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TITLE: Development of a Novel Vector for Multiple CDC Category A Pathogens

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**14. ABSTRACT**

The long-term goal of this project is to develop and evaluate human cytomegalovirus (HCMV) as an effective large capacity persistent vaccine vector to provide protective immunity for multiple bioterrorist agents and emerging diseases. The aim of the current proposal was to determine the potential utility of HCMV as vaccine vector for CDC category A agents using rhesus cytomegalovirus (RhCMV) vaccine vectors in combination with the monkeypox (MPV)-rhesus macaque (RM) model. RhCMV is highly homologous to HCMV, and the MPV:RM model recapitulates all aspects of smallpox infection of humans. Specific Aim 1 was to generate a panel of RhCMV/MPV vectors expressing MPV antigens A29L, A35R, M1R and B6R in either the wild type RhCMV vector, or in a vector lacking MHC immunomodulatory genes. Vectors have been constructed, genetically characterized and electroporated into RhCMV permissive cells to reconstitute recombinant viruses. MPV antigen expression of vectors is currently being confirmed. Specific Aims 2 and 3 were to establish the pathobiology of WT MPV infection in RMs, and to monitor the immunological consequences of WT MPV infection. To date, four RMs have been experimentally inoculated intra-bronchially with MPV Zaire strain. Two with 2 x 10^7 plaque forming units (PFU) and two with 2 x 10^5 PFU, to define a lethal dose by this route of infection and to characterize the virus/host interactions. A summation of the ongoing studies is provided. Together, completion of these three specific aims will form the foundation for future studies designed to determine the efficacy of the RhCMV/MPV vectors at inducing a protective immune response to MPV challenge in RMs.
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INTRODUCTION:
The aim of the current study is to ascertain the potential utility of human cytomegalovirus (HCMV) as a safe, potent, large capacity vaccine vector for CDC category A agents. HCMV possesses a number of unique characteristics, which potentially make HCMV an ideal vaccine vector to induce protective immunity against acute and chronic bioterrorist weapons. These characteristics include the lack of significant disease associated with infection, persistence of the virus for the lifespan of the host, an ability to re-infect HCMV seropositive individuals, and a capacity to induce a large memory T cell response. In the present study, we will determine the utility of the CMV vector approach using the highly homologous rhesus cytomegalovirus (RhCMV) vaccine vector in combination with the monkeypox (MPV)-rhesus macaque (RM) model. The MPV:RM model is the only animal model that recapitulates all aspects of smallpox virus infection in humans. Since MPV also infects humans and causes symptoms that are indistinguishable from smallpox, MPV was also recently added to the list of category A select agents. Vaccinia virus (VV) is an effective vaccine against smallpox virus and MPV, but has significant side effects in a portion of the human population. Modified vaccinia ankara (MVA) vaccination has recently been shown to be partially protective against MPV in non-human primates, but required two vaccinations in order to induce protection. In the event of an actual smallpox outbreak, this approach would be unfeasible since there would be no time for multiple vaccinations to be made in close contacts and the effectiveness of this highly attenuated vaccine for post-exposure vaccination is uncertain at best. Therefore, development of long-term protection against smallpox virus and MPV in humans with a safe vaccine that can be administered in a single dose would be of significant value. Recently, subunit DNA vaccine vectors expressing multiple VV genes (A27L, A33R, L1R, and B5R) were shown to protect mice and non-human primates from lethal challenge with MPV (1, 2). Specific Aim 1 of the current study is to generate RhCMV vectors expressing the MPV protective antigens (A29L, A35R, M1R and B6R) in both a wild type RhCMV vector as well as in a vector deleted for immunoregulatory regions. Specific Aim 2 and 3 are designed to further characterize the virological and immunological aspects of MPV infection of RMs to further develop the MPV:RM model.

BODY:
"SA1: We will generate RhCMV-MPV vectors expressing the VV protective antigens that correlate to MPV-A29L, A35R, M1R and B6R.
SA1A: We will first construct a wild type (WT) RhCMV vector expressing the MPV protective antigens.
SA1B: We will develop an RhCMV vector that lack the MHC immunomodulatory genes expressing the MPV protective antigens to determine if we can increase immunogenicity as well as increase vector space for other antigen expression cassettes."

The goals of Specific Aim 1 are nearing completion, resulting in construction of two series of RhCMV vectors each expressing one of four individual MPV genes (A29L, A35R, M1R and B6R). These MPV genes are the correlates of VV genes that were shown to induce a protective immune response to MPV in RMs using a DNA vaccine approach (2). Two different RhCMV genetic backgrounds were used for construction of these vectors comprised of either the complete WT genome (designated WTRhCMV/MPV vectors), or a genome deleted for major immunomodulators of the virus (US2-US11 homologous region, Rh182-189) (designated
ΔRhCMV/MPV vectors). A comparison of the anti-MPV immune response and level of protection elicited by immunization with the two RhCMV vectors in future in vivo studies will enable determination of the role of these immunomodulators for induction of a protective immune response. The original aim proposed construction of two single vectors (WT and ΔRhCMV-based) each expressing all of the 4 MPV genes. However, to avoid any potential genetic instability problems arising from oversized genomes, we elected to construct 4 individual vectors each expressing a single MPV gene for a total of 8 vectors (1 series WT-based, the other series ΔRhCMV-based). Although this alternative approach has resulted in a considerable (4-fold) increase in the amount of work, we believe it to be the more prudent approach as it will avoid future concerns regarding stability of the RhCMV/MPV vectors.

A schematic of the RhCMV/MPV vectors is shown in Figure 1. MPV replicates in the cytoplasm, and an initial concern was the possibility that the MPV genes contained cryptic splice sites that would prevent production of full length proteins following gene expression in the nucleus (the site of expression of CMV encoded genes). Plasmids containing epitope tagged versions of the MPV genes under control of the EF1α constitutive promoter were constructed. MPV protein expression was assessed by transient transfection of these plasmids into RM fibroblasts and analysis by western immunoblot. All 4 cloned MPV genes expressed full length proteins following transient transfection indicating the lack of cryptic splice sites (data not shown).

The MPV gene cassettes were inserted into RhCMV vectors using bacterial artificial chromosome (BAC)-based linear recombination technology. Briefly, recombination cassettes comprised of the EF1α expressed epitope-tagged MPV genes combined with a selectable marker (kanamycin resistance; KanR) were inserted at the desired site within the RhCMV BAC genome using linear recombination. Recombinant RhCMV BAC clones were then selected on the basis of kanamycin resistance. The KanR marker is flanked by frt recombination sequences, which enables removal of the KanR by FLP recombinase leaving only the MPV gene and a single frt site within the recombinant RhCMV BAC genome. The entire ΔRhCMV/MPV series and WTRhCMV/MPVA29L vectors have been constructed and characterized; construction of the remaining WTRhCMV/MPV vectors is nearing completion. Restriction enzyme digestion analysis shows the lack of aberrant genomic rearrangements in the recombinant RhCMV BAC vectors (compare EtBR stained gel of RhCMV vectors with wild type RhCMV) (Figure 2A). Southern analysis using a probe directed against the KanR shows insertion of the recombination cassette within the RhCMV BAC genomes (Figure 2B). The KanR marker was subsequently removed by FLP induction. PCR analysis using primers flanking the site of MPV cassette insertion show amplification of an insert of the predicted size within the targeted genomic region, and removal of the KanR marker (observed as a 1.5kb band shift) (Figure 2C). Recombinant BACs were recently transfected into permissive RM fibroblasts to reconstitute RhCMV virus. A number of these BACs have already given rise to virus (as observed by virus-associated cytopathic effect in the culture), and will shortly be confirmed for MPV protein expression prior to in vitro growth characterization. We have previously shown that RhCMV vectors expressing simian immunodeficiency virus antigens inserted at identical sites within the RhCMV genome grow normally, and we expect a comparable outcome with the RhCMV/MPV vectors. Hence, we are now positioned to determine the efficacy of RhCMV vectors to elicit a protective immune response in the MPV:RM model system.
"SA2: We will establish the pathobiology of WT MPV infection of nonhuman primates."

A. Experimental inoculation and clinical evaluation
During the first year of support we evaluated our intrabronchial infection model for monkeypox virus pathogenesis in RMs. Our initial experimental RM infection study involved two animals, each inoculated with $2 \times 10^7$ plaque forming units (PFU) of MPV Zaire (MPVZ). Both animals developed respiratory disease by day 6 post-infection (pi), which was characterized as intermittent cough and elevated temperatures. On day 7 pi, animal coughs were more persistent and their oxygen saturation levels declined to 95%. On day 9 pi, one animal developed pox lesions on its trunk and lower extremities, while the second animal had no visible lesions on examination. Both animals exhibited lower oxygen saturation levels. On day 10 pi, oxygen saturation levels were further reduced and the animals were humanely euthanized and tissue samples were harvested for histopathological examination and virus load determination.

Since the $2 \times 10^7$ PFU dose was pathogenic in both animals, we sought to define a nonpathogenic dose. As other reports had shown that $2 \times 10^5$ PFU is non-pathogenic by intravenous and intratracheal inoculation (2, 4), we infected two animals with this dose intrabronchially. One animal developed a fever on day 4 pi and the second animal presented with oral and facial pox lesions. By day 5 pi, both animals exhibited episodes of coughing and both displayed oral pox lesions. By day 8 pi, the animals exhibited numerous lesions in and around their mouths and both had persistent cough and symptoms of pneumonia by clinical examination. On day 11 pi, the lesions on both animals were progressing, and their oxygen saturation levels decreased to 90%. By day 12 pi, the oxygen saturation levels dropped to 85%. During days 13 through 14, animal conditions stabilized. By day 16 pi, one animal developed complication associated with secondary bacterial infection in the lungs, despite administration of antibiotics, and was humanely euthanized. The second animal is resolving the pox lesions and lung infection.

B. Gross and histopathological examination
Pathological examination of the two RMs inoculated with $2 \times 10^7$ PFU euthanized at day 10 pi revealed widespread pox lesions in their oral cavity and on several internal organs. The lungs of both animals were heavy and congested, and exhibited significant inflammation, consistent with bronchopneumonia and appeared essentially as described by Zaucha et al (5). Additional findings were also consistent with those previously reported (5).

In the second cohort, the animal that was euthanized at day 18 pi exhibited numerous lesions in the oral cavity, in particular several large lesions on the tongue, which coincided with the animal not eating hard food (Figure 3). This animal also suffered from bronchopneumonia.

C. Virus isolation
Peripheral blood mononuclear cells (PBMCs) yielded recoverable virus by coculture in all four animals. Virus was also isolated from the fluids recovered by bronchoalveolar
lavage (BAL) and from lung tissue, tracheal bronchial lymph nodes and splenocytes. Further evaluation of viral load is being performed by real-time PCR.

"SA3: We will monitor the immunological consequences of WT MPV infection of nonhuman primates.

SA3A: We will determine the antiviral CD4+ and CD8+ T cell responses to the MPV structural proteins, A29L and A35R, in MPV-infected RM.

SA 3B: We will determine the antiviral antibody responses mounted against the MPV structural proteins, A29L and A35R, in MPV-infected RM."

Before we could determine the antiviral CD4+ and CD8+ T cell responses to MPV, we had first to define the host specific responses to experimental MPV infection. We accomplished this by flow cytometric analysis utilizing immunological reagents that are well characterized for RMs (3).

In the first cohort, we observed an increase in CD4+ central memory T cells at day 7 pi, which was followed by an increasing response in CD8beta+ cells of the central and effector memory populations in day 7 and day 10. Since these animals were euthanized on day 10 pi, we could not evaluate host responses past this date.

In the second cohort of animals, we were fortunate that both animals survived past day 10, which enabled collection of data on host response to infection to at least day 18 for one animal. The data are quite consistent with that of the first cohort. Specifically, CD4+ central memory T cells increased on day 7 pi, which was followed by an increase on day 11 of CD4+ effector memory T cells (Figure 4A), CD8beta+ T cell of the effector memory type (Figure 4B) and both marginal zone and memory CD20+ B cells (Figure 4C). The B cell responses were much more dramatic in the second animal cohort, which is likely due to the fact that the inoculum utilized in the first cohort overwhelmed the animals and hindered the ability of the animals to respond effectively to the MPV infection.

The surviving animal and subsequent animals inoculated with MPV will be monitored for host responses to the MPV antigens targeted in Specific Aim 1.

KEY RESEARCH ACCOMPLISHMENTS:
• Construction and in vitro characterization of all ΔRhCMV/MPV vaccine vectors and WTRhCMV/A29L; other vectors nearing completion.
• Defining pathogenic outcome of intrabronchial MPV-inoculation
• Characterization of host immune responses to pathogenic MPV infection

REPORTABLE OUTCOMES:
None.

CONCLUSION:
We have accomplished or are nearing completion of the specific aims proposed in the initial application. Specific Aim 1 will result in two series of RhCMV recombinants
expressing 4 MPV orthologues of VV genes shown to confer protection against MPV disease in RMs following direct DNA injection (2). Our decision to construct individual vectors each expressing a single MPV ORF increased considerably the level of work entailed for this aim. However, we anticipate that this more prudent approach will pay-off in the long term, as it assures genetic stability of the viruses. The construction of the ΔRhCMV/MPV vectors will enable determination of the role of these virally encoded immunomodulators for induction of a protective immune response against MPV.

Specific Aims 2 and 3 have served to establish the intrabronchial inoculation MPV:RM challenge model, in terms of pathogenesis, virological aspects of infection as well as in the resultant anti-MPV immune response. We are now positioned to ascertain the capacity of the RhCMV/MPV vectors to elicit a protective immune response to MPV challenge in this animal model. RhCMV and HCMV are highly homologous. Hence, a demonstration of efficacy in the RM model can be rapidly translated to production of an HCMV-based MPV vaccine for potential use against these category A pathogens in humans.

REFERENCES:

SUPPORTING DATA:
Figure 1: Schematic of RhCMV/MPV vectors
Figure 2: Genomic analysis of RhCMV/MPV vectors
Figure 3: Gross oral lesion from monkey experimentally inoculated with MPVZ
Figure 4: T and B cell responses to MPV infection
FIGURE 1. Schematic of RhCMV/MPV vectors

rh213 | Rh214

RhCMV genome (WT or ΔR182-189)

EF1α | MPV gene | pA

EF1α promoter
epitope tag
polyadenylation site
(A29L, A35R, M1R or B6R)
Figure 2

A

B

1.5kb —

12kb —

10kb —

5kb —
Figure 2: Genomic characterization of RhCMV/MPV BAC vectors. Linear recombination was used to insert a cassette, composed of an EF1α promoter-driven MPV gene and a frt-flanked KanR marker, into the RhCMV BAC (either WTRhCMV or ∆RhCMV). The KanR marker was subsequently removed by FLP recombinase. A) Restriction enzyme digestion analysis shows the lack of aberrant genomic rearrangements in the recombinant RhCMV BAC vectors. B) Southern analysis using a probe directed against the KanR shows insertion of the recombination cassette within the RhCMV BAC genome. C) PCR analysis using primers flanking the site of MPV cassette insertion show amplification of an insert of the predicted size (*) within the targeted genomic region, and FLP-mediated removal of the KanR marker (observed as a 1.5kb band shift). PCR amplification of this region from the WTRhCMV results in a band <500bp which runs within the primer band.
Figure 3: Gross oral lesion (ulcerative stomatitis) from monkey experimentally inoculated with human monkeypox virus (Zaire strain).
**Figure 4.**

**A.**

Effect of MPV infection on CD4+ proliferation

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<th>% Expression</th>
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**B.**

Effect of MPV on CD8 Proliferation

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<th>% Expression</th>
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<td>3.3</td>
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<tr>
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13
**Figure 4 (cont.)**

**Figure 4:** Time course of host specific T and B cell responses to MPV infection from the second animal cohort. CD4+ T cell responses (A), CD8beta + T cell responses (B) and CD20+ B cell responses (C) are graphically represented as percent of lymphocytes expressing Ki-67 or CD69. CD4+ and CD8beta+ T cells are classified as central memory (CM), effector memory (EM) and naïve based on the presence of memory markers CD28 and CD95. CD20+ B cells are classified as naïve, marginal zone-like and memory based on the expression of CD27 and IgD.