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Combination of MIDGE-Th1 DNA vaccines with the cationic lipid SAINT-18: Studies on formulation, biodistribution and vector clearance

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ABSTRACT

We have previously shown that the combination of MIDGE-Th1 DNA vectors with the cationic lipid SAINT-18 increases the immune response to the encoded antigen in mice. Here, we report on experiments to further optimize and characterize this approach. We evaluated different formulations of MIDGE-Th1 vectors with SAINT-18 by assessing their influence on the transfection efficiency in cell culture and on the immune response in mice. We found that high amounts of SAINT-18 in formulations with a w/w ratio MIDGE Th1/SAINT-18 of 1:4.8 are beneficial for cell transfection *in vitro*. In contrast, the formulation of HBsAg-encoding MIDGE-Th1 DNA vectors with the lowest amount of SAINT-18 (w/w ratio MIDGE Th1/SAINT-18 of 1:0.5) resulted in the highest serum IgG1 and IgG2a levels after intradermal immunization of mice. Consequently, latter formulation was selected for a comparative biodistribution study in rats. Following intradermal administration of both naked and formulated MIDGE-Th1 DNA, the vectors localized primarily at the site of injection. Vector DNA levels decreased substantially over the two months duration of the study. When administered in combination with SAINT-18, the vectors were found in significantly higher amounts in draining lymph nodes in comparison to administration of naked MIDGE-Th1 DNA. We propose that the high immune responses induced by MIDGE-Th1/SAINT-18 lipoplexes are mediated by enhanced transfection of cells *in vivo*, resulting in stronger antigen expression and presentation. Importantly, the combination of MIDGE-Th1 vectors with SAINT-18 was well tolerated in mice and rats and is expected to be safe in human clinical applications.

1. Introduction

DNA vaccines have been described to induce both relevant cell-mediated and humoral immune responses against numerous viral, bacterial and parasitic pathogens in a number of animal models [1,2] but less so in clinical trials. Their success has been hampered mainly by low immunogenicity in humans [3]. In order to improve the immunogenicity of DNA vaccines, a variety of strategies is being applied, primarily addressing delivery systems to increase the DNA uptake into target cells by physical [4,5] or chemical methods [6]. Complexes of DNA with cationic lipids or polymers have been reported to improve immune responses by facilitating the transfer of DNA vectors across membranes and protecting the DNA from degradation by nucleases [7].

We have developed DNA vectors exclusively comprising the expression cassette and having a reduced size in comparison to plasmids, the Minimalistic Immunogenically Defined Gene Expression (MIDGE) vectors [8]. MIDGE-Th1 vectors are linear double-stranded DNA molecules, which are closed with single-stranded hairpin loops at both ends and contain a peptide nuclear localization sequence covalently bound to one of the loops. Immunization with MIDGE-Th1 vectors induced strong humoral and cellular immune responses [9–11] which were significantly increased by formulation with the cationic lipid SAINT-18 [12]. A SAINT-18 molecule contains two hydrophobic tails and one cationic pyridinium head group and builds complexes with the negatively

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charged DNA [13]. These complexes, called lipoplexes, allow cell surface adsorption and subsequent transfection of cells.

It has been established that plasmid vectors primarily localize at the site of injection after intramuscular [14–17] or intradermal (i.d.) injection [15,18]. For some cationic lipids and polymers, the impact on the biodistribution of plasmid vectors has also been described [19–22]. However, the influence of SAINT-18 on the biodistribution of DNA vectors has not been reported so far.

Here, we characterize different formulations of MIDGE-Th1 vectors with SAINT-18, describe their transfection efficiency in cell culture and their immunogenicity in mice. We selected the most immunogenic formulation and compared biodistribution and clearance of formulated to naked MIDGE-Th1 vectors after i.d. administration to rats as an essential step toward clinical testing.

2. Materials and methods

2.1. Synthesis of MIDGE-Th1 vectors

The synthesis of MIDGE-HBsAg-Th1 vectors (1878 bp) encoding the HBsAg coding sequence, subtype *ayw*, has been described previously [10]. MIDGE-synSHBsAg-Th1 vectors (1881 bp) were produced in the same manner using the HBsAg coding sequence, subtype *adw2* (GenBank: [23]), which was optimized for human codon usage by GeneArt (Regensburg, Germany). For transfection of cells, MIDGE-eGFP-Th1 vectors (1943 bp) encoding enhanced Green Fluorescent Protein (GenBank:) were prepared.

2.2. Determination of transfection efficiency

Transfection of cells with DNA/SAINT-18 lipoplexes has been described before [13]. Here, CHO-K1 cells (ATCC CCL-61) were grown in complete Glasgow's Minimum Essential Medium (GMEM) containing 5% (v/v) fetal bovine serum (FBS), 60 μg/μl gentamicin and 2 mM L-glutamine at 37 °C in 5% CO_2. Prior to transfection, cells were detached and suspended in GMEM w/o FBS. Formulations of 10 μg MIDGE-eGFP-Th1 vectors with 1, 2.5, 5, and 10 μl of 7.5 mM SAINT-18 (1-methyl-4-(cis-9-dioleyl) methyl-pyridinium-chloride, Synvolux Therapeutics B.V.) were prepared in water resembling w/w ratios MIDGE-Th1/SAINT-18 of 1:0.5–1:4.8. N/P ratios were defined as the charge ratio between cationic nitrogen residues in SAINT-18 and anionic phosphate groups in the DNA. N/P ratios were calculated assuming that 645 g/mol correspond to each positive nitrogen residue containing SAINT-18 molecule and 330 g/mol corresponds to the average mass of a nucleotide bearing one negative phosphate group. MIDGE-Th1/SAINT-18 lipoplexes were incubated for 15 min, before adding 1.5×10^6 CHO-K1 cells suspended in GMEM w/o FBS. As negative controls, cells were not transfected (Ctrl.) or mock-transfected with SAINT-18 only. Transfections were performed in triplicates. After 2.5 h, about 9×10^4 cells were seeded on 12-well plates in complete GMEM. After 48 h, the transfection efficiency was determined by analysis of eGFP expression of 5×10^3 cells using flow cytometry (Guava EasyCyte, Merck Millipore, Billerica, USA). Cells were harvested and mixed with 1% (v/v) propidium iodide to stain dead cells. The transfection efficiency was indicated as percentage of eGFP-expressing cells of the gated living population and mean fluorescent intensity (MFI) of the gated living population was measured.

2.3. Particle size and zeta-potential measurement

Particle size and zeta-potential of lipoplexes were measured by laser light scattering using a Nicomp submicron particle analyzer (Nicomp 380/ZLS, Santa Barbara, USA). The mean diameter was obtained from the volume distribution curves produced by the particle analyzer. For zeta-potential measurements, lipoplexes were diluted with water.

2.4. Mouse immunogenicity study

Female BALB/c mice, 81–82 days of age at the first immunization, were supplied by Charles River Laboratories (Sulzfeld, Germany). All mice were maintained according to Good Laboratory Practice (GLP) regulations at LPT (Laboratory of Pharmacology and Toxicology, Hamburg, Germany). Mice ($n = 6$ per group, allocated by means of computer generated randomization program) were immunized i.d. at the dorsal tail base with formulations of 10 μg MIDGE-HBsAg-Th1 vectors (5 μl of DNA concentrated at $c = 2$ mg/ml or 2.5 μl of DNA concentrated at 4 mg/ml) with 1, 2.5, 5, and 10 μl of 7.5 mM SAINT-18 in water (total injection volume for formulations prepared with DNA concentrated at 2 mg/ml: 6, 7.5, and 10 μl; total injection volume for formulations prepared with DNA concentrated at 4 mg/ml: 3.5, 5, 7.5 and 12.5 μl) on test days 1 and 21. On test day 35, sera were obtained and HBsAg specific IgG1 and IgG2a antibodies were determined as previously described [12] using HBsAg coated ELISA plates (Enzygnost Anti-HBs II, Dade Behring, Marburg, Germany). HRP-conjugated Rat-anti-mouse IgG1 and IgG2a (559626 and 553391, BD, Heidelberg, Germany), diluted 1:3000 and 1:1000 respectively, served as detection antibodies. Mouse-anti-HBsAg IgG1 and IgG2a (MA1-19263 and MA1-19264, Affinity BioReagents, Rockford, USA) were used as standard. The detection limit was 39 pg/ml for IgG1 and IgG2a.

2.5. Rat biodistribution study

Female and male Wistar rats, 58–61 days (male) or 72–76 days (female) of age at the first administration, respectively, were supplied by Charles River Laboratories. Rats were maintained according to GLP regulations at LPT. Rats ($n = 30$ per group equally divided per sex and allocated by means of computer generated randomization program) were injected i.d. at the dorsal tail base with 83 μg MIDGE-synSHBsAg-Th1 vectors ($c = 2$ mg/ml; $\sim 4.3 \times 10^{13}$ copies) formulated either with 8.3 μl of 7.5 mM SAINT-18 (w/w ratio of 1:0.5) or with 8.3 μl water (total injection volume: 50 μl). This was the maximum feasible dose based on the maximum volume that can be injected i.d. into rats (50 μl). 24 h, 14 days and 60 days after single administration, 10 animals per group (equally divided per sex) were sacrificed after blood collection from the retrobulbar venous plexus. The following tissues were harvested and stored at −80 °C until quantitative real-time PCR (qPCR) analysis: inguinal lymph nodes (LNI), spleen, mesenteric lymph nodes (LNM), liver, kidney, heart, lung, muscle (left femur), ovaries, testes, axillary lymph nodes (LNA), brain, skin at the injection site and bone marrow (left femur). Special care was taken during preparation of the tissues to avoid cross contamination. For each tissue, a clean set of instruments that had been disinfected with 70% Ethanol, immersed with DNA*ZapTM* (Ambion, Austin, USA) and rinsed with sterile water, was used.

2.6. Extraction of total DNA and qPCR assay

Total DNA (containing host cell genomic DNA and MIDGE-Th1 vectors) was extracted from 10 to 20 mg aliquots of frozen tissues, from 250 μl blood and 250 μl bone marrow using the KingFisher Cell and Tissue DNA Kit and the KingFisher Blood DNA kit, respectively (Thermo Fisher Scientific, Vantaa, Finland). The DNA concentration was determined using DropSense96 (Trinean, Gentbrugge, Belgium). The qPCR primer/probe set was designed to specifically detect and amplify a 77 bp DNA sequence of the MIDGE-Th1 vector. The forward primer (5′-GTCGTTTAGTGAA CCGTCAGATCA) anneals to the CMV-promoter region whereas the