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Sharwani, Khalid, Sharma, Ravi, Krishnan, Madhan, Jones, Terry, Mayora-Neto, Martin, Cantoni, Diego, Temperton, Nigel J., Dobson, Susan L, Subramaniam, Krishanthi, McNamara, Paul S and others (2021) Detection of serum cross-reactive antibodies and memory response to SARS-CoV-2 in pre-pandemic and post-COVID-19 convalescent samples. *The Journal of Infectious Diseases*

DOI

<https://doi.org/10.1093/infdis%2Fjiab333>

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Enhancement of Humoral Immune Responses to a Human Cytomegalovirus DNA Vaccine: Adjuvant Effects of Aluminum Phosphate and CpG Oligodeoxynucleotides

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A human cytomegalovirus (HCMV) glycoprotein B (gpUL55) DNA vaccine has been evaluated in BALB/c mice. Intramuscular immunization of these mice with pRc/CMV2-gB resulted in the generation of high levels of gpUL55-specific antibody (geometric mean titer [GMT] 1:8900) and neutralizing antibody (GMT 1:74) after 2 booster doses given 5 and 10 weeks after primary inoculation. Emulsifying the construct with the aluminum phosphate gel adjuvant Adju-Phos before immunization enhanced gpUL55-specific antibody responses (GMT 1:17800, $P=0.04$). Co-immunization with CpG oligodeoxynucleotides was shown to enhance levels of neutralizing antibodies generated by immunization of mice with a pRc/CMV2-gB/Adju-Phos emulsion ($P=0.04$). The results provide a rationale for evaluating combinations of other HCMV proteins for incorporation into a multi-target DNA vaccine, and for the optimization of adjuvant usage, to elicit enhanced levels of neutralizing antibodies. **J. Med. Virol. 70:86–90, 2003.** © 2003 Wiley-Liss, Inc.

KEY WORDS: cytomegalovirus; DNA vaccine; CpG ODN; adjuvant

INTRODUCTION

The β -herpesvirus, human cytomegalovirus (HCMV), infects most individuals during their lifetime, yet results in disease only in those whose immune system is immature, impaired by immunosuppressive drugs or the human immunodeficiency virus (HIV). The virus infects 0.3–2.4% of neonates, making it the most important cause of intrauterine infection [Stagno, 1990]. Women who enter pregnancy seronegative for HCMV have a 1% chance of developing primary HCMV infection

while pregnant [Griffiths and Baboonian, 1984; Stagno et al., 1986]. The development of a cytomegalovirus (CMV) vaccine for the prevention of primary HCMV infection is thus a major public health priority. A recent report from the Institute of Medicine, Washington, D.C., strongly supports the development of a HCMV vaccine based on the economic impact of the disease caused by this virus [Stratton et al., 2001]. In a study reported recently by our group, using a mathematical modeling approach, we calculated that the critical vaccination proportion required for eradication of HCMV in the developed world lies between 59% and 62%. This finding demonstrates that HCMV could be eradicated from the population, given the current routine pediatric immunization rates ($\sim 90\%$) by a vaccine with only 65–68% efficacy at preventing primary infection [Griffiths et al., 2001].

Of the large number of unique proteins encoded within the HCMV genome [Chee et al., 1990; Prichard et al., 2001], only a small proportion are thought to be important targets of the protective immune responses [Plotkin, 1999, 2001]. The neutralizing antibody response against HCMV is directed predominantly against a single protein, glycoprotein B (gpUL55) [Utz et al., 1989; Britt et al., 1990], while the tegument protein pp65 (ppUL83) is a major target of the cellular immune response [Riddell et al., 1991; McLaughlin-Taylor et al., 1994; Wills et al., 1996]. These antigens

Grant sponsor: Royal Free Peter Samuel Fund; Grant sponsor: University of London Central Research Fund; Grant sponsor: Peel Medical Research Trust.

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Accepted 30 October 2002

DOI 10.1002/jmv.10357

Published online in Wiley InterScience
(www.interscience.wiley.com)

have formed the basis of most experimental vaccine candidates thus far, which encompass their use within recombinant viral vectors [Berencsi et al., 1993, 2001], recombinant protein vaccines [Pass et al., 1999], and peptide vaccines [Diamond et al., 1997].

Over the last decade, DNA vaccines have been shown to be effective inducers of cellular and humoral responses against many viral antigens [Davis and McCluskie, 1999; Robinson and Pertmer, 2000]. This relatively new technology has been employed to produce candidate vaccines against CMV [Pande et al., 1995; Gonzalez Armas et al., 1996; Endresz et al., 1999, 2001; Hwang et al., 1999; Morello et al., 2000; Schleiss et al., 2000; Temperton, 2002]. Many groups have attempted to enhance the immune response to DNA vaccines by the coadministration of various immunomodulators (cytokines, chemokines, costimulatory molecules), the delivery of plasmids in liposomes, and the use of experimental adjuvants [Gurunathan et al., 2000]. However, these experimental adjuvants are currently unlicensed for use in humans. It has recently been shown that negatively charged aluminum salts, which are currently licensed for use in humans, can be employed to enhance substantially, antibody responses to DNA vaccine-encoded genes [Ulmer et al., 1999]. CpG motifs within plasmid backbones or oligodeoxynucleotides (ODNs) are potent immunostimulators of the mammalian immune system [Krieg, 2000]. Synthetic CpG ODNs with a nuclease-resistant phosphorothioate backbone have been employed as adjuvants for successful enhancement of both humoral and cellular responses to protein antigens. These ODNs also have adjuvant effects when coadministered with DNA vaccines [Klinman et al., 1997, 1999, 2000]. Since CpG ODNs trigger B-cell proliferation and secretion of immunoglobulin, they may be particularly useful to enhance the anti-gpUL55 antibody responses [Krieg et al., 1995].

In this study, we have evaluated the adjuvant effects of aluminum phosphate and coadministered CpG ODN on the generation of gpUL55-specific and neutralizing antibody responses after inoculation of mice with an HCMV gpUL55 DNA vaccine.

MATERIALS AND METHODS

Plasmid Construction

A complete gpUL55 DNA vaccine construct was generated by polymerase chain reaction (PCR) amplification of the entire open reading frame (ORF) from strain AD169 using BIO-X-ACT™ DNA polymerase (Bioline, London, UK) with primers, incorporating restriction enzyme sites to facilitate cloning (shown underlined): gBA (5'-gAAgCTTCgACgCgCCTCATCgC-TgCT-3') and gBB (5'-gTCTAgACCTCCTggTTCAGAC-gTTCT-3'). The product was cloned into pGEM-T (Promega, Southampton, UK); the resulting clones were subjected to nucleotide sequence analysis in both directions to confirm that no point mutations had been introduced during the amplification process. The gpUL55

gene was then subcloned into the high-level nonfusion expression vector pRc/CMV2 (Invitrogen, Groningen, the Netherlands). Recombinants were grown in *Escherichia coli* DH5 α cells, which facilitate the production of high-levels of supercoiled plasmid necessary for DNA vaccination purposes, and high-quality plasmid DNA was purified using Qiagen plasmid kits according to the manufacturer's instructions (Qiagen, Crawley, UK).

DNA Transfection

HeLa cells were transfected transiently with pRc/CMV2-gB and the parental plasmid using the Lipofectamine™ 2000 reagent (Invitrogen) according to the manufacturer's protocol. For immunofluorescence experiments, these cells were spotted onto wells in multi-spot microscope slides (Hendley, Essex, UK) and fixed in 100% acetone at -20°C before binding with the 7.17 anti-gB monoclonal antibody (Mab) [Utz et al., 1989].

Adjuvants

Aluminum phosphate gel adjuvant (Adju-Phos, Superfos Biosector A/S, Vedbaek Denmark) was mixed with plasmid or plasmid/CpG ODN, for a final aluminum concentration of 450 $\mu\text{g}/\text{ml}$. CpG ODN 1668: 5'-TCC-ATG-ACG-TTC-CTG-ATG-CT-3' was synthesized with a nuclease-resistant phosphorothioate backbone (Operon, Alameda, CA). In this study, 10 μg of this ODN was combined with 50 μg pRc/CMV2-gB before being emulsified in Adju-Phos.

Immunizations of Mice

Groups of five 5-week-old female BALB/c mice were immunized intra-muscularly once with 50 μg pRc/CMV2 (parental plasmid for mock infection), 50 μg pRc/CMV2-gB, 50 μg pRc/CMV2-gB emulsified with Adju-Phos or 50 μg pRc/CMV2-gB/CpG ODN 1668 emulsified with Adju-Phos. Booster immunizations were given 5 and 10 weeks after initial inoculation with identical plasmid/adjuvant formulations. Serum samples were obtained before immunization, and at weeks 2, 6, and 11, and were stored at -20°C in 0.1-ml aliquots before use.

Determination of HCMV-gpUL55-Specific Antibody Responses

Anti-gpUL55 antibody responses in BALB/c mice immunized with pRc/CMV2 constructs were analyzed by endpoint dilution immunofluorescence, using a recombinant baculovirus expressing HCMV gpUL55 [Wells et al., 1990] (a kind gift from W. Britt, University of Alabama). Sf21 insect cells were infected with recombinant baculovirus at a multiplicity of infection (MOI) of 10. After 48-hr incubation at 28°C , 3×10^4 cells in a 15- μl volume were spotted onto wells in multispot microscope slides (Hendley, Essex, UK) and fixed in 100% acetone at -20°C . Doubling dilutions of mouse sera (1:40–1:40960) were prepared, and 15 μl of each dilution was added to wells containing either infected or uninfected control Sf21 cells. After incubation in a

humidified chamber at 37°C for 40 min, slides were washed once for 5 min in 1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS), twice in PBS, and air-dried; 15 µl of FITC-conjugated goat antimouse immunoglobulins diluted 1:10 in PBS (DAKO, Glostrup Denmark) was added to each well. The slides were incubated in a humidified chamber at 37°C for 40 min, washed once for 5 min in 1% BSA in PBS, twice in PBS, and air-dried. After mounting slides with Citifluor (Citifluor Ltd, Leicester, UK), they were viewed under fluorescence microscope and the last serum dilution at which positive staining cells were visible was taken as the antibody titer.

Determination of Neutralizing Antibody Titers

Human MRC5 fibroblasts (passages 20–30) were seeded in 8 well chamber slides (2×10^4 cells/well) 24 hr before testing. In this study, 20 µl mouse serum (dilutions 1:4–1:256 in minimal essential medium [MEM] + 10% fetal calf serum [FCS]) was mixed with 15 µl of a constant titer of AD169 virus stock (50 immediate-early antigen-producing units (IEU)/well) and 5 µl guinea pig complement (Sigma). After 1-hr incubation (37°C, 5% CO₂), the virus/serum mixture was added to the cell monolayer and incubated for 1 hr. After aspiration and the addition of fresh MEM, the slides were incubated at 37°C for a further 18–24 hr. After fixing with acetone the cells were incubated for 30 min with a MAb against the 72-kD IE protein, washed 3 times in PBS and then incubated for 30 min with FITC-conjugated sheep antimouse IgG (Sigma) (diluted 1:100). After washing, the cells were air-dried, mounted with Citifluor and positive nuclei scored by fluorescent microscopy (full field at 20× magnification). The serum dilution producing 50% inhibition of IEU input virus infectivity, as compared with an untreated control, was taken as the neutralizing titer.

Statistical Analysis

Comparisons between groups for statistical significance were performed with the Mann-Whitney test. All *P*-values of <0.05 were regarded as significant.

RESULTS

Effect of Adju-Phos on Antibody Responses to a gpUL55 DNA Vaccine

Serum antibody titers were measured at pre-inoculation and at weeks 2, 6, and 11 after first inoculation. Antibody to gpUL55 was not detected in any pre-inoculation sera, 2-week sera, or sera from mice immunized with the parental vector pRc/CMV2. Intramuscular immunization of BALB/c mice with pRc/CMV2-gB (50 µg) resulted in the generation of gpUL55-specific antibodies in 5/5 mice immunized. The administration of a second booster dose augmented significantly the anti-gpUL55 response (*P* = 0.01) compared with the first boost with 4/5 mice generating titers of 1:10240 (Fig. 1). The geometric mean antibody titer after

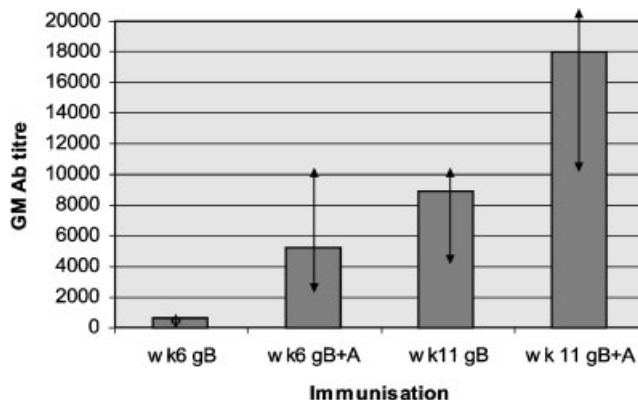


Fig. 1. Effect of the aluminium phosphate gel adjuvant, Adju-Phos, on HCMV gB-specific antibody titres elicited by immunisation of BALB/c mice with the pRc/CMV2-gB DNA vaccine (booster doses given at weeks 5 and 10 postprime). The antibody titre, measured by endpoint dilution immunofluorescence, was the last serum dilution at which positive staining cells were visible. Geometric mean antibody titres represented by histograms and arrow-ended lines cover the range of titres generated in individual mice. Wk, week; A, Adju-Phos; GM, geometric mean.

the second booster dose (1:8900) was almost 14-fold greater than that obtained after the first boost. All four BALB/c mice immunized with three doses of pRc/CMV2-gB (initial prime + 2 booster doses) developed antibodies capable of neutralizing AD169. The geometric mean neutralizing antibody titer was 1:74, with titers for individual mice ranging from 1:32 to 1:256 (Fig. 2).

In an attempt to boost levels of gB antibodies induced by DNA immunization, pRc/CMV2-gB DNA was emulsified with aluminum phosphate gel adjuvant (Adju-Phos) before use. Two of the five mice given pRc/CMV2-gB + Adju-Phos developed gpUL55-specific antibodies at week 6 with a geometric mean titer of 1:5120, which was greater than the geometric mean antibody titer of 1:640 obtained in mice given DNA vaccine alone (Fig. 1). By week 11, all five mice had developed antibodies to gpUL55 with a geometric mean titer of 1:17800 (1:20480 in 4/5 mice) representing a 2-fold increase in titer over those obtained with the gpUL55 DNA vaccine alone (*P* = 0.04). In contrast, neutralizing antibody titers measured at week 11 in mice given pRc/CMV2-gB + Adju-Phos were not significantly different to those from mice given pRc/CMV2-gB alone (*P* = 0.3; Fig. 2).

Effect of CpG ODN on Antibody Responses

At week 11 after the initial inoculation (booster doses given at weeks 5 and 10), mice immunized with a CpG ODN/pRc/CMV2-gB/Adju-Phos emulsion developed neutralizing antibody titers that were not significantly different from those in mice immunized with pRc/CMV2-gB alone (geometric mean titer 1:97 vs 1:74, *P* = 0.6; Fig. 2). Interestingly, neutralizing antibody titers generated by CpG ODN/pRc/CMV2-gB/Adju-Phos immunization were significantly higher than those generated by pRc/CMV2-gB/Adju-Phos (1:97 vs 1:37, *P* = 0.04; Fig. 2). No increase in gB-specific antibody titer

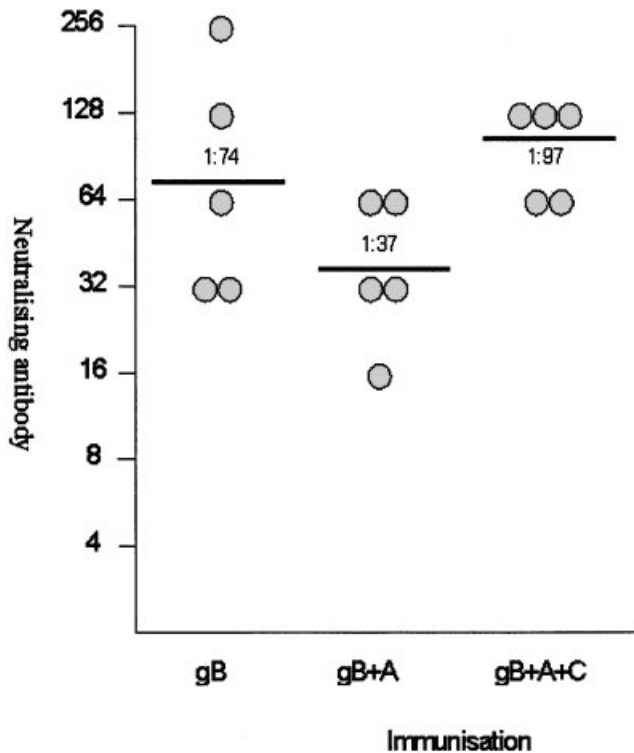


Fig. 2. Neutralising antibody (NA) titres developed at week 11 after immunisation of BALB/c mice with 50 µg pRc/CMV2-gB + 2 booster doses given at weeks 5 and 10 after initial inoculation. NA titre is the serum dilution producing 50% inhibition of virus infectivity compared with controls. Horizontal lines represent geometric means; filled circles represent NA titres developed in individual mice. gB, DNA vaccine pRc/CMV2-gB; A, Adju-Phos aluminium phosphate gel adjuvant; C, CpG ODN.

was found using CpG ODN/pRc/CMV2-gB/Adju-Phos as immunogen compared with pRc/CMV2-gB/Adju-Phos ($P = 0.35$) or pRc/CMV2-gB alone ($P = 0.21$) (data not shown).

DISCUSSION

We have developed a prototype HCMV DNA vaccine, pRc/CMV2-gB, and evaluated its immunogenicity in a BALB/c mouse system. In the absence of exogenous adjuvants, the vaccine was shown to be highly immunogenic, generating strong anti-gpUL55 and neutralizing antibody responses comparable to those obtained by other groups for candidate HCMV vaccine constructs evaluated in mice and humans [Endresz et al., 1999, 2001; Frey et al., 1999; Hwang et al., 1999; Gonczol and Plotkin, 2001].

Consistent with previous studies evaluating the immunogenicity of DNA vaccines delivered with conventional aluminum adjuvants, the formulation of our vaccine with aluminum phosphate gel adjuvant (Adju-Phos) was found to enhance gpUL55-specific antibody responses significantly, with antibody titers of 1:20480 elicited in 4/5 mice given three doses of the DNA vaccine. This approach represents a procedurally simple method of enhancing antibody responses to a DNA vaccine-encoded antigen in a mouse model system. Interestingly,

Ulmer et al. [1999] also showed that this adjuvant is able to boost humoral immune responses to DNA vaccines in non-human primates. In contrast, we found no significant difference in neutralizing antibody titer in mice immunized with a pRc/CMV2-gB/Adju-Phos emulsion compared with groups immunized with pRc/CMV2-gB alone, although further studies with more mice are required to confirm this observation.

It is thought that emulsifying CpG ODN with vaccine antigen maintains closeness and thus augments the adjuvanticity and immunostimulatory potential of the CpG species [Klinman et al., 1999]. Mice immunized with a CpG ODN/pRc/CMV2-gB/Adju-Phos emulsion showed a significant increase in neutralizing antibody titer compared with mice given pRc/CMV2-gB/Adju-Phos alone. In contrast, immunization of mice with pRc/CMV2-gB + CpG ODN emulsified with Adju-Phos did not result in an enhancement of gpUL55-specific antibody responses compared with pRc/CMV2-gB + Adju-Phos or pRc/CMV2-gB alone. Such a result may be attributable to the fact that HCMV gpUL55 encoded by the pRc/CMV2-gB plasmid is already highly immunogenic in BALB/c mice making the immunostimulatory effects of CpG coadministration, at the concentrations used, difficult to quantify in the numbers of mice used. Weeratna et al. [1998] reported that co-immunization of CpG ODNs synthesized with phosphorothioate backbones and DNA vaccines results in an ODN dose-dependent reduction in gene expression from the plasmid and postulate that this may be due to competitive interference at binding sites on the surface of target cells. Our data do not appear to support these observations, at least at the ODN concentrations used, since mice immunized with pRc/CMV2-gB + CpG ODN emulsified with Adju-Phos developed gpUL55-specific titers of 1:20480 at week 11 after initial inoculation.

In conclusion, we have shown that coadministration of Adju-Phos and/or CpG ODN can enhance the humoral immune responses engendered by an HCMV gpUL55-based DNA vaccine. The results provide a rationale for evaluating combinations of other HCMV proteins, such as gH/gL [Spaete et al., 1993; Urban et al., 1996] and gM/gN [Mach et al., 2000], in such a model system to determine whether enhanced levels of neutralizing antibodies can be achieved.

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