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DNA Vaccination by Electroporation and Boosting with Recombinant Proteins Enhances the Efficacy of DNA Vaccines for Schistosomiasis Japonica▽

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Schistosomiasis japonica is an endemic, zoonotic disease of major public health importance in China. Control programs combining chemotherapy and snail killing have not been able to block transmission of infection in lakes and marsh regions. Vaccination is needed as a complementary approach to the ongoing control programs. In the present study, we wanted to determine if the efficacies of DNA vaccines encoding the 23-kDa tetraspanin membrane protein (SjC23), triose phosphate isomerase (SjCTPI), and sixfold-repeated genes of the complementarity determining region 3 (CDR3) in the H chain of NP30 could be enhanced by boosting via electroporation in vivo and/or with cocktail protein vaccines. Mice vaccinated with cocktail DNA vaccines showed a significant worm reduction of 32.88% ($P < 0.01$) and egg reduction of 36.20% ($P < 0.01$). Vaccine efficacy was enhanced when animals were boosted with cocktail protein vaccines; adult worm and liver egg burdens were reduced 45.35% and 48.54%, respectively. Nearly identical results were obtained in mice boosted by electroporation in vivo, with adult worm and egg burdens reduced by 45.00% and 50.88%, respectively. The addition of a protein vaccine boost to this regimen further elevated efficacy to approximately 60% for adult worm burden and greater than 60% for liver egg reduction. The levels of interleukin-2, gamma interferon, and the ratios of immunoglobulin G2a (IgG2a)/IgG1 clearly showed that cocktail DNA vaccines induced $CD4^+$ Th1-type responses. Boosting via either electroporation or with recombinant proteins significantly increased associated immune responses over those seen in mice vaccinated solely with DNA vaccines. Thus, schistosome DNA vaccine efficacy was significantly enhanced via boosting by electroporation in vivo and/or cocktail protein vaccines.

Schistosomiasis is an important parasitic disease affecting more than 200 million people, with 779 million individuals at risk of infection worldwide (26). In China, about 238 million people distributed in 449 counties are at risk of infection with schistosomiasis. The majority of these individuals reside in 90 counties located in Hubei, Hunan, Anhui, Jiangsu, Jiangxi, and Yunnan provinces, where control of this disease remains a problem (11). Combination treatment of humans and livestock with praziquantel and killing of the snail intermediate host have been successful strategies in some areas of China. However, control remains problematic in the aforementioned 90 counties in the lakes and marsh regions and high reinfection rates are maintained that require frequent drug treatment. The potential risk of drug resistance cannot be ruled out (35). For these reasons, vaccine development as a complementary approach for the control of schistosomiasis is necessary.

Several types of vaccines have been developed and tested against *Schistosoma japonicum* infection (36). These vaccines include DNA, recombinant protein, synthetic peptide, and multivalent vaccines. Several schistosomiasis japonica vaccine candidates have been tested in small and large animals (pigs and water buffalo), including the 23-kDa tetraspanin membrane protein (SjC23), triose phosphate isomerase (TPI), glutathione *S*-transferase (GST), paramyosin, fatty acid binding protein (FABP), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and gynecophoral canal protein (GCP) (36). Each of these vaccines induces partial protection against challenge infection as measured by reduction in total worm burdens: 26.90% to 58.60% for SjC23, 27.90% to 51.20% for TPI, 37.19% to 44.44% for GST, 35.00% to 41.10% for paramyosin, 33.80% for FABP, and 32.40% for GCP (5, 36, 38, 40). Only two vaccines approached 50% efficacy, SjC23 and SjCTPI (5, 38).

DNA vaccination was introduced in 1990 with a study that demonstrated the induction of protein expression upon direct intramuscular injection of plasmid DNA into myocytes (32). The advantages of DNA vaccines over traditional, attenuated, or subunit vaccines are the low cost of production, thermal

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stability, and their ability to induce a wide variety of long-lived cellular and humoral immune responses. In our laboratory, the coding regions for *Schistosoma japonicum* (Chinese strain) 23-kDa membrane protein (SjC23) and triose phosphate isomerase (SjCTPI) were cloned into eukaryotic expression plasmid pcDNA3.1 as a DNA vaccine vector. Several different groups have shown that each of these DNA vaccines induces partial protection in animals, with worm reduction rates ranging from 20% to 58.6%, depending on the animal species challenged and the group performing the study (5, 37, 38, 40, 41).

Cocktail vaccines that include more than one vaccine antigen have been reported to induce higher levels of efficacy than univalent vaccines (13). For example, mice vaccinated with a cocktail DNA vaccine against *Toxoplasma gondii* produced high levels of antiparasite antibody, coincident with lymphocyte proliferation and production of gamma interferon (IFN-γ) following challenge with the parasite (14). Unlike *Toxoplasma*, schistosomes are large, multicellular organisms that undergo several different developmental stages as they mature to egg-laying adult worms in the mammalian host. Therefore, utilization of a vaccine that incorporates multiple schistosome antigens might be capable of inducing immune responses against multiple stages of the parasite, providing a longer time window to harm and/or kill developing worms. In this respect, we previously demonstrated that vaccination with a DNA vaccine that combined pcDNA3.1-SjC23 and pcDNA3.1-SjCTPI plasmids provided greater efficacy than that in animals vaccinated with either single-DNA vaccine. Worm and egg burdens were reduced 41.5% and 61.4% in animals vaccinated with both plasmid DNA vaccines compare to 28.1% and 37.9% for animals vaccinated with pcDNA3.1-SjC23 and 29.1% and 44.2% for animals vaccinated with pcDNA3.1-SjCTPI (39). Thus, combination DNA vaccines are a valid approach for enhancing vaccine efficacy against *Schistosoma japonicum*.

In addition to using cocktail DNA vaccines, studies suggest that prime-boost regimens enhance vaccine efficacy over that seen when only DNA is administered. Prime-boost regimens prime recipients with DNA vaccines and then boost with recombinant proteins or subunit vaccines (20, 23). This approach makes sense, as DNA vaccines consistently drive induction of strong cellular immune responses, while subunit protein vaccines elicit strong humoral responses. Prime-boost vaccine strategies have been successfully applied to many different types of diseases, including AIDS, tuberculosis, and malaria (7, 8, 28). In an earlier study we examined the potential of prime-boost vaccine regimens. We found that mice primed with pcDNA3.1-SjC23 and then boosted with SjC23 recombinant protein had worm and egg numbers reduced by 36.9% and 30.7%, respectively, which was significantly higher than the 26.9% worm and 22.2% egg reductions seen in mice primed and boosted with pcDNA3.1-SjC23. In addition, SjC23-specific antibody responses were higher in the prime-boost group, suggesting that prime-boost regimens had superior efficacy and enhanced immunogenicity compared with DNA-only immunization (24).

Results from several trials of DNA vaccines against *Schistosoma japonicum* have reported worm reduction rates of 27 to 52%, with only two vaccines achieving >50% efficacy. The overall lower efficacy of DNA vaccines may be partially due to insufficient uptake of DNA plasmid by muscle cells, resulting in poor immune responses to vaccine antigens. In this regard, in vivo electrotransfer, electropermeabilization, or electroporation of plasmid DNA has resulted in increased DNA uptake and subsequently enhanced protein expression in treated muscle cells (1, 16). For influenza virus vaccines, when mice were immunized with a plasmid DNA vaccine by electroporation, they subsequently exhibited 100% protection against lethal challenge with H5N1 avian influenza virus, completely blocking virus replication in the lungs (34). In addition, delivery of DNA vaccines by electroporation has been shown to elevate DNA vaccine efficacy over conventional delivery of DNA vaccines for tumors, malaria, and hepatitis (2, 6, 30).

In the present study, we prepared cocktail DNA vaccines by mixing pcDNA3.1-SjC23, pcDNA3.1-SjCTPI, and pcDNA3.1-$(CDR3)_6$, and we also prepared cocktail recombinant protein vaccines consisting of SjC23, SjCTPI, and NP30 and then compared the protective efficacies of these "cocktail" vaccines delivered as conventional DNA/DNA, DNA/electroporation, DNA/recombinant protein, and DNA/electroporation/recombinant protein combinations against *Schistosoma japonicum* challenge infection in BALB/c mice.

MATERIALS AND METHODS

Parasites and animals. Cercariae were collected from *S. japonicum* (Chinese strain)-infected *Oncomelania hupensis* obtained from the Jiangsu Institute of Parasitic Diseases. Female BALB/c mice, 4 to 5 weeks of age, were purchased from Shanghai SLAC Laboratory Animal Co. Ltd. and used for vaccine trials according to the guidelines of the Animal Research Advisory Committee of the institute.

Preparation of cocktail DNA vaccines. The sixfold-repeated genes of the complementarity-determining region 3 (CDR3) in the heavy (H) chain of NP30 were previously cloned into pcDNA3.1 (29). The genes of pcDNA3.1-SjC23, pcDNA3.1-SjCTPI, and pcDNA3.1-$(CDR3)_6$ were purified using Mega plasmid DNA kits (Qiagen, Germany) and diluted into sterile saline solution to a final concentration of 1.5 mg/ml. The cocktail DNA vaccines used for immunization were produced by mixing pcDNA3.1-SjC23, pcDNA3.1-SjCTPI, and pcDNA3.1-$(CDR3)_6$ at equal volumes. The vector plasmid pcDNA3.1 was used as a negative control.

Preparation of cocktail protein vaccines. The genes of SjC23-HD (large hydrophilic domain) and SjCTPI were subcloned into plasmid pGEX-5X-1. The recombinant plasmid was transformed into *Escherichia coli* BL21(DE3) followed by expression of the protein when induced by isopropy-β-D-thiogalactoside. The recombinant proteins were purified using the Bulk and Redipack GST purification modules (Amersham Biosciences, Sweden), and the specificity was identified by serum from patients and rabbits infected with *Schistosoma japonicum* through Western blotting and then adjusted to concentrations of 1.5 mg/ml. The antiidiotypic monoclonal antibody of *Schistosoma japonicum* NP30 was kindly provided by Guan Xiaohong, Nanjing Medical University, and diluted to a final concentration of 1.5 mg/ml. Finally, equal volumes of purified recombinant proteins of SjC23-HD, SjCTPI, and NP30 were mixed to form the cocktail protein vaccines.

Electroporation in vivo. Electroporation in vivo was carried out according to the method described by Aihara and Miyazaki (1). A pair of electrode needles 5 mm apart was inserted into the muscle to cover the DNA injection sites, and electric pulses were delivered using an electric pulse generator (programmed control stimulator YC-2; Chengdu Instrument Factory, Chengdu, China). Four pulses of 100 V each were delivered to each injection site at the rate of one pulse per second, with each pulse lasting for 40 ms. Then, four pulses of the opposite polarity were applied.

Immunization and challenge infection. (i) Experiment I. A total of 126 female 4- to 5-week-old BALB/c mice were divided randomly into nine groups (A to I) and vaccinated intramuscularly with 100-μl volumes at weeks 0, 3, and 6 for experiment I. Groups boosted with recombinant proteins were injected at week 9 subcutaneously with recombinant protein solutions mixed with complete Freund's adjuvant. Group A, the injection control, was immunized with saline solution; group B, the pcDNA3.1 control, was immunized with pcDNA3.1; group C, the pcDNA3.1 control, was then boosted with cocktail protein vaccines; group

TABLE 1. Protective efficacies of different vaccination regimens against *Schistosoma japonicum* challenge infection (experiment I)[a]

Group	No. of mice	Total no. of worms	% Worm reduction	No. of eggs in liver	% Egg reduction
A (blank control)	11	25.82 ± 3.25		62,001 ± 9,814	
B (pcDNA3.1)	10	25.40 ± 2.46		61,761 ± 13,787	
C (pcDNA3.1 and cocktail protein vaccines)	12	21.25 ± 3.12	17.70	56,182 ± 9,404	9.39
D (cocktail DNA vaccines)	9	17.33 ± 2.74	32.88	39,559 ± 10,772	36.20
E (cocktail DNA and protein vaccines)	9	14.11 ± 3.55	45.35	31,908 ± 5,553	48.54
F (pcDNA3.1 with EP)	10	25.30 ± 3.80		58,272 ± 11,680	
G (pcDNA3.1 with EP and cocktail protein vaccines)	13	21.15 ± 3.44	18.09	54,257 ± 15,117	12.49
H (cocktail DNA vaccines with EP)	10	14.20 ± 3.12	45.00	30,453 ± 8,114	50.88
I (cocktail DNA with EP and protein vaccines)	12	11.08 ± 3.42	57.09	25,259 ± 8,436	59.26

[a] Values for worm and egg numbers are means ± standard deviations. Worm and egg reduction rates of each vaccine group were compared to group A (blank control). Statistically significant differences in egg reduction rates were seen in group E versus group C ($P < 0.01$) or group D ($P < 0.05$). Worm and egg burdens were significantly reduced in group H versus group D ($P < 0.05$); also, worm and egg burdens were significantly reduced in group I compared to group E ($P < 0.05$) or group H ($P < 0.05$).

D received cocktail DNA vaccines; group E received cocktail DNA vaccines and then was boosted with recombinant protein vaccines; group F received pcDNA3.1/EP control injected intramusculary, followed by electroporation in vivo (EP); group G received pcDNA3.1 control with EP and then was boosted with recombinant protein vaccines; group H received cocktail DNA vaccines with EP; group I received cocktail DNA with EP and then was boosted with recombinant protein vaccines at week 9.

(ii) Experiment II. A second vaccine efficacy trial was carried out with 96 5- to 6-week-old female BALB/c mice divided randomly into eight groups (a to h). Vaccination volumes, sites, and regimens were identical to those described for experiment I. Group a received a pcDNA3.1 control; group b received pcDNA3.1 control with EP and then was boosted with cocktail recombinant protein vaccines; group c received cocktail DNA vaccines; group d received cocktail DNA vaccines and then was boosted with cocktail recombinant protein vaccines; group e received cocktail DNA vaccine with EP; group f received cocktail DNA with EP and then was boosted with cocktail recombinant protein vaccines; group g received pcDNA3.1-SjCTPI plasmid DNA; group h received pcDNA3.1-SjCTPI with EP.

For both experiments I and II, mice were challenged with 40 ± 1 cercariae of *S. japonicum* 4 weeks after the final DNA immunization or 2 weeks after the protein boost, by abdominal skin penetration.

Determination of protective efficacy. Six weeks postchallenge, mice were euthanized and perfused to determine adult worm burdens, and sections of livers were removed and digested to determine liver egg burdens. Mouse livers were removed and weighed and then digested overnight with 5% potassium hydroxide (5 ml) at 37°C. The worm reduction rates were calculated as follows: [(average worm burden in control group – average worm burden in test group)/(average worm burden in control group)] × 100%. The egg reduction rate was calculated as [(eggs per gram in control group – eggs per gram in test group)/(eggs per gram in control group)] × 100%.

Antibody detection. For antibody detection, serum samples were collected 2 days before vaccination and before challenge. Purified, recombinant SjC23, SjCTPI, and NP30 were diluted with carbonate buffer (pH 9.6) to 5 μg/ml, mixed at equal volumes, and then plated at 100 μl/well overnight at 4°C. Plates were then washed three times with phosphate-buffered saline (PBS) containing 0.05% Tween 20 (PBST) and then blocked with 0.3% (wt/vol) bovine serum albumin in PBS for 1 h at room temperature. After blocking, plates were washed three times with PBST and then incubated with mouse serum which was diluted 1:400 for immunoglobulin G (IgG) detection and 1:200 for IgG1 and IgG2a detection with PBS at 37°C for 1 h. Prevaccination and infected mouse sera were used as negative and positive controls, respectively. Horseradish peroxidase-conjugated goat anti-mouse IgG, IgG1, and IgG2a were obtained from Southern Biotech (Birmingham, AL). The plates were washed five times with PBST, followed by incubation with horseradish peroxidase-conjugated goat anti-mouse IgG, IgG1, and IgG2a (diluted 1:5,000) for 1 h at 37°C. Plates were washed five times with PBST and then developed with tetramethylbenzidine substrate (Jingmei Biotech, Beijing, China) for 5 to 10 min, and the reaction was stopped by the addition of sulfuric acid (2 mol/liter). The optical density was read at 450 nm using a microplate reader (Autobio, Zhengzhou, China).

Cytokine measurements. Three days before challenge, two mice from each group were sacrificed, spleens were removed, and single-cell suspensions were prepared. Splenocytes from two mice were pooled, and then red blood cells were lysed with lysis buffer (NH_4Cl at 139.6 mmol/liter, Tris at 16.96 mmol/liter; pH 7.2), washed, counted, and then cultured in triplicate in 96-well plates (Corning) at 6×10^5 cells/well in RPMI 1640 medium supplemented with 10% fetal calf serum for 72 h at 37°C with 5% CO_2. Cells were stimulated with soluble egg antigen (SEA) at 100 μg/ml, concanavalin A (ConA) at 10 μg/ml, or medium alone. Culture supernatants were harvested and kept at −80°C until detection and assayed by flow cytometry analysis with a BD FACSCalibur flow cytometer for interleukin-2 (IL-2), IL-4, and IFN-γ by using a mouse/rat soluble protein CBA Flex kit (BD Biosciences), according to the manufacturer's instructions.

Statistics. Values for worm and egg burdens and antibody levels were compared using a one-way analysis of variance, and a P value of <0.05 was considered statistically significant.

RESULTS

Determination of protective efficacy against challenge infection. Efficacies of the different vaccination regimens were calculated as differences in adult male and female worm burdens as well as reductions in liver egg burdens 42 days postchallenge. The results of the two experiments are presented in Tables 1 and 2. In experiment I (Table 1), mice inoculated intramuscularly with cocktail DNA vaccines (group D) showed a significant worm reduction of 32.88% ($P < 0.01$) and egg reduction of 36.20% ($P < 0.01$) compared to mice injected with saline or pcDNA3.1 control (groups A and B). Reductions in worm and egg burdens were enhanced when these groups were boosted with cocktail protein vaccines (group E), with adult worm and egg reduction rates of 45.35% and 48.54%, respectively. Interestingly, nearly identical results were obtained in mice boosted with cocktail DNA vaccines administered by electroporation (group H), with adult worm and egg burdens reduced by 45.00% and 50.88%, respectively. There was a significant difference in both worm and egg reduction rates between group D and group E ($P < 0.05$) and between group D and group H ($P < 0.05$). A further significant increase in vaccine efficacy was seen when we employed each of the three vaccination methods on the same group of mice (group I). Mice immunized with cocktail DNA vaccines, boosted with cocktail DNA vaccines by electroporation, and then given a final boost with cocktail protein vaccines had adult worm and liver egg burdens reduced by 57.09% ($P < 0.01$) and 59.26% ($P < 0.01$), respectively, compared to control groups A and B. There were significant differences in both worm and egg burdens between group I and group H ($P < 0.05$) and between group I and group E ($P < 0.05$).

Similar results were obtained in experiment II (Table 2). We

TABLE 2. Protective efficacies of different vaccination regimens against *Schistosoma japonicum* infection (experiment II)[a]

Group	No. of mice	Total no. of worms	% Worm reduction	No. of female worms	% Female-worm reduction	No. of eggs in liver	% Egg reduction
a (pcDNA3.1)	10	22.40 ± 2.59		10.10 ± 3.38		64,849 ± 11,452	
b (pcDNA3.1 with EP and cocktail protein vaccines)	11	19.09 ± 3.54	14.77	7.00 ± 2.44	30.69	51,479 ± 9,833	20.62
c (cocktail DNA vaccines)	9	15.11 ± 2.09	32.54	5.33 ± 3.35	47.19	36,275 ± 13,069	44.06
d (cocktail DNA and protein vaccines)	9	12.56 ± 1.94	43.95	3.78 ± 1.39	62.60	27,513 ± 8,181	57.57
e (cocktail DNA vaccine with EP)	9	13.11 ± 2.71	41.47	4.67 ± 1.73	53.80	30,592 ± 3,379	52.83
f (cocktail DNA with EP and protein vaccines)	10	9.10 ± 3.21	59.38	2.80 ± 1.23	72.28	21,081 ± 11,856	67.49
g (TPI)	10	16.30 ± 2.36	27.23	6.50 ± 1.51	35.64	47,180 ± 10,735	27.25
h (TPI with EP)	11	12.91 ± 2.59	42.37	5.09 ± 2.17	49.60	37,127 ± 10,953	42.75

[a] Values for worm and egg counts are means ± standard deviations. Adult worm and liver egg reduction rates were calculated for each group in comparison to group a. Statistically significant differences in adult worm and liver egg burdens were seen for the following comparisons: between group d and group b ($P < 0.01$) or group c ($P < 0.05$); between group e and group c ($P < 0.05$); between group h and group g (TPI) ($P < 0.01$); and between group f and group d ($P < 0.05$) or group e ($P < 0.01$).

observed that adult worm and liver egg burdens were significantly reduced in mice immunized with cocktail DNA vaccines (group e), 41.47% and 52.83%, respectively, or univalent DNA vaccine pcDNA3.1-SjCTPI (group h), 42.37% and 42.75%, respectively, followed by electroporation in vivo, compared to group c (32.54% and 44.06%) and group g (27.23% and 27.25%). As observed in experiment I, the best protective efficacy was seen in group f mice immunized with cocktail DNA vaccines followed by electroporation in vivo and boosted with cocktail protein vaccines. In group f, the adult worm, female worm, and egg reduction rates were 59.38%, 72.28%, and 67.49%, respectively, compared to the pcDNA3.1 control group.

Determination of vaccination-induced antigen-specific antibodies. To evaluate vaccine-specific antibody responses, serum was collected from each mouse and analyzed by enzyme-linked immunosorbent assay (ELISA) for antibody titers against the mixture of vaccine antigens (see Materials and Methods). The levels of total IgG and the IgG isotypic responses of mice in the various groups from the two experiments are shown in Fig. 1 and 2. The detection of the IgG, IgG1, and IgG2a levels was repeated in the two experiments and showed similar results.

In experiment I (Fig. 1), mice immunized with cocktail DNA vaccines and/or cocktail protein vaccines produced vaccine antigen-specific IgG, while sera from control groups did not. Interestingly, group E, vaccinated using cocktail DNA priming and protein boosting, had a significantly higher antibody response (A_{450}, 2.63) than group D, which was vaccinated solely with cocktail DNA vaccines (A_{450}, 1.37). Similarly, there was a significantly elevated vaccine specific antibody response in group H mice vaccinated with cocktail DNA vaccines followed by electroporation in vivo (A_{450}, 2.04) compared to mice solely vaccinated with cocktail DNA vaccines (group D).

Mice immunized with cocktail DNA vaccines and then boosted with cocktail protein vaccines produced vaccine-specific IgG1 and IgG2a antibodies, with ratios of IgG2a/IgG1 of 0.525, 1.829, and 0.712 in groups C, D, and E and 0.394, 3.518, and 0.914 in groups G, H, and I, respectively. Levels of total IgG and IgG1 were increased significantly compared to control

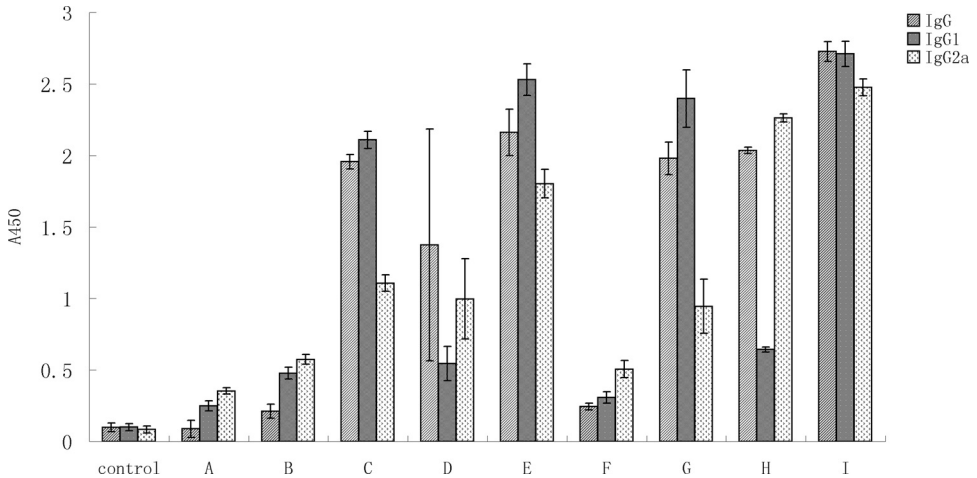

FIG. 1. Levels of IgG, IgG1, and IgG2a antibodies to vaccine antigen(s) in experiment I. Sera were collected as described in Materials and Methods and tested by ELISA for IgG, IgG1, and IgG2a antibodies against pooled vaccine antigens. Levels of IgG in group E (cocktail DNA and protein vaccines) were significantly higher than in group D (cocktail DNA vaccines) ($P < 0.01$); levels of IgG in group H (cocktail DNA vaccines with EP) were significantly higher than in group D ($P < 0.01$); levels of IgG in group I (cocktail DNA with EP and protein vaccines) were significantly higher than in group E ($P < 0.01$) or group H ($P < 0.01$).

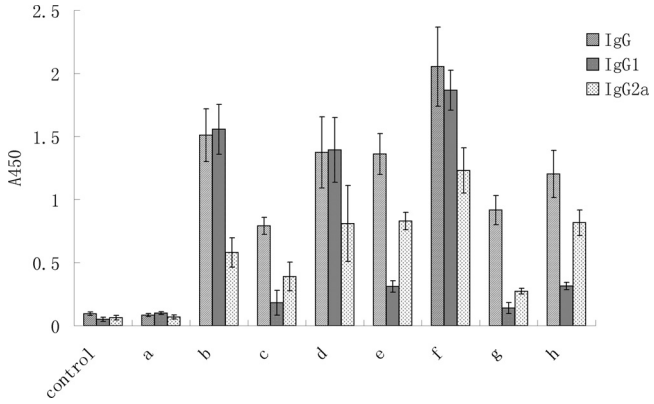

FIG. 2. Levels of IgG, IgG1, and IgG2a antibodies to vaccine antigen(s) in experiment II. An ELISA to determine levels of vaccine antigen(s)-specific IgG, IgG1, and IgG2a was conducted as described in Materials and Methods. Levels of IgG in group d (cocktail DNA and protein vaccines) were significantly higher than in group c (cocktail DNA vaccines) ($P < 0.01$); levels of IgG in group e (cocktail DNA vaccine with EP) were significantly higher than in group c ($P < 0.01$); levels of IgG in group h (TPI with EP) were significantly higher than in group g (TPI) ($P < 0.05$); levels of IgG in group f (cocktail DNA with and protein vaccines) were significantly higher than in group d ($P < 0.01$) and group e ($P < 0.01$).

groups when cocktail proteins were used for boosters. Further, the levels of IgG2a were significantly increased compared to the group primed with cocktail DNA vaccines and then boosted by electroporation in vivo.

In experiment II (Fig. 2), similar tendencies were observed in terms of IgG2a/IgG1 ratios. Group-specific ratios were as follows: group b, 0.373; group c, 2.152; group d, 0.581; group e, 2.669; group f, 0.659; group g, 1.943; group h, 2.599. Compared to the nonelectroporation group, there were significant elevations of IgG levels in the group primed with cocktail DNA vaccines followed by boosting via electroporation. We also observed elevations in vaccine-specific IgG levels in mice boosted with cocktail protein vaccines, compared to boosting with cocktail DNA vaccines.

Vaccine-specific cytokine production induced by various vaccination regimens. In addition to measuring antigen-specific antibody responses, we also assayed for production of IL-2, IFN-γ, and IL-4 in response to stimulation with SEA or ConA. Splenocytes were harvested as described in Materials and Methods and then pulsed with medium (blank), ConA, or SEA for 72 h and supernatants collected. Levels of IL-4 in all groups from both trials were less than 10 pg/ml (the minimum limit of detection using this kit is 10 pg/ml). This is consistent with earlier studies with TPI and the 23-kDa integral membrane protein candidate vaccine antigens driving Th1-type but not Th2-type immune responses (9, 10). Levels of IL-2 and IFN-γ for each of the two experiments are presented in Tables 3 and 4, respectively.

In experiment I levels of IL-2 in response to ConA or SEA in each of the three control groups (A, B, and F) were undetectable or minimal (Table 3). Similar to the results for vaccine efficacy, the highest levels of IL-2 were seen in groups H and I, the groups boosted via electroporation or with electroporation followed by a final boost with cocktail recombinant vaccine proteins (group I), with the highest levels of IL-2 detected

TABLE 3. Boosting with cocktail DNA vaccines by electroporation or with cocktail protein vaccines leads to enhanced production of IL-2 and IFN-γ (experiment I)

Group	IL-2 (pg/ml)[a]			IFN-γ (pg/ml)[a]		
	Blank	ConA	SEA	Blank	ConA	SEA
A	<10	11.38	10.01	<10	<10	<10
B	<10	<10	11.11	<10	<10	17.58
C	<10	18.50	24.90	<10	19.54	36.70
D	<10	20.90	59.21	<10	120.72	492.31
E	<10	23.97	68.02	<10	123.86	503.90
F	<10	<10	<10	<10	11.95	39.55
G	<10	21.46	52.94	<10	27.58	83.15
H	<10	39.15	79.28	<10	300.79	625.55
I	<10	43.92	112.02	<10	97.05	664.23

[a] Data are average values of readings from triplicate wells for each group.

from splenocytes of group I animals. Results for IFN-γ followed a similar pattern to those detected for IL-2, with the three control groups (A, B, and F) having negligible levels of IFN-γ production and groups H and I having the highest levels of IFN-γ production. Interestingly, levels of IFN-γ were also significantly elevated in groups D (cocktail DNA vaccines) and E (cocktail DNA vaccines and protein cocktail boost). The levels of IL-2 and IFN-γ were highest in the groups that received a vaccine boost via electroporation.

Experiment II compared the three optimal vaccination regimens determined in the trials in experiment I, as well as two groups, g and h, for which SjCTPI DNA was the only vaccine antigen (Table 4). Levels of IL-2 from SEA-stimulated splenocytes were highest in group f, the group with the highest level of protective efficacy. Group f received cocktail DNA vaccines as a prime, followed by cocktail DNA vaccines electroporated with a final boost of cocktail protein vaccines. Interestingly, groups g and h, which both utilized only SjCTPI DNA vaccine, elicited fairly high levels of IL-2. Consistent with data throughout this study, in group f in this experiment (group I in experiment I) the highest levels of IFN-γ were induced. Splenocytes from mice vaccinated only with SjCTPI (g and h) had elevated levels of both IL-2 and IFN-γ. The levels of IFN-γ for these two groups (g and h) were not as significantly elevated as the levels of IL-2.

TABLE 4. Boosting by electroporation or with cocktail protein vaccines leads to enhanced production of IL-2 and IFN-γ (experiment II)

Group	IL-2 (pg/ml)[a]			IFN-γ (pg/ml)[a]		
	Blank	ConA	SEA	Blank	ConA	SEA
a	<10	37.54	13.11	<10	38.97	26.56
b	<10	91.99	111.48	<10	35.35	197.98
c	<10	45.39	234.68	<10	49.32	439.27
d	<10	75.33	270.74	<10	75.33	546.32
e	<10	89.65	254.71	<10	96.71	896.43
f	<10	43.17	431.73	<10	92.67	1,398.04
g	<10	31.72	110.30	<10	65.04	265.04
h	<10	74.32	231.28	<10	83.43	318.23

[a] Data are average values of readings from triplicate wells for each group.

DISCUSSION

Schistosomiasis is an infectious disease that is widespread in tropical and subtropical countries. China is one of the countries where schistosomiasis is endemic. Current schistosomiasis control strategies in China are based on chemotherapeutic treatment of infected individuals and eradication of the snail intermediate hosts (25). In the lakes and marsh regions of China, seasonal transmission means that a large percentage of individuals residing in these areas will be reinfected on an annual or frequent basis, requiring constant monitoring of infection and drug treatment with praziquantel. Annual administration of praziquantel, the drug of choice for more than 30 years, provides an opportunity for schistosomes to become resistant to this drug. In other countries where praziquantel is administered in mass chemotherapy programs, resistance has been reported (3, 12). Thus, the development of alternative, long-term control measures, such as vaccines, is required. Control of schistosomiasis in the lakes and marsh regions of China as well as other Southeast Asian countries and in Saharan Africa will require development of interventions that reduce the numbers of infecting parasites such that the numbers of egg-laying adult parasites are significantly reduced, leading to reductions in the numbers of eggs shed into the environment and reducing transmission of the disease.

The use of partially protective vaccines administered in conjunction with praziquantel treatment has been considered an ideal control measure for some time. To date, a number of candidate vaccines have been tested in various animal models of schistosomiasis japonica, including several different DNA vaccines (SjFABP, SjGCP, SjGST, SjC23, and SjCTPI). Each of these DNA vaccines provided partial protection against *Schistosoma japonicum* infection in mice (36). The purpose of this study was to determine if administration of partially protective DNA vaccines via different regimens would significantly elevate vaccine efficacy over that previously reported. *Schistosoma japonicum* is a complex helminth parasite with multiple life cycle stages in the mammalian definitive host. Additionally, the parasite has a complex outer membrane structure which helps parasites evade immune system recognition. Therefore, for this study, we decided to vaccinate mice with cocktail vaccines that allowed us to target parasite membranes (SjC23), a glycolytic enzyme (SjCTPI), and a gut-associated antigen (CDR3). Vaccination with cocktail DNA vaccines provided a 32.88% worm reduction rate and 36.20% egg reduction rate, which was slightly higher than efficacy levels for univalent DNA vaccines. However, the amount of DNA administered (50 μg of each DNA vaccine) in this study was half the amount used in previous studies (100 μg) (37, 41).

The alterations in DNA vaccination that we employed in this study were either to deliver DNA vaccines via electroporation or to perform a final boost with recombinant protein antigens. Electroporation is a novel, excellent, and nonviral system for delivering DNA into muscle. Immunization with cocktail DNA vaccines followed with a boost of cocktail vaccines delivered by electroporation significantly enhanced vaccine efficacy over that seen when cocktail DNA vaccines were administered without electroporation. Worm and egg reduction rates increased to 45.00% and 50.88%, respectively, significantly higher than the efficacy levels observed in mice immunized with cocktail DNA vaccines delivered by injection ($P < 0.05$). Experiment II provided nearly identical results, showing significantly increased vaccine efficacy when electroporation was used to deliver the cocktail DNA vaccine boost, rather than intramuscular injection. Examination of splenocyte responses to ConA or parasite antigens demonstrated that mice vaccinated via the regimen of cocktail DNA vaccines with EP produced higher concentrations of IL-2 and IFN-γ than cells from mice immunized with DNA vaccines delivered by injection. Thus, administration of DNA vaccines by electroporation significantly enhances vaccine efficacy and immunogenicity. The reason for this may be electroporation in vivo is a more efficient way to get DNA into muscle cells, which would augment expression of vaccine antigens, providing greater immune system stimulation (27). Another possibility is that the application of an electric field directly in the tissue results in an inflammatory response that aids priming of immune responses against DNA-encoded antigens (30). The electroporation protocol that we employed here has been successfully utilized for DNA vaccines for several different diseases (19).

Prime-boost strategies were developed to improve overall vaccine efficacy following priming with plasmid DNA. Prime-boost strategies deliver DNA vaccines for the priming immunization and then boost with proteins, peptides, or live attenuated viruses (22). Results for this immunization strategy have been highly encouraging in both augmenting and modulating vaccine-induced immunity against many diseases. In the present study, we primed with cocktail DNA vaccines against *Schistosoma japonicum* and then boosted with cocktail protein vaccines. In this study we found that mice vaccinated via the prime-boost regimen had enhanced protection against challenge infection compared to mice vaccinated via the DNA-DNA regimen. Adult worms and liver egg burdens were reduced 32.88% and 36.20%, respectively, in DNA-DNA-treated mice, compared to 45.35% and 48.54%, respectively, in prime-boost-vaccinated mice (experiment I, with similar results in experiment II).

In addition to increasing vaccine efficacy, vaccination via prime-boost significantly increased vaccine-specific antibody responses over those seen in mice solely vaccinated with plasmid DNA vaccines. The positive impact of protein boosting on vaccine-specific antibody responses has been demonstrated previously (7, 8, 20, 23, 28). These enhanced vaccine responses are likely due to increased antigen presentation. In contrast to proteins expressed in the cytosol following DNA injection, injected proteins are endocytosed and presented on major histocompatibility complex class II molecules, facilitating humoral responses (15). In addition, antibody maturation through increased avidity may also play a role in mice immunized by DNA priming followed by protein boosting. Interestingly, protein boosting had a greater effect on vaccine-specific antibody production in animals whose first boost was via electroporation compared to the groups of mice that received cocktail DNA vaccines by injection in both experiments. Taken together with the efficacy results, electroporation has a beneficial impact on enhancing vaccine-specific immune responses. As mentioned earlier, one possible mechanism is that electroporation-mediated tissue damage may act by recruiting mononuclear cells to the site of antigen expression, as observed by Gronevik et al. (10, 17).

Several groups have suggested that cell-mediated immunity involving IFN-γ-activated cells may be one mechanism of protective immunity in the mouse schistosome challenge model (4, 9, 21). In this regard, in vitro studies have demonstrated that schistosomula, the presumed target stage for both SjC23 and SjCTPI vaccines, were killed by macrophages or endothelial cells activated via combinations of cytokines, including IFN-γ and IL-12 (33). Therefore, Th1-type immune responses seem to be important in the induction of vaccine-induced resistance to schistosomiasis in mice. In the present study, the levels of IL-2 and IFN-γ and the ratios of IgG2a/IgG1 clearly showed that vaccination with these DNA vaccines induced $CD4^+$ Th1-type responses.

While boosting via electroporation or with recombinant proteins significantly increased vaccine efficacy and associated immune responses over those seen in mice vaccinated solely with cocktail DNA vaccines, the vaccine regimen that combined each of these approaches induced the highest levels of protective efficacy, inducing 60% or greater reductions in adult worm and liver egg burdens. The results of these trials show that there are better ways to deliver schistosome vaccines in mice and that these methods should rapidly be attempted in large animals, such as water buffalo, to determine if these vaccination regimens approach are effective in large animals which have been shown to play large roles in the transmission of schistosomiasis.

With regard to schistosomiasis in humans, serious symptoms of schistosomiasis generally appear in people who harbor large numbers of parasites, and thus a vaccine able to effectively reduce a patient's worm burden by 50% or more could dramatically reduce the number of severe cases of the disease, decreasing morbidity. Furthermore, as with vaccination of water buffalo, a 50% efficacious vaccine for human schistosomiasis would decrease transmission of the disease (18). In this study, two large experiments were carried out that compared multiple vaccination parameters. We found that vaccination with cocktail plasmid DNA vaccines that included a boost administered by electroporation increased vaccine efficacy by greater than 40%. Importantly, the addition of a protein cocktail vaccine boost to this effective regimen elevated efficacy to approximately 60% for adult worm burden reduction and greater than 60% for liver egg reduction. Employment of either vaccine regimen in water buffalo alone, combined with praziquantel treatment, should result in significant decreases in transmission of the disease in the lakes and marsh regions, based on mathematical models of transmission in these regions of China (31). Importantly, if these vaccines were to be utilized in humans as well as bovines, then one would expect to see a dramatic drop in transmission of schistosomiasis as well as a reduction in infection prevalence and intensity. We believe that if either of these two improved vaccination regimens are shown to augment vaccine efficacy in water buffalo, and these findings are taken together with previous work by our groups, a standard vaccination protocol for schistosomiasis in China and eventually other regions of the world will be developed.

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