Abstract

Background: DNA vaccination is a convenient means of immunizing animals with recombinant parasite antigens. DNA delivery methods are believed to affect the qualitative nature of immune responses to DNA vaccines in ways that may affect their protective activity. However, relatively few studies have directly compared immune responses to plasmids encoding the same antigens after injection by different routes. Therefore, the purpose of this study was to explore the influence of the route of administration on antibody responses to plasmids encoding antigens from the filarial nematode parasite Brugia malayi.

Methods: Four B. malayi genes and partial genes encoding paramyosin (BM5), heat shock protein (BMHSP-70), intermediate filament (BMIF) and a serodiagnostic antigen (BM14) were inserted in eukaryotic expression vectors (pJW4303 and pCR™3.1). BALB/c mice were immunized with individual recombinant plasmids or with a cocktail of all four plasmids by intramuscular injection (IM) or by gene gun-intradermal inoculation (GG). Antibody responses to recombinant antigens were measured by ELISA. Mean IgG1 to IgG2a antibody ratios were used as an indicator of Th1 or Th2 bias in immune responses induced with particular antigens by IM or GG immunization. The statistical significance of group differences in antibody responses was assessed by the non-parametric Kruskal-Wallis test.

Results: Mice produced antibody responses to all four filarial antigens after DNA vaccination by either the IM or GG route. Antibody responses to BM5 paramyosin were strongly biased toward IgG1 with lower levels of IgG2a after GG vaccination, while IM vaccination produced dominant IgG2a antibody responses. Antibody responses were biased toward IgG1 after both IM and GG immunization with BMIF, but antibodies were biased toward IgG2a after IM and GG vaccination with BMHSP-70 and BM14. Animals injected with a mixture of four recombinant plasmid DNAs produced antibodies to all four antigens.

Conclusions: Our results show that monovalent and polyvalent DNA vaccination successfully induced antibody responses to a variety of filarial antigens. However, antibody responses to different antigens varied in magnitude and with respect to isotype bias. The isotype bias of antibody responses following DNA vaccination can be affected by route of administration and by intrinsic characteristics of individual antigens.
Background

*Brugia malayi* is a mosquito-borne nematode parasite and a cause of lymphatic filariasis in humans [1]. The parasite is transmitted when third stage larvae (L3) of *B. malayi* enter the human host following a bite by an infective mosquito. Control is based on treatment of microfilaria (MF) carriers and anti-mosquito measures that decrease transmission; no vaccines are available for prevention of infection.

Prior studies have shown that a degree of protective immunity to filariasis can be induced in animals by vaccination with irradiated L3 [2,3]. The potential for use of live filariasis vaccines in humans is limited because of safety issues and limited availability of larvae. Several laboratories are working to develop effective recombinant antigen-based vaccines that would be more practical and effective than live parasite vaccines.

DNA vaccination is a promising approach that may have advantages over vaccination with live parasites or protein antigens. DNA vaccines have been shown to be an effective means of generating cellular and humoral immune responses, and they have conferred protection against a wide range of infectious agents including viruses, parasites, and bacteria in animal models (reviewed in [4]).

We have previously reported that vaccination with recombinant *B. malayi* paramyosin (BM5) protein induced partial immunity to challenge infections in jirds [5]. More recently, we reported that mice injected IM with plasmid DNA encoding BM5 developed antigen-specific humoral and cellular immune responses [6]. However, this vaccination failed to protect jirds from challenge infections. These results raised the issue of how to optimize DNA vaccination to induce protective immunity.

Considerable effort has been expended toward improving the efficacy of DNA vaccines through vector design, optimization of immunization schedules, and by combining DNA vaccination with other vaccine types (reviewed in [7]). The most widely used DNA vaccination methods are intramuscular injection of plasmid DNA in an aqueous solution (IM) and ballistic intradermal injection of plasmid DNA-coated gold particles by gene gun (GG). Intramuscular injection and gene gun inoculation differ in the efficiency of DNA delivery [8,9]. Following IM injection, cells take up DNA from the extracellular space by poorly understood mechanisms [10,11], while the more efficient GG immunization directly transfects cells by depositing DNA-coated gold beads into the cytoplasm of antigen presenting cells [12,13]. Some reports have claimed that the route of DNA delivery has a major impact on the type of immune responses induced by DNA vaccines, with GG inducing Th-2 biased immune responses dominated by IgG1 subclass antibodies and IM inducing Th-1 biased responses dominated by IgG2a antibodies (9, 14, 15). However, most studies of the effects of route of administration for DNA vaccination have been performed with viral antigens, and none have employed nematode antigens. Therefore, the present study was designed to examine how the route of DNA delivery affects antibody responses to filarial nematode antigens in mice. We also examined the issue of whether antibody responses to individual filarial antigens are affected when mice are injected with a cocktail of plasmids encoding several antigens (polyvalent DNA vaccination).

Methods

Selection of Recombinant Antigens and Preparation of Plasmid DNA for Immunization

Genes and partial genes for *B. malayi* paramyosin (BM5) [16], intermediate filament (BMIF) [17], a heat-shock protein (HSP-70) (GenBank number:AY383564), and an antigen used for immune diagnosis (BM14) [18] were chosen for immunization. Two eukaryotic expression vectors, pJW4303 and pCR™3.1, were used for DNA vaccination. We have previously shown that both vectors were capable of inducing immune responses to BM5 in mice [6]. The same study showed that mice injected with these vectors with no inserted gene sequences did not produce antibodies to *B. malayi* antigens. For this reason, control vaccinations with vector alone were not repeated in the current study.

BM5, BMIF and HSP-70 were inserted into pJW4303 with methods previously described in detail [6]. Briefly, cDNAs were recovered from pBluescript by EcoRI digestion and directly ligated into the EcoRI site of pJW4303, downstream from a tissue plasminogen signal peptide sequence and upstream of a bovine growth hormone transcription termination sequence. The cDNA of BM14 was produced by PCR amplification with specific primers and directly ligated into pCR™3.1 creating BM14/pCR3 recombinant plasmid by methods previously described in detail [6]. The orientation and reading frame of all recombinant plasmids were confirmed by DNA sequencing.

Plasmid DNA for injection was prepared using the Qiagen Plasmid Maxikit (Hilder, Germany) according to the manufacturer's instructions. The quantity and purity of isolated plasmid DNA was assessed spectrophotometrically, and the ratio of OD 260 to OD 280 of DNA preparations used for immunization was > 1.8. Purified plasmid DNA was dissolved in 10 mM Tris EDTA, pH 8.0, and diluted in PBS to obtain a final concentration of 1 mg/ml for injection.
Intramuscular and intradermal inoculation of DNA

For IM injection, a 1 cc insulin syringe with a 28 1/2-gauge needle was used to inject mice with 50 µl of a 1 mg/ml solution of plasmid DNA in PBS into quadriceps muscles in both rear legs. For GG inoculation, DNA was precipitated onto gold beads (1.0 µm) with a DNA loading ratio of 10 µg/mg gold according to the manufacturer's instructions (Bio-Rad Laboratories, Hercules, CA). A helium pulse gun (Bio-Rad) was used with discharge pressure of 250 psi to delivery 5 µg of plasmid DNA in a single shot into freshly shaven abdominal skin for single antigens. For some experiments, all four plasmids were combined, and four nonoverlapping shots were administered with the gene gun to deliver a total of 5 µg of each plasmid in the cocktail.

Vaccination Protocol

Four-week-old female BALB/c mice were vaccinated with 100 µg plasmid DNA in PBS by IM injection or 5 µg plasmid DNA by GG at 0 and 4 wk. Sera were collected at 2, 6, and 8 wk after the first immunization.

Antibody Assays

The proteins encoded by cDNAs used for DNA vaccination have been previously expressed in various expression vectors and purified [5,16-18]. Antibody responses to purified recombinant antigens (BM5/MBP, BMIF/GST, BMHSP/His, BM14/His) were assessed in mice by ELISA. Microtiter plates (Dynatech Technologies, Chantilly, VA) coated with purified recombinant filarial antigens (2 µg/ml in carbonate buffer, pH 9.6) were incubated with mouse sera diluted 1/100 in PBS/T/FCS in duplicate wells for 2 hr at 37°C. Bound antibody was detected after incubation with peroxidase-conjugated goat anti-mouse IgG, IgG1, or IgG2a antibodies (Southern Biotechnology Associates, Birmingham, AL) for 1 hr at 37°C. Optical density at 490 nm (OD) was read versus a PBS blank at 490 nm with a Biotek 312e Micro plate ELISA reader. Sera that produced net OD values greater than the mean OD plus 3 SD obtained with a panel of normal mouse sera were considered to have significant antibody responses.

IgG subclass ELISAs were normalized as previously described (18). Briefly, microtiter plates were coated with 10 µg/ml of either purified IgG, IgG1 and IgG2a mouse proteins (Southern Biotechnology Associates) overnight at 4°C, and then incubated with serial dilution of goat anti-mouse horseradish peroxidase (HRP)-conjugated antibodies to total IgG, IgG1 or IgG2a antibodies (Southern Biotechnology Associates, Birmingham, AL) for 1 hr at 37°C. Optical density at 490 nm (OD) was read versus a PBS blank at 490 nm with a Biotek 312e Micro plate ELISA reader. Sera that produced net OD values greater than the mean OD plus 3 SD obtained with a panel of normal mouse sera were considered to have significant antibody responses.

Figure 1

This figure shows serum IgG isotype antibody responses in mice vaccinated with BM5 by intramuscular (IM) N = 7 and Gene Gun (GG), N = 8 immunization. Antigen-specific ELISAs were performed with sera collected 8 wk after the first immunization. A: IgG1 and IgG2a results shown are mean OD ± SE for IM and GG mice; B: IgG1 to IgG2a ratios are shown for mice immunized with BM5 by IM and GG routes. Values shown are mean PD ± SE. The ratios in the two groups were significantly different (p < 0.05).
BMIF DNA Vaccination

Mice immunized with BMIF antigen by either IM or GG produced very similar levels of total IgG and IgG2a antibodies to BMIF. Higher IgG1 antibody responses to BMIF were observed in mice immunized by GG (Figure 2A), but the ratios of IgG1 to IgG2a in GG and IM vaccinated mice were not significantly different. The ratios of IgG1 to IgG2a from IM and GG immunizations were > 1 (GG = 7.5, IM = 2.5) (Figure 2B), signifying more IgG1 than IgG2 in both groups. As observed after BM5 immunization, GG immunization with BMIF DNA produced more pronounced isotype skewing than IM vaccination (Figure 2A).

BMHP DNA Vaccination

Gene gun and IM DNA immunization with BMHSP induced very similar IgG antibody response to BMHSP with strong IgG2a responses and very weak IgG1 responses (Figure 3A). The ratios of IgG1 to IgG2a antibodies were very low (< 0.3) after both GG and IM immunization with DNA encoding this antigen (Figure 3B).

BMI14 DNA Vaccination

Intramuscular and GG DNA immunization with BM14 antigen produced antibody isotype responses similar to those observed after BMHSP vaccination, with stronger IgG2a than IgG1 (IgG1/IgG2a < 0.5) (Figure 4A). However, IM vaccination induced stronger antibody responses, with more total IgG and IgG1 antibodies to BM14 antigen, than those produced by GG immunization (Figure 4B).

Antibody Responses to Plasmid Mixtures

The effect of mixing plasmids on DNA vaccination was determined by examining antibody responses to antigens encoded by plasmids combined in a four antigen DNA cocktail vaccine. Most mice vaccinated with the plasmid mixture produced IgG antibodies to each of the antigens.
encoded by plasmids in the cocktail. Polyvalent vaccination did not significantly alter the isotype bias of antibody responses to individual antigens (data not shown). Specific antibodies were detectable 2 wk after the first IM injection in most cases, and antibody levels increased after booster injections of plasmid DNA (Figure 5). Responses to polyvalent vaccination by GG were similar, but these took longer to develop (data not shown).

IgG antibody responses induced by the combination vaccine were compared to those obtained after injection with single plasmid components of the combination vaccine. However, polyvalent vaccination did not alter the isotype bias of antibody responses to individual antigens (data not shown). Antibody responses to three of the antigens (BM5, BM1F and BM14) were weaker following cocktail vaccination than after vaccination with single plasmids. However, antibody responses to BMHSP were the same after monovalent and polyvalent vaccination. Antibody responses to BM14 were lower than those to other antigens in the cocktail.

**Discussion**

**Effect of Route of DNA Delivery on Antibody Responses to B. malayi Antigen(s) in Mice**

One of the objectives of this work was to compare antibody responses induced by IM or GG immunization with plasmids encoding several *B. malayi* antigens. Several factors have been reported to influence the strength and nature of immune responses in mice after DNA vaccination [20,21]. Prior reports have emphasized the effects of routes and methods of DNA delivery as factors that affect antibody responses because of differences in efficiency of gene transfer and types of cells transfected [9,15,22,23]. Early studies showed that IM injection results in low-level transfection of myocytes [24], whereas intradermal injection is believed to directly transfec antigen-presenting cells [25,26].

Our results showed that mice produced antibody responses to target filarial antigens after DNA vaccination by either IM or GG intradermal injection. Intramuscular injection tended to induce stronger antibody responses. However, the plasmid DNA dose was 20 times higher in i.m immunized mice. Therefore, GG induced higher antibody responses per unit plasmid DNA. Similar results have been reported in mice immunized with other antigens [15,25].

Apart from antibody levels, several studies have reported that the route of DNA delivery can affect the protective efficacy of DNA vaccines, which does not always correlate with antibody titers. For example, DNA encoding a *Plasmodium yoelii* antigen (HEP17) protected mice equally well when given by the GG or IM routes, while DNA encoding two other antigens (CSP and SSP2) induced better protection by the IM route than by the GG route [27]. On the other hand, Leitner et al., reported that DNA encoding CSP was protective in a different malaria model only after GG vaccination [28]. Thus, neither route was conclusively favored in all cases; different injection methods may provide optimal immunogenicity for different antigens. Therefore, our results and the work of others suggest that it is not possible to reliably predict the optimal route for a given antigen to achieve desired immune responses. There is currently no substitute for empirical testing of each antigen.

Some prior studies have shown that GG vaccination tends to induce helper T-cell responses of the Th2 phenotype with a bias toward IgG1 antibody responses, while IM injection of DNA tends to produce a Th1-phenotype with a bias toward IgG2a [9,29]. In the present study, this pattern was observed for only one of four antigens tested (BM5). Other recombinant plasmids produced antibody responses biased toward IgG1 or IgG2a independent of route. These results suggest that the isotype profile of antibodies generated by DNA immunization depends not only on the route of DNA delivery but also on the intrinsic nature of antigens used for immunization.

**Antibody Responses to a Polyvalent DNA Vaccine**

A second goal of our study was to compare antibody responses induced by monovalent and polyvalent DNA vaccines. One of the potential advantages of DNA vaccination is the ability to develop combination vaccines.
consisting of multiple plasmids expressing different antigens. Polyvalent vaccines may be needed to achieve high levels of protection against complex pathogens like helminth parasites. It is important, therefore, to determine whether co-injection of multiple plasmids successfully induces antibody to each component antigen encoded by the plasmid mixture. We confirmed this to be the case in mice injected with a cocktail of plasmids encoding four B. malayi antigens. Total IgG antibody responses to three of these antigens (BMIF, BM14 and BMHP) tended to be weaker than those in mice injected with single plasmids.

In conclusion, we have studied murine antibody responses to four B. malayi antigens following DNA vaccination by the IM and GG routes. Our results show that antibody responses induced by DNA vaccines can be affected by the route of injection and also by the intrinsic nature of the antigens encoded. We also showed that a tetravalent DNA vaccine induced antibodies to all four B. malayi antigens encoded by the plasmids in the cocktail. Although these findings represent progress, we realize that much more work is needed before practical DNA vaccines for filariasis become a reality. Our next priority will be to determine whether the antigens employed in this study (alone or in combination) have protective activity against B. malayi in animals. In addition, more work is needed to optimize vaccination protocols, and many other candidate antigens need to be tested. We believe these results will be of interest to those working to develop DNA vaccines against complex pathogens such as filarial nematodes.

Conclusions
The type of antibody responses induced by DNA vaccines can be affected by the route of injection and also by the intrinsic nature of antigens employed. Polyvalent DNA vaccines can induce antibodies to each component antigen.

List of abbreviations
IM intramuscular injection
GG Gene gun-intradermal inoculation
MF Microfilaria
BM5 Brugia malayi paramyosin
BMHSP-70 Brugia malayi 70 kDa heat shock protein
BMIF Brugia malayi intermediate filament
BM14 Brugia malayi serodiagnostic antigen

Competing interests
None declared.

Authors' contributions
Benwen Li had primary responsibility for the study (planning, data analysis, and writing); Amy Rush cloned the recombinant sequences into vaccine plasmids and performed ELISA assays; Shaorong Zhang performed immunizations and some of the data analysis; Kurt Curtis performed immunizations and prepared recombinant antigens for ELISA assays; Gary Well helped to plan the study, analyze results, and write the manuscript.

Acknowledgements
We thank Dr J. Mullins, University of Washington, Seattle, WA, for his generous gift of plasmid pJW4303.

References
1. Ottesen EA, Duke BO, Karam M, Bebbehahi K. Strategies and tools for the control/elimination of lymphatic filariasis. Bull WHO 1997, 75:491-503.
2. Yate JA, Higash GI. Brugia malayi: vaccination with 60cobalt-attenuated infective stage larvae protects jird against homologous challenge. Am J Trop Med Hyg 1985, 34:1132-37.
3. Weil G, Li BW, Lifitis F, Chandrashekar R. Brugia malayi: Antibody responses to larval antigens in infected and immunized jirds. Exp Parasitol 1992, 74:315-23.
4. Montgomery DL, Ulmer JB, Donnelly JJ, Liu MA. DNA Vaccines. Pharmacol Ther 1997, 74:195-205.
5. Li BW, Chandrashekar R, Weil G. Vaccination with recombinant filarial paramyosin induces partial immunity to Brugia malayi infection in jirds. J Immunol 1993, 150:1881-85.
6. Li BW, Zhang SR, Curtis KC, Weil G. Immune responses to Brugia malayi paramyosin in rodents after DNA vaccination. Vaccine 1999, 18:76-81.
7. Leitner W, Yang H, Restifo NP. DNA and RNA based vaccines: principles, progress and prospects. Vaccine 2000, 18:765-77.
8. Robinson HL. Nucleic acid vaccines: an overview. Vaccine 1997, 15:785-87.
9. Pettner TM, Roberts TR, Haynes JR. Influenza virus nucleoprotein-specific immunoglobulin G subclass and cytokine responses solicited by DNA vaccination are dependent on the route of vector DNA delivery. J Virol 1996, 70:619-25.
10. Wolf JA, Malone RW, Williams P, Chong W, Accadi G, Jani A, Felgner PL. Direct gene transfer into mouse muscle in vivo. Science 1990, 247:1463-68.
11. Robinson HL. DNA vaccines: basic mechanism and immune responses [Review]. Int J Mol Med 1999, 4:549-55.
12. Eisenbraun MD, Fuller DH, Haynes JR. Examination of parameters affecting the elicitation of humoral immune responses by particle bombardment-mediated genetic immunization. DNA Cell Biol 1993, 12:791-97.
13. Tang DC, De Vit M, Johnston SA: Genetic immunization is a simple method for eliciting an immune response. Nature 1992, 356:152-54.
14. McCluskie MJ, Millan CLB, Gramzinki RA, Robinson HL, Santoro JC, Fuller JT, Widera G, Haynes JR, Purcell RH, Davis HL. Route and method of delivery of DNA vaccine influence immune responses in mice and non-human primates. Mol Med 1999, 5:287-300.
15. Boyle J, Silva A, Brady JL, Lew A: DNA immunization: Induction of higher avidity antibody and effect of route on T cell cytotoxicity. Proc Natl Acad Sci USA 1997, 94:1426-31.
16. Li BW, Chandrashekar R, Alvarez R, Lifitis F, Weil GJ. Identification of paramyosin as a potential protective antigen against Brugia malayi infection in jirds. Mol Biochem Parasitol 1991, 49:315-24.
17. Chandrashekar R, Curtis KC, Ramzy RM, Lifitis F, Li BW, Weil GJ. Molecular cloning of Brugia malayi antigens for diagnosis of lymphatic filariasis. Mol Biochem Parasitol 1994, 64:261-71.
18. Chandrashekar R, Curtis KC, Li BW, Weil GJ: Molecular characterization of Brugia malayi intermediate filament protein which is an excretory product of adult worms. *Mol Biochem Parasitol* 1995, 73:231-39.

19. Coffman RL, Seymour BWP, Leibman DA: The role of helper T cell products in mouse B differentiation and isotype regulation. *Immunol Reviews* 1988, 102:5-28.

20. Donnelly JJ, Ulmer JB, Shiver JW, Liu MA: DNA vaccines. *Annu Rev Immunol* 1997, 15:617-48.

21. Davis HL, McCluskie JM: DNA vaccines for viral diseases. *Microb Infect* 1999, 1:7-23.

22. Feltquate DM, Heaney S, Webster RG, Robinson HL: Different T helper cell types and antibody isotypes generated by saline and gene gun DNA immunization. *J Immunol* 1997, 158:2278-84.

23. Fynan EF, Webster RG, Fuller DH, Haynes JR, Santoro JC, Robinson HL: DNA vaccines: protective immunizations by parenteral, mucosal, and gene-gun inoculations. *Proc Natl Acad Sci USA* 1993, 90:11478-82.

24. Wolff JA, Williams P, Acsadi G, Jiao S, Jani A, Chong W: Conditions affecting direct gene transfer into rodent muscle in vivo. *Biotechniques* 1991, 11:474-85.

25. Raz E, Carson DA, Parker SE, Parr TB, Abai AM, Aichinger G, Gromkowski SH, Singh M, Lew D, Yankaukshas MA: Intradermal gene immunization: The possible role of DNA uptake in the induction of cellular immunity to viruses. *Proc Natl Acad Sci USA* 1994, 91:9519-23.

26. Cardoso AI, Sixt N, Vallier A, Fayolle J, Buckland R, Wild TF: Measles virus DNA vaccination: Antibody isotype is determined by the method of immunization and the nature of both the antigen and the coinmunized antigen. *J Virol* 1998, 72:2516-18.

27. Hoffman SL, Doolan DL, Sedegah M, Gramzinski R, Wang H, Gowda K, Hobart P, Margalith P, Norman J, Hedstrom RC: Nucleic acid malaria vaccines. *Current status and potential*. *Ann N Y Acad Sci* 1995, 772:88-94.

28. Leitner WW, Seguin MC, Ballou WR, Seitz JP, Schultz AM, Sheehy MJ, Lyon JA: Immune responses induced by intramuscular or gene gun injection of protective deoxyribonucleic acid vaccines that express the circumsporozoite protein from Plasmodium berghei malaria parasites. *J Immunol* 1997, 159:6112-19.

29. Davis HL, Mancini M, Michel ML, Whalen RG: DNA-mediated immunization to hepatitis B surface antigen: longevity of primary response and effect of boost. *Vaccine* 1996, 14:910-15.
