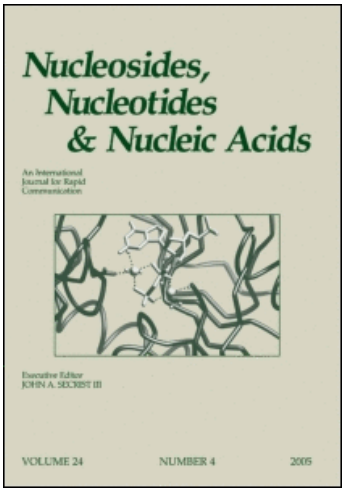

Nucleosides, Nucleotides and Nucleic Acids
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597286

Polyethylelimine Masks Mitogenic Effect of DNA

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To cite this Article Laktionov, Pavel P. , Rykova, Elena Yu. and Vlassov, Valentin V.(1997) 'Polyethylelimine Masks Mitogenic Effect of DNA', Nucleosides, Nucleotides and Nucleic Acids, 16: 10, 1883 — 1891
To link to this Article: DOI: 10.1080/07328319708002541
URL: http://dx.doi.org/10.1080/07328319708002541

NUCLEOSIDES & NUCLEOTIDES, 16(10&11), 1883-1891 (1997)

POLYETHYLENIMINE MASKS MITOGENIC EFFECT OF DNA

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Abstract. The effect of polyethylenimine on the mitogenic stimulation of murine splenocytes by DNA and oligonucleotides has been investigated. It was found that polyethylenimine masks the mitogenic stimulation effect of the compounds.

INTRODUCTION

In order to develop antisense approaches and methods of gene therapy of practical value, it is necessary to elaborate effective procedures for delivery of oligonucleotides and polynucleotides into cells in conditions allowing to eliminate undesirable side effects of these compounds. It is known that bacterial DNA[1] and some oligonucleotides, in particular GC-rich oligonucleotides[2], cause mitogenic stimulation of lymphocytes. The lymphocytes activation stimulates immune reactions which are not always desirable. When antisense oligonucleotides or DNA constructs are used to suppress lymphokines production or arrest multiplication of viruses proliferating in immune cells, the stimulation of lymphocytes proliferation should be avoided.

Polyethylenimine is a versatile vector for delivering nucleic acids into cells in vitro and in vivo[3]. No evidence of acute nor chronic toxicity has been reported when polyethylenimine was administered in animals at doses required for delivery of oligonucleotides and nucleic acids (LD 50 > 4g/kg). The cytotoxicity data obtained

with PEI/DNA complexes demonstrated that the complex has no detectable effect on cells metabolism at concentrations exceeding the concentration required for successful transfection of cells in culture.

We investigated the effect of polyethylenimine on the lymphocyte activation properties of DNA and oligonucleotides and we have found that the complex formation eliminates the mitogenic effect of the compounds.

MATERIALS AND METHODS

The oligomer used in this study was 5'GAAGGGAGGAp synthesized on the ASM-102U synthersizer BioSet (Russia) using phosphoramidite chemistry[4]. The oligonucleotide (ODN) was purified by electrophoresis, suspended in phosphate buffer saline and sterilized by filtration through 0,22 μm membrane filter. Radioactive label was introdused into oligonucleotide by 5'-terminal phosphate exchange. [γ-^{32}P] ATP with specific radioactivity $3 \cdot 10^3$ Ci/mmole BioSan (Russia) was used. pUC19 DNA was purified by method[5] followed by agarose gel electrophoresis. DNA was stored as a solution in phosphate buffer saline and sterilized by filtration. Mice used in the study were 8-week old CBA mice purchased from ICIG (Russia). DNase 1, DPFF grade, was from Worthington Biochemical CO. (USA), concanavalin A (Con A) and lipopolysaccharide E.coli-0111:B4 (LPS) were from Pharmacia (Finland), polyethylenimines (PEI) Mol. Wt. 50.0000 (PEI 50) and Mol. Wt. 800.0000 (PEI 800) were from Fluka (Switzerland), RPMI 1640 was from Flow (Scotland), foetal calf serum was from BioSan (Russia), [^{3}H] thymidine ([^{3}H]T) was from Amersham (England). PEI/polynucleotides particles were prepared according to the protocol described in the paper of Boussif et al [3]. Briefly, for a triplicate experiment, in the case of 2 μg/well dose of DNA, 6 μg of plasmid was dissolved in 30 μl of 150 mM NaCl in an Eppendorf tube, vortexed and quickly collected by centrifugation. In a second Eppendorf tube, 16 μl of PEI stock solution (10 mM amine nitrogen concentration) was mixed with 14 μl of 150 mM NaCl solution, vortexed and collected by centrifugation. The PEI solution was added to the plasmid solution and

mixed vigorously for 10 min. 20 μl of the prepared PEI/DNA solution was added to each cell culture well. The same protocol was used for preparing the complexes of oligonucleotide with PEI using PEI/oligonucleotide ratio 1 μl/μg. To isolate spleen lymphocytes, mice were killed by cervical dislocation, spleen was removed aseptically and the lymphocytes were prepared as described previously[6]. Cells were suspended in RPMI 1640 medium supplemented with 10% foetal calf serum ($2 \cdot 10^6$ cells/ml), dispensed in 180 μl volume in wells of 96-well plate Costar (USA) and cultivated in CO_2 -incubator (37^0C, 5% CO_2). Mitogens, DNA and oligonucleotides dissolved in 20 μl RPMI 1640 medium were added to cells simultaneously. In some experiments DNA was digested with DNAse I (10 ng /ng DNA) for 2 h, 37^0C. 54 hours later the cells were washed three times by centrifugation with the growth medium without mitogens, each well was pulsed with 0.5 μCu of [^{3}H]T and 18 hours later the [^{3}H] incorporation was determined. Cells were harvested by extensive pipetting, transferred to filters and radioactivity was estimated using a scintillation β- counter 1211 RacBeta, Pharmacia (Finland). Cell viability was determined using the trypan-blue exclusion test.

To show that PEI does not prevent oligonucleotides absorbtion by splenocytes, spleen lymphocytes were prepared as described above and incubated with 5μM concentrations of free [^{32}P]-oligonucleotide or oligonucleotide complexed with PEI. Cells were cultivated with oligonucleotides in wells of 12-well plate Costar (USA) (0.25 ml, $2 \cdot 10^6$ cells/ml) 2 hours in serum-free RPMI 1640 medium. After incubation cells were harvested, washed three times with RPMI 1640 medium prewarmed to 37^0C and cell bound radioactivity was estimated using β - counter 1211 RacBeta, Pharmacia (Finland). The oligonucleotide was shown to be undegraded under described conditions.

RESULTS AND DISCUSSION

Plasmid- mediated transfection has a great advantage as compared to the methods based on viral vectors, because it raises none of the problems related to the

biological vectors derived from infections agents. The efficiency of non - viral delivery techniques is steadily improving.

However, serious limitation of plasmid gene-delivery techniques for in vivo application may arise from the recently discovered mitogenic properties of the bacterial DNA which was explained by the high content of nonmethylated GC sequences in these DNA[2]. Bacterial plasmid vectors may display the lymphocyte activating properties, typical of bacterial DNA. This polyclonal activation effect of the plasmid DNA, although being helpful for gene immunization in vivo, is not desirable in other cases where DNA is introduced in organism. When gene transfection and injections of antisense oligonucleotides are used for inhibiting gene expression in the cells of immune system, for treatment of autoimmune disorders or inhibiting lymphocyte-specific viruses, it is necessary to suppress unspecific immune stimulation effects.

Recent studies[7,3] have shown that some cationic lipids and polyethylenimine polymer turned out to be efficient gene - transfection agents. Luciferase reporter gene transfer with the polyethylenimine (PEI) polycation into a variety of cell lines and primary cells occurs with high efficiency with no signs of cytotoxicity. PEI was successfully used for delivery of oligonucleotides into cells[3].

Since polycations and lipopolyamines change the mechanism of interaction of nucleic acids with cells, one could expect that these materials may affect the ability of the mitogenic DNA to trigger cell proliferation. We investigated effect of two variants of polyethylenimine polymers, with Mol. Wt. of 50 and 800 kDa on DNA-activated proliferation of spleen cells. The data shown in Figure 1 evidence that naked plasmid DNA activates [^{3}H] thymidine incorporation in the primary spleen lymphocytes culture at DNA doses, typically needed for successful DNA transfection. Mitogenic effect is efficiently inhibited by DNAse treatment of the preparation before adding to the culture medium, ensuring the activation effect not to be from contaminating LPS (Figure 1).

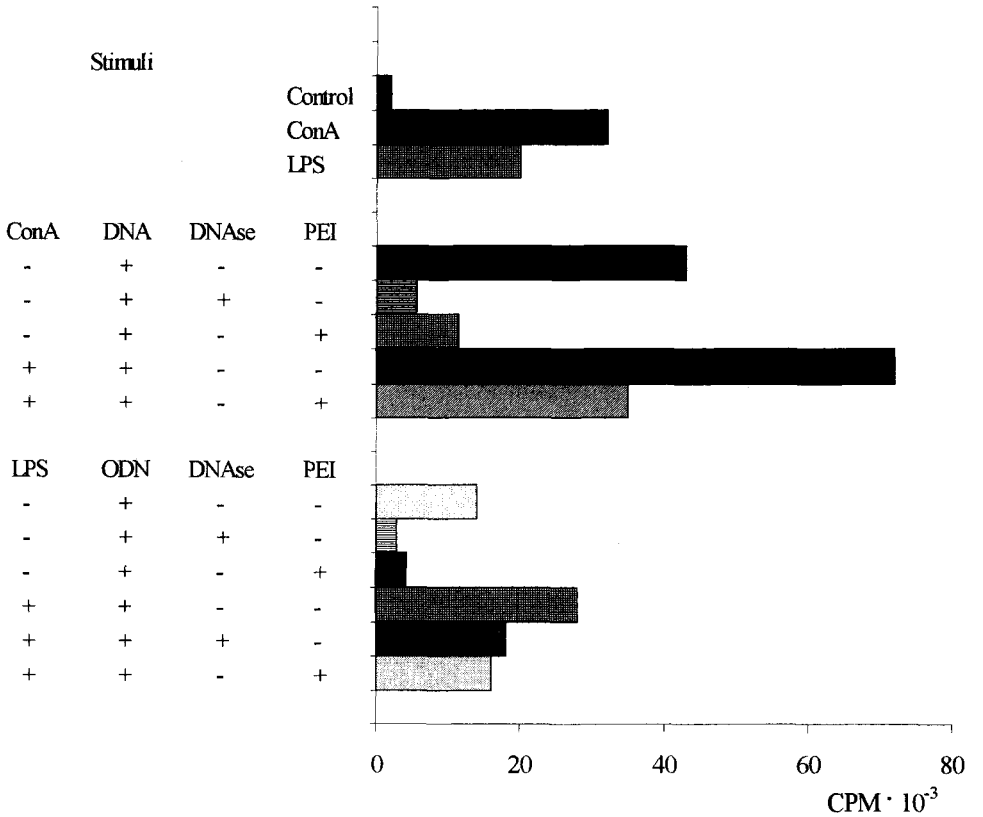

FIGURE 1. Effect of PEI on proliferative response of CBA mice spleen lymphocytes to mitogenic DNA and the oligonucleotide (GAAGGGAGGA)p. CBA spleen cells were cultured for 72 hours in the presence of naked plasmid DNA (0,5 μg/well), oligonucleotide (6 μg/well), or in the presence of the complexes of the compounds with PEI 50. Where indicated, DNA was digested with DNAse. ConA (1μg/ml) and LPS (1μg/ml) were added to the lymphocytes simultaneously with nucleic acids material. The results are the mean ± SD of triplicate cultures and are representative of three separate experiments.

The data shown in the Table 1 evidence, that PEI/DNA particles, prepared according to the standard protocol using both PEI polymers, demonstrate a decreased ability to cause polyclonal activation of the primary spleen lymphocytes as compared to the naked mitogenic DNA. In these conditions the activation effect of the naked plasmid DNA was highest for the most effective transfection doses (2 and 0,5

TABLE 1 Proliferative response of CBA splenocytes to stimulation with mitogenic DNA

	[3H]T incorporation (cpm x10^{-3})		
treatment	control	PEI 50	PEI 800
control	2,1±0,2	2,9±0,2	3,4±0,3
DNA 2μg/well	55,0±4,8	17,5±2,6	13,4±1,9
DNA 0.5μg/well	43,0±5,1	12,2±0,7	10,8±1,5
DNA 0.2μg/well	16,5±2,1	3,8±0,3	4,1±0,5
oligo 2μg/well	12,0±1,9	1,8±0,6	3,3±0,6

CBA spleen cells (1x10^6/ml) were cultured for 72 h in the presence of plasmid DNA, oligonucleotide (GAAGGGAGGA)p or complexes of the compounds with PEI at indicated doses. PEI/DNA complexes were obtained with PEI 50 and PEI 800 as described in Materials and Methods. The results are the mean ± SD of triplicate cultures and are representative of three separate experiments.

μg/well). The same doses of DNA in the form of PEI/DNA particles had 4-fold lower stimulation effect. At lower dose of the DNA (0,2 μg/well) which corresponds to concentrations typically used for transfection, the complete abrogation of the DNA stimulatory influence over spleen lymphocytes (Table 1) was observed.

To ensure the effect of the polycations was not related to cell toxicity, we assayed cell viability for every experimental point. It was found to be 85-90% at the third day of culture growth. Adding of PEI/DNA particles into the lymphocytes culture did not interfere with activities of stimulation agents of different nature : T-cell activator concanavalin A and B-cell mitogen bacterial LPS. As it is seen in the Figure 1, the naked plasmid DNA has an additive stimulation effect with Con A, which is not observed when PEI/DNA complex is used. This fact is in agreement with the data of Messina et al[8] and Krieg et al[2] who postulated a direct T-cell independent

mitogenic effect of bacterial DNA on target B-lymphocytes. The independence of the presence of PEI/DNA complex on the lymphocyte stimulation effect of Con A indicates that PEI/DNA particles do not affect on lymphocyte metabolism.

Costimulatory action of a mitogenic oligonucleotide and LPS is illustrated by the data shown in figure 1. We have identified a mitogenically active phosphodiester oligonucleotide (GAAGGGAGGA)p while screening through a number of G-rich oligonucleotides containing G - triplets. This oligonucleotide has a considerable stimulating effect on [^{3}H] thymidine incorporation by spleen lymphocytes culture in vitro, although the stimulation was lower as compared to the bacterial DNA (Figure 1). The oligonucleotide, PEI/oligonucleotide complex and the DNAse digested oligonucleotides displayed effects on the splenic lymphocytes culture similar to that of the corresponding DNA preparations (data not shown). The mitogenic oligonucleotide and LPS used together cause nearly additive lymphocyte - stimulation effect, while PEI/oligonucleotide complex and LPS have stimulating effect equal to that of the LPS alone. From these data it follows also that the PEI/oligonucleotide complex do not interfere with the LPS activation signal to increase splenic lymphocytes proliferation.

It was shown that oligonucleotides and DNA interact with serum proteins and that electrostatic interactions play an important role in the complex formation[9]. The data of interactions of oligonucleotides with cell surface proteins[10,11] propose the interactions of oligonucleotides with cellular receptors as possible mechanism of action of oligonucleotides to the cell. Yamamoto supposed that oligonucleotides containing palindromic sequences activates spleen cells after penetration into the cells, because lipofection of synthetic oligodeoxyribonucleotide to murine splenocytes with lipofectin enhanced interferon production and natural killer activity[12]. It should be noted that N-[1- (2,3-dioleyloxy) propyl] - N, N, N - trimethylammonium chloride (lipofectin) can not only enhance the transport of oligonucleotides into cells, but change its hydrophobic properties promoting interactions with cell membranes and, probably, membrane proteins. PEI as a highly branched polymer with highest cationic charge density potential can ensnare DNA and, obviously, prevent the electrostatic

interactions of DNA with proteins. It was shown that DNA is liberated from the DNA/PEI complex only after the complex is taken up by cells[3]. Our data demonstrate that PEI/oligonucleotide and PEI/DNA complexes have no mitogenic effect. This effect of PEI is not originated from cytotoxicity of the compound or some inherent lymphocyte proliferation inhibiting property of PEI polymers. It was shown that PEI do not prevent cellular uptake of oligonucleotide by splenocytes. The experiments with [^{32}P]-oligonucleotide demonstrated that spleen lymphocytes absorb free oligonucleotides 1.5-2 times less as regards to oligonucleotides bound with PEI polymer. As far as PEI - complexed DNA cannot efficiently interact with specific proteins and DNA is liberated from the DNA/PEI complex into the cells, we can propose that the mitogenic activation of lymphocytes by naked plasmid DNA is induced by the interaction of nucleic acids with some cell surface receptors responsible for triggering processes. Our data of PEI inhibiting mitogenic effect of DNA, indicate the possibility of receptor - triggered mechanism of stimulation of spleen cell proliferation by plasmid DNA as well as G-rich oligonucleotides.

The obtained data indicate, that polyethylenimine polymer has an additional advantage as a vector for gene therapy, because it can prevent undesirable mitogenic properties of mitogenic DNA.

ACKNOWLEDGEMENT: This work was supported by INTAS grant 94-3214.

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Received January 15, 1997
Accepted June 11, 1997