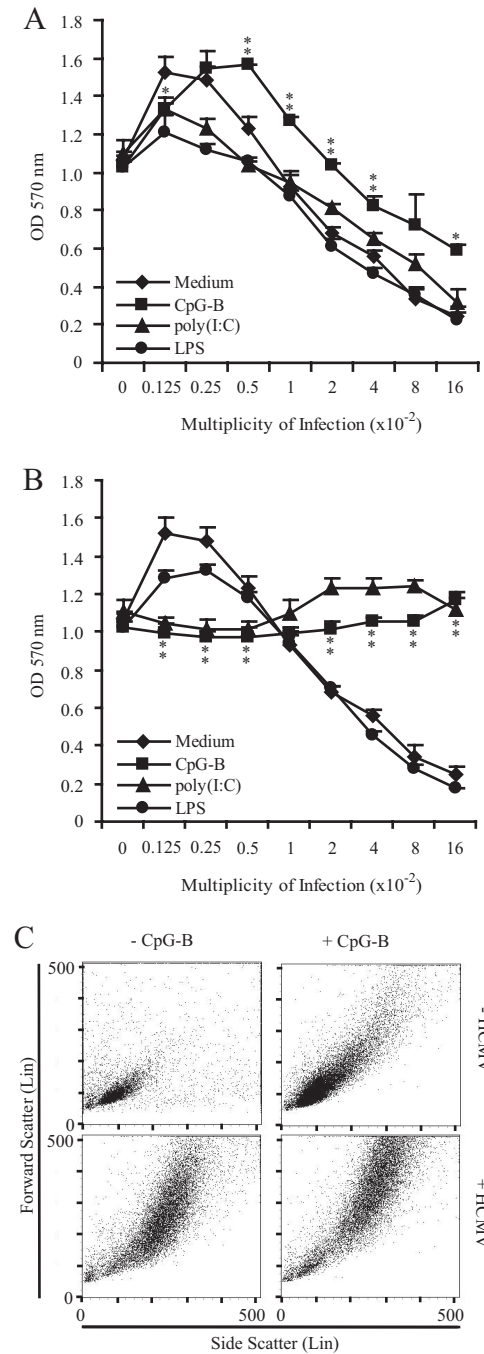


FIGURE 6. Cellular growth and survival of HCMV-infected NHDF. A and B, NHDF were uninfected or infected with HCMV AD169 at various MOIs as indicated, and added CpG-B (10 μM), poly(I:C) (50 μg/ml), LPS (100 ng/ml), or medium only during the 2-h viral exposure (B) or 2 h after removal of virus (A). Cellular growth and survival were determined by MTT assay at day 7 of culture and data are given as mean OD 570 nm ± SD of triplicates. *, $p < 0.01$ and **, $p < 0.001$ for statistical significance from Student's *t* test for two-tailed test shown for comparing CpG-B treatments with medium controls. C, FACS analysis of uninfected and 3 days HCMV AD169-infected (MOI 0.01) NHDF with or without CpG-B (10 μM) added 2 h postinfection. Data are representative of three (A and B) and four (C) independent experiments.

metabolism in the absence of HCMV (Fig. 6, A and B). To further elucidate the CpG-B-mediated increase in MTT metabolism on HCMV-infected NHDF, cell death was analyzed by Annexin V-FITC and propidium iodide staining with flow cytometry analysis. HCMV-induced cell death was reduced by

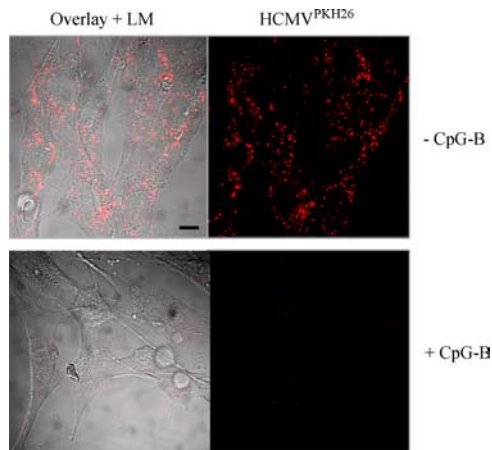


FIGURE 7. CpG blocks cellular uptake of HCMV. NHDF were incubated with or without CpG-B (10 μ M) during infection with PKH26-labeled HCMV AD169 (HCMV^{PKH26}) at an MOI of 1.6. Following infection for 1 h, cells were fixed with paraformaldehyde and analyzed by confocal microscopy for uptake of virus. Confocal images are shown as PKH26 labeling separately and overlaid with differential interference microscopy images. Scale bar represents 10 μ m. The experiment shown is representative for four independent experiments.

CpG-B by 64% (from 11% to 4%) at day 3 postinfection, confirming a strong prosurvival CpG-B activity. Also, at day 7 postinfection, CpG-B still lowered the virus-induced cell death by 18% (from 40% to 33%), despite the obvious higher cytopathic effect in the CpG-B-treated HCMV-infected cells.

If CpG-B or poly(I:C) was added at the time of HCMV infection, a marked inhibition of HCMV-induced effects on cell viability/metabolic activity was observed (Fig. 6B). Both CpG-B and poly(I:C) reduced the MOI-dependent response to the level of uninfected NHDF (Fig. 6B).

In light of the potent antiviral activities of CpG-B and poly(I:C) during infection (Fig. 5B), these observations suggested that the TLR ligands might block at a very early point in the HCMV entry/replication cycle. We therefore investigated whether CpG-B might be functioning by blocking initial viral binding or uptake into fibroblasts when this ODN was added concurrently with infection. HCMV particles were directly labeled with the fluorescent membrane dye PKH26, and viral uptake was examined by confocal microscopy. Upon HCMV infection, viral particles were taken up by NHDF (Fig. 7). Addition of CpG during initial HCMV infection almost completely abolished cellular uptake (Fig. 7). Consistent with what was observed for viral production (Fig. 5B), this inhibition was not ODN sequence specific because CpG-A and the control ODN GpC showed a comparable potency in blocking HCMV entry (data not shown). In addition, poly(I:C), but not LPS, also blocked HCMV uptake to the cells (data not shown). Parallel treatment with PKH26 without HCMV served as negative control, and verified that only virion-associated dye was internalized by the NHDF (data not shown).

HCMV can bind its fibroblast-expressed extracellular receptor at 4°C, whereas viral entry/internalization requires significantly higher temperature (5). When HCMV binding was allowed to proceed at 4°C and then CpG-B was added when increasing the temperature to 37°C, viral production was still almost abolished (>99.9% inhibition reduction of viral titers). This confirmed that CpG-B blocked the HCMV entry to the cells, in agreement with recent findings of Lukanini et al. (38).

Discussion

In this study, we show an opposing and time-dependent role of CpG in modulating HCMV infection of fibroblasts. Addition of CpG concurrent with initial HCMV infection is a potent antagonist, blocking the uptake of HCMV particles into cells at a post-receptor binding step. In contrast, when CpG-B is added at time points shortly following viral entry; this TLR9 ligand significantly enhances virus production and cell survival. Importantly, the proviral action of class B CpG correlated completely with the ability to up-regulate TLR9 expression upon infection, strongly suggesting a TLR9-dependent mechanism of action.

Whether TLR9 is expressed in fibroblasts is still somewhat controversial, and previous studies have reported both the presence (34–36) and absence (37–39) of TLR9 in human fibroblasts. This reporting is not surprising because the level of TLR9 in uninfected fibroblasts is quite low. However, our data show that TLR9 is clearly up-regulated in fibroblasts following HCMV infection, suggesting that TLR9-dependent signaling may be important for antiviral defense in cells of stromal lineage. This suggestion is especially relevant given that stromal cells are known to be the major target for MCMV infection in the spleen and produce virtually all the initial type I IFN in response to MCMV infection in vivo in this organ (18, 46, 47).

Whether the HCMV-induced TLR9 in fibroblasts represents a cell-intrinsic antiviral immune response or a viral strategy to usurp aspects of a host defense pathway remains unclear. The proviral action of CpG we have observed makes it tempting to speculate that HCMV has targeted aspects of TLR9 signaling to promote cell survival and enhance viral replication in infected stromal cells. HCMV activates and depends on the PI3K/Akt pathway for productive replication in fibroblasts (48), and CpG-B has the potential to induce TLR9-dependent PI3K/Akt activation (49). The fact that both HCMV and CpG activate the PI3K/Akt pathway in fibroblasts may explain why CpG-B enhances the proviral function of HCMV in these cells. The CpG class-specific differences in proviral activity suggest that the function of HCMV DNA-mediated TLR9 activation may be cell type-dependent. This action is supported by the fact that CMV does not productively replicate in plasmacytoid DCs, although the virus induces a TLR9-dependent innate cytokine response in these cells (15, 50). However, further studies are needed to define whether the proviral CpG-B effect reflects an HCMV-induced immune evasion in infected fibroblasts, and to prove the TLR9 dependence of this activity.

Addition of class B CpG 2 h postinfection potently enhances HCMV spread in skin fibroblasts, but not in oral mucosa fibroblasts isolated from the same patient, and this dichotomy correlated with HCMV-induced TLR9 expression. Such site-specific phenotypic variation in the expression and production of immunoregulatory factors (cytokines, chemokines, and costimulatory molecules, e.g., CD40) by fibroblasts is well described (51, 52). Significant differences in the gene expression profiles of stromal cells is commonly observed in various human diseases; playing an important role in immunological recruitment and T cell survival in chronic inflammatory diseases such as rheumatoid arthritis (53). An interesting aspect of the lack of TLR9 expression by oral mucosa fibroblasts is the fact that CMV infection is likely to occur via this route, for instance in HCMV transmission from mother to child through breastfeeding (54).

Only a few reports have indicated potential negative side effects of CpG administration, including activation of malignant B cells (55, 56) and morphological changes in lymphoid organs (57). The threat of enhancing autoimmune diseases and pathogen-induced septic shock is also a concern for CpG therapy in humans (33).

Interestingly, Olbrich et al. (58, 59) reported that CpG-B-mediated effects on Friend retrovirus infection in mice was also dichotomous, with higher viral loads observed whether CpG pretreatment was performed, and enhanced antiviral immune responses if CpG was added postinfection. Addition of CpG-B has also been shown to reactivate latent HIV from human T cells in vitro (60). These studies, although varied, indicate that the use of CpG in immune therapy could have potential deleterious effects in some settings. If our observed in vitro proviral CpG activities translate to in vivo effects on HCMV-infected stromal cells, the use of CpG as immune adjuvant for treatment of immunosuppressed individuals at high risk for clinical HCMV infection should be taken into careful consideration.

HCMV uses several strategies to inhibit cellular apoptosis, likely contributing to increased cellular viability, enhanced viral production, and inhibition of the host immune response (2, 3). CpG likely enhances the release of infectious HCMV by enhancing survival of infected cells, thereby increasing the capacity of the cell to produce new virions. CpG has been shown to promote survival of neutrophils (61), and enhance the survival and proliferation of B cells (25, 27, 29, 55) and plasmacytoid DCs (62).

The CpG-mediated proviral activity is sequence specific, with class B CpG being the strongest enhancer of HCMV production. The CpG classes have been shown to differ with respect to type I IFN induction and cell type activation in a TLR9-dependent manner (29). CpG-B and CpG-C are both potent B cell activators, whereas CpG-C also induces IFN- α production in PBMC (29, 30). Fibroblasts produce high amounts of IFN- β (63), and cell-intrinsic antiviral HCMV immunity in fibroblasts is mainly IFN- β -mediated (10, 11). Thus, although CpG induces more IFN- α than IFN- β in leukocytes (31), IFN- β is expected to be the main antiviral IFN in HCMV-infected fibroblasts. IFN- β inhibits HCMV replication in fibroblasts (10, 64), and our data clearly show that CpG-C is equivalent to CpG-B in proviral activity if the "antagonizing" IFN- β effects are neutralized. Class A CpG is the most potent type I IFN inducer of the three classes when hematopoietic cells are analyzed (29–31), but our study indicates a general lack of CpG-A response in HCMV-infected fibroblasts. This finding may compare with the CpG class-specific effects on B cells, with a high level of activity seen for CpG-B and CpG-C, but not CpG-A (29, 30). As a therapeutic drug, CpG-C is recommended on the basis of its Th1 adjuvant activity (29). From our data we suggest that class A CpG should be considered for a therapeutic drug to avoid the potential proviral HCMV action of CpG.

Immune protection from HSV-2 infections in mice pretreated with CpG has been ascribed to TLR9-mediated immunomodulating activities and not to blocking of viral entry to vaginal epithelial cells (26). Our study shows that CpG can directly inhibit cellular uptake of a herpesvirus, consistent with recently published results (38). In HCMV antisense ODN studies by Azad and Anderson et al. (65, 66), viral blocking was observed in HCMV-infected NHDF with unspecific synthetic ODNs at concentrations above 3 μ M. Our results with CpG inhibition of HCMV uptake at quite low concentrations suggest that a similar mechanism is occurring in our system. Synthetic PTO ODNs have also been reported to interfere with cellular uptake of HIV (67) and HSV-2 (68, 69), whereas HSV-1 was relatively resistant (69), but the specific mechanism for PTO ODN action is not known. It has been postulated that the susceptibility of enveloped viruses to inhibition by sulfated polysaccharides, such as dextran sulfate, correlates with their sensitivity to blocking by PTO ODNs (69). Both cellular binding and entry of HCMV depends on specific viral envelope glycoproteins, and sulfated polysaccharides can block the binding of viral envelope glycoproteins to cell membrane receptors (re-

viewed in Ref. 70). Consequently, the CpG-mediated block to viral uptake may act by similar mechanisms as postulated for other PTO ODNs. Thus, in contrast to the sequence-specific proviral action of CpG-B when added postinfection, the PTO ODN-mediated blocking of HCMV entry to the fibroblasts seems to be both DNA sequence- and TLR9-independent, as recently also suggested by Lugini et al. (38).

For the outcome of CpG-mediated influence on HCMV infection in fibroblasts the time of exposure seems crucial. We report that CpG-B exposure postinfection significantly enhances viral spread, whereas all CpG classes block viral entry when present during infection. Harwani et al. (39) recently showed that CpG-C also has some inhibitory effect on HCMV spread in fibroblasts when added prior to infection.

When CpG-B is added 2 h postinfection and left in culture for 7 days, it is present for at least two HCMV replication cycles. Because a strong proviral CpG effect is observed, it indicates that CpG-B cannot block viral entry during secondary rounds of spread in cultured fibroblasts. The inability of CpG to block HCMV spread in infected NHDF is likely due to the fact that HCMV spreads largely cell-to-cell after initial infection (71, 72).

In total, this study indicates that three distinct TLR ligand-mediated pathways influence HCMV infection in fibroblasts, and potentially other stromal cell lineages. First, CpG-B exhibits proviral activity by enhancing fibroblast survival resulting in increased viral production. Second, viral spread is inhibited by TLR3 and TLR4 ligands through an IFN- β -dependent mechanism. And finally, viral entry in fibroblasts is blocked by CpG (dsDNA) and poly(I:C) (dsRNA) most probably by a TLR-independent mechanism. It is intriguing to think that CMV may have evolved specific strategies to use TLR-dependent signaling to its advantage during initial infection of stromal cells, as it has recently been shown that the initial IFN- $\alpha\beta$ response to CMV infection in vivo is independent of TLR signaling (18). Taken together this adds new and important information to the study of TLR ligand-mediated viral influence and in particularly to the role of CpG in HCMV infections.

Disclosures

The authors have no financial conflict of interest.

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