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SHORT COMMUNICATION

Amphotericin B abolishes the cytotoxic effect of Toll-like receptor 9 agonist CpG Oligonucleotides in breast cancer cell line MCF-7

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ABSTRACT

Background: Toll-like receptors (TLRs) are key sensors of microbial components and triggers signals responsible for the activation of innate immune responses. Unmethylated cytosine-guanosine oligodeoxynucleotides called CpG ODNs act as a ligand for TLR9. **Aims and Objectives:** The present study was conducted to determine whether the presence of antimycotic amphotericin B (AmB) used in the culture medium has any profound effect on MCF-7 cells treated with CpG. **Materials and Methods:** MCF-7, a breast adenocarcinoma cell line, was cultured and maintained in the media with the absence of AmB (Group 1) and presence of AmB at 0.25 µg/ml (Group 2). The morphology of MCF-7 cells grown in these two groups of media was assessed, and the cytotoxicity of CpG treated in both the groups was evaluated by MTT assay. **Results:** Visualizing the monolayer of MCF-7 cells grown in two groups of media revealed identical morphology, growth pattern, and confluence. MCF-7 cells in Group 1 media treated with the CpG ODN at 1, 2, and 3 µM for 24 h showed a dose-dependent cytotoxicity in 13%, 18%, and 22% of the cells, respectively, whereas treatment with CpG ODN at 1, 2, and 3 µM for 48 h demonstrated a better cytotoxicity of 16%, 27%, and 37% of cells, respectively. On the other hand, MCF-7 cells in Group 2 media treated with the CpG ODN at 1, 2, and 3 µM for 24 h and 48 h showed poor cytotoxic effect. **Conclusion:** While comparing the effect of CpG ODN in Group 1 (AmB−) versus Group 2 (AmB+) for 24- and 48-h duration in MCF-7 cells evidenced that the response of CpG ODN was significantly different between the two groups ($P < 0.01$). The results strongly evidenced that the presence of AmB in culture media during the maintenance of cell line shown to decrease the cytotoxic effects of CpG ODN. Hence, the present study suggests to avoid using AmB in cell culture, particularly while conducting *in vitro* studies related to TLR signaling.

KEY WORDS: Cell Culture; Toll-like Receptor; CpG oligonucleotides; Antimycotic Agent; Amphotericin B; Cytotoxic Activity

INTRODUCTION

Cell culture has been used as a model system to study the biochemical characteristics of cell and study the effects of a drug (or) toxic compound. To maintain the animal cell culture free from bacterial and fungal contamination, antibiotics (AB) - penicillin and streptomycin (50-100 µg/ml) and fungicides/antimycotic (AM) - amphotericin B (AmB) at a concentration of 0.25-2.5 µg/ml are included in tissue culture medium.[1] In general, at this working concentration, AB penicillin and streptomycin (50-100 µg/ml) and fungicides - amphotericin (0.25-2.5 µg/ml) are nontoxic to animal and/or mammalian cell line.[2,3] Toll-like receptors (TLRs) are key sensors of microbial components and triggers signals responsible for the activation of innate immune response.[4] There are 10 TLRs expressed in humans,

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namely, TLR1-10. Each TLR recognizes different molecules derived from bacteria, viruses, and fungal products through pathogen-associated molecular patterns.[4] Unmethylated cytosine-guanosine oligodeoxynucleotides called CpG-ODNs act as a ligand for TLR9.[5] Cohen et al. showed that ABs reduce the mRNA expression in embryonic stem cells in *in vitro*.[6] Sau et al. demonstrated that AmB induces release of pro-inflammatory cytokines from murine peritoneal macrophage and HEK293 cell lines through TLR-mediated signaling.[7]

AmB (fungizone), a polyene antifungal, is an amphipathic fermentation product of the Gram-positive bacterium, first isolated from *Streptomyces nodosus* in 1955.[8] AmB has remained the prominent chemotherapeutic drug used for severe systemic fungal infections for more than half a century.[9] As AmB is a microbial product and amphiphilic like lipopolysaccharide, it can trigger TLR2 and TLR4 signaling.[7] Experiments conducted earlier in our laboratory to determine the cytotoxic activity of TLR9 ligand CpG in MCF-7 cell line evidenced that cells grown and maintained in the presence and absence of AmB showed variation in dose- and time-dependent effect on cell viability. Hence, we performed a study to assess whether AmB interferes with the cytotoxic effect of CpG on MCF-7 cells cultured *in vitro*. Accordingly, we compared the effects of CpG ODN on the viability of cells cultured and maintained in two types of media. Cells were cultured in Group 1 media, which contained penicillin and streptomycin (pen-strep) alone, or in Group 2 media containing pen-strep and AmB mixture. An empiric observation made in our study suggests that the response of CpG is reduced when AmB is used in culture medium for growing MCF-7 cell line.

MATERIALS AND METHODS

Culture and Maintenance of Cell Lines

The human breast adenocarcinoma cell line (MCF-7) was procured from National Centre for Cell Science, Pune, India. MCF-7 cell line was cultured in minimum essential medium (MEM) purchased from Himedia, Mumbai, supplemented with 10% fetal bovine serum (FBS), certified US origin (Gibco) with AB - penicillin (100 units/ml) and streptomycin (100 µg/ml), and AM - AmB purchased from Himedia, Mumbai.

Cells were grown in MEM media containing 10% fetal bovine serum and AB - penicillin (100 units/ml) and streptomycin (100 ug/ml) alone (Group 1), and cells were grown in MEM media containing 10% FBS, AB - penicillin (100 units/ml) and streptomycin (100 µg/ml), and AM - AmB - 0.25 µg/ml (Group 2). The cell lines were maintained in a CO_2 incubator (Vue wireless radio) at 37°C in a 5% CO_2 atmosphere with 95% humidity.

Synthesis of CpG ODN

CpG ODN 2006 sequence 5'-tcgtcgttttgtcgttttgtcgtt-3' (24 mer), phosphorothioated sequence[10] was synthesized from Integrated DNA Technologies (IDT Inc, USA). Lyophilized ODNs were dissolved in endotoxin-free water and prepared as a 100 µM stock, which was aliquoted and stored at −20°C.

In Vitro Cell Viability/Cytotoxicity Assay

Briefly, seeding was done in 96-well plates with MEM media (in the absence of AB and AM agent) and 10% FBS at a density 2×10^4 cells/well. Once a confluent cell monolayer was observed, media from the wells were removed, and CpG ODN was treated at a concentration 1, 2, and 3 µM in triplicates. Quadruplicate wells with cells alone and media alone were included in the experiment. Triton-X-100 (2%) was included in triplicates as a positive control. The plate was incubated for 24 and 48 h, and the cell morphology was assessed with a phase-contrast microscope before treating with MTT.

MTT Assay

The cytotoxicity of CpG ODN 2006 on MCF-7 cells was determined by the MTT (3-(4, 5-dimethylthiazol-2yl)-2, 5-diphenyltetrazolium bromide) assay as described by Marshall et al.[11]. After 24 and 48 h of incubation with CpG ODN, supernatant was removed from each well, and MTT (0.5 mg/ml) was added to each well and incubated at 37°C for 4 h. Formation of formazan crystals was confirmed by visualizing under inverted phase-contrast microscope. Then, MTT solution was removed, and crystals were dissolved with 150 µl of dimethyl sulfoxide and isopropanol (1:1 ratio). OD values are obtained by measuring in ELISA reader at 595 nm with reference 655 nm.

% of viability = (Test O.D/Control O.D) * 100

Statistical Analysis

All data were expressed as the mean ± standard deviation ($n = 3$). Student's *t*-test was used to compare the statistical significance between two groups ($^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$). Statistical analysis was performed using GraphPad prism 7.

RESULTS

This study was conducted to examine whether the presence of AM AmB used in the culture medium for maintenance of cell line has any significant effect on MCF-7 cells treated with CpG ODN. Accordingly, we compared the effects of CpG ODN on the viability of cells cultured in two groups of media. First, we examined the morphology of MCF-7 cells cultured

in Group 1 media compared to Group 2. Figure 1 shows the phase-contrast images of MCF-7 cultured in T-25 flask. In terms of cell morphology, growth pattern, and confluency, there was no significant difference between the cells grown in these two different types of media.

Later, we examined the cytotoxic effects of CpG ODN on MCF-7 cells cultured in these two groups of media. The cytotoxic effects of CpG ODN on MCF-7 cells cultured in Group 1 and Group 2 media were evaluated by MTT assay (Figure 2a and b). MCF-7 cells maintained in Group 1 media and treated with the CpG ODN for 24 h at 1, 2, and 3 μM showed a dose-dependent cytotoxicity in 13%, 18%, and 22% of the cells, respectively. CpG ODN at 1 and 3 μM caused a significant cytotoxic effect ($P < 0.05$) compared to control (untreated) cells whereas treatment with CpG ODN at 1, 2, and 3 μM for 48 h demonstrated a better cytotoxicity in 16%, 27%, and 37% of cells, respectively, and showed a significant cytotoxic response at 2 μM ($P < 0.01$). On the other hand, MCF-7 cells treated with the CpG ODN in Group 2 media for 24 h at 1, 2, and 3 μM showed cytotoxicity in 12%, 8%, and 15% of the cells, respectively. CpG ODN treated at 3 μM alone showed a significant difference ($P < 0.05$). Interestingly, cells treated with CpG ODN for 48 h at 1, 2, and 3 μM revealed no cytotoxic effect in any of the tested concentrations, and there was a further increase in cell proliferation to 100%, 102%, and 109%, respectively, compared to control cells.

The study results demonstrate that MCF-7 cells evidenced a clear, dose-dependent, and time-dependent cytotoxicity of CpG ODN in Group 1 media (AB alone) but not in Group 2 media (AB + AmB). While comparing the effect of CpG ODN in Group 1 (AmB−) versus Group 2 (AmB+) for 24 h in MCF-7 cells evidenced that the response of CpG ODN at 2 μM (18% vs. 8%) and 3 μM (22% vs. 15%) was significantly different ($P < 0.01$) between the two groups, i.e., in the absence and presence of AmB. Similarly, while comparing the response of CpG ODN at 1, 2, and 3 μM for 48 h in Group 1 versus Group 2 showed highly significant difference between the two groups ($P < 0.01$). Since these data were obtained from three independent experiments conducted in triplicates, our results strongly evidenced that the presence of AmB in culture media during the maintenance of cell line shown to decrease the cytotoxic effects of the CpG ODN.

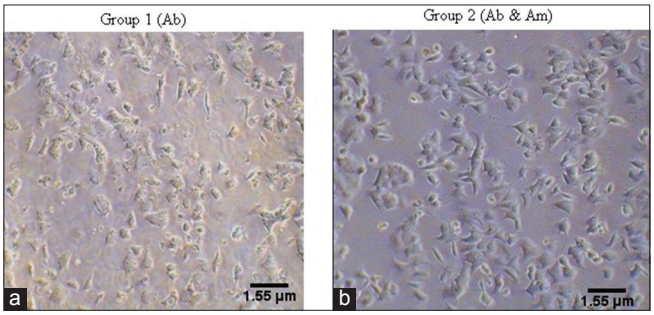

Figure 1: (a and b) Cell morphology of MCF-7 cell line grown in two different group. MCF-7 cells passage number 37 were grown for 6 days in Group 1 (left panel) or Group 2 (right panel) media, with media changed once in 3 days

DISCUSSION

The effect of TLR9 ligand CpG on viability of MCF-7 cells grown in two types of media (Group 1 and Group 2) was compared. Figure 2a demonstrates that MCF-7 cells grown in Group 1 media show a clear dose- and time-dependent cytotoxic effect of CpG on MCF-7 cells (Figure 2a).

In contrast, the data on Group 2 media (Figure 2b) show that the antimycotic (AmB) interferes with cytotoxic effect of CpG, even when used at 10-fold less than therapeutic concentration ~2 μg/ml found in human serum.[12] The present study results correlate with Mathieson et al.[13] showed that antimycotics and AB alter the proteome of MCF-7 cell *in vitro*. Arning et al. found elevated plasma levels of

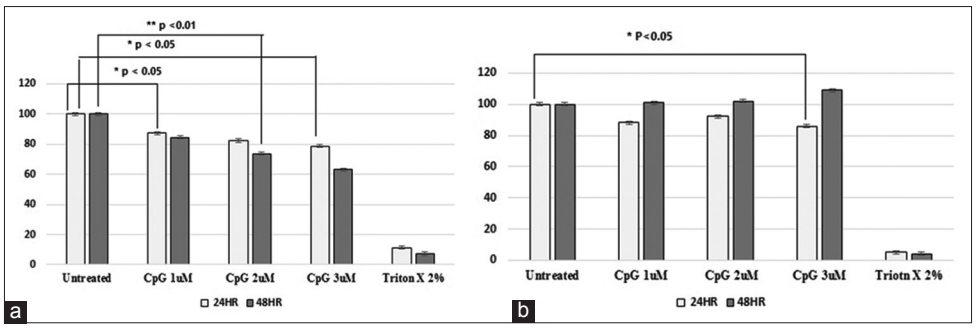

Figure 2: (a) Cytotoxic effects of cytosine-guanosine oligodeoxynucleotides (CpG ODN) on MCF-7 cells grown in Group 1 media. In Group 1, MCF-7 cells treated with CpG ODN at a concentration of 1, 2, and 3 μM for 24 h showed 87%, 82%, and 78% cell viability, whereas cells incubated for 48 h showed 84%, 73%, and 63% respectively. CpG ODN treated for 24 h at 1 and 3 μM showed statistically significant cytotoxicity (*$P < 0.05$) compared to untreated cells. For 48 h, CpG ODN at 2 μM alone showed statistically significant cytotoxicity (**$P = 0.01$). Cells treated with Triton-X-100 (2%) showed only 11% and 7% cell viability for 24 and 48 h in Group 1 media. (b) Cytotoxic effects of CpG ODN on MCF-7 cells grown in Group 2 media. In Group 2, MCF-7 cells treated with CpG ODN at a concentration of 1, 2, and 3 μM showed 88%, 92%, and 85% cell viability for 24 h and 101%, 102%, and 109% cell viability for 48 h. CpG ODN at 3 μM showed statistically significant cytotoxicity (*$P < 0.05$) at 24 h. Cells treated with Triton-X-100 (2%) only showed 5% and 4% cell viability for 24 h and 48 h in Group 2 media

interleukin (IL)-6, tumor necrosis factor-alpha (TNF-α), and IL-1-Rα in patients administered with AmB.[12] Sau et al. evidenced that AmB-induced release of pro-inflammatory cytokines TNF-α and IL-6 is dependent on expression of TLR2, CD14 coreceptor, and adaptor protein MYD88. In addition, TLR4 may also function as a signaling receptor for AmB to induce cytokine release.[7]

While comparing the effect of CpG ODN in Group 1 (AmB−) versus Group 2 (AmB+) for 24 and 48 h duration in MCF-7 cells evidenced that the response of CpG ODN was significantly different between the two groups ($P < 0.01$). Hence, our results clearly demonstrate that AmB abolishes the cytotoxic activity of CpG in MCF-7 cells. Our results corroborate with a recent report which pointed out that AmB acts as TLR2− and TLR4 agonist and the cytokines elicited in human peripheral blood mononuclear cells upon treatment with AmB was similar to that of a known TLR agonist monophosphoryl lipid-A suggestive of TRIF-based TLR4 signaling mediated by AmB.[14] Thus, the diminished effect of CpG in AmB treated MCF cells is likely due to the activated TLR2 and TLR4 signaling and release of pro-inflammatory cytokines. Very low dose of AmB is used as a potential adjuvant in vaccine preparations.[15] Hence, the present study suggests the necessity to avoid using AmB for sterility in animal cell culture, especially dealing with the treatment of CpG in cell lines.

CONCLUSION

While comparing the effect of CpG ODN in Group 1 (AmB−) versus Group 2 (AmB+) for 24 and 48 h duration in MCF-7 cells evidenced that the response of CpG ODN was significantly different between the two groups ($P < 0.01$). The results strongly evidenced that the presence of AmB in culture media during the maintenance of cell line shown to decrease the cytotoxic effects of CpG ODN. Hence, the present study suggests to avoid using AmB in cell culture, particularly while conducting *in vitro* studies related to TLR signaling.

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