Modulation of the Immune Response to the Severe Acute Respiratory Syndrome Spike Glycoprotein by Gene-Based and Inactivated Virus Immunization

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Received 14 May 2005/Accepted 12 August 2005

Although the initial isolates of the severe acute respiratory syndrome (SARS) coronavirus (CoV) are sensitive to neutralization by antibodies through their spike (S) glycoprotein, variants of S have since been identified that are resistant to such inhibition. Optimal vaccine strategies would therefore make use of additional determinants of immune recognition, either through cellular or expanded, cross-reactive humoral immunity. Here, the cellular and humoral immune responses elicited by different combinations of gene-based and inactivated viral particles with various adjuvants have been assessed. The T-cell response was altered by different prime-boost immunizations, with the optimal CD8 immunity induced by DNA priming and replication-defective adenoviral vector boosting. The humoral immune response was enhanced most effectively through the use of inactivated virus with adjuvants, either MF59 or alum, and was associated with stimulation of the CD4 but not the CD8 response. The use of inactivated SARS virus with MF59 enhanced the CD4 and antibody response even after gene-based vaccination. Because both cellular and humoral immune responses are generated by gene-based vaccination and inactivated viral boosting, this strategy may prove useful in the generation of SARS-CoV vaccines.

The severe acute respiratory syndrome coronavirus (SARS-CoV) has emerged as a respiratory pathogen caused by a newly recognized human coronavirus (30, 32, 45, 50). In contrast to previously described coronaviruses, this disease syndrome is highly lethal and is accompanied by significant pulmonary and systemic pathology that has prompted a search for preventive vaccines. Several studies have now demonstrated that it is possible to elicit protective immune responses to viruses in animal models (7, 8, 17, 61, 75). Protection against pulmonary viral replication is mediated by antibodies in a murine vaccine model, which are necessary and sufficient for protection (75). As multiple isolates of this virus have become available, increased molecular heterogeneity has become apparent (12, 19, 37, 76, 79). This sequence variability is observed in a variety of gene products. Of relevance to the development of SARS-CoV vaccines, there is amino acid sequence variability in S, found in alternative human strains and in animals, notably the palm civet (54, 63). It has been recognized recently that certain variants, including more recent specific human isolates, as well as the palm civet isolates, are resistant to neutralization by antibody (73), raising concerns that vaccines based on the original Urbani strain or closely related isolates may not provide complete protection against those that may evolve in the future.

Depending on the method of vaccination, different types of immune responses can be elicited by alternative vectors or proteins with adjuvants. While immunization with proteins or inactivated viruses using adjuvants primarily induces humoral immunity, gene-based vaccination with plasmid DNA and/or replication-defective adenoviral vectors elicits stronger cellular immunity, in addition to humoral responses of various degrees, depending upon the antigen (3, 5, 9, 18, 25, 26, 28, 33, 38, 41, 46, 52, 57, 58, 60, 66, 68). The effects of combined immunization with gene-based vaccination and protein boosting are less well understood in terms of the balance of cellular and humoral immunity. Though DNA priming and protein boosting have been shown to increase antibody responses (14, 20, 29, 34, 65), the effects of protein adjuvants on different DNA and recombinant adenoviral vector (rAd) immunization regimens have not been explored fully. Whether the balance of CD4 and CD8 immunity is altered or the order of immunization affects this response is unknown. In this report, we have evaluated the immune response to these by different combinations of priming and boosting with DNA, adenovirus, and inactivated viral vaccines. The ability to boost gene-based vaccines with the adjuvanted inactivated virus shows clear enhancement of the CD4 and antibody responses. The CD8 responses are not similarly enhanced after such a boost. In contrast, DNA priming followed by rAd boosting with vectors encoding S allow induction of a strong CD8 response. The ability to combine different vaccine modalities may increase the breadth of the immune response and contribute to the development of an effective SARS-CoV vaccine.

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FIG. 1. Analysis of cellular and humoral immunity after DNA, adenoviral, or inactivated SARS virus, with or without adjuvant. Mice in each test group ($n = 10$) received injections with DNA (25 μ g), rAd-S (10^9), or with inactivated SARS virus (5 μ g), with or without adjuvants as indicated in each column. The control group that was sham injected contained five mice. Immunizations were performed twice at 4-week intervals. Intracellular cytokine staining and serum analysis were performed 10 days after the last injection. Each symbol represents the percent positive cells in the CD4⁺ (left panel) or CD8⁺ (middle panel) T-cell population for one animal. The mean ICS response for the responding animals is indicated by horizontal bars. P values represent comparisons of groups by Student's t test (tail = 2, type = 2). Mouse sera were tested by ELISA against SARS-S protein performed 10 days after the last injection (right panel). The mean value of the optical density at 450 nm (O.D.450) from the animals' sera within the test group is represented by the solid bar, with the upper error bar as the standard deviation.

MATERIALS AND METHODS

Generation of immunogens. (i) DNA vector. The SARS S-expressing vector has been previously described (74, 75). Basically, a gene encoding the SARS-CoV spike (S) protein was synthesized using human-preferred codons and expressed in a mammalian expression vector that contains the cytomegalovirus enhancer/promoter and splice donor and the human T-cell leukemia virus type 1 R region (4).

(ii) Inactivated SARS virus. An inactivation method was developed for SARS-CoV before initiating purification steps. SARS-CoV harvested from Vero cells was inactivated with β -propiolactone (Ferrak, Berlin Chemie, Germany) at a final concentration of 0.05% for 16 h at 4°C, followed by hydrolysis of any residual β -propiolactone by elevating the temperature to 37°C for 3 h. Then replication incompetence of the viruses was confirmed by an inactivation assessment. An aliquot of the inactivated harvest was passaged twice in Vero cells to exclude the presence of residual infectious virus. A confluent Vero cell culture was incubated in a T175 flask with 10 ml inactivated harvest in 90 ml cell culture medium at 37°C. After 4 days, the supernatant was transferred to a second confluent Vero cell culture and cells were incubated for 1 h at 37°C. Subsequently, the supernatant was replaced by fresh medium and the cell culture was further incubated at 37°C for 4 days. Vero cells from the first passage were further incubated with fresh cell culture medium for another 4 days at 37°C. The cell cultures were evaluated for the presence of cytopathic effect which was caused by the SARS-CoV infection.

(iii) Recombinant adenoviral vector encoding S. Replication-defective rAd encoding S (rAd-S) was generated by a modification of a previously published method (10, 33, 42, 57, 70, 72). Briefly, the synthetic SARS S adapted from the sequence described above (terminated at amino acid 1229) was subcloned into the shuttle plasmid pAdAdaptCMVmcS. 293 cells were cotransfected with 2 μ g of purified, linearized shuttle plasmid, pADAPT, with an ADV cosmid by calcium phosphate transfection. After 7 to 12 days, the supernatant containing recombinant adenovirus was collected from the cell lysate, freezing and thawing at least three times. The production of recombinant adenovirus was scaled up by infection of 293 cells with the virus-containing supernatant. The viruses were purified by cesium chloride, aliquoted (10^{12} particles/ml), and stored in phosphate-buffered saline (PBS) with 13% glycerol at -20°C for future use.

Adjuvants and ovalbumin. MF59 (Chiron Vaccines) was used at a 1:1 dilution with the immunogens right before immunization. CpG oligodeoxynucleotides (CpG) 1826 or CpG 7909 (25 μ g in 0.05 ml; Coley Pharmaceutical Group,

Wellesley, MA) was mixed with the inactivated SARS virus (5 μ g in 0.05 ml) per mouse right before immunization. Alum (Pierce, Rockford, IL) was used at a 1:1 ratio (vol/vol) with each immunogen. In the experiment using ovalbumin (Sigma-Aldrich, St. Louis, MO), 50 μ g of ovalbumin in 0.05 ml was mixed with adjuvants immediately before injection (total of 0.1 ml) into each mouse.

Immunization. In this study, experiments were performed with different primary immunizations, either one rAd, three DNA, or three DNA with one rAd injection. After the primary injections, animals were boosted with inactivated SARS viruses with or without adjuvants. Each test group contained 10 BALB/c mice, and control groups contained 5 mice. Immunizations were performed at 3-week intervals, and intracellular cytokine staining was performed 10 days after the last injection. For the experiment with DNA immunizations, each animal was injected with 50 μ g of the plasmid DNA in 100 μ l of PBS in the quadriceps muscle for a total of three injections. For experiments with rAd immunization, the replication-defective adenoviral vector encoding the SARS spike was injected (10^9 particles) intramuscularly in 100 μ l of PBS. In each experiment, the inactivated SARS virus boost-immunization was performed 3 weeks after the last prime immunization. Five micrograms of inactivated SARS virus was mixed immediately with or without different adjuvants in a final volume of 100 μ l in PBS before intramuscular injection into each animal. Negative control groups were immunized with empty vector plasmid or control rAd following the same schedule as the test groups in each experiment. Animal experiments were carried out in compliance with all relevant federal and National Institutes of Health policies. Because of the large number of animals and because the immunization regimens vary depending on the combination of immunogens, it was not technically possible to perform all comparisons within the same experiment; however, because the animals were inbred and the vaccines were stable, some comparisons between groups could be made.

Assessment of cellular immune responses by intracellular cytokine staining. CD4⁺ and CD8⁺ T-cell responses were evaluated using intracellular cytokine staining (ICS) by flow cytometry for gamma interferon (IFN- γ) and tumor necrosis factor alpha (TNF- α) as previously described (28, 67, 75) with peptide pools (17- to 19-mers overlapping by 10 amino acids; 2.5 μ g/ml each) covering the SARS-CoV spike protein. Cells were then fixed, permeabilized, and stained using rat monoclonal anti-mouse CD3, CD4, CD8, IFN- γ , and TNF- α antibodies (BD-Pharmingen). The IFN- γ - and TNF- α -positive cells in the CD4⁺ and CD8⁺ cell populations were analyzed with the program FlowJo (Tree Star, Inc.).

FIG. 2. Comparison of adenoviral and inactivated SARS virus boosting, with or without adjuvant following DNA priming. Mice in each test group ($n = 10$) received three injections at 3-week intervals with DNA (25 μ g) and were boosted with rAd-S (10^9) or with inactivated SARS virus (5 μ g), with or without adjuvants 3 weeks after the last DNA injection. The control group that was sham injected contained five mice. Intracellular cytokine staining was performed 10 days after the last injection, and immunoglobulin levels were measured as described in Materials and Methods. Each symbol represents the percent positive cells in the CD4⁺ (left panel) or CD8⁺ (middle panel) T-cell population for one animal. The mean ICS responses for the responding animals are indicated by horizontal bars. P values represent comparisons of groups by Student's t test (tail = 2, type = 2). Mouse sera were tested by ELISA against SARS-S protein performed 10 days after the last injection (right panel). The mean value of the optical density at 450 nm (O.D.450) from the animals' sera within the test group is represented by the solid bar, with the upper error bar as the standard deviation.

Analysis of the humoral immune response. Mouse anti-SARS-S immunoglobulin G (IgG) enzyme-linked immunosorbent assay (ELISA) titer was measured using a modified lectin capture method described previously (28, 67, 75), except the Myc-tagged, transmembrane domain-truncated SARS-CoV spike protein (SARS-SΔTM-Myc) was used for capture. Serial dilutions of the mouse serum were tested to ensure reliability of the results. For simplicity, only the 1:1,000 dilution data are shown in the figures, with error bars indicating the standard deviations.

Statistical analysis. Each individual animal immune response was counted as an individual value for statistical analysis. The significance of the cellular and humoral immune responses was calculated by Student's t test (tails = 2, type = 2) as indicated by the P value.

RESULTS

To analyze the immune response elicited by alternative vectors encoding S and inactivated virus, mice were immunized with DNA or with inactivated SARS virus alone or mixed with MF59 adjuvant, an oil squalene-in-water emulsion (47), compared to an adenoviral vector encoding S alone (Fig. 1). CD4, CD8, and antibody responses were assessed 10 days after the final boost by each vaccine. Intracellular cytokine staining on unstimulated cells was used to standardize for background staining in the assay, and there was little variation in unimmunized mice. DNA vaccination induced a low level of CD4 immunity and was similar to inactivated SARS or adenoviral vector alone, in contrast to the inactivated SARS virus with MF59, which induced an increase in CD4 immunity (Fig. 1, left panel). In contrast, adenoviral immunization induced a substantial CD8 response while inactivated SARS virus with MF59 induced a negligible CD8 response. The immunoglobulin response paralleled that of the CD4 response, with the highest levels in the inactivated SARS plus MF59 group. A similar

CD4 and IgG response was observed with the inactivated SARS virus without adjuvant, though of lower magnitude. Interestingly, the adenoviral immunization alone also elicited a significant increase in the IgG titers.

Effect of priming and boosting of gene-based and inactivated SARS virus vaccine. The effect of priming and boosting was next analyzed using different combinations. In all cases, stimulation of the T-cell responses was markedly increased compared to immunization with single agents alone. In particular, DNA priming followed by adenoviral boosting induced a substantial CD4 response that was lower in magnitude than DNA priming and inactivated virus, either with or without adjuvant, and the MF59 adjuvant gave the optimal CD4 ICS response (Fig. 2, left panel). In contrast, the DNA prime/rAd boost induced predominantly a CD8 response, with more than 4% positive cells by intracellular cytokine staining (Fig. 2, middle panel), while the DNA priming followed by protein boosting with or without adjuvant stimulated minimal CD8 responses. In all cases, a substantial immunoglobulin response was induced, and there were no significant differences between these groups (Fig. 2, right panel). These findings suggest that the prime and boost combinations are highly effective at inducing cellular and humoral immunity, although the cellular response appears to be biased toward CD8 cells for adenoviral boosting and toward CD4 for inactivated virus boosting.

CpG oligonucleotides do not cross-prime the CD8 response following inactivated virus boosting. Since CpG oligodeoxynucleotides have been shown to facilitate cross-priming that enhances cellular immunity in the absence of protein adjuvants (1, 51, 78), the ability of these oligonucleotides to enhance the CD8 responses was evaluated after DNA prim-

FIG. 3. Comparison of inactivated SARS virus boosting with MF59 and CpG after DNA priming. Mice in each test group ($n = 10$) received three injections at 3-week intervals with DNA (25 μ g) and were boosted with inactivated SARS virus (5 μ g), with or without MF59 and the specified CpG adjuvants, 3 weeks after the last DNA injection. The control group that was sham injected contained five mice. Intracellular cytokine staining was performed 10 days after the last injection, and immunoglobulin levels were measured as described in Materials and Methods. Each symbol represents the percent positive cells in the CD4⁺ (left panel) or CD8⁺ (middle panel) T-cell population for one animal. The mean ICS responses for the responding animals are indicated by horizontal bars. *P* values represent comparisons of groups by Student's *t* test (tail = 2, type = 2). Mouse sera were tested by ELISA against SARS-S protein performed 10 days after the last injection (right panel). The mean value of the optical density at 450 nm (O.D.450) from the animals' sera within the test group is represented by the solid bar, with the upper error bar as the standard deviation.

ing and inactivated SARS virus boosting. Specifically, we asked whether CpGs improved cellular immunity in the presence of MF59 following a DNA/inactivated virus prime-boost vaccination. All combinations of priming and protein boosting yielded substantial CD4 responses, as in the previous experiment, compared to no adjuvant, but this response was not increased by the inclusion of either of two previously identified oligodeoxynucleotides, CpG1826 or CpG7909 (Fig. 3, left panel). In addition, these CpGs did not increase the CD8 response; a minor diminution was noted with both CpGs that was statistically significant for CpG1826 (Fig. 3, middle panel). However, the IgG responses were substantial and the CpG7909 oligodeoxynucleotide group was slightly higher than either the MF59-alone group or the CpG1826 oligodeoxynucleotide group ($P = 0.043$ and 0.044 , respectively) (Fig. 3, right panel).

The ability of MF59 and CpG to influence immune responses after DNA priming and rAd boosting was next assessed to evaluate whether the CD8 response could be reelicited once a potent gene-based vaccine had been administered. Secondary boosting with the different combinations of inactivated SARS virus and adjuvant with and without CpG revealed a similar effect, with no substantial modulation of the CD4 response (Fig. 4, left panel), and a slight decrease in the CD8 response was observed in the presence of CpG (Fig. 4, middle panel). No significant difference was observed in the CD8 response between inactivated SARS virus with or without MF59 in these studies. Again, a strong IgG response was observed and did not appear influenced by the inclusion of MF59 or CpG adjuvant (Fig. 4, right panel). This response was not unexpected, given that the CD4 responses were substantially

increased in all groups compared to the control (Fig. 4, left panel, column 1 versus 2 to 5).

The boosting effect of inactivated SARS virus was next evaluated after rAd priming alone, and no significant effect was observed for the CD4 response (Fig. 5, left panel). A small increase in the CD8 response was observed by inclusion of the MF59 adjuvant with inactivated SARS compared to the inactivated virus alone. This increase was affected by the CpGs (Fig. 5, middle panel). The IgG responses were similarly not significantly different among these groups (Fig. 5, right panel).

Boosting of gene-based vaccine responses by inactivated virus and adjuvant is antigen specific. To determine whether the effects of rAd priming and inactivated SARS boosting were antigen specific or adjuvant dependent, we evaluated the response to inactivated SARS virus or to an unrelated protein, ovalbumin, comparing MF59 or alum adjuvants. The enhanced CD4 response was observed only in the presence of inactivated SARS virus with both the MF59 and alum adjuvants (Fig. 6, left panel, columns 1 and 3). In both cases, this effect was antigen specific, as ovalbumin did not elicit a similar response (Fig. 6, left panel, columns 2 and 4). No significant differences were observed in the CD8 responses using either adjuvant comparing the inactivated virus to the ovalbumin protein control (Fig. 6, middle panel). The IgG responses were similar to those observed with CD4 and were seen in the presence of the inactivated SARS virus but not ovalbumin with either adjuvant (Fig. 6, right panel). Therefore, the protein boost effect was antigen specific and elicited by two independent adjuvants.

FIG. 4. Effects of secondary boosting by inactivated SARS virus and MF59 with and without CpG oligos: lack of cross-priming of CD8 responses. Mice in each test group ($n = 10$) received three injections at 3-week intervals with DNA (25 μ g) and were boosted with rAd-S (10^9) 3 weeks later. These animals subsequently received a secondary boost with inactivated SARS virus (5 μ g), with or without MF59 and the specified CpG adjuvants, 3 weeks after the last rAd injection. The control group that was sham injected contained five mice. Intracellular cytokine staining was performed 10 days after the last injection, and immunoglobulin levels were measured as described in Materials and Methods. Each symbol represents the percent positive cells in the CD4⁺ (left panel) or CD8⁺ (middle panel) T-cell population for one animal. The mean ICS responses for the responding animals are indicated by horizontal bars. *P* values represent comparisons of groups by Student's *t* test (tail = 2, type = 2). Mouse sera were tested by ELISA against SARS-S protein performed 10 days after the last injection (right panel). The mean value of the optical density at 450 nm (O.D.450) from the animals' sera within the test group is represented by the solid bar, with the upper error bar as the standard deviation.

FIG. 5. Effect of inactivated SARS virus boosting after adenoviral priming. Mice in each test group ($n = 10$) received an injection of rAd-S (10^9) and were boosted 3 weeks later with inactivated SARS virus (5 μ g), with or without MF59 and the specified CpG adjuvants. The control group that was sham injected contained five mice. Intracellular cytokine staining was performed 10 days after the last injection, and immunoglobulin levels were measured as described in Materials and Methods. Each symbol represents the percent positive cells in the CD4⁺ (left panel) or CD8⁺ (middle panel) T-cell population for one animal. The mean ICS responses for the responding animals are indicated by horizontal bars. *P* values represent comparisons of groups by Student's *t* test (tail = 2, type = 2). Mouse sera were tested by ELISA against SARS-S protein performed 10 days after the last injection (right panel). The mean value of the optical density at 450 nm (O.D.450) from the animals' sera within the test group is represented by the solid bar, with the upper error bar as the standard deviation.

FIG. 6. Antigen specificity of the inactivated SARS virus boosting. Animals were immunized with rAd-S (10^9) as in Fig. 5 and boosted with inactivated SARS virus ($5\text{ }\mu\text{g}$) or ovalbumin ($5\text{ }\mu\text{g}$; negative control) with MF59 or alum adjuvants as indicated, 4 weeks later. The cellular immune responses of these animals were analyzed with ICS 10 days after the final immunization by flow cytometry, and immunoglobulin levels were measured as described in Materials and Methods. Each symbol represents the percent positive cells in the CD4⁺ (left panel) or CD8⁺ (middle panel) T-cell population for one animal. The mean ICS responses for the responding animals are indicated by horizontal bars. *P* values represent comparisons of groups by Student's *t* test (tail = 2, type = 2). Mouse sera were tested by ELISA against SARS-S protein performed 10 days after the last injection (right panel). The mean value of the optical density at 450 nm (O.D.450) from the animals' sera within the test group is represented by the solid bar, with the upper error bar as the standard deviation.

DISCUSSION

The effects of gene-based vaccination on immune responses are becoming increasingly understood. For example, DNA priming followed by adenoviral boosting for several different antigens can elicit strong CD4 and CD8 cellular immune responses (9, 25, 26, 28, 46, 52, 57, 66, 68). In contrast, protein vaccines typically utilize adjuvants that elicit strong CD4 responses and minimal CD8 responses (21, 31, 40, 55, 71, 77). More recently, Toll receptor agonists, particularly CpGs, have been shown to improve the cellular immune responses elicited by proteins, particularly in murine models and in some non-human primate systems (11, 15, 27, 44, 53). Because we have found previously that the increase in SARS S antibody titers generated by DNA/rAd or rAd immunization do not broaden protection against civet and recent SARS-CoV isolates (73), we have focused on gene-based vaccination and protein adjuvant combinations that increase the antibody response at the same time they maintain or enhance balanced T-cell immunity.

Genetic vaccination in combination with inactivated virus boosting showed that boosting with adjuvanted proteins induces a qualitatively different immune response, with increased CD4 responses relative to CD8 and strong antibody responses; gene-based responses, particularly those using rAd vector boosting, elicit strong CD8 responses and less marked CD4 responses, although the immunoglobulin response is substantial. These disparate effects are likely due to the nature of the interaction of the various vaccines with dendritic cells. rAd is thought to infect immature dendritic cells, which upon maturation can present synthesized antigens within class I major histocompatibility complex proteins to stimulate CD8 immu-

nity, while alum and other protein adjuvants typically boost CD4 and humoral responses through class II major histocompatibility complex pathways. When inactivated virus with adjuvant was used to boost animals primed with rAd vectors, an antigen-specific increase in the CD4 response was observed (Fig. 6). In contrast, the CD8 response was not affected either by the antigen or by the adjuvant and was also similar in magnitude to the stimulation seen without boosting (Fig. 1, middle panel, lane 5). We interpret this finding to indicate that the adjuvant effect after rAd priming, which induces a CD8 response, is CD4 specific. This finding is consistent with its ability to boost the T-dependent antibody response.

These results suggest that the final boost of a gene-based vaccine with adjuvanted protein can lead to a CD4-enhanced response and diminish the CD8 response, although the antibody responses in many instances can be comparable. In contrast, DNA priming followed by rAd boosting causes stronger antigen-specific CD8 expansion. Though the mechanism of this selective activation is not completely understood, this effect is most likely due to the ability of rAd to synthesize the gene product within dendritic cells that promote better class I antigen presentation. In the case of the inactivated virus, such a response would require uptake of preformed protein antigens. Cross-presentation would therefore be required to induce a CD8 response, which is less efficient. These responses are typically Th1 responses, based on the isotypes of the antibodies and the pattern of cytokine secretion, and we have not observed changes in isotype ratios that indicate a shift to a Th2 response with any of the alternative immunizations.

The ability to stimulate the CD4 responses may be desir-

able if the immune response is largely dependent on neutralizing antibodies; however, in the case of SARS, several S glycoproteins have been identified that are resistant to neutralization. In some instances, it appears that these S glycoproteins either emerged more recently or have been derived from palm civet viruses. The S glycoprotein recognizes a cellular receptor, the human ACE-2 protein (35). The region sensitive to neutralization maps to the receptor binding domain (36, 56, 73). Recent data suggest that the antibody-resistant strains preferentially recognize the civet ACE-2 and may not have adapted as effectively as the isolates that were identified during the outbreaks in late 2003 and 2004 (36). The ability to induce a strong humoral response would be desirable if it is possible to generate antibodies to these new strains; however, it is not possible to elicit such antibodies using homologous immunization in animals with this same strain. Therefore, it would appear desirable presently to elicit cellular immunity by vaccination. Whether there are alternative means to combine the protein with different Toll receptor agonists, including novel CpGs or small molecule Toll receptor agonists such as imiquimod or resiquimod (6, 22, 43, 49, 62), is yet unknown but would be highly desirable. Though the coupling of CpGs directly to inactivated virus or proteins might enhance cellular immunity similar to gene-based vaccination, the present data suggest that the simple addition of the CpG to the adjuvant does not provide the type of cross-priming in a murine model that would be desirable for improvement of CD8 responses. In this study, inactivated virus rather than purified S protein was used as an immunogen. Though inactivated virus contains additional virus proteins and contains more epitopes than purified S protein, the inactivated virus was studied here for two reasons. First, the inactivated viral particles contain native SARS S protein, the most relevant antigenic structure with respect to neutralizing antibodies. Previous studies have shown that this antigen is capable of inducing such neutralizing antibody responses (13, 23, 48, 59, 69, 75), and the structure found on the virion presents the relevant native conformation. Second, similar vaccines are being tested or are under development for human clinical studies (2, 39). Therefore, the ability to perform a prime-boost immunization with clinically relevant products and to analyze their immunogenicity affords an opportunity to examine synergy between these different vaccine candidates that have clinical implications. The neutralization of the prototypic human SARS coronavirus is encouraging, and the neutralization titer has also correlated with ELISA titers for the Urbani strain (73, 75). It may be tempting to pursue a vaccine strategy based on antibody neutralization, but it is important to recognize that vaccines for animal coronaviruses which rely on humoral immunity have not proven efficacious (16, 24, 64). Current animal models of SARS infection that faithfully replicate human disease do not exist. Moreover, it is not possible to propagate viruses which preferentially utilize the palm civet ACE-2 receptor that are insensitive to antibody neutralization (36, 73). It is presently not possible to evaluate relative vaccine efficacy; however, a variety of novel approaches, including adaptation of virus to different species, the development of transgenic animals expressing the human ACE-2 receptor, and the use of aged animals in challenge models, may assist in this effort in the future. Further analysis of vaccine candidates with alternative immunologic profiles in

relevant animal models will assist in the selection of vaccines that will be most appropriate for development in humans.

ACKNOWLEDGMENTS

We thank Cheng Cheng and James Lee for assistance in the laboratory, Ati Tislerics for assistance with manuscript preparation, Toni Garrison and Brenda Hartman for figure preparation, and members of the Nabel lab for helpful discussions and advice.

This research was supported in part by the Intramural Research Program of the U.S. National Institutes of Health, Vaccine Research Center, NIAID, and by the Chiron Corporation with support from the 6th Framework Program of the European Commission (FP6-511065).

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