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Recombinant Technology

Spermine compaction is an efficient and economical method of producing vaccination-grade DNA

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Abstract

Plasmid DNA inoculations can induce both humoral and cellular immunity, and this technique is now being employed in developing vaccination regimens for a large number of applications. DNA vaccination studies require the preparation of large amounts of purified plasmid DNA with low endotoxin contamination, and the cost burden for multiple injections, multiple animal or large animal studies is significant. We recently reported that selective compaction with spermine can be used to purify large quantities of DNA. We wanted to determine whether this method would produce DNA suitable for vaccination. Endotoxin levels for spermine-compacted DNA were 0.3 ± 0.01 endotoxin units (EU)/μg, well within the accepted range (less than 3 EU/μg) for in vivo use. When injected intramuscularly into mice, column-purified and spermine-compacted DNA induced an equivalent antigen-specific CD8+ T-cell response. The labor and time involved in purifying 5 mg of DNA by each method were similar, but the cost of spermine-compacted DNA was only 20% of the cost of column-purified DNA. We conclude that spermine compaction is an efficient and economical method for preparing vaccination-grade DNA.

Keywords: Plasmid DNA; Vaccination; Purification; Cytotoxic lymphocytes

1. Introduction

The past decade has seen an explosion in the application of plasmid DNA immunization. Plasmid DNA has been used to induce antibody and cellular responses to a variety of viral, bacterial and protozoan antigens in several species (e.g. fish, ferrets, dogs, cats, mice, cattle, fowl and primates including humans) (Anderson et al., 1996; Kowalczyk and Ertl, 1999). The prolific use of DNA vaccines can be largely attributed to the relative ease with which recombinant DNA can be manipulated and the simplicity of its administration. Additionally, plasmid DNA offers several advantages over other classical vaccines such as purified protein(s) or viral vectors. Unlike purified protein vaccines, plasmid-encoded antigens are expressed endogenously and thus efficiently induce cytotoxic T cells (CTL) (Ulmer et al., 1993). Recombinant viral vaccine vectors can also induce strong CTL;

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however, the response to the recombinant antigen is often low within the hierarchy of responses to other viral antigens (Yewdell and Bennink, 1999). The immunodominance of irrelevant viral antigens can be overcome by first priming with DNA that expresses the desired recombinant antigen (Schirmbeck et al., 1995). The DNA prime/recombinant virus boost strategy has been successfully employed to induce CTL in many vaccine model systems including the SIV rhesus model (Hanke et al., 1999). However, large amounts of DNA (3–5 mg per animal) are often needed to induce robust SIV-specific CTL responses (Lu et al., 1996; Egan et al., 2000). Large quantities of single plasmids are also needed for veterinary large animal studies, and for comparisons of large treatment group numbers of small rodents.

The success of DNA vaccination has led to a new difficulty: methods that were designed to produce small amounts of DNA for laboratory use must be adapted to produce pharmacological quality and quantities. Of particular concern is achieving a level of purity suitable for use in most animal models. Specifically, the removal of contaminants such as bacterial proteins, RNA and endotoxin is essential for most in vivo applications. Purity is especially critical when mice or primates, which are highly sensitive to bacterial lipopolysaccharide endotoxin (LPS), are immunized with DNA (Wicks et al., 1995; Boyle et al., 1998). However, obtaining this level of purity often becomes increasingly problematic when plasmid DNA is produced on the scale needed to carry out such studies as described above.

The most common method currently used to obtain large quantities of ''vaccination-grade'' DNA uses salts to precipitate DNA from lysates of bacterial cultures. Enzymatic inactivation of LPS and RNA is then required, followed by column purification (e.g. Qiagen™). The final material contains very low levels of LPS or RNA and is very effective for inducing antigen-specific antibody and CTL responses after intramuscular injection (i.m.). This material is effective for vaccination, but is expensive to produce. Murphy et al. (1999) recently described an inexpensive and rapid method for purification of DNA that employs small cationic molecules such as spermine or spermidine to selectively precipitate plasmid DNA from bacterial lysates. These ''selective compaction agents'' bind to the major and minor grooves of double-stranded DNA in direct contact with the negatively charged phosphate groups. The resultant charge neutralization enables formation of compact DNA structures both within and between plasmids. This results in a 10^6-fold reduction of the volume that DNA occupies in solution. This final plasmid DNA precipitation then occurs in the absence of salts, which may carry down contaminating RNA, proteins or LPS. In traditional methods of precipitating DNA, cesium chloride gradient centrifugation is required to remove these contaminants. In the commonly used commercial column purifications, the contaminants are removed by specific enzyme incubations.

Because spermine compaction enables production of large quantities of DNA cheaply and efficiently, we wanted to determine whether it could be used to produce vaccination-grade DNA. Here we demonstrate that plasmid DNA purified by spermine compaction is comparable in purity to material purified by a method using enzymatic treatment and chromatographic isolation (Qiagen). The immunogenicity of both materials was also equivalent, as assessed by their ability to induce antigen-specific cytotoxic T-cell (CTL) responses and IFN-γ producing CD8+ cells. The time involved in preparing 5 mg of DNA by each method was similar, but the cost of spermine-compacted DNA was only one fifth of the cost of column-purified material.

2. Materials and methods

2.1. Mice and plasmid DNA vector vaccine construct

B6 and BALB/c mice (4–6-week-old females) were purchased from Jackson laboratories and maintained at the OHSU Department Animal Care (DAC) facilities. The pTH.HM plasmid used in this study (generously provided by Dr. Tomas Hanke, Oxford) contains a mini-gene encoding several defined HIV, SIV and malaria CTL epitopes for humans, primates and H-2^d mice (Hanke et al., 1998b, 1999). The epitope RGPGRAFVTI derived from the V3 region of the HIV envelope glycoprotein is presented by the H-$2D^d$ molecule. CTL recognizing this epitope are readily induced in BALB/c mice after immunization with plasmid DNA or MVA containing this mini-gene (Hanke et al., 1998a). The pOVA.E4REV plasmid

was constructed by inserting into the A site of the commercially available vector pIRES (Clotech) a PCR amplicon encoding the ORF of ovalbumin (OVA) derived from a recombinant OVA vaccinia virus (VV-OVA) (a kind gift from Dr. J. Yewdell). The B site contains the E4 orf4 gene from human Adenovirus 5 in the reverse direction (E4REV). DH5-α competent cells were transformed separately with the two different constructs and maintained as glycerol stocks (− 70 °C) prior to culturing and large-scale plasmid DNA purification.

2.2. DNA purification

Bacteria harboring the plasmids were selected on LB agar plates containing ampicillin [120 μg/ml]. A single colony was selected and incubated in a 37 °C shaker (225 rpm) for 12 h in 5 ml of LB broth plus ampicillin [120 μg/ml] (LBB + amp). This was used to inoculate 500 ml of LBB + amp that was subsequently incubated as above for an additional 12 h. The culture was divided into two equal parts and processed for purification of plasmid DNA.

One half was subjected to the Qiagen endotoxin-free Maxi Kit procedure (Qiagen, Chatsworth, CA) according to the manufacturer's instructions. Briefly, this involved standard alkaline lysis and treatment with RNase followed by filtration (QIA-filter). This was followed by an incubation to remove endotoxin, then loading onto and elution from the "Qiagen-tip" (DNA-binding column). DNA was then precipitated by isopropanol, centrifuged, washed, centrifuged again and air-dried.

The remaining culture material was processed using selective compaction with spermine tetrahydrachloride (Murphy et al., 1999). Cells were centrifuged and then washed once with 25 ml of TE pH 8.0. Immediately after washing and re-suspension, the cells were subjected to standard alkaline lysis in the absence of ribonuclease-A (RNase A) (Sambrook et al., 1989). The mixture was neutralized with the addition of 25 ml ice-cold 3 M potassium acetate pH 5.5 and then incubated on ice for 30 min. The flocculent precipitate was removed first by filtration through a QIAfilter (Qiagen™) and subsequent centrifugation for 30 min at 12,000 × *g*. Clarified material was precipitated by the addition of isopropanol (0.7 volumes) and 4 °C centrifugation for 30 min at 12,000 × *g*. The pellet was washed twice with 70% ethanol and dried before dissolving in ice-cold 2.5 M LiCl. Bacterial RNA was precipitated by 4 °C centrifugation at 10,000 × *g* for 15 min. The RNA depleted supernatant was then subjected to precipitation with isopropanol as above. The pellet was washed three times with 70% ethanol and dried before dissolving in 5 ml of 10 mM Tris–HCL pH 7.9. In a final step, the plasmid DNA was compacted by spermine–HCL [1.5 mM] (Sigma) addition and 4 °C centrifugation at 10,000 × *g* for 10 min. Both preparations were re-suspended to a final volume of 1 ml in sterile PBS and stored at − 20 °C.

2.3. Quantification and analysis of DNA purity

Nucleic acid was quantified by spectrophotometric measurement. Optical density (OD) was measured at 260 and 280 nm and OD 260/280 nm ratios calculated. Both preparations were then re-suspended to a final concentration of 1 μg/μl in sterile PBS. Twenty micrograms of material from each of the preparations was analyzed by electrophoreses through a 1.0% ethidium stained agarose gel. To detect minute amounts of degraded RNA in the samples, eletrophoretic analysis was performed in a 1.0% agarose gel stained with SBRY gold™ (Molecularprobes).

The Pyrochrome test kit (Assoc. of Cape Cod) was used to determine the levels of contaminating endotoxin in each of the purified plasmid DNA samples. A sample of 200 μl containing 10 μg of material was added to a separate tube containing 200 μl of pyrochrome solution. After vortexing, the mixtures were incubated for a total of 26 min at 37 °C. Eighty-nine microliters of stop solution (50% acetic acid) was then added and OD 405-nm readings were recorded on a diode array spectrophotometer (Beckman DU 7500). A standard curve with known amounts of endotoxin was generated using the same procedure. Concentrations of contaminating endotoxin units (EU) were then calculated for each of the purified plasmid DNA preparations. Samples were analyzed in duplicate experiments to determine mean EU values.

2.4. Immunizations and detection of antigen-specific responses

Mice were immunized with 100 μg of plasmid DNA by intramuscular (i.m.) injection on one occa-

sion. Animals received either material obtained from the Qiagen Kit or the spermine compaction procedure. Under anesthesia (isofluorane), animals were shaved and injected in the area of the left and right tibialis anterior muscles. Each injection site received 50 μl of DNA [1 μg/μl] in sterile PBS. Ten-day post-DNA immunization animals were either sacrificed and splenocytes obtained or used in a prime-boost experiment. Mice immunized with the pOVA.E4REV construct were given an intraperitoneal (i.p.) booster immunization with VV-OVA [1×10^5 pfu] and sacrificed 7-day post-boost. Whole spleens were remove by aseptic dissection and placed into sterile HBSS containing 1.0% fetal bovine serum (FBS), penicillin, streptomycin and glutamine (PSG). Cell suspensions made by passing spleens through a mesh screen (Falcon) were enumerated and placed into culture medium (RPMI-1640 supplemented with 10% FBS, PSG and 5×10^{-5} M 2-mecaptoethanol) at a density of 4×10^6 cells/ml.

Splenocyte cultures used for CTL assays were stimulated for 5 days in the presence of the HIV envelope peptide (RGPGRAFVTI) [2 μg/ml] and supernatant from the X63-6353 cell line as a source of IL-2 (Karasuyama and Melchers, 1988). Cells were harvested and enumerated after 5 days of peptide stimulation and used as effectors in a ^{51}Cr-release assay. Effectors were diluted sequentially three-fold in U-bottom wells (96-well plate Costar) to yield effector to target (E/T) ratios of 90, 30 and 10:1. Targets were P815 cells labeled for 1 h with ^{51}Cr [100 μCi/1×10^6 cells], and pulsed with peptide [2 μg/ml] or not. Ten thousand targets per well were added to the effectors. After 6 h of incubation at 37 °C, 25 μl of culture supernatant was harvested from each well and transferred to Lumaplates and activity measured using a TopCount (Packard Instruments). Spontaneous and total ^{51}Cr-release were determined from wells in which the target cells were incubated in medium alone or 5% Triton X-100, respectively. Percent specific lysis was calculated as [(sample release − spon-spontaneous release)/(total release − spontaneous release)] × 100. The spontaneous release for all samples was below 10% of the total release.

Splenocytes used for intracellular cytokine staining (ICCS) detection were cultured for 5 h with or without SIINFEKL peptide [1 μg/ml] in the presence of the protein transport inhibitor GolgiPlug™ (BD-Pharmingen). Cells were washed, treated with Fc Block™ (BD-Pharmingen) and stained with anti-CD8 Tricolor (Caltag). Cells were then fixed, permeabilized and stained for intracellular IFN-γ using the XMG1.2 monoclonal antibody fluorescein conjugate or isotype control (BD-Pharmigen). Flow data was collected using a BD FACSCalibur™ cytometer and data analysis was performed using FlowJo™ software (Treestar).

3. Results and discussion

3.1. Recovery of material

In order to compare the efficiency of plasmid DNA recovery for the two different purification procedures, a bacterial culture harboring the pTH.HM plasmid was divided equally and processed for plasmid DNA isolation using the Qiagen and spermine compaction methods. The amount of nucleic acid recovered was determined by spectrometry OD reading at 260 nm. Purity was examined by determining the OD 260 to 280 nm ratio. Although the purity of the preparations were similar (1.82 ± 0.06 and 1.79 ± 0.05, Qiagen kit and spermine compaction, respectively), there was a considerable difference in the total amount of nucleic acid isolated. The Qiagen Endotoxin-free Maxi kit produced approximately 600 μg of nucleic acid. However, nearly an eight-fold greater amount of nucleic acid was isolated using the spermine compaction method (5.0 mg). We note that, to ensure cost-efficiency, Qiagen recommends volumes of starting material designed to ensure that the "Qiagen-tip" column is saturated with DNA; larger columns ("Mega" or "Giga" columns) could have been used to obtain a 5 mg yield.

To determine if the increased recovery of nucleic acid in the spermine compaction preparation was due to efficient precipitation of plasmid DNA and not due to contaminating RNA, samples from both purification preparations were examined by electrophoreses through agarose gels stained with dyes to detect nucleic acids. Fig. 1a shows the profile of the nucleic acid material in the two preparations using EtBr stain. The gel was purposely overloaded in order to detect small amounts of RNA. It should be noted that the lower band of the samples representing super-coiled plasmid DNA is brighter and larger in the spermine

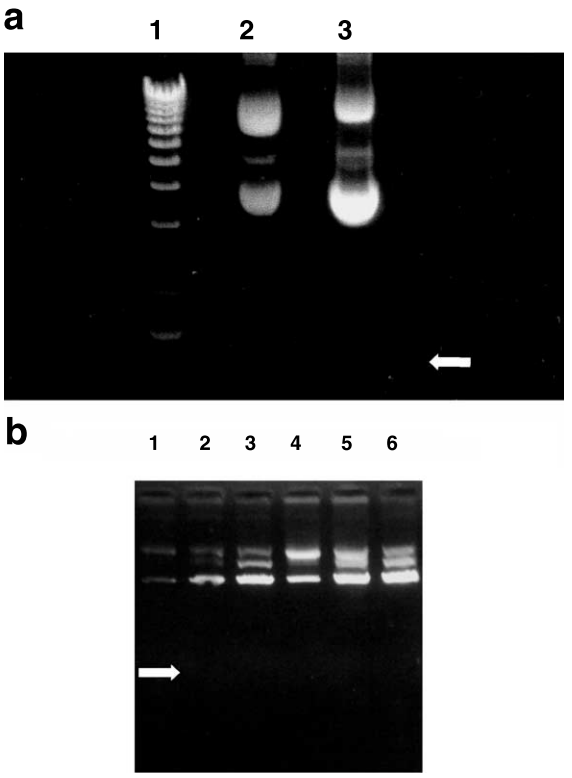

Fig. 1. (a) Comparison of nucleic acid constituents by agarose gel electrophoretic analysis. Twenty micrograms of material from each of the preparations was analyzed by electrophoreses through a 1.0% ethidium stained agarose gel. Lane 1 is a 1-kb DNA ladder (Gibco). Lanes 2 and 3 are material from the Qiagen and spermine compaction preparations, respectively. The arrow indicates the position at which contaminating bacterial RNA typically runs in a plasmid preparation. (b) Agarose gel electrophoretic analysis by SYBR gold staining. Samples were examined on a 1.0% agarose gel stained with SBYR gold diluted 1:10,000. Lanes 1–3 contain approximately 250 ng of plasmid DNA sample from preparations Qiagen, spermine and spermidine, respectively. Lanes 4–6 are the same sample preparations as 1–3, respectively, with three times the material loaded per lane. The arrow indicates the position at which contaminating bacterial RNA typically runs in plasmid preparation.

preparation, compared to the column preparation, giving the impression that there is more spermine purified DNA loaded. However, the reciprocal case is true for the upper band representing nicked linear DNA, therefore, the total amount of DNA loaded is closely equivalent for both sample preparations based on spectrometry reading. In Fig. 1b, using more sensitive nucleic acid stain, SBYR shows that highly degraded RNA was not present in the samples. Since very little genomic DNA and no contaminating RNA were observed in either sample, we concluded that the nucleic acids were predominately plasmid DNA. Therefore, the spermine compaction method was markedly more efficient for isolation of plasmid DNA when compared to the Qiagen kit for an equal amount of culture material. Although the manufacturer recommends the column we used for this quantity of culture, for a moderate copy number plasmid, a larger capacity Qiagen column or successive runs with the same could have been used to recover a greater portion of the total DNA in the sample. The spermine compaction method however does not involve a trade-off between yield and cost-efficiency, and will reliably precipitate most of the available DNA without increasing the cost.

3.2. Purity

The level of endotoxin contamination in plasmid preparations can vary widely depending on the method of purification. For example, plasmid DNA purified by sequential centrifugation through two cesium chloride gradients (2× CsCl) will typically contain around 2.5 EU/μg (Davis et al., 1996). Qiagen reports results of less than 0.1 EU/μg when plasmid DNA is prepared with the Endo-free kit (Qiagen, 2000). Plasmid DNA with endotoxin levels of 3 EU/μg or less has been successfully used for immunization of mice (Davis et al., 1996). We measured the endotoxin in our two preparations to determine if the level of contamination fell within an acceptable range for in vivo use. Our Qiagen kit performed well within the manufacture's claims [0.011 (± 0.001) EU/μg]. Although the amount of endotoxin in the spermine compaction preparation was higher [0.3 (± 0.01) EU/μg], this is well below the level that would warrant concern for in vivo use in a mouse model, where concentrations up to 10-fold higher have been used.

3.3. Immunogenicity

We next tested the ability of the two DNA preparations to induce an antigen-specific CTL response. Our plasmid DNA vaccine encodes an HIV envelope peptide epitope within a polyepitope mini-gene. This vaccine has been shown to induce

peptide-specific CTL in BALB/c mice after a single injection. The two different plasmid DNA preparations were tested by i.m. injection into BALB/c mice to determine the level and consistency of CTL induced. Four mice were used in each treatment group and each received 100 μg of plasmid DNA from either the Qiagen or spermine compaction preparation. Ten-day post-immunization splenocytes were harvested and then stimulated with peptide in culture for 5 days. A chromium release assay was performed to measure the level of CTL activity induced in each of the vaccinated animals. The results are shown in Fig. 2. Both preparations induced robust CTL activity to an equivalent extent in each of the four animals.

A second side-by-side comparison of the two purification methods using a different plasmid-encoded antigen was performed. The pOVA.E4REV construct was isolated by both of the purification methods and used in a DNA prime recombinant virus boost immunization regimen, a commonly used method for inducing robust antigen-specific T-cell responses. ICCS detection was performed to accurately quantify the number of antigen-specific CD8+ T cells induced by vaccination. Fig. 2b shows the results of this experiment. A significant six- and eight-

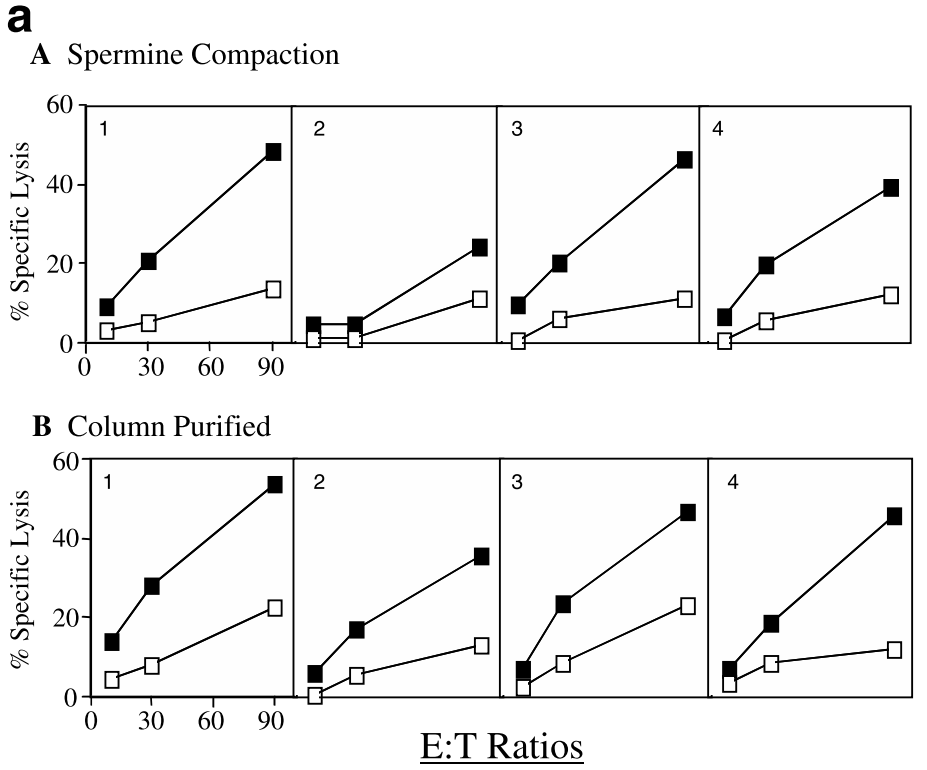

Fig. 2. (a) Comparison of CTL activity specific for HIV envelope peptide epitope after i.m. immunization with pTH.HM purified by either the spermine compaction (panel A) or Qiagen method (panel B). BALB/c mice ($n=4$) were immunized with 100 μg of plasmid DNA from either preparation. Splenocytes were harvested 10 days after immunization and stimulated in vitro with peptide (RGPGRAFVTI) for 5 days. Percentage of specific lysis was determined using ^{51}Cr P815 cells (H-2^{d}) peptide pulsed (closed squares) or not (open squares) as target cells. Results are expressed as a mean for triplicate wells. (b) Comparison of SIINFEKL-specific CD8 T-cell responses by intracellular cytokine staining after immunization with PBS (MOCK) or plasmid DNA (pOVA.E4REV), purified by either the spermine compaction or Qiagen column method and VV-OVA boost. B6 mice ($n=4$) were immunized with 100 μg of plasmid DNA or PBS ($n=3$), 7 days after mice were immunized i.p. with recombinant ovalbumin vaccinia virus [1.0×10^5 pfu]. Splenocytes were harvested 10 days after and stimulated for 5 h with 1 μg/ml SIINFEKL peptide or PBS in the presence of the protein transport inhibitor BFA. Shown in the upper right quadrants are the percent CD8 positive cells staining positive for IFN-γ. Staining results from a naive mouse are shown for background comparison.

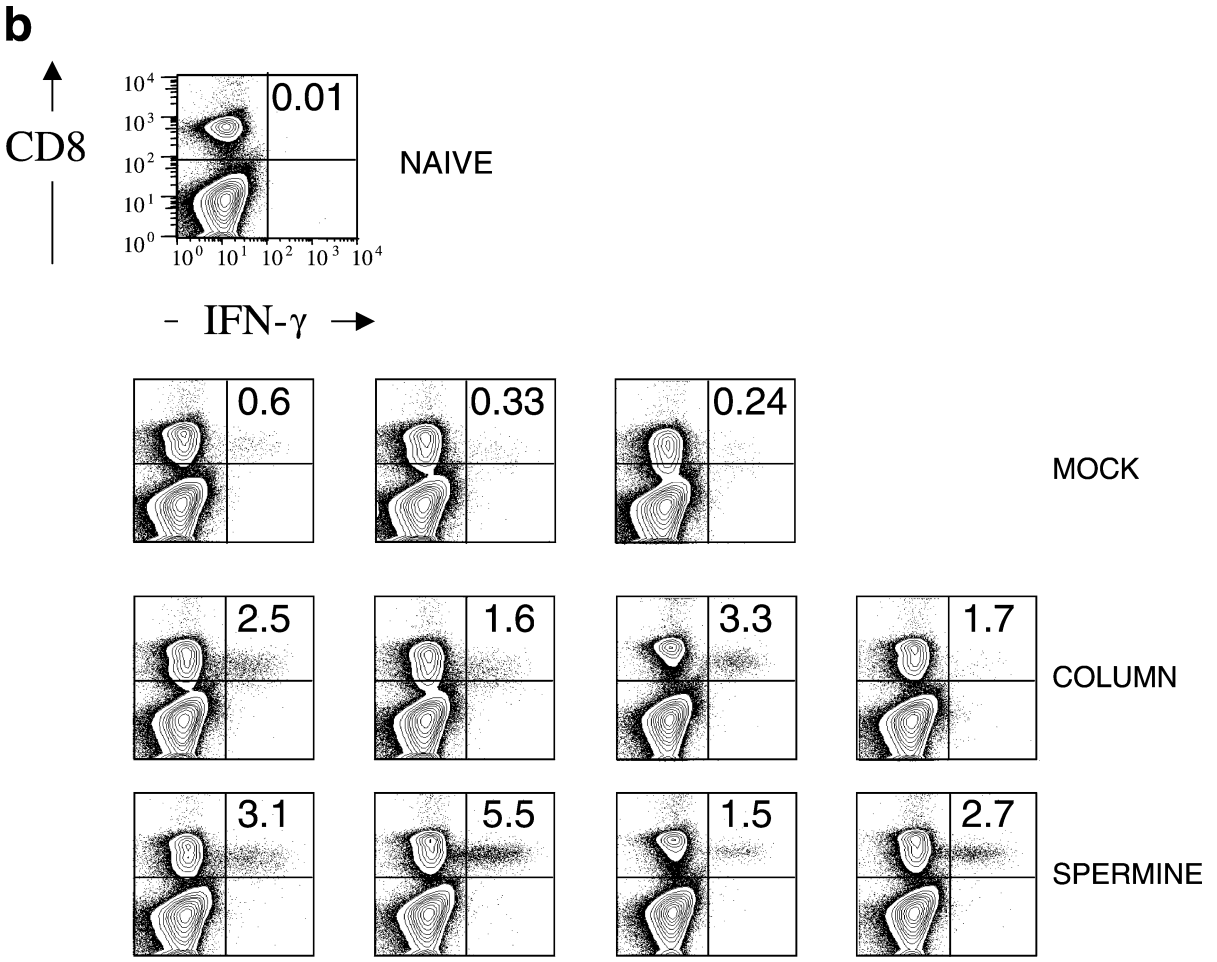

Fig. 2 (*continued*).

fold increase in SIINFEKL-specific CD8+ T cells was produced in animals primed with DNA purified by the Qiagen and spermine methods, respectively, when compared to unprimed (mock) animals. However, the difference in antigen-specific responses between the two purification methods is not statistically significant ($p = 0.34$). Similar results were observed when the same samples were analyzed by K^b-SIINFEKL tetramer staining (data not shown). Additionally, we have used spermine and spermidine compaction to purify three further plasmids ranging in size from 2 to 6.5 kb with similar efficiency of recovery and success in antigen-specific CD8+ T-cell generation.

3.4. Cost and time

It is evident from these results that spermine compaction can be used to prepare vaccination-grade DNA. As both methods of DNA preparation showed similar efficacy at inducing an immune response, the choice between the methods will depend on their relative costs and time involved. We have found that the labor involved in producing DNA to be similar between the two methods. Both methods employ an alkaline lysis step for the initial isolation of nucleic acid, and both employ a filter to remove the initial flocculant precipitate. Thereafter, the spermine method employs three precipitations (two isopropanol

and one LiCl) to remove contaminating RNA and several ethanol washes, before the final sperimine precipitation. In total, there are five centrifugations, which constitute the main labor time. In contrast, the column purification method requires only two centrifugations, but needs additional incubations with specific reagents to remove RNA and endotoxin, and also the column purification. The main labor time is involved in equilibrating, loading, washing, and eluting from the column, the time involved is increased by the larger volumes required for large quantities of DNA. The time from alkaline lysis to final DNA preparation for both methods is almost identical (approximately 5 h), and the amount of labor directly involved is comparable. The advantage of spermine compaction is that DNA is produced for approximately one fifth of the cost of using the commercial columns. We calculate the cost of producing 5 mg of DNA by spermine to be US$15.73. The manufacturers recommend two Qiagen Mega-columns to purify 5 mg of DNA, at a cost (for endotoxin-free material) of US$92.

We see the main application for this method to be when large amounts of DNA are required, as scaling up of the spermine compaction methods involves very little increase in cost or effort. Such quantities are frequently required for vaccine studies even in mice, for instance, in experiments requiring multiple injections or a large number of animals. However, the method may be of particular value in large animal applications, such as in veterinary use, in agriculture and aquaculture, and in non-human primate studies. With further modification and study, it may even prove useful for human clinical application.

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