# Enhancement of Transduction Efficiency and Antitumor Effects of IL-12N220L-expressing Adenovirus by Co-delivery of DOTAP

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**ABSTRACT**

**Background:** Adenovirus (Ad) vectors have been widely used for many gene therapy applications because of their high transduction ability and broad tropism. However, their utility for cancer gene therapy is limited by their poor transduction into cancer cells lacking the primary receptor, coxsackievirus and adenovirus receptor (CAR). **Methods:** To achieve CAR-independent gene transfer via Ad, we pretreated Ad with 1,2-dioleoyl-3-trimethylammonium propane (DOTAP) and analyzed their transduction efficiency into cancer cells *in vitro* and *in vivo* comparing with the virus alone. **Results:** Treatment of DOTAP significantly increased adenoviral gene transfer in tumor cells *in vitro*. Moreover, DOTAP at an optimum dose (10 $\mu$g/ml) enhanced IL-12 transgene expression by fivefold in tumor, and twofold in serum after intratumoral injection of adenovirus expressing IL-12N220L (Ad/IL-12N220L). In addition, cotreatment of DOTAP decreased tumor growth rate in the Ad/IL-12N220L-transduced tumor model, finally leading to enhanced survival rate. **Conclusion:** Our results strongly suggest that DOTAP could be of great utility for improving adenovirus-mediated cancer gene therapy. **(Immune Network 2007;7(4):179-185)**

**Key Words:** Adenovirus, DOTAP, liposome, tumor

## Introduction

Adenovirus vectors (Ads) are widely used in many gene therapy applications because of their several prominent advantages over other vectors. Ad can produce high levels of transgene expression compared with other established gene transfer methods, including retroviruses and cationic lipids. In addition, Ad is capable of efficiently delivering gene into various cell types (either dividing or non-dividing cells). Adenoviral DNA is not integrated into the host genome, thereby resulting in a low risk of insertional mutagenesis. Furthermore, Ad can accommodate the large-size transgene and is easy to manipulate by classical recombinant DNA techniques. Finally, the production of high titers of Ad, which is necessary for clinical trials, is well established (1,2).

Ads have been commonly used to transfer tumor suppressor genes, suicide genes, anti-angiogenic factors, prodrug activating genes and immunostimulatory genes for cancer gene therapy (3,4). However, the utility of Ad is limited by their low transduction efficiency in certain types of cancer cells due to the low expression level of the primary adenovirus receptor, coxsackievirus and adenovirus receptor (CAR) (5). Although CAR is expressed ubiquitously on most normal epithelial tissues, its lack or down-regulation has been reported in various tumor types (6-8).

There have been many studies designed to improve Ad-mediated gene therapy in cells expressing a low level of CAR. One of them is the modification of Ad through the attachment of ligand for cellular receptors and the incorporation of chimeric envelope glycoprotein.

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Moreover, biochemical reagents such as polybrene, lipofectamine, protamine sulfate and DOTAP have been used to facilitate the entry of virus particles into the target cells. Complexing adenovirus with polycations has been shown to be effective in increasing Ad-mediated gene transfer (9-13). *In vitro* studies have demonstrated that polycations can efficiently increase Ad delivery into various tumor cells (14-17). However, *in vivo* studies showing same dramatic effects as *in vitro* results are lacking (14,15,18,19). Therefore, the low efficiency of polycation-mediated Ad delivery *in vivo* remains to be a major obstacle to achieving its successful application for cancer gene therapy.

DOTAP which is widely known as transfection lipid consists of a monocationic trimethylammonium head group and two unsaturated hydrocarbon chains. Previous studies revealed that DOTAP can efficiently transduce plasmid DNA to various cells *in vitro* and improve immune response and protective immunity by co-immunization with DNA or recombinant tumor-associated antigen (20-22). Additionally, it was reported that DOTAP can improve transduction efficiency of Ad in several tumor cells *in vitro* and enhance pulmonary gene transfer *in vivo* (15,23). However, its therapeutic application usage, especially in cancer gene therapy, has not been well studied.

In this study, the effect of DOTAP on adenoviral transduction in tumor cells is investigated using Ad expressing IL-12N220L (Ad/IL-12N220L) which was known to have potent antitumor effects (24). Our study's results show that the combined use of DOTAP and Ad greatly improved transduction in mouse and human melanoma which are resistant to adenoviral infection. Furthermore, DOTAP improved the therapeutic antitumor effect of Ad/IL-12N220L in mouse melanoma model. These results suggest that DOTAP can be a promising strategy for improving Ad-based cancer gene therapy.

## Materials and Methods

*Mice*. Six- to eight-week old C57BL6 mice were purchased from Charles River Breeding Laboratories (Shizuoka, Japan). The mice were housed in a specific pathogen-free environment in an internally approved vivarium at the institute.

*Cell lines*. B16F10, the mouse melanoma cell line, A375, the human melanoma cell line were purchased from American Type culture Collection (Manassas, VA). These were maintained in DMEM supplemented with 10% fetal bovine serum (Hyclone, Logan, UT) and 1% w/v each of penicillin/streptomycin (Life Technologies, Inc., Grand Island, NY) per 100 ml.

*Cationic liposome*. 1,2-dioleoyl-3-trimethylammonium propane (DOTAP) was purchased from Roche (Mannheim, Germany).

*Construction of replication-defective adenoviral vectors*. Recombinant replication-defective adenoviruses (Ads) were generated according to the AdEasy™ Vector System (Q Biogene) and Ad/IL-12N220L was constructed as described earlier (24). Briefly, the cDNAs of murine IL-12N220L and EGFP were subcloned into the adenoviral shuttle vector, pShuttleCMV. After recombination with the adenoviral backbone vector, pAdEasy in *Escherichia coli* BJ5183, the recombinant adenoviruses were generated and expanded in 293 cells.

*In vitro adenovirus transduction*. Cells were seeded into 48-well plates at $3\times10^4$ cells/well and were incubated overnight at 37°C. Adenovirus was diluted in DMEM without serum to achieve a 2× virus dilution. DOTAP was similarly diluted into DMEM without serum to yield 2× dilutions. Adenovirus and DOTAP mixtures were combined at a 1 : 1 ratio and were allowed to incubate for 30 min at room temperature. Cell monolayers were washed with PBS and overlaid with 150 $\mu$l of virus/DOTAP mixture. After 2 h incubation in a $CO_2$ incubator at 37°C, the cells were washed with PBS to remove the complexes, and 300 $\mu$l fresh serum-containing medium was added. The cells were then incubated for an additional 46 h before assessing GFP expression. Gene transduction efficiency was assessed by flow cytometry on a FACScalibur using CellQuest software (Becton Dickinson, Tokyo, Japan), acquiring 10,000 events by forward and side scatter gating to exclude cell debris.

*In vivo adenovirus transduction*. A dose of $5\times10^5$ B16F10 cells in 100 $\mu$l of PBS was subcutaneously injected into the right hind flank of C57BL/6 syngenic mice. After palpable tumor formation (a mean diameter of 7 mm), the animals were intratumorally injected with $1\times10^8$ PFU of Ad/IL-12N220L pre-incubated with 50 $\mu$l PBS or 5, 10, 20 $\mu$g/ml of DOTAP in equal volume of PBS. The serum samples were taken 24 h and 48 h after viral injections. At 48 h after viral injection, the mice were sacrificed, and the tumors of sacrificed

animals were disaggregated in PBS with a tissue homogenizer for 1 min on ice. After centrifugation, the supernatant was harvested. IL-12 concentration in the tumor and serum samples was detected by IL-12p70 ELISA kits (R&D System, Minneapolis, MN) according to the manufacturer's instructions. The results are plotted as mean±SEM of three animals/data point.

*Antitumor effects in the in vivo tumor model*. B16F10 cells were seeded at an initial density of $2\times10^6$ in a 100 mm dish and were incubated overnight at 37°C. The cells were infected with adenovirus at a multiplicity of infection (MOI) of 100 in 4 ml serum-free medium at 37°C for 2 h. The culture medium was replaced, and the cells were additionally incubated for 4 h. Infected cells were trypsinized, washed, and injected into C57BL/6 mice subcutaneously. At 24 h after tumor injection, the serum samples were taken and the expression level of IL-12 in the serum was measured by IL-12p70 ELISA kits. Tumor size was measured at least twice in a week with a digital caliper for two-dimensional longest axis (L in mm) and shortest axis (W in mm), and tumor volume was calculated using the following formula: volume in $mm^3=(L\times W^2)/2$. Mice bearing tumors that exceeded 15 mm in two perpendicular diameters or 20 mm in one diameter were sacrificed for ethical reasons according to institutional guidelines.

*Statistical analysis*. We used student's t-test to measure statistical difference between groups. For all cases, differences were considered significant when the p values were $<0.05$.

## Results

*DOTAP increased the transduction efficiency of adenovirus into tumor cells*. To investigate the effect of DOTAP on the efficiency of adenovirus-mediated gene expression, mouse (B16F10) and human (A375) melanoma cells were infected with Ad/EGFP pre-incubated with DOTAP at different concentrations. When the expression level of GFP was analyzed by flow cytometry at 48 hr after infection, DOTAP augmented GFP expression by facilitating the Ad transduction in a dose-dependent manner (Fig. 1). In particular, transgene expression was increased up to by 5 (B16F10), 3.5 (A375)-fold at 10 $\mu$g/ml of DOTAP compared to the virus alone. At optimal concentrations of DOTAP, we did not observe any cytotoxicity under the light

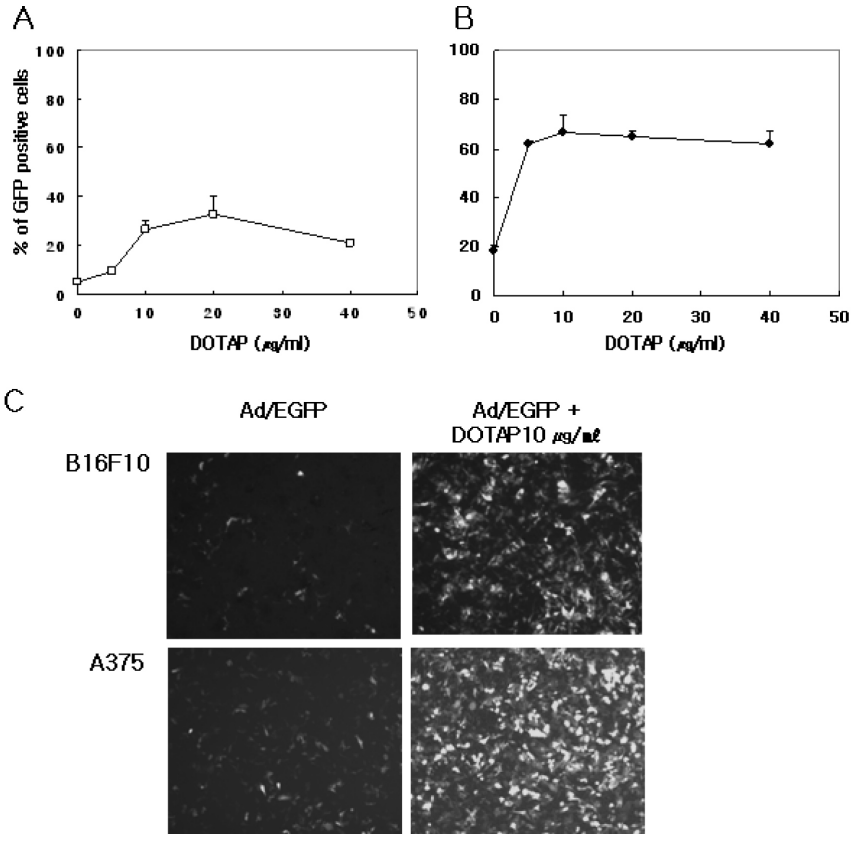


**Figure 1.** Enhanced adenovirus transduction of mouse (B16F10) and human (A375) melanoma cells using DOTAP. Ad/EGFP was admixed with indicated amounts of DOTAP. B16F10 (A) and A375 (B) were respectively transduced at an MOI of 200, 10 for 2 h, incubated for an additional 46 h, and the percentage of GFP expressing cells was determined by FACS analysis. Representative samples (C) were visualized microscopically for GFP under standard excitation/emission parameters. Each data point represents the average of triplicate wells±SEM.

microscopy. Based on the optimal dose DOTAP (10 $\mu$g /ml), the transduction efficiency of Ad with DOTAP was assessed at various doses of Ad (MOI of 10-500). As shown in Fig. 2, DOTAP allowed higher levels of transduction at all doses of Ad tested. These results indicate that the cotreatment of Ad with DOTAP can markedly enhance the transduction efficiency of Ad into melanoma without notable cytotoxicity.

*DOTAP augmented adenovirus transduction into tumors following intratumoral injection*. The effect of DOTAP on *in vivo* gene transfer of Ad in tumors was evaluated using s.c. B16F10 tumors in C57BL/6 mice. Mice bearing tumors were intratumorally injected with $3\times10^8$ pfu Ad/IL-12N220L pre-incubated with or without 5, 10, 20 $\mu$g/ml of DOTAP, respectively. When IL-12 levels in serum and tumor were determined by IL-12 p70 ELISA, DOTAP enhanced *in vivo* IL-12 expression in a dose-dependent manner (Fig. 3). Based on this *in vitro* result, the optimum dose of DOTAP was established at 10 $\mu$g/ml. At this concentration DOTAP improved IL-12 expression by twofold in serum at 24 hr after viral injection, and fivefold in tumor at 48 hr. Collectively, these results demonstrated that DOTAP could augment the transduction efficacy of adenoviral vector in B16F10 melanoma both *in vitro* and *in vivo*.

*Enhanced antitumor effect of Ad/IL-12N220L by cotreatment of DOTAP in the mouse B16F10 tumor model*. We previously found that Ad encoding IL-12N220L (Ad/IL-12N220L), which selectively decreases the secretion of p40 as a natural antagonist of IL-12, showed potent antitumor effects even when compared to that of encoding native IL-12 (24). To test the effect of DOTAP on Ad/IL-12N220L-mediated antitumor activity, B16F10 cells were infected by rAd/IL-12N220L *in vitro* with or without DOTAP and then were subcutaneously injected into syngenic C57BL/6 mice. When the level of IL-12 in serum was determined by ELISA at 24 hrs after tumor injection, the expression level of this transgene increased 40-fold by DOTAP, which is higher than even that by adenovirus alone (Fig. 4A). In addition, mice bearing B16F10 transduced with Ad/IL-12N220L + DOTAP showed the further retardation of tumor growth at a great degree (Fig. 4B, $p<0.05$) and significantly increased the survival rate compared to those with only Ad/IL-12N220L (42.8% versus 21.4%, Fig. 4C). Taken together, cotreatment with DOTAP increased the transduction efficiency of Ad in tumors

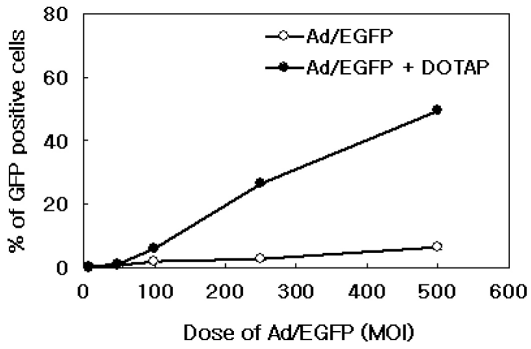


**Figure 2.** Effect of adenovirus dose on DOTAP-mediated adenovirus transduction. Transductions were performed at various doses of Ad/EGFP (10, 50, 100, 250, 500 MOI) complexed with DOTAP (10 $\mu$g/ml) and compared with parallel infections with Ad/EGFP alone. At 48 h the percentage of GFP expressing cells was determined by FACS analysis. Each data point represents the average of triplicate wells±SEM.

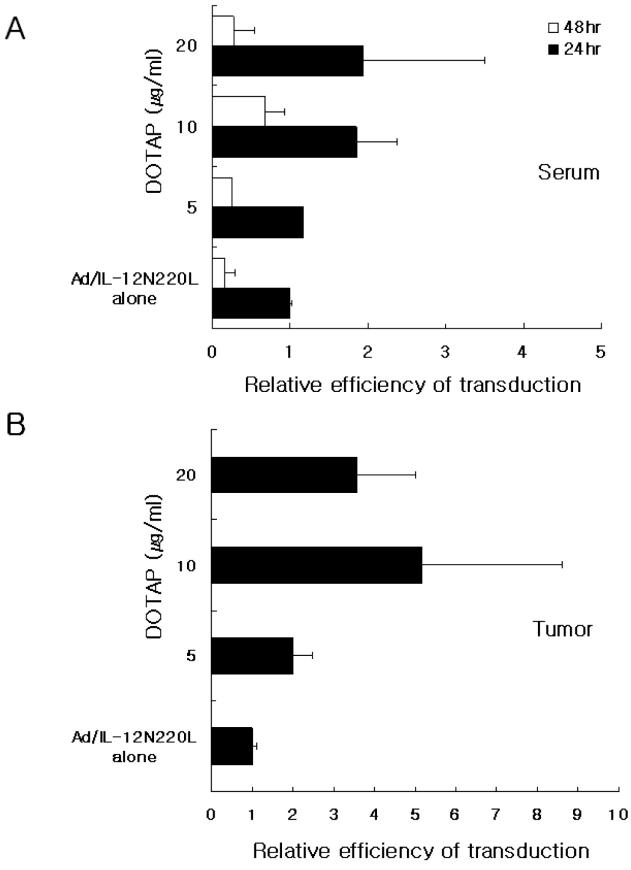


**Figure 3.** *In vivo* IL-12 levels in serum (A) and tumor (B) following intratumoral injection of Ad/IL-12N220L with DOTAP. Recombinant adenovirus encoding IL-12N220L ($3\times10^8$ pfu) with or without DOTAP was injected into B16F10 established tumors. The serum samples were taken 24 and 48 hr after viral injections. Then 48 h after viral injection, the mice were sacrificed and the tumor samples were taken. The results are expressed relative to values with infection with adenovirus alone, and are presented as mean±SEM of three mice.

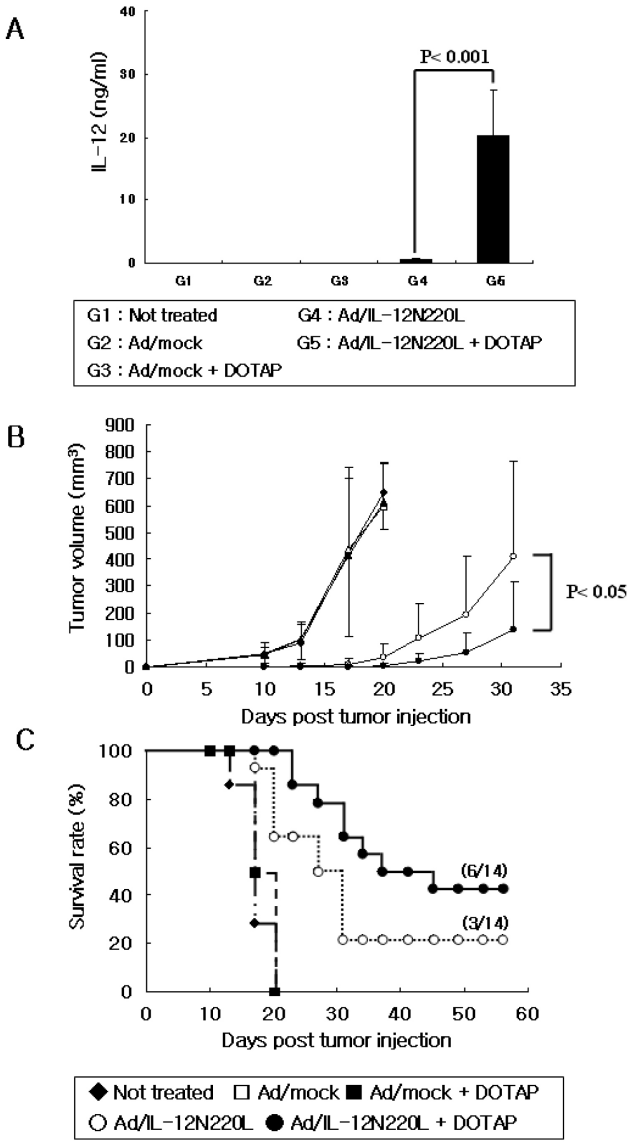


**Figure 4.** The enhanced antitumor effects by cotreatment of DOTAP. B16F10 cells were infected with adenovirus with or without DOTAP 10 $\mu$g/ml at an MOI of 100 for 6 hr *in vitro*. The infected cells were trypsinized, washed, and injected into C57BL/6 mice subcutaneously. At 24 h after tumor injection, the serum samples were taken and the expression level of IL-12 in the serum (A) was measured by ELISA. The tumor volume (B) and survival rate (C) were determined at least twice in a week. The results are presented as mean±SEM of 6∼8 mice, while the p values was determined by students's t-test (two-tailed).

and the higher production of therapeutic molecule (IL-12N220L), leading to the enhanced antitumor effects of Ad-transduced tumor vaccine.

## Discussion

Loss of CAR expression is frequently observed in various cancer cell lines and clinical cancer specimens (7,25,26), hampering efforts to perform efficient adenoviral gene therapy in cancer patients. It was reported that several polycationic liposomes could increase Ad-mediated gene delivery into the cells lacking CAR (10-19). In this paper, we tested one of polycation liposomes, DOTAP in adenovirus delivery into mouse and human melanoma cell lines resistant to Ad infection. We found that the transduction efficiency of Ad pretreated with DOTAP was three to fivefolds higher than that of Ad only (Fig. 1). In addition, DOTAP significantly enhanced the therapeutic gene (IL-12) expression in both tumor and serum after intratumoral injection of adenovirus encoding IL-12N220L (rAd/IL-12N220L) (Fig. 3). It was reported that high dose of Ad could cause adverse effects such as liver injury and induce humoral immune response against Ad which interferes with repeated Ad administration (1,27). Based on our study's results, DOTAP is expected to reduce toxicity and vector immunity in Ad-based cancer gene therapy by enhancing the transduction efficiency and lowering the titer of Ad.

The mechanism for this augmentation is not known. However, one of the possible explanations for this observed phenomenon is that polycationic compounds such as DOTAP serve as electrostatic bridges between the virus and the target cells. Mammalian cells possess significant negative surface charge due to glycosylated phospholipids on cell membrane (28,29), and mammalian viruses also exhibit a net negative charge at a physiologic pH (30). It is therefore possible that the added polycationic compounds during virus adsorption diminish electrostatic repulsion between negatively charged mammalian cells and eukaryotic viruses.

Given these *in vitro* and *in vivo* results, we hypothesized that DOTAP, in conjunction with the adenovirus vector, would improve the therapeutic efficacy of IL-12 gene-based cancer therapy. In this study, we used genetically engineered IL-12N220L which is superior to IL-12 in cancer immunotherapy as proven previously (24). To test whether the increased IL-12 expression by DOTAP could elicit enhanced antitumor effect *in vivo*, we used the *in vitro* engineered tumor model with Ad. Compared to the mice injected with Ad/IL-12N220L-infected cells, those with Ad/IL-12N220L + DOTAP infected cells showed higher IL-12 expression in serum, and further retarded tumor growth rate followed by higher survival rate (Fig. 4). When we tested the

therapeutic effects of Ad/IL-12N220L with DOTAP following intratumoral injection in the established tumor model, we also found that the IL-12 level in serum 24 hr after Ad injection was two to fourfold higher in the DOTAP-treated groups compared with only the virus-treated group. However, Ad/IL-12N220L both with and without DOTAP dramatically retarded the tumor progress, leading to no significant difference in the aspects of tumor progression and survival rate in both groups (data not shown). This differential effect of DOTAP on serum IL-12 level and tumor progression may have resulted from the threshold of IL-12 doses for its biological activity *in vivo*. Even though we could not see the therapeutic advantages of DOTAP cotreatment with Ad/IL-12N220L in our experimental setting, it is notable that DOTAP may significantly reduce the Ad dose requiring same therapeutic effects and it will be also beneficial for decreasing the production of neutralizing antibody against Ad.

In summary, this study provides evidence that adenovirus transduction efficacy can be improved *in vitro* and *in vivo* by the simple addition of DOTAP. DOTAP also enhanced Ad-mediated therapeutic gene (Ad/IL-12N220L) expression and resulted in a dramatic antitumor effect. Therefore, DOTAP could be a powerful tool for enhancing the Ad-mediated therapeutic efficacy of Ad/IL-12N220L in cancer gene therapy. Furthermore, DOTAP may be utilized in other cancer gene therapies such as cytokines, chemokines, and suicide genes, especially in *ex vivo* transduced tumor cell-based cancer therapy.

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