Plasmid DNA production for gene therapy and therapeutic or preventive vaccination in humans: a challenge for the pharmaceutical industry

REVIEW

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ABSTRACT

The field of DNA vaccine technology has experienced an increasing development during the last 10 years, being an attractive, cost effective and simple choice for researchers. Here, we focus on the relevant principles for designing an efficient pharmaceutical-grade plasmid DNA manufacturing process for human use. Plasmid DNA constructs pIDKE2S (coding for the E2 protein of the hepatitis C virus), $pVEGF_{121}$ (coding for the human vascular endothelial growth factor 121) and the pCGV (coding for Gumboro's disease virus antigens as DNA immunogen in poultry) were analysed as study cases.

Key words: DNA vaccine, plasmid, preparative purification

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RESUMEN

Producción de ADN plasmídico para la terapia génica y vacunas terapéuticas o preventivas en seres humanos: un reto para la industria farmacéutica. En los últimos 10 años, la tecnología de la vacunación con ADN se ha desarrollado de manera vertiginosa, debido a que muchos investigadores la consideran una opción atractiva, sobre todo por ser relativamente sencilla y económica. En este trabajo se discuten los aspectos que pudieran ayudar al diseño de un proceso de producción eficiente de ADN plasmídico con calidad farmacéutica para su empleo en seres humanos. En el estudio se emplearon el plasmidio pIDKE2S (codificante para la proteína E2 del virus de la hepatitis C), el pADN-VEGF (codificante para el factor de crecimiento del endotelio vascular) y el pCGV (codificante para la proteína gumboro para la profilaxis de la enfermedad de Gumboro) en forma de vacuna de ADN.

Palabras clave: vacuna de ADN, plasmidio, purificación a gran escala

Introduction

Gene therapy development in the late 60´s and the finding demonstrating successful direct transfection of animal cells with plasmid DNA *in vivo* [1] in the early 90´s, constituted starting points for a new technology: naked DNA immunization.

It is currently an expanding field, with preclinical studies being conducted in animal models for obtaining humoral and cellular protective immune responses against diseases of viral [2-6], bacterial [7], tumoral [8-10] or parasitic [11] origin, with particular emphasis on inducing cytotoxic CD8+ T lymphocytes (CTL) [2, 12, 13].

Here, we discuss the main issues in the challenge of producing plasmid DNA according to the regulatory authorities' recommendations [14] for this type of vaccine.

Certain characteristics of plasmid DNA-based therapy and vaccination

The DNA immunization technology is attractive compared to other vaccine technologies, having the following potencial advantages:

· These vaccines are immunologically similar to live-attenuated vaccines, but they lack infection risk.

· They can be combined with other immunogens in the same preparation, thus generating multivalent immunity against several disease entities, or against different antigens of the same entity.

· They show greater genetic and thermal stability than protein vaccines.

· New vaccines with a well established genetic identity can be produced in a shorter time.

· Vaccines can be developed against etiological agents that cannot be propagated in cultures.

· It is possible to generate long-lasting and helper CD4+ T lymphocyte-mediated immune responses.

· The immune responses can be modulated by relatively simple procedures.

· Immunity is induced despite the presence of maternal antibodies.

Plasmid DNA purification

The plasmid DNA purification technique at the laboratory level allows us to obtain DNA for experiments such as sequencing, gene mapping and others, equally sensitive and with genetic material purity as its main limitation. It mainly comprises several procedures involving operations such as ul-

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tracentrifugation in CsCl gradients [15] and liquid-liquid extraction, concentration, or precipitating with organic solvents as a means of concentration or purification [16]. These methods based on polyethylene glycol precipitation are difficult to scale up, including open operations that are not considered sanitary, although they have been reported effective for DNA immunization studies in animals [17].

Therefore, some of the main pharmaceutical and biotechnological companies around the world such as Vical Inc. (USA), Wyeth-Lederle (formerly Apollon Inc., USA) and Therexsys Ltd. (UK), and independent groups from colleges such as the *Centre for Bioprocess Engineering* (University of Birmingham, UK), have worked in establishing technological processes for the high-scale production of plasmid DNA for therapeutic use or as vaccines in humans.

To meet potential market demands of a given DNA vaccine, a large-scale purification process has to be reproducible, fulfilling all requirements of the regulatory agencies [14] and assuring an estimated yield ranging from 10 to 500 mg per dose, with doses of up to 5 mg used in humans occasionally [18, 19].

Requirements for vectors employed for DNA therapy and immunization

It is strictly necessary that the plasmid vector to be used for immunization must have been sequenced and characterized by restriction analysis and electrophoresis.

Vectors developed in our laboratory for DNA immunization in humans meet all the requirements for these applications; that is to say, they show a high copy number in *Escherichia coli*; they carry the human cytomegalovirus immediate early promoter (hCMVI/E), the most widely used promoter due to its strength, promiscuity and low homology with mammalian sequences; they are compact enough, lacking sequences that are not essential for their function, therefore, minimizing the possible homology with the host. Moreover, they only carry the *E. coli* plasmid replication origin and the kanamycin resistance gene as the selection marker in bacteria.

Quality specifications of the product

The plasmid DNA purification process for vaccine application in humans has to be designed so that the resulting plasmid fulfills the quality standards established for this type of product. Purity, identity, efficacy, potency and safety are the fundamental aspects.

As for other products, potency and safety are related to purity, due to the undesired or non specific effects induced by contaminants present in the product.

While some of these requirements can not be assumed, such as dose and administration route for plasmid DNA, there are regulations that must be observed and fulfilled [14].

Biological activity assays should be carried out to compare the product of the gene produced by the expression vector with the natural product *in vitro*. If possible, an animal model for *in vivo* testing should be available.

Contaminant studies have to be focused on the presence of pyrogens, protein traces, cellular DNA and RNA molecules, microbial contamination and others [14].

Bacterial host strains

There is no consensus on the most convenient genotypic or phenotypic characteristics of bacterial strains for the large-scale production of plasmid DNA.

The selection criteria for *E.coli* strains as hosts for producing plasmids are based, among others, on sustained plasmid stability, its high cellular density, a low probability for promoting genetic modification of the plasmid molecule and its compatibility with further purification steps. Among the most widely reported *E.coli* K-12 strains in use are: TG 1, XL-1 *Blue*, JM109, DH5a and DH10 B (GIBCO BRL).

In our lab, the best results have been obtained with the *E. coli* DH 10B strain, grown in *Terrific Broth* (TB) medium. Other authors have reported that, by optimizing this step involving the host cell, the plasmid and the fermentation medium, up to 50 mg of plasmid DNA per liter of culture can be obtained [20, 21].

Cell bank systems

Establishing cell banks, both primary and working cell banks, is one of the critical steps for plasmid DNA production. The procedure is well documented, and not exclusively used for this type of production [22].

Primary and working cell banks are microbiologically-pure cultures, entirely composed of host cells transformed with the given plasmid. These cultures must be characterized to exhaustion, and more precisely, on their viability, purity, plasmid stability, restriction and target gene. Kinetic properties as specific growth speed and duplication time must be measured. Cell banks are kept at -70 ºC or in liquid nitrogen, by using glycerol or DMSO as cryopreservatives.

Certain me considerations on the main steps of the production process

The following constructs were employed as models to study the main steps of the production process:

· pIDKE2S: carrying the gene coding for the E2 protein of the hepatitis C virus (HCV);

· $pVEGF_{121}$: carrying the gene coding for the human vascular endothelial growth factor isoform 121;

· pCGV: carrying the gene coding for Gumboro's disease virus antigens as DNA immunogen in poultry.

A general process for plasmid production is depicted in the diagram of figure 1.

The pIDKE2S (4,558 bp) [23] is a vector for DNA immunization against the E2 protein of the HCV (aa. 384-717 of the viral protein from a Cuban HCV isolate). At present, there are more than 170 million people infected with the HCV around the world, and there is no vaccine available against this pathogen [24]. In particular, the viral E2 protein is an attractive vaccine candidate, carrying several epitopes for humoral and cellular immune responses and the putative viral region interacting with the cellular receptor for the virus. The sequence coding for the E2 protein in the pIDKE2S construct is under the transcriptional control

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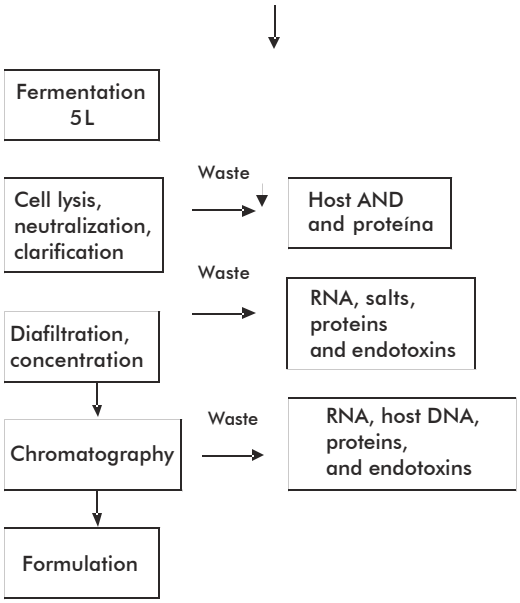

Figure 1. High-scale plasmid DNA' purification process.

of the hCMVI/E promoter and transcription termination/ mRNA polyadenylation signals from the simian vacuolating virus 40 (SV40). It also comprises a plasmid replication origin (ori), functional in *E. coli*, and the kanamycin resistance gene.

On the other hand, the $pVEGF_{121}$ construct expressing the human vascular endothelial growth factor 121 is employed in gene therapy for treating ischemic cardiopathy and hind limb ischemia that are considered health problems worldwide [25]. This protein displays a pro-angiogenic activity. The $pVEGF_{121}$ vector (4 039 bp) carries the $VEGF_{121}$ gene under the same transcriptional regulatory sequences as in the pIDKE2S construct, having the same plasmid backbone sequences.

The third model construct employed was the pCGV plasmid (3 600 bp), for prophylactic DNA immunization against Gumboro´s disease. This disease causing immunosuppression in poultry populations, is widely distributed and highly important in poultry farming. The pCGV plasmid carries the transcriptional control elements for their expression in mammalian cells as described for the pIDKE2S and the pVEGF121 constructs, also carrying the plasmid replication origin from the pBR322 vector, the ampicillin resistance gene and the *Nco* I-*Sal* I fragment of 688 bp from the pGC plasmid inserted into the *Xba* I- *Sal* I of the pAEC-Δ2 (kindly donated by A. Herrera; CIGB, Havana, 1999).

For producing plasmids pIDKE2S and $pVEGF_{121}$, 5L of culture medium were fermented during 6 h. At the beginning of the logarithmic phase, the growth temperature was shifted from 37 ºC to 42 ºC, producing an increase in plasmid yields. This is based on a G to A mutation present on the replication origin of pUC plasmids [26], generating conformational changes in the RNA II molecule that limits its attachment to the RNA I. The formation of the RNAI-RNA II complex restricts the star of plasmid replication [27]. Thus, this mutation circumvents the negative copy number regulation inside the cell, increasing the number of plasmid molecules at 42 °C and even more at 45 ºC [27].

Fermentation conditions for cells transformed with the pCGV construct were: 5L of culture medium, shaking 100 rpm, 0.5 vvm, pH 8.0, 37 ºC for 6 h to a final $O.D._{530\ nm}$ of 10. pH was controlled by adding H_3PO_4 40% and NaOH [40].

Cell lysis, primary processing and concentration

Cells were lysed by alkaline lysis procedures and completely homogenized by using a blade shaker, to avoid viscosity resulting from adding the neutralization solution (NaOH 0.2 M, SDS 1%). Moreover, pH was kept below 13 to avoid DNA hydrolysis and to reduce operation time. In spite of these limitations, this is a germ-free, scalable and completely affordable operation [21-23]. pH control is critical in this process, since pH values above 12.5 can irreversibly denature plasmid molecules as previously reported [21, 24]. After lysis, neutralization was achieved by adding potassium acetate and acetic acid at a 1.6 M concentration and pH of 5, followed by filtration through Miracloth membranes (CALBIOCHEM, Germany) for removing denatured host genomic DNA and cell debris. This step is alternative to centrifugation, saving time and preventing plasmid degradation by the host nucleases found in the cell lysate. Clarified lysates were concentrated and diafiltrated through hollow fiber cartridges of 0.1 µm, enabling a gross elimination of RNA molecules [26] as the major contaminants present in the preparation, as well as proteins, endotoxins and salts.

Final purification and formulation

For obtaining pure plasmid DNA, a Q-Sepharose anion exchange chromatography was conducted (Amersham Pharmacia Biotech, USA) for resolving proteins and nuclease contaminants. By adding salts to the equilibrated solution, such as NaCl at a 0.7 M concentration, non-specific attachment of contaminants to the gel matrix were reduced and furtherly washed off with the same salt solution. This favored the attachment of plasmid DNA molecules, being eluted at 1M NaCl in a TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0).

Immediately afterwards, a gel filtration chromatography through a 400 nm poresized Sephacryl S-1 000 gel matrix (Amersham Pharmacia Biotech, Sweden) was carried out, equilibrated with a 0.9% NaCl solution. This is a time consuming, limited capacity operation, since the sample volume applied to the column has to be lower than 10% of the matrix volume to achieve and adequate resolution. In spite of these limitations, a recovery of up to 50% can be achieved with this type of chromatography technique, wich is also free from pyrogens, proteins, chromosomal DNA and RNA traces. Plasmid DNA was obtained at optimal concentrations for the dose to be applied (0.5 to 1 mg/mL) in gene therapy and immunization studies. Besides, DNA obtained after size exclusion chromatography was mostly supercoiled in structure, over 90%, and showing purity standards for human administration and also as a vaccine, as reported for this type of product [23].

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Quality control

The production process established has to be reproducible and must fully comply with current quality specifications. It is generally stated that manufacturing high quality biological products does not depend on certifying their quality, but on validating the process for producing them.

Methods have to be available for controlling the master and working cell banks of bacterial host strains, according to their identity, genotype, phenotype, duplication time and morphology parameters, metabolic properties and checking plasmid DNA identity by restriction and sequencing. All of these assure the quality and consistency of the final product [14, 20].

Analytical methods have to be developed for addressing the molecular identity, purity, sterility and potency of plasmid DNA at the end of the process.

The identity of the plasmid under study was assessed by restriction analysis and sequencing (data not shown). DNA concentration was determined by spectrophotometry according to absorbance at 260 nm. Moreover, purity considered as the relative DNA/protein content was estimated by the same method, and expressed as the ratio of absorbance at 260 nm and 280 nm respectively (A_{260}/A_{280}), with a value of 1.8 as expected [20, 22, 29-33].

Other contaminants such as genomic DNA and RNA were analyzed by electrophoresis in agarose 0.8% gels. Protein concentration was determined by the bicinchoninic acid assay [35].

The amount of final plasmid DNA in a supercoiled conformation is a relevant issue. Purified lots were analysed by agarose gel electrophoresis, denoting over 90% of plasmid DNA in a supercoiled conformation (Figure 2). This is related to the potency of the product, since linear topology isoforms are more susceptible to degradation by nucleases *in vitro* [36-38]. Up to now, several reports indicate a similar behavior between both topological conformations *in vivo*. According to these studies, final DNA fulfills all the quality specifications established for the product (Table 1).

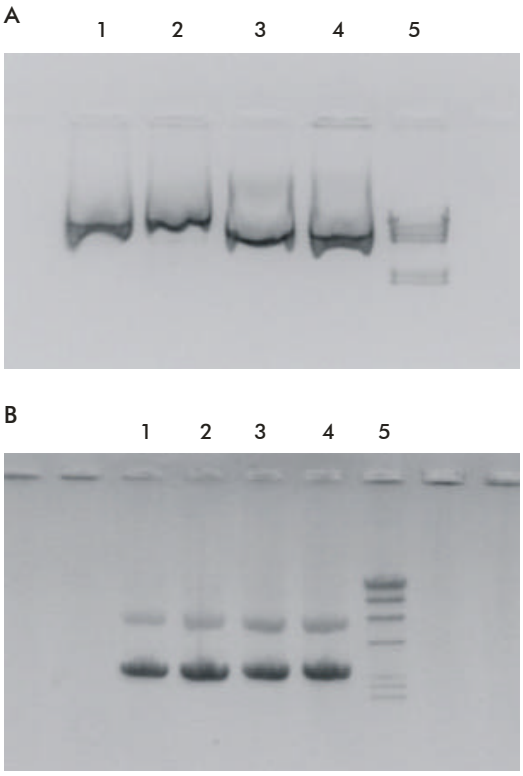

Figure 2. A: Electrophoresis of pure final lots in 0.8 % agarose gels. Lanes 1 and 2: 1 μg of pIDKE2S, lots 1 and 2, respectively; lanes 3 and 4: 1 μg of $pVEGF_{121}$, lots 1 and 2, respectively; lane 5: Lambda *Hind* III DNA molecular weight marker. B: Electrophoresis of pure pCGV final lots in 0.8 % agarose gels. Lanes 1 to 4: 1 μg of plasmid DNA, lots 1 to 4, respectively; lane 5: Lambda *Hind* III DNA molecular weight marker.

Conclusions

Today, the efficient high-scale production of pharmaceutical grade plasmid DNA constitutes an engineering task demanding experience and a high technical level. However, it is possible to establish a cost-effective technology, giving suitable and reproducible results and complying with regulatory agencies requirements.

An average yield of 120 mg of plasmid DNA per lot was obtained, free of unspecific contaminants.

The process established for obtaining plasmid DNA for therapy and vaccination fulfills the requirements established for this kind of product (Table 1).

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Table 1. Average results from four lost of plasmids.

Steps	pVEGF	pIDKE2S	pCGV
Recovery	150±0.12 mg/lot	110±0.11 mg/lot	123±0.12 mg/lot
Purity	≥ 90% supercoiled DNA	≥ 90% supercoiled DNA	≥ 90% supercoiled DNA
Protein	Not detected	Not detected	Not detected
Pyrogen	Satisfactory	Satisfactory	Satisfactory
Sterility	Bacterial colony growth not detected	Bacterial colony growth not detected	Bacterial colony growth not detected
Chromosomal DNA	Not detected	Not detected	Not detected
Identity	4 039 pb	4 558 pb	3 600 pb
Organic solvent traces (Absolute ethanol)	40 ppm	40 ppm	40 ppm
Organoleptic properties	Particles not observed in the sample	Particles not observed in the sample	Particles not observed in the sample
pH	6.6	6.6	6.6

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