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An oral mucosal DNA vaccine for SARS coronavirus infections

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Main Body

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Summary

In this study, different forms of SARS coronavirus (SARS-CoV) spike protein-based vaccines for generation of neutralizing antibody response against SARS-CoV were compared using a mouse model. High IgG levels were detected in mice immunized with intraperitoneal (i.p.) recombinant spike polypeptide generated by Escherichia coli (S-peptide), mice primed with intramuscular (i.m.) tPA-optimize800 DNA vaccine (tPA-S-DNA) and boosted with i.p. S-peptide, mice primed with i.m. CTLA4HingeSARS800 DNA vaccine (CTLA4-S-DNA) and boosted with i.p. S-peptide, mice primed with oral live-attenuated Salmonella typhimurium (Salmonella-S-DNA-control) and boosted with i.p. S-peptide, mice primed with oral live-attenuated S. typhimurium that contained tPA-optimize800 DNA vaccine (Salmonella-tPA-S-DNA) and boosted with i.p. S-peptide, and mice primed with oral live-attenuated S. typhimurium that contained CTLA4HingeSARS800 DNA vaccine (Salmonella-tPA-S-DNA) and boosted with i.p. S-peptide. No statistical significant difference was observed among the Th1/Th2 index among these six groups of mice with high IgG levels. Sera of all six mice immunized with i.p. S-peptide, i.m. DNA vaccine control and oral Salmonella-S-DNA-control showed no neutralizing antibody against SARS-CoV. Sera of the mice immunized with i.m. tPA-S-DNA, i.m. CTLA4-S-DNA, oral Salmonella-S-DNA-control boosted with i.p. S-peptide, oral Salmonella-tPA-S-DNA, oral Salmonella-tPA-S-DNA boosted with i.p S-peptide, oral Salmonella-CTLA4-S-DNA and oral Salmonella-CTLA4-S-DNA boosted with i.p. S-peptide showed neutralizing antibody titers of <1:20-1:160. Sera of all the mice immunized with i.m. tPA-S-DNA boosted with i.p. S-peptide and i.m. CTLA4-S-DNA boosted with i.p. S-peptide showed neutralizing antibody titers of >or=1:1280. The observation in this study may have major practical value, such as immunization of civet cats, since production of recombinant proteins from E. coli is far less expensive than production of recombinant proteins using eukaryotic systems.

Introduction

SARS – Overview

Severe acute respiratory syndrome (SARS), which happened in 2003 in 30 countries across five continents, was the first coronavirus caused epidemic leading to 774 deaths out of 8096 cases,[1-4] with around 10% fatality rate. A novel coronavirus, SARS coronavirus (SARS-CoV), was subsequently found to be the causative agent for the disease based on the fulfillment of Koch's postulates.[1,5-6] SARS-CoV was shown to be transmitted from exotic game food animals to human. The lack of recognition of the virus, the high transmission rate in the "super-spreading events",[7-11] together with the ease of international air travel in modern world help in the rapid global dissemination of the disease. The discovery of the very similar bat SARS-CoV,[12-13] and the small scale SARS re-emergence in late 2003 after resumption of Southern China's wild life market,[14-15] show the possibility for SARS to return if the conditions are fit for the introduction, mutation, amplification and transmission of the virus.[12-15]

Prevention of SARS

Although lots of drugs have been tested for their antiviral activity against SARS-CoV, the actual effect of the antiviral drugs is still unknown. Therefore, prevention of SARS through infection control and vaccine would still be the most effective way against global spreading of future SARS outbreak.

In animal coronavirus infections, it has been shown that the spike proteins of coronaviruses were highly immunogenic, and immunization of animals using spike protein-based vaccines were able to produce neutralizing antibodies that were effective in prevention of infections caused by the corresponding coronaviruses. For SARS-CoV infection, it has been shown that nucleotides 952–1530 of the spike protein gene of SARS-CoV encoded a 193-amino acid fragment responsible for attaching to the receptor for SARS-CoV, angiotensin-converting enzyme 2.[16] Furthermore, we, and also others, have shown that patients with SARS produced antibody response against the spike protein of SARS-CoV,[17-19] and it has been demonstrated that the spike protein is the major target for passive immunization.[20-21] In studies that determine the relative importance of humoral and cell mediated immunity for protection against SARS-CoV infection, it was confirmed that neutralizing antibody, when administered by passive immunization, was crucial in conferring protection,[22] whereas T-cell immunity was unable to lead to protection.[23] In addition, for vaccine candidates against SARS-CoV, spike protein-based DNA vaccines appeared to be a promising group of vaccine shown to produce protective immunity against SARS-CoV infections, whereas recombinant spike protein vaccines produced by *Escherichia coli* were not efficient in terms of generation of protective immunity as compared to those generated from eukaryotic systems such as transfection of cell lines.[22-33] However, multiple doses of intramuscularly (i.m.) administered DNA vaccine or recombinant protein generated from the eukaryotic systems are quite expensive, and therefore may not be practical in developing countries. No data on less expensive modalities of immunization, such as DNA vaccine followed by boosters of recombinant vaccine produced by E. coli or oral mucosal DNA vaccines,[34-37] are available.

In this study, we compared the different forms of SARS-CoV spike protein-based vaccines for generation of neutralizing antibody response against SARS-CoV using a mouse model. The relative effectiveness of recombinant spike polypeptide vaccine produced by *E. coli*, two different types of intramuscular spike polypeptide DNA vaccine with and without boosters of recombinant spike polypeptide vaccine produced by *E. coli* and two different types of oral mucosal spike polypeptide DNA vaccine with and without boosters of recombinant spike polypeptide vaccine produced by *E. coli* are compared.

Materials and methods

Animals

Male BALB/c (H-2^d) mice (6–8 weeks old, 18–22 g) were used in all animal experiments. They were housed in cages, under standard conditions with regulated day length, temperature and humidity, and were given pelleted food and tap water ad libitum.

Recombinant SARS-CoV S polypeptide vaccine from E. coli

To produce a plasmid for protein expression, primers (LPW742 5'-CGCGGATCCGAGTGACCTTGACCGGTGC-3' and LPW931 5'-CGGGGTACCTTAACGTAATAAAGAAACTGTATG-3') were used to amplify the gene encoding amino acid residues 14- 667 of the spike protein of the SARS-CoV by RT-PCR. This portion of the spike protein was used because it contains the receptor-binding domain within the S1 domain that is highly immunogenic, whereas the complete spike protein was not expressible in *E. coli*. The PCR product was cloned into the *Bam*HI and *Kpn*I sites of vector pQE-31 (Qiagen, Hilden, Germany). The resultant clone was digested by *Pst*I, and the *Pst*I fragment which contained the gene encoding amino acid residues 250- 667 of the spike protein was cloned into expression vector pQE-30 (Qiagen, Hilden, Germany) in frame and downstream of the series of six histidine residues. The $(His)_6$-tagged recombinant spike polypeptide (S-peptide) was expressed and purified using the Ni^{2+}-loaded HiTrap Chelating System (Amersham Pharmacia, USA) according to the manufacturer's instructions.

Human codon usage optimized SARS-CoV DNA vaccines

To enhance the expression of S polypeptide in human cells, the 2 SARS-CoV DNA vaccines, tPA-optimize800 (tPA-S-DNA) and CTLA4HingeSARS800 (CTLA4-S-DNA), were constructed using the concept of human codon usage optimization[38] with QUIKChange Multi Site-Directed Mutagenesis Kit (Strategene, USA) according to manufacturer's instructions in the laboratory of Dr. David Ho. The synthetic polypeptides were cloned into pcDNA3.1(+) (Invitrogen, CA, USA).

Oral mucosal tPA-optimize800 and CTLA4HingeSARS800 DNA vaccines

The oral mucosal tPA-optimize800 and CTLA4HingeSARS800 DNA vaccines (*Salmonella*-tPA-S-DNA and *Salmonella*-CTLA4-S-DNA) were prepared by transforming into auxotrophic *S. typhimurium* aroA strain SL7207 (*S. typhimurium* 2337-65 derivative *hisG46*, DEL 407 [aroA::Tn10 Tc-s], a gift from Dr Bruce Stocker) by electroporation.[39]

Transfection of 293 cells with tPA-optimize800 and CTLA4HingeSARS800

Two hundred and ninety-three cells were plated at 1×10^7 cells per well in Dulbecco's modified Eagle's medium with 10% fetal calf serum in a six-well plate on the day before transfection. On the day of transfection, each well was transfected with 1 μg plasmid encoding eukaryotically expressed SARS-CoV S polypeptide (tPA-S-DNA or CTLA4-S-DNA) or pcDNA3.1(+) (S-DNA-control) with FuGENE 6 Reagent (Boehringer Mannhein, Germany) according to manufacturer's instructions. Forty-eight hours after transfection, the cells were harvested and lysed by freezing and thawing 3 times. After centrifugation at 14000 rpm, the supernatant was used for the detection of SARS-CoV S polypeptide by Western blot assay using pre-immune rabbit serum and hyperimmune polyclonal serum from rabbit immunized with S-peptide.

Western blot analysis

Ten microliters of supernatant of 293 cell lysates obtained from 293 cells transfected with tPA-S-DNA, CTLA4-S-DNA or S-DNA-control was loaded into each well of a sodium dodecyl sulfate (SDS)–8% polyacrylamide gel and subsequently electroblotted onto a nitrocellulose membrane. The blot was incubated separately with 1:1000 dilution of pre-immune rabbit serum or hyperimmune polyclonal serum from rabbit immunized with S-peptide. Antigen–antibody interaction was detected with an ECL fluorescence system.

Immunization schedule

Seventy-two BALB/c mice were used for the immunization experiments. The immunization schedule is summarized in Table 1. On days 0, 14 and 28, 6 mice were immunized intraperitoneally (i.p.) with S-peptide [0.5 µg per mouse (Group 1, Table 1)]. On day 0, 6 mice were immunized i.m. (tibialis anterior muscle) with S-DNA-control [100 µg per mouse (Group 2, Table 1)] and 12 mice each were immunized i.m. with tPA-S-DNA [100 µg per mouse (Group 3, Table 1)] or CTLA4-S-DNA [100 µg per mouse (Group 5, Table 1)]. On days 28 and 42, 6 of the 12 mice in the two DNA vaccine groups were boosted with i.p. S-peptide [0.5 µg per mouse (Groups 4 and 6, Table 1)]. On day 0, 12 mice each were immunized orally with *S. typhimurium* aroA strain (*Salmonella*-S-DNA-control) [6 × 10^9 bacterial cells per mouse (Group 7, Table 1)], *Salmonella*-tPA-S-DNA [6 × 10^9 bacterial cells per mouse (Group 9, Table 1)] or *Salmonella*-CTLA4-S-DNA [6 × 10^9 bacterial cells per mouse (Group 11, Table 1)]. On days 28 and 42, 6 of the 12 mice in the three groups were boosted with i.p. S-peptide [0.5 µg per mouse (Groups 8, 10 and 12, Table 1)].

Measurement of serum antibodies against SARS-CoV S polypeptide

Mice from each group were bled on the day before immunization and 42 days after the last dose of vaccine in the corresponding group. The blood was centrifuged at 2700 × *g* for 20 min and the supernatant (serum) was stored at -70 °C before antibody measurement.

Mouse sera (diluted with PBS-2% skim milk, 1:10 for IgM, 1:80 for IgG, 1:1280 for IgG1, 1:40 for IgG2a, 1:10 for IgG2b and 1:320 for IgG3) were added to ELISA plates precoated with S-peptide (80 ng per well for IgM, IgG, IgG2a, IgG2b and IgG3 and 10 ng per well for IgG1). The plates were incubated at 37 °C for 1 h. After washing with washing buffer 5 times, 100 µl HRP-conjugated goat anti-mouse IgM and IgG, rabbit anti-mouse IgG1, IgG2a and IgG3 and rat anti-mouse IgG2b antibody (Zymed Laboratories Inc., USA) diluted according to manufacturer's instructions using PBS-2% skim milk were added to the corresponding wells accordingly and incubated at 37 °C for 1 h. IgM and total IgG levels were assayed to assess the primary and secondary immune response, while the IgG subtypes were used to determine whether the humoral response was inclined towards the Th1 (IgG2a and IgG2b) or Th2 (IgG1 and IgG3) pattern. After washing with washing buffer 5 times, 100 µl diluted 3,3′,5,5′- tetramethylbenzidine was added to each well and incubated at room temperature for 15 min. One hundred microliters of 0.3 M sulphuric acid was added and the absorbance at 450 nm of each well was measured. Each sample was tested in duplicate and the mean absorbance for each serum was calculated. The serum antibody level of a particular mouse was defined as the absorbance obtained from the serum taken 42 days after the last dose of the vaccine minus that of the corresponding mouse taken the day before immunization. The Th1/Th2 index of each mouse is calculated by the following formula: IgG2a × IgG2b/IgG1 × IgG3.

Neutralizing antibody assay

All work with infectious virus was performed inside a type II Biosafety Cabinet, in a Biosafety Containment level III facility, and with powered air-purifying respirators (HEPA Airmate, 3M, St. Paul, MN, USA). Initial screening of mouse sera against the prototype SARS-CoV strain no. 39849 was performed in 96-well microtiter plates seeded with fetal rhesus kidney-4 cells. Two-fold dilutions of mouse sera (from 1:20 to 1:1280) were tested in duplicate against 100 $TCID_{50}$ of SARS-CoV. A corresponding set of cell controls with sera but without virus inoculation was used as controls. The cells were scored for the inhibition of the CPE at 48 h. The titer of neutralizing antibody is defined as the maximum dilution of serum at which the percentage of CPE is less than or equal to 50%.

Measurement of lymphocyte proliferation index (LPI)

On day 60, single-cell suspensions of spleen cells from the 6 mice of each group were depleted of erythrocytes by adding freshly prepared Gey's solution. The cells were resuspended in RPMI 1640 medium (Gibco BRL, Rockville, MD) supplemented with 15% fetal calf serum and inoculated into microwell plates at 5×10^5 cells per well in triplicate. Cells were stimulated with phytohaemagglutinin at 5 μg per well (positive control), S-peptide at 0.1 μg per well or RPMI medium (negative control). Cells were cultured at 37 °C 5% CO_2 for 3 days, and ^{3}H-labelled thymidine (Amersham Life Science, Buckinghamshire, UK) was added at 1 μCi per well for the last 18 h. Cells were harvested onto glass microfibre filter (Whatman International Ltd., UK) using a Model CH1 cell harvester (Insel, Hampshire, UK) and radioactivity was measured by a liquid scintillation counter (Beckman, Fullerton, CA, USA). The S-peptide-specific LPI of a particular sample is defined as the ratio of the difference of radioactivity between the sample and the negative control and that between the positive and negative controls.

Interleukin-4 (IL-4) and interferon-γ (IFN-γ) assays

On day 60, spleens from the 6 mice in each group were harvested. Single-cell suspensions were prepared and cells from mice within the same group were pooled. 2×10^6 cells were cultured in 1 ml RPMI 1640 medium supplemented with 10% fetal calf serum and 5×10^{-5} M 2-mercaptoethanol in 24-well plates. S-peptide was added at final concentrations of 0.5, 1.0 and 2.5 μg/ml. Supernatant (200 μl) from each sample was collected at 24, 48 and 72 h for cytokine measurement. Supernatant from each sample was added to the IL-4 Mouse Biotrak ELISA 96-well microtiter plate (Amersham Life Science, Buckinghamshire, UK) and IFN-γ Mouse Biotrak ELISA 96 well microtiter plate (Amersham Life Science, Buckinghamshire, UK) according to the manufacture's instruction. Each well was measured at 450 nm, using TMB buffer as a blank. Each pooled sample was tested in triplicate and the mean absorbance for each pooled sample was calculated.

Statistical analysis

Comparison was made among the serum antibody levels and LPI of the various groups of mice using one-way ANOVA. $P < 0.05$ was regarded as statistically significant.

RESULTS

SARS-CoV spike polypeptide expression in 293 cells transfected with tPA-optimize800 and CTLA4HingeSARS800

The supernatant of 293 cell lysates obtained from 293 cells transfected with tPA-S-DNA, CTLA4-S-DNA or S-DNA-control were separated on SDS–polyacrylamide gels followed by Western blot analysis with sera from pre-immune rabbit serum or hyperimmune polyclonal serum from rabbit immunized with S-peptide. Prominent immunoreactive protein bands of about 90 and 110 kDa were visible on the Western blot that used cell lysates obtained from 293 cells transfected with tPA-S-DNA and CTLA4-S-DNA, respectively, as the antigen and hyperimmune polyclonal serum from rabbit immunized with S-peptide as the source of antibody (Figure 1, lanes 1 and 2). These sizes were consistent with the expected size of 91.4 and 108.1 kDa for the corresponding spike polypeptides.

Antibody response

The antibody levels of the 12 groups of mice on day 42 were summarized in Figure 2. No IgG was detected in mice of Groups 2, 3, 5, 7, 9 and 11, whereas high IgG levels were detected in mice of Groups 1, 4, 6, 8, 10 and 12. No statistical significant difference was observed among the Th1/Th2 index among these six groups of mice with high IgG levels.

Neutralizing antibody assay

The number of mice with different neutralizing antibody titers immunized with different forms of spike polypeptide-based vaccines against SARS-CoV was shown in Table

2. Sera of all the six mice immunized with i.p. S-peptide, i.m. S-DNA-control and oral Salmonella-S-DN A-control (Groups, 1, 2 and 7) showed no neutralizing antibody against SARS-CoV. Sera of the mice immunized with i.m. tPA-S-DNA, i.m. CTLA4-S-DNA, oral *Salmonella*-S-DNA-control boosted with i.p. S-peptide, oral *Salmonella*-tPA-S-DNA, oral *Salmonella*-tPA-S-DNA boosted with i.p S-peptide, oral *Salmonella*-CTLA4-S-DNA and oral *Salmonella*-CTLA4-S-DNA boosted with i.p. S-peptide (Groups, 3, 5 and 8–12) showed neutralizing antibody titers of <1:20–1:160. Sera of all the mice immunized with i.m. tPA-S-DNA boosted with i.p. S-peptide and i.m. CTLA4-S-DNA boosted with i.p. S-peptide (Groups 4 and 6) showed neutralizing antibody titers of ≥ l:1280.

Lymphocyte proliferation index

The S-peptide-specific LPI of the 12 groups of mice on day 60 were summarized in Figure 3. Significant lymphocyte proliferation was detected in Groups 1, 3, 4, 5, 6, 8, 9, 10, 11 and 12, compared to the control groups (Groups 2 and 7).

Interleukin-4 and interferon-γ assays

At 24 h, IL-4 was undetectable in all 12 groups of mice for all three concentrations of S-peptide (data not shown). At 48 h, IL-4 was detectable only in mice of Groups 6, 8 and 12 (data not shown). At 72 h, IL-4 was detectable in mice of Groups 1, 5, 6, 8, 9, 10 and 12 (Figure 4).

At 24 h, the IFN-γ levels of the 12 groups of mice are shown in Figure 4. IFN-γ was detectable in mice of Groups 1, 3, 4, 5, 6, 8, 9, 10, 11 and 12. At 48 and 72 h, the IFN-γ levels mice of Groups 1, 4, 5, 6, 8, 10, 11 and 12 were all >6000 pg/ml (data not shown).

Discussion

Several different potential vaccines were developed to investigate their protection against SARS in this study. Among all the combinations of vaccines, mice primed with SARS-CoV human codon usage optimized spike polypeptide DNA vaccines and boosted with S-peptide produced by *E. coli* generated the highest titer of neutralizing antibody against SARS-CoV. It has been observed, and is confirmed in the present study, that S-peptide produced by *E. coli* did not induce neutralizing antibody against SARS-CoV infection (Table 2, Group 1). On the other hand, recombinant spike polypeptide generated by eukaryotic systems such as transfection of COS7 and BHK21 cells or DNA vaccine was able to elicit high neutralizing antibody titer against SARS-CoV infection.[23,29,32] This was probably because when S-peptide produced by *E. coli* was used, the three dimensional folding and/or the glycosylation of the S-peptide was not optimal for the generation of neutralizing antibodies. In this study, we documented that although recombinant S-peptide produced by *E. coli* itself was not able to generate neutralizing antibody against SARS-CoV infection, mice primed with spike polypeptide DNA vaccine and boosted with S-peptide from *E. coli* were able to generate high titer of neutralizing antibody against SARS-CoV (Table 2, Groups 4 and 6). This indicates that the type of vaccine used for priming is crucial in determining the type of immune response developed. Subsequent doses will booster the immune response generated by the first dose of vaccine. Of note is that the humoral immune response developed in mice primed with spike polypeptide DNA vaccine and boosted with S-peptide from *E. coli* was not particularly of the Th1 type as compared to that developed in mice immunized with S-peptide from E. coli alone. This indicates that a Th1 type immune response may not be essential for the generation of neutralizing antibodies against SARS-CoV.

The present observation may have major practical value, such as immunization of civet cats, as production of recombinant proteins from *E. coli* is far less expensive than production of recombinant proteins using eukaryotic systems such as transfection of cell lines or DNA vaccines. Although it has been shown that DNA vaccines are able to generate both humoral and cellular immunity successfully for various pathogens in mice, one of the major limitations for its clinical use is its ineffectiveness when it is used in humans, unless a large amount of DNA is used for immunization.[40-41] As for the production of recombinant spike polypeptide generated by eukaryotic systems such as transfection of COS7 and BHK21 cells[32] or using the baculovirus system,[33] although the conformation and/or glycosylation of the spike polypeptide produced can theoretically be more similar to the native viral spike protein, it is not easy to scale up the production of such recombinant proteins to industrial levels. Prokaryotic system was used to express two antigenic determinants that were identified through testing by convalescent serum, however, neutralizing antibodies were not produced in all animals, which maybe due to the conformational change of the S polypeptide through expression by *E. coli*, and that the carrier protein may not be a good one for immunogens like small peptides.[42] Therefore, the large amount of S-peptide that is produced by *E. coli* in a relatively inexpensive way could be used as boosters instead of being injected alone as vaccine. This principle can also be examined in vaccination for other pathogens, where “more effective” modalities of vaccination, such as DNA vaccine, can be used for priming, and the “less expensive” recombinant protein produced by *E. coli*, instead of eukaryotic systems, can be used as boosters.

Spike polypeptide DNA vaccines delivered by the live-attenuated *Salmonella* system did not induce good neutralizing antibody against SARS-CoV infection. We have previously shown that hepatitis B virus DNA vaccine presented by the live-attenuated *Salmonella* system generated good cytotoxic T lymphocyte response, but minimal antibody response, against hepatitis B virus in a mouse model.[34-35] Furthermore, we found that this immune response was able to down-regulate transgene expression in hepatitis B virus surface antigen transgenic mice.[36] Subsequently, we reported a comparison of the efficacy of DNA vaccine, DNA vaccine delivered by the live-attenuated *Salmonella* system and recombinant protein vaccine for generation of protective immune response against *Penicillium marneffei*, a thermal dimorphic fungus infecting 10% of HIV positive patients in China and Southeast Asia, in a mouse model.[37] Results showed that, similar to hepatitis B virus DNA vaccine presented by the live-attenuated *Salmonella* system, *P. marneffei* DNA vaccine delivered by the live-attenuated *Salmonella* system did not generate good antibody response, whereas intramuscular DNA vaccine generated the best protective immunity against *P. marneffei* infection, implying that both cellular and humoral immune response are important for protection against *P. marneffei* infection [29]. In the present study, it was observed that, in line with the results of hepatitis B virus DNA vaccine and *P. marneffei* DNA vaccine delivered by the live-attenuated *Salmonella* system, spike polypeptide DNA vaccines delivered by the live-attenuated Salmonella system did not induce good antibody response (Figure 2 and Table 2, Groups 9 and 11). Although the mice developed high antibody levels against the spike protein after boosting with S-peptide (Figure 2, Groups 10 and 12), the antibodies were not neutralizing in our cell culture system (Table 2, Groups 10 and 12). This may be due to the ineffectiveness of the DNA vaccine delivered by the live-attenuated Salmonella system in priming the development of neutralizing antibodies in the correct configuration, while the “non-neutralizing” antibodies against the spike protein were only elicited in response to the subsequent recombinant S-peptide.

Conclusions

Among all the vaccine combinations, sera of all the mice immunized with intramuscular tPA-optimize800 DNA vaccine boosted with intraperitoneal recombinant spike polypeptide generated by *E. coli* and intramuscular CTLA4HingeSARS800 DNA vaccine boosted with intraperitoneal recombinant spike polypeptide showed very high neutralizing antibody titers of ≥1:1280, showing that protective immunity could be generated by priming with DNA vaccines and boosted with S-peptide produced by *E. coli*.

Implications

The present study shows a novel type of immunization schedule that is able to produce protective immunity in animals, which a “more effective” modality of vaccination, such as DNA vaccine, can be used for priming, and the “less expensive” recombinant protein produced by *E. coli*, instead of that produced by the eukaryotic systems, can be used as boosters. This study could have major practical values, such as immunization of wild animals like civet cats, since this new method is far less expensive than immunization using recombinant proteins generated by eukaryotic systems.

Dissemination

The present results have been disseminated in the form of two publications, one in an international referred journal and one in a PhD thesis. The results have also been presented in the Health Research Symposium 2007. The symposium, entitled “Building bridges between research, practice & policy”, was held on 29 September 2007. The Symposium has invited renowned international and local speakers to highlight the overall impact of health services research in the past decade focusing on health gain, clinical effectiveness and improvements in service delivery and quality. It has also served as a forum for participants to share experience and to present the outcomes of their health services research. The Symposium has been attended by health research practitioners, policy makers and health service users from all sectors of the community, including public and voluntary organisations, hospitals, academic and government bodies. In addition, the findings have also been presented in the 11th Research Postgraduate Symposium 2006 held by the Li Ka Shing Faculty of Medicine, The University of Hong Kong. The symposium has been attended by postgraduate students of HKU, health research practitioners and professors of biomedical research from all around the world. Due to the importance of the results, the World Health Organization has invited Professor PCY Woo as a consultant in the Technical Meeting on animal models for SARS vaccines held in London, United Kingdom in August 2005, in which Professor Woo was the only Hong Kong participant in that meeting.

Publications

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Table 1 Immunization schedule for different forms of S polypeptide based vaccines against SARS-CoV

Groups	First dose (Day 0)			Second dose			Third dose		
	Vaccines	Routes of administration	Dose per mouse	Vaccines	Routes (days) of administration	Dose per mouse	Vaccines	Routes (days) of administration	Dose per mouse
1	S polypeptide	Intraperitoneal	50 μg	S polypeptide	Intraperitoneal (14)	50 μg	S polypeptide	Intraperitoneal (28)	50 μg
2	pcDNA3.1(+)	Intramuscular	100 μg	-	-	-	-	-	-
3	tPA-optimize800 DNA vaccine	Intramuscular	100 μg	-	-	-	-	-	-
4	tPA-optimize800 DNA vaccine	Intramuscular	100 μg	S polypeptide	Intraperitoneal (28)	50 μg	S polypeptide	Intraperitoneal (42)	50 μg
5	CTLA4HingeSARS800 DNA vaccine	Intramuscular	100 μg	-	-	-	-	-	-
6	CTLA4HingeSARS800 DNA vaccine	Intramuscular	100 μg	S polypeptide	Intraperitoneal (28)	50 μg	S polypeptide	Intraperitoneal (42)	50 μg
7	*S. typhimurium* aroA strain	Oral	6×10^9 bacterial cells	-	-	-	-	-	-
8	*S. typhimurium* aroA strain	Oral	6×10^9 bacterial cells	S polypeptide	Intraperitoneal (28)	50 μg	S polypeptide	Intraperitoneal (42)	50 μg
9	Mucosal tPA-optimize800 DNA vaccine	Oral	6×10^9 bacterial cells	-	-	-	-	-	-
10	Mucosal tPA-optimize800 DNA vaccine	Oral	6×10^9 bacterial cells	S polypeptide	Intraperitoneal (28)	50 μg	S polypeptide	Intraperitoneal (42)	50 μg
11	Mucosal CTLA4HingeSARS800DNA vaccine	Oral	6×10^9 bacterial cells	-	-	-	-	-	-
12	Mucosal CTLA4HingeSARS800 DNA vaccine	Oral	6×10^9 bacterial cells	S polypeptide	Intraperitoneal (28)	50 μg	S polypeptide	Intraperitoneal (42)	50 μg

Table 2 Neutralizing antibody titers for different forms of S polypeptide based vaccines against SARS-CoV

Groups	Neutralizing antibody titers (no. of mice)							
	<1:20	1:20	1:40	1:80	1:160	1:320	1:640	≥1:1280
1 (S-peptide)	6	0	0	0	0	0	0	0
2 (S-DNA-control)	6	0	0	0	0	0	0	0
3 (tPA-S-DNA)	0	2	0	0	4	0	0	0
4 (tPA-S-DNA boosted with S-peptide)	0	0	0	0	0	0	0	6
5 (CTLA4-S-DNA)	0	4	2	0	0	0	0	0
6 (CTLA4-S-DNA boosted with S-peptide)	0	0	0	0	0	0	0	6
7 (*Salmonella*-S-DNA-control)	6	0	0	0	0	0	0	0
8 (*Salmonella*-S-DNA-control boosted with S-peptide)	0	2	0	4	0	0	0	0
9 (*Salmonella*-tPA-S-DNA)	4	2	0	0	0	0	0	0
10 (*Salmonella*-tPA-S-DNA boosted with S-peptide)	2	1	2	1	0	0	0	0
11 (*Salmonella*-CTLA4-S-DNA)	5	1	0	0	0	0	0	0
12 (*Salmonella*-CTLA4-S-DNA boosted with S-peptide)	2	2	1	0	1	0	0	0

Legends to Figures

Figure 1 Prominent immunoreactive protein bands of about 90 and 110 kDa were visible on the Western blot (lanes 1 and 2), indicating antigen–antibody interactions between the 293 cell lysates obtained from 293 cells transfected with tPA-optimize800 and CTLA4HingeSARS800, respectively, and hyperimmune polyclonal serum from rabbit immunized with $(His)_6$-tagged recombinant S polypeptide. No antigen–antibody interactions were observed between the 293 cell lysates obtained from 293 cells transfected with tPA-optimize800 or CTLA4HingeSARS800 and the pre-immune rabbit serum (lanes 3 and 4).

Figure 2 Serum antibody levels (O.D. 450) at day 42 in the 12 groups of Balb/c mice immunized with the various vaccines. The 12 groups correspond to the 12 groups of mice described in Table 1 (bar = average of six mice, error bar = 1 standard deviation).

Figure 3 SARS-CoV spike polypeptide-specific lymphocyte proliferation index of Balb/c mice immunized with the various vaccines. The 12 groups correspond to the 12 groups of mice described in Table 1 (bar = average of six mice, error bar = 1 standard deviation).

Figure 4 IL-4 (at 72 h) and Interferon-γ (at 24 h) levels of splenic cell culture supernatant in Balb/c mice immunized with the various vaccines. The 12 groups correspond to the 12 groups of mice described in Table 1.