Induction of Protective Immunity Against Brucellosis following Immunization using Alginate Microspheres

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The development of safe and efficacious immunization systems to prevent brucellosis is needed to overcome the disadvantages of the currently licensed vaccine strains. A Novel method of immunization was examined using microspheres containing a non immunogenic eggshell precursor protein of the parasite Fasciola hepatica (vitelline protein B, VpB) conjugated to a live B. melitensis attenuated mutant and subsequently encapsulated into alginate microcapsules. The immunogenicity and protective efficacy of the capsules were evaluated in mice. A single immunization dose in BALB/c mice with encapsulated B. melitensis conferred a significant enhancement of protection compared to the un-encapsulated vaccine against wild type B. melitensis 16M challenge. VpB capsules also induced a higher and sustained IgG response compared to the un-encapsulated vaccine. In addition, cytokine secretion from spleen cells of mice vaccinated with the encapsulated mutant revealed elevated secretion of gamma interferon (INFγ), Interleukin 12 (IL-12) and Interleukin 2 (IL-2), but no Interleukin 4 (IL-4), suggesting an induction of a T helper 1 (Th1) response. Together, these results suggest that microencapsulation of live Brucella produces an enhanced delivery vaccine system against brucellosis increasing the efficacy of poorly-performing un-encapsulated vaccine candidates.

Evaluation in Chickens of a Live Attenuated NS1 Mutant Avian Influenza Virus Vaccine

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A proper vaccination program can play a critical role in prevention and control of avian influenza (AI) in commercial poultry. Low pathogenic (LPAI) H5 and H7 AI subtypes cause serious economic losses to the poultry industry and have the potential to mutate to highly pathogenic AI (HPAI) strains. Due to trade implications, differentiation of infected from vaccinated animals (DIVA) is an important issue in the control of AI. Therefore, the development and characterization of vaccine candidates with DIVA properties is critical in improving vaccination programs. Keeping these aspects in mind, we investigated the role of an NS1 mutant virus as a potential DIVA live attenuated vaccine. Influenza virus NS1 protein plays a major role in blocking the host's antiviral response and, as a non-structural protein, is only expressed during active virus replication and therefore is absent in purified vaccine preparations. Using an eight-plasmid reverse genetics system, we have recovered moderately virulent parental (rH5N3) and NS1 mutant (rH5N3/NS1-143) viruses. rH5N3/NS1-143 expresses only the first 143 amino acids of the NS1 protein compared to the 232 of the parental rH5N3. The growth properties of rH5N3 and rH5N3/NS1-143 were compared in cell culture and in different age embryonated chicken eggs. Our results confirm that NS1 is involved in down regulation of interferon as shown by INF mRNA expression analysis and by the inability of rH5N3/NS1-143 to efficiently grow in older age, interferon competent, chicken embryos. To evaluate the potential use of rH5N3/NS1-143 as a live attenuated vaccine, 6-week old chickens were inoculated with rH5N3 and rH5N3/NS1-143 viruses. rH5N3/NS1-143 showed partial attenuation, when compared to rH5N3, as determined by histological lesions of tracheae and lungs. In addition, rH5N3/NS1-143 infected chickens developed a strong antibody response to NP and/or M1 but not to NS1. Interestingly, challenge of rH5N3 and rH5N3/NS1-143 inoculated chickens with rH5N3, 4-weeks post inoculation, indicated that rH5N3/NS1-143 confers only partial protection with regards to virus replication regardless of the strong immune response to NP and/or M1. Studies are in progress to determine if a killed, inactivated rH5N3/NS1-143 vaccine confers protection and can be used as a DIVA vaccine.

IgA Inducing Protein: A new role for dendritic cells in B cell modulation

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Over the last several years there has been a great deal of progress in characterizing the role of dendritic cells (DC) in the activation and modulation of B cells. DC-secreted IL-12 has been shown to have an important role in DC-induced B cell differentiation. DC-secreted chemokines can induce B cell trafficking to the lymph nodes. DC-produced survival factors such as BAFF and APRIL have been shown to be essential for B cell maturation, but have also been implicated in class-switch recombination and B cell lymphoma survival. Recently added to this list of DC-derived factors effecting B cells is IgA-inducing protein (IGIP). Here we characterize DC production of IGIP in response to stimulation via various activation pathways, and examine the DC-B cell interactions necessary to induce class switching and differentiation in vitro. Monocyte derived DC were cultured in vitro with TLR agonists (3,4,5, and 9) and other cytokines, including CD40L, GM-CSF, IL-4 and Flt-3L, as well as the neuropeptide vasoactive intestinal peptide (VIP). Under appropriate in vitro stimulation conditions, IGIP mRNA expression could be up-regulated as much as thirty five-fold above non-stimulated samples within 12-48 hours. IGIP expressing DC were also able to induce IgA class-switch recombination as measured by secreted IgA in cultures using magnetically sorted tonsilar B cells in a DC-B cell co-culture.

Determination of a Dengue 2 Type-specific Neutralizing Epitope on the Envelope Protein Domain III (ED3)

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Dengue is the most important mosquito-borne viral disease affecting humans with an estimated 2.5 billion people living in regions at risk for dengue epidemic transmission. Dengue is caused by four related but antigenically distinct viruses termed DENV-1,-2,-3, and -4. Currently a dengue vaccine is not available and the development of an effective vaccine will depend, in part, on the induction of neutralizing antibodies. The DENV major envelope glycoprotein (E) can be divided into three structural domains designated domain I (ED1), domain II (ED2), and domain III (ED3). ED3 contains the most distal projecting loops from the virion surface and has been shown to predominantly elicit DENV type-specific neutralizing antibodies. ED3 has been implicated as the primary receptor binding motif and monoclonal antibodies (mabs) specific for ED3 block virus adsorption to susceptible cells more efficiently than mabs specific for ED1 or ED2. Therefore antibodies specific for this domain are likely to neutralize the virus by blocking attachment to the cell receptor. Based on the lack of cross-protective immunity among the four dengue viruses and the current understanding of disease pathogenesis, it is believed that a dengue vaccine will need to be tetravalent and ideally elicit DENV type-specific neutralizing antibodies. In this study the epitope of five DENV-2 type-specific neutralizing mabs (3H5, M8051122, 9F16, 2Q1899, and 9F11) was determined. A recombinant ED3 (rED3) comprising amino acids 295-395 of the E protein from DENV-2 strain New Guinea C (NGC) was constructed. A total of 40 single amino acid substitutions were introduced by site-directed mutagenesis into the rED3 at 27 different surface accessible residues. The dissociation constant (Kd) of each rED3 mutant was determined with each of the five mabs by titration in an indirect ELISA. Mutants M301G, K305G, K305A, E383G, and P384A resulted in a greater than 10-fold increase in Kd (i.e. greater than a 10-fold decrease in binding affinity) with all five mabs compared to the wild-type rED3. This suggests that residues M301, K305, E383, and P384, which cluster on the upper lateral face of ED3, represent the epitope of all five DENV-2 type-specific mabs. These four residues are conserved among DENV-2 strains and are found in a region of ED3 that is proposed to bind to the host cell receptor.

Effector CD4+ T lymphocytes Resolve Acute HSV-1 Infection at both Genital and Neuronal Tissues.

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Classically, it is thought that $CD8^+$ T cells are predominantly responsible for eliminating viral infections. Indeed, it has been previously demonstrated that $CD8^+$ T cells are important for clearance of infectious herpes simplex virus (HSV) from the sensory ganglia in primary infection. We present evidence for $CD4^+$ T cell-mediated clearance from the genital tract, as well as for $CD4^+$ T cell protection of neuronal tissue. In immunocompetent mice, HSV-specific $CD4^+$ T cells were present in both secondary lymphoid and neuronal tissues during primary HSV-1 infection, peaking between days 6 and 8 post-infection. Further, the $CD4^+$ T cells present in lymphoid and neuronal tissues showed distinct patterns of the activation markers CD25, CD44, and CD69 post-infection. Utilizing a model in which $CD4^+$ T cells were adoptively transferred into genetically B- and T cell-deficient $Rag1^{-/-}$ mice, we showed that $CD4^+$ T cells were sufficient for clearance of HSV-1 from the genital tract. Additionally, compared with animals that did not receive T cells, animals that received $CD4^+$ T cells had dramatically lower viral titers in the spinal cord and dorsal root ganglia, indicating that $CD4^+$ T cells may function in protection of neuronal tissue. Interferon-gamma (IFN-γ) has previously been shown to play a role in T cell-mediated protection against genital HSV-2 infection. To examine the cell types responding to IFN-γ, IFN-γ receptor (IFN-γR) chimeric mice were created that expressed IFN-γR on both parenchymal and hematopoietic cells, on parenchymal cells only, on hematopoietic cell only, or did not express IFN-γR. Chimeric mice that expressed IFN-γR on parenchymal cells demonstrated lower viral titers in the genital tract and in neuronal tissues, as opposed to mice that either did not express IFN-γR, or those that expressed the receptor only on hematopoietic cells. In summary, $CD4^+$ T cells are important for the resolution of primary HSV-1 infection at both genital and neuronal sites. Preliminary results suggest that this resolution may be mediated, at least in part, by IFN-γ action on parenchymal cells.

Importance of toll-like receptor 4 in immunity against Rickettsia conorii

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Toll-like receptors (TLR) are important in inducing innate and adaptive immune responses as well as skewing the local cytokine profiles in numerous infectious diseases. The importance of TLR in rickettsial diseases, however, has not been described. We have previously shown that adoptive transfer of dendritic cells that had been stimulated with non-rickettsial LPS, and thereby ligating TLR-4, was sufficient to partially protect mice from lethal challenge with Rickettsia conorii. To extend our previous data, we have utilized C3H/HeJ mice, which possess a mutation in TLR-4, to more fully understand the significance of TLR-4 ligation in rickettsial immunity and pathogenesis. We demonstrated that mice lacking TLR-4 (C3H/HeJ) are significantly more susceptible to R. conorii infection than their wild-type counterparts (C3H/HeN). In fact, upon i.v. inoculation with R. conorii, TLR-4 deficient mice succumb rapidly within 6 days while greater than 50% of C3H/HeN mice survive infection. These survival data further correlate with rickettsial loads ands immunohistochemical staining of rickettsial antigens throughout disease in the pathologic organs. Histologic evaluation of H&E stained organs revealed startling differences. Spleens obtained from C3H/HeJ mice on d 5 p.i. exhibited significant cell death, which was further corroborated by significantly decreased splenocyte counts in C3H/HeJ mice when compared to C3H/HeN mice. Additionally, spleens that exhibited marked cell death also possessed significant fibrin deposition, visualized by PTAH staining. Determination of serum cytokine levels in mice revealed that mice possessing TLR-4 produced significantly greater quantities of Th1 cytokines, IL-12p40 and IL-12p70 as well as IL-17 and TNF-α. In conclusion, we have demonstrated that TLR-4 plays an important role not only in immunity to rickettsiae, but also in the pathologic disease process.

Identification of novel vaccine adjuvants engineered from Mycoplasma genitalium: New prospective for immunomodulating compounds.

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Effective adjuvants for topically-applied vaccines have been derived previously from conserved pathogen associated molecular patterns (PAMPS) to elicit, in many cases, a localized Th1-biased inflammatory response. Mycoplasma genitalium (MG) is a sexually transmitted pathogen associated with symptomatic urethritis in men and mucopurulent cervicitis in women. We hypothesized that a local infection associated with inflammation may provide novel PAMPs for adjuvant development, and have begun evaluation of selected MG target proteins. Because MG causes inflammatory urethritis and cervicitis, we set out to define inflammatory components of MG and evaluate their utility as adjuvants for locally applied vaccines. In this regard, two membrane lipoproteins and two heat shock proteins were selected for testing to determine if they elicit a Th1-biased inflammatory response. The impact of purified recombinant MG protein exposure was quantified first using a mouse vaginal model and confirmed using cultured human vaginal and cervical epithelial cells. Six hours after intravaginal application of MG Heat-shock protein 60 (Hsp60) in the mouse, IL6, G-CSF, KC, and RANTES were all significantly increased in vaginal lavages. Cytokine levels returned to baseline by 24h indicating a transient effect and a lack of prolonged inflammation. Hsp60 significantly increased secretion of TNF-alpha, IL6, IL8, and IL1-alpha from human cervical and vaginal epithelial cells that peaked 6h after application. Interestingly, MG Hsp10 did not induce significant Th1 cytokine secretion in cultured epithelia or in mouse vaginal lavages at any tested time points. Testing of both lipoproteins revealed that both MG307 and MG309 induced robust Th1 responses from the mouse and cultured human epithelia. Specifically, KC, G-CSF, RANTES, and IL1-beta were significantly increased in mouse lavages six hours following application in a pattern distinct from MG Hsp60. Lipoprotein MG307 induced significant levels of IL6 and TNF-alpha secretion from cultured vaginal cells, with a significant increase in IFN-gamma from cervical epithelia. Similar to MG307, MG309 induced IL6 and TNF-alpha secretion from cervical and vaginal epithelia. MG309 also induced significant levels of IL8 thereby further differentiating the innate responses to select MG proteins. Of the recombinant MG proteins studied, the most robust response was observed for lipoprotein MG309, and thus we tested whether local application would create an HSV2-resistant environment to evaluate its potential as a topical microbicide. In preliminary studies, intravaginal application of MG309 did not provide significant protection from infection, time to disease onset, or increase the survival time in mice experimentally infected with HSV2. Investigations into the inflammatory components of MG will provide opportunity to define the basic etiologies of MG-associated urethritis and cervicitis, and concomitantly facilitate the development of more effective vaccine adjuvants. Further, it is possible to adapt MG-derived pro-inflammatory components for use as topically-applied immunomodulating microbicides, and is an attractive alternative to address the growing epidemic of sexually transmitted infections.

The ΔfbpA vaccine from Mycobacterium tuberculosis induces a strong expression of T-box expressed in T cells (T-bet) in immune T cells and drives a stronger Th1 response compared to the BCG vaccine.

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Tuberculosis is the leading cause of death due to an infectious disease. The BCG vaccine only protects children against tuberculosis with variable efficacy, and newer vaccines have been sought after. We described previously an fbpA vaccine derived from M. tuberculosis (MTB) H37Rv that was more effective than BCG in mice against tuberculosis (Copenhaver et al, Infection & Immunity, 2004, 72:7084). We have investigated the molecular basis of protection induced by the fbpA vaccine. C57Bl/6 mice were immunized intraperitoneally with either BCG or fbpA vaccine, and splenic T cells were primed using macrophages or dendritic cells infected with either fbpA or BCG. T cells were then stained for transcriptional markers of Th1 (T-bet), Th2 (GATA3) or Th3 (Foxp3) and analyzed by flow cytometry. Cytokine synthesis was detected by ELISA. fbpA infected macrophages induced a larger expansion of T-bet+ CD4+ T cells than BCG, in parallel with an increased level of IFN-γ secretion. Neither GATA3 nor Foxp3 expression were significant for either vaccines (less than 5%). The frequency of IFN-γ+ T cells was then determined in the spleens of mice immunized with the vaccines. fbpA vaccine again induced a larger expansion of CD4+ IFN-γ+ T cells in the spleens over time compared to BCG vaccine. Since IFN-γ is the key determinant of resistance to tuberculosis, the increased efficacy of fbpA vaccine thus appears to be due to its ability to prime a stronger in vitro and in vivo T-bet (Th1) response, leading to higher IFN-γ production in mice.

Genitally-Applied Vaccine Adjuvants; Development of a Screening Method for Immunomodulatory Activity and Immunotoxicity

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The increasing rate of sexually transmitted infections (STI) and their epidemic prevalence in the adult population indicates a clear need for improved vaccines. Topical vaccines offer advantages over traditional, injected vaccines including ease of application, reduced chance of blood-borne pathogen transmission and increased compliance. Success with such topical vaccines is dependant, in part, upon improved adjuvants. These adjuvants should elicit cytokine/chemokine profiles and innate immune cell recruitment thatHHHHH then primes acquired immunity to increase vaccine efficacy without inducing immunotoxicity. To this end we have developed an innovative methodology for screening genitally-applied compounds for potential immunomodulatory activity and immunotoxicity. Vaginal and ectocervical epithelial cells isolated from human donor tissue were immortalized and utilized for these experiments. Epithelial cells are appropriate for these studies because they likely will be the first cell type contacted by genitally-applied compounds and are highly immunoresponsive. Based on our previous studies, Poly I:C (PIC; a toll-like receptor 3 agonist) was utilized because it has been used as an adjuvant and it modulates innate immune responses that create a resistant state to our prototypical STI, herpes simplex virus type 2 (HSV-2). Other potential adjuvants also were tested to determine if they produced similar antiviral resistance with no treatment, vehicle treatment, and non-immunomodulatory microbicides utilized as controls. To evaluate the test compounds, samples of supernatant from treated human vaginal epithelial cultures were collected 6h (early) and 24h (late) after treatment. Cytometric bead array showed PIC significantly ($p<0.01$) increased IFNγ and MIP-1β at 6h, IL-8 at 24h, and IL-6, GM-CSF, and TNFα at 6 and 24h consistent with a TH1 immune response. As an indicator of immunotoxicity, modulation of toll-like receptor (TLR) expression was analyzed by quantitative PCR. Unexpectedly, PIC application altered TLR expression, significantly increasing expression of TLR 2 and 3 potentially indicating heightened responsiveness to viral glycoproteins and double-stranded RNA, respectively. The impact of genitally-applied, potential adjuvants or microbicides also was addressed by immune cell recruitment in transepithelial migration assays using immortalized human cervicovaginal epithelial cells grown inverted on transwells. After establishing multilayer polarized cultures, apical cell surfaces (basal chambers) were treated with the test compounds. Eighteen hours after treatment, neutrophils were added to the apical chamber (basolateral epithelial surface), allowed to migrate for 4h and then counted. Significant ($p<0.05$) transepithelial migration of neutrophils into the basal chamber of PIC- and fMLP-treated (a known neutrophil chemotactic tripeptide) cultures was observed. Neutrophil transepithelial migration was not significantly ($p>0.05$) elicited by carrageenan (a non-immunomodulatory microbicide) treatment. These data indicate that genitally-applied immunomodulatory vaccine adjuvants can be screened effectively to determine if the compound will produce an immunological response appropriate to enhance resistance to STI and effectively immunize a host.

West Nile Viruses with Mutations in NS2A Are Attenuated In Vivo

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West Nile virus (WNV) has caused thousands of documented cases of human disease and hundreds of deaths since its introduction into North America in 1999. Within the last seven years, the geographic distribution of this virus has expanded to include all of the continental United States as well as extending north to Canada and south to Mexico and the Caribbean. Currently, there is no FDA-licensed vaccine available for human use to combat WNV-induced disease. We sought to identify changes to the WNV genome that could allow the virus to persist in vivo. WNV subgenomic replicons were forced to adapt to replicate within cells using antibiotic selective media, thereby modeling a persistent infection in cell culture. Cell-adapted replicons selected in this way had become noncytopathic. To characterize these noncytopathic replicons, genomes were harvested from the replicon-bearing cells and sequenced to detect any changes made to their genome. Multiple point missense mutations and one insertion were observed in many, but not all, of the nonstructural genes. However, several of these mutations failed to produced the noncytopathic phenotype when introduced individually into the parental replicon. Here, we report the detailed examination of two of these point missense mutations in NS2A that did render the replicon noncytopathic: D73H and M108K. WNV harboring these either of these NS2A mutations replicated more poorly than wt WNV and no longer caused cytopathic effect in cell culture. WNV NS2A D73H and WNV NS2A M108K were also attenuated in vivo. Furthermore, mice infected with these attenuated NS2A mutant viruses seroconverted, suggesting that these mice could be protected from challenge with wt WNV. Unfortunately, single point mutations can easily revert to a wt phenotype. One mouse out of the fifteen studied died after infection with WNV NS2A D73H. Sequence analysis of virus harvested from this mouse upon death revealed a reversion back to the parental Asp residue at the mutated locus, transforming this attenuated virus into lethal wt WNV. The NS2A D73H and M108K mutations described here may not be sufficient vaccine candidates when incorporated into wt WNV alone, but could be used in conjunction with other alterations to the genome in incorporated into existing vaccine candidates to produce a safe and immunogenic live attenuated WNV vaccine for human use.

Prognostic indicators of successful vaccination against Mycobacterium tuberculosis

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Tuberculosis (TB) is still a major health problem worldwide with more than 2 billion people infected per year. Cell mediated immunity plays a huge role in the outcome of the disease, where a Th1 type response based on the activation of CD4+T cells and CD8+T cells and release of cytokines such as IFNγ and cytotoxic granules such as granulysin and perforin, play a major role. Measurements of protection after TB vaccination include the standard skin test, IFNγ measures and T cell proliferation assays. These methods are not adequate correlates of protection. The lack of better vaccines than the gold standard BCG and the lack of good correlates of protection demand that new biomarker molecules are explored. Our group recently cloned a bovine homologue of granulysin (Bo-lysin), a potent cytotoxic granule able to kill intracellular mycobacteria, that may be a good candidate as a biomarker molecule for TB vaccination. In the present study we examined the kinetics of Bo-lysin, compared to perforin and IFNγ in vitro, after an antigen specific stimulation with PPD and mitogen in different bovine T cell populations. Results comparing the mRNA fold increase in both BCG vaccinated and non-vaccinated cattle demonstrated that Bo-lysin expression is very high after 24 h in both CD4+ and CD8+ T cells, when stimulated with PPD. Perforin levels were over 100 fold after 48h after stimulation with mitogen, but not present until 72 h when stimulated with PPD in both CD4+ and CD8+ T cells. Perforin was detected by FACS in the different T cell populations only 5 days after stimulation. IFNγ was observed as early as 1 day post-stimulation, being characterized by a biphasic pattern. The bo-lysin expression pattern suggests that signaling required to activated transcription of this cytotoxic granule is different than perforin and IFNγ. The absence of expression of granulysin by γδ T cells (lower than 2 fold) suggests a typical antigen presentation process may be required for granulysin expression as compared to perforin and IFNγ. Results shown here provide support for further investigations of granulysin and perforin as candidate biomarkers of protection after vaccination against TB.
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Infectious DNA is more immunogenic than attenuated virus carrying mutations affecting its infectivity

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Flavivirus infectious DNA (iDNA) offers many advantages for the development of live vaccines. As an infectious clone, it allows direct manipulation of viral genomes for purposes of rational attenuation. Being a special occasion of DNA vaccines, it can be used directly for immunization yielding infection virus in vivo. We utilized West Nile infectious DNA to evaluate attenuating effects of mutations targeting two positions within the “hinge” region of the viral envelope glycoprotein. Amino acid residues at the E positions 138 and 276 were changed to those found at the corresponding positions (138 and 279) in the E protein of Japanese Encephalitis virus vaccine SA-14-14-2 (E138K and K276M). We hypothesized that these mutations would exert the same attenuating effect with a minimal effect on the immunogenicity. Both mutations affected virus growth properties in mammalian, but not in mosquito cells. We utilized pH dependent fusion of C6/36 cells to determine effect of these mutations on the functionality of the E protein. The E138K mutation strongly influenced the pH-mediated fusion activity of the virus leading to a shift in the fusion threshold. The K276M mutation did not produce any effect. Further, we compared the residual virulence and immunogenicity of the wild type, single and double mutant viruses upon inoculation as infectious viruses and directly as iDNA by needle injection or using Gene Gun. While all mutant viruses were significantly attenuated when administered as infectious viruses, a less attenuated phenotype of the K276M mutant was revealed after intradermal immunization with K276M iDNA using Gene Gun. As expected, all mutant viruses induced WN specific immune response in immunized animals the magnitude of which clearly correlated with the ability of virus to replicate in mammalian cells. Surprisingly, the magnitude of WN-specific immune response was substantially higher after immunization directly with mutant iDNA, which after i.d. immunization using Gene Gun appeared essentially independent on virus growth characteristics. Such outcome may have resulted from the involvement of mechanisms controlling immune response to DNA vaccines and indicates that use of iDNA may substantially broaden the range of usable attenuating mutations.

The ∆fbpA vaccine from Mycobacterium tuberculosis H37Rv is more efficiently processed than BCG for antigen 85B presentation in macrophages and dendritic cells

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Tuberculosis is the leading cause of death due to an infectious disease. Newer vaccines have been sought after since the BCG vaccine is safe but has a variable efficacy. We showed recently that it is an inefficient presenter of antigens in macrophages that may impact on its vaccinogenicity (Singh et al, J. Immunology, 2006, 177; 3250-3259). We developed a ∆fbpA vaccine derived from M. tuberculosis H37Rv that is more effective than BCG in the mouse model against tuberculosis (Copenhaver et al, Infection & Immunity, 72: 7084). We have now compared the ability of C57Bl/6 mouse bone marrow derived macrophages and dendritic cells (DCs) to present antigens from BCG and ∆fbpA vaccines using an antigen 85B specific BB7 T cell hybridoma developed by Harding and Boom (Cell. Immunology, 2000; 10:63-74). DCs and macrophages were infected with either BCG or ∆fbpA and allowed to present the Ag85B epitope to the T cells and IL-2 release from the BB7 T cells was determined by ELISA. While ∆fbpA infected macrophages rapidly presented Ag85B (>1000 pg/mL IL-2 by 4 hr), BCG infected macrophages were less efficient to prime IL-2 (< 100 pg/mL IL-2 by 4 hr). Immature DCs were unable to process BCG but processed ∆fbpA after a lag time. DCs induced to mature with TNFα or LPS still processed BCG less efficiently than ∆fbpA vaccine. To investigate the basis of better intracellular processing of ∆fbpA vaccine, infected DCs were stained for phagosome maturation markers and antigen processing molecules colocalizing with gfp expressing strains of BCG or ∆fbpA and analyzed by laser confocal microscopy. ∆fbpA vaccine showed a stronger colocalization with LAMP1, Cat-D, Cat-S and MHC-II in contrast with BCG that excluded Cat-S, MHC-II and LAMP1, to variable extents. Since Cat-D, Cat-S and MHC-II molecules are involved in antigen processing, efficient generation of Ag85B epitope in ∆fbpA vaccine seems to correlate with a better targeting of antigen processing mechanisms in DCs. Furthermore, Ag85B is an immunodominant epitope of M. tuberculosis and DNA encoding Ag85B is protective against tuberculosis in mice. Thus, our studies indicate that M. tuberculosis derived novel vaccines like ∆fbpA can be better vaccines than BCG vaccine due to differences in antigen processing in macrophages and DCs. This project was supported by NIH NIAID 49534.

Non-glycosylated NS1 fully attenuates West Nile virus for neuroinvasiveness

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West Nile virus (WNV) is a flavivirus and a member of the Japanese encephalitis (JE) serogroup. All members of the JE serogroup except JEV contain three highly conserved glycosylation (Asn-X-Ser/Thr) sites in the NS1 protein whereas JEV and other arthropod-borne flaviviruses contain two of these three sites. In an effort to attenuate the highly virulent NY99 strain, the three glycosylation sites were ablated using site-directed mutagenesis to change the Asn to an Ala for each of the three sites. In changing all three sites we were able to attenuate the mouse neuroinvasiveness of the virus 100-fold compared to the NY99 parental strain. Interestingly, construction of a virus with mutations ablating the glycosylation site in the E protein together with the ablation of the three NS1 glycosylation sites completely attenuated this virus with an LD50 of > 100,000. Although this virus was highly attenuated, the NS1 mutant virus reverted to virulence and reversion at the first NS1 glycosylation site seen in virus isolated from the brain of all mice that succumbed to infection. In order to diminish reversion, two new viruses were generated where the first NS1 glycosylation site now contained two or three amino acid substitutions in the glycosylation motif while the second and third glycosylation site remained a single Asn to Ala amino acid change. These viruses were highly attenuated for mouse neurovirulence and neuroinvasiveness, 3,000-fold and >100,000-fold, respectively, compared to the parental NY99 strain. Furthermore, in comparison to the Ala mutations, changing the first glycosylation site only with these two and three amino acid mutations also attenuated the virus.

Optimization of the conditions for secretion by the Burkholderia mallei type three secretion system and use of the effector proteins in an optimized BALB/c infection model

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Burkholderia mallei, the etiologic agent of glanders disease is a gram-negative, capsulated, non-spore forming, non-motile bacillus. B. mallei was used as a biological weapon during the American Civil War, World Wars I and II, and reported to be weaponized by the Soviet Union in Afghanistan. Recently, B. mallei has come under renewed investigation as a possible biological threat and is classified as a category B agent. Currently, there is no effective vaccine for glanders in animals or humans. Scientific reports describing pathogenesis associated with glanders is limited to identification of a type III secretion system (TTSS) and capsule production. Characterization of effector proteins has been widely investigated in other organisms but limited in the case of Burkholderia species. Through a plasmid based yplA reporter system, we established an approach for TTSS secretion characterization with the goal to utilize these effectors as putative protective epitopes in a BALB/c mouse model. The reporter system is based on the ability to secrete YplA, a Yersinia TTSS effector phospholipase protein, production of the secreted YplA reporter protein was confirmed by immunoblots of culture supernatants and by lipase activity on indicator agar plates. BALB/c mice vaccinated with a linear expression element homologous to the B. mallei TTSS effector protein BopA showed increased survival when challenged with wild type B. mallei intraperitoneally. Additionally, survival of BALB/c mice vaccinated with non viable B. mallei was increased compared to non vaccinated mice, demonstrating the feasibility of our mouse model. Further, our results indicate that a TTSS reporter system can be used to assay secretion in B. mallei.

Mutational Analysis of the West Nile Virus NS4B Protein

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West Nile Virus (WNV) is a member of the genus Flavivirus in the family Flaviviridae. The WNV genome is a positive-sense RNA molecule approximately 11kb in length encoding a single polyprotein that is cleaved by a combination of viral and host proteases to produce three structural and seven nonstructural proteins. NS4B is a small hydrophobic protein approximately 27kD in size that is hypothesized to participate both in viral replication and evasion of host innate immune defenses. Utilizing site-directed mutagenesis of a WNV NY99 infectious clone, amino acid substitutions were introduced into the NS4B protein primarily targeting two distinct regions. The first was a stretch of amino acid residues located in the N-terminal domain exhibiting a high degree of conservation among both mosquito- and tick-borne flaviviruses. The second set of residues localized to a central hydrophobic region where mutations have been frequently observed in various attenuated or passage-adapted flavivirus strains. Out of eight engineered substitutions, the NS4B P38G substitution was found to be associated with temperature-sensitive and small-plaque phenotypes. Furthermore, this mutation was found to attenuate neuroinvasiveness greater than 10,000,000-fold compared to the wild-type NY99 virus in a mouse model. Although this mutant exhibited viremias that reached significant titers, the titers were lower than those associated with wild-type NY99 WNV infection. Following extended incubation at 41°C, mutants encoding putative compensatory substitutions in the NS4B protein were selected and exhibited a reduction in the temperature-sensitive phenotype and return to a virulent phenotype in the mouse model.

Improving yield and testing safety of a novel pseudo-infectious virus vaccine candidate for West Nile (WN) encephalitis.

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Safe and effective vaccines have only been produced for a handful of diseases caused by flaviviruses. These include inactivated vaccines (INV) and live-attenuated vaccines (LAV). In general LAV suffer from concerns of residual virulence and/or reversion to virulence, and INV can be difficult to produce due to low potency, requiring large-scale production and removal of contaminants that can produce adverse reactions. In order to blend the replicative capacity of LAV with the safety of INV, we have engineered a new type of vaccine candidate consisting of a pseudo-infectious flavivirus (RepliVAX) that contains a large deletion in a key structural protein, C. Our WN RepliVAX grows well in C-expressing cells, and does not produce infectious progeny in normal cells. However, when RepliVAX infects normal cells, it produces an extracellular form of E that is known to be an effective immunogen. Inoculation of baby mice with RepliVAX demonstrated that no mice were killed with any RepliVAX dose tested (up to 2x106 IU), whereas less than one infectious unit of WT WNV killed 50% of the baby mice. Inoculation of adult mice with RepliVAX demonstrated a similar level of attenuation/safety. Importantly, mice vaccinated with as few as 30,000 IU of RepliVAX produced high levels of WNV-neutralizing antibodies and were protected from encephalitis (Mason et al. 2006, Virology 351:432). The genetic stability of RepliVAX and the C-expressing cells used to produce it are important issues for both vaccine production and safety. With respect to the latter issue, genetic recombination between the C gene expressed in these cells and RepliVAX could produce a virulent virus, and thus is key to RepliVAX safety. To reduce chances of recombination a cell line was generated that encoded a C gene designed to be incapable of recombining with the RepliVAX genome. To demonstrate that recombination could not occur between this cell line and RepliVAX, and to test the long-term stability of the cell line, these cells were repeatedly passed and used to propagate RepliVAX for 10 sequential passages. The cell lines maintained their phenotype, and RT-PCR failed to reveal any changes in the cell-encoded C. After 10 passages of RepliVAX on these cells, we were unable to detect any recombination by either phenotypic (blind passage on Vero cells) or genetic (RT-PCR) analyses. Interestingly, passage 10 RepliVAX demonstrated larger foci and increased growth kinetics when compared with passage 0. RT-PCR analyses revealed mutations in RepliVAX that altered the proteolytic cleavage sites bordering the truncated C protein. Further selection of better-growing mutants and purposeful incorporation of these mutations into second-generation RepliVAX will allow us to improve our vaccine. Taken together our results demonstrate that WN RepliVAX is a safe, potent, and effective type of vaccine that is broadly applicable to the development of vaccines for flavivirus diseases.

Characterization of the human immune response to Francisella tularensis following Live Vaccine Strain vaccination

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Francisella tularensis (Ft) is the causative agent of human tularemia. In the US, the live vaccine strain (LVS) remains unlicensed due to its poor characterization, high virulence in animal models, and unknown mechanism(s) of attenuation. Immune responses from clinical samples obtained following LVS vaccination are being characterized using an inclusive clone set consisting of ORFs from Ft subspecies tularensis (Type A) and Ft subspecies holarctica strain (Type B).

Ft subspecies tularensis (Schu 4) and LVS recently have been sequenced, and we have sequenced the genome of a virulent Ft subspecies holarctica strain (OSU18). These data provide the unique opportunity to characterize comprehensively the humoral and cell-mediated immune responses of LVS-vaccinated individuals. Through the generation and use of clone sets, the antigenicity of each Ft ORF can be determined. In the whole ORF clone set, each ORF is expressed with an N-terminal 6x-His tag under a lac-inducible promoter. In addition to the whole ORF clone set, a shotgun clone set, generated from sequencing products, is being utilized to detect Ft immunoreactive proteins. Use of both clone sets provides the opportunity to substantiate results obtained independently from each clone set. Clones are analyzed for immunoreactivity by probing with sera from individuals vaccinated with either 109 or 105 cfu of LVS via percutaneous or subcutaneous administration, respectively. Furthermore, peripheral blood mononuclear cells from LVS-vaccinated individuals will be used in separate screens using the whole ORF clone set to identify proteins recognized by the cell-mediated immune response. These data will bring LVS closer to licensure in the US, and will serve as initial steps in the development of a subunit vaccine for tularemia.

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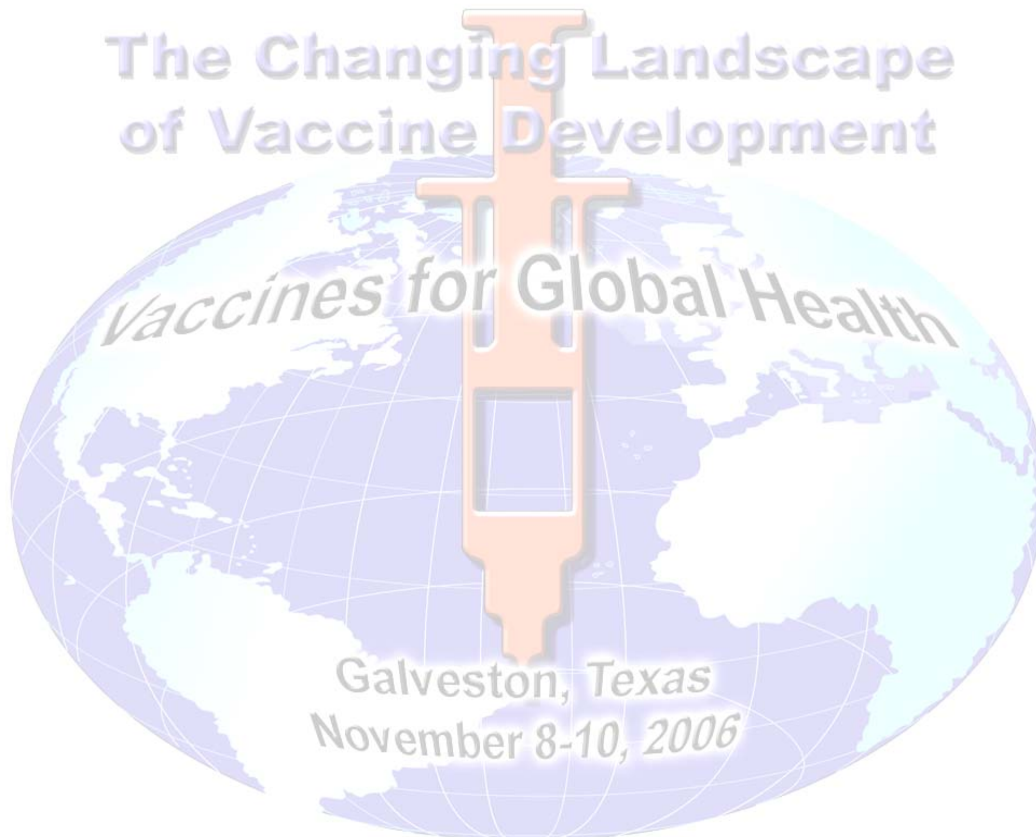

Emergence and Pathogenesis of Venezuelan Equine Encephalitis Virus Subtype IE

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Venezuelan equine encephalitis virus (VEEV) is a highly infectious alphavirus endemic in parts of Central and South America; however, recent human epidemics and equine epizootics indicate that VEEV poses as a serious public and veterinary health threat. The etiologic agents during major VEEV outbreaks are subtype IAB or IC, while subtype IE strains are considered enzootic, equine-avirulent, and incapable of exploiting horses as amplification hosts. However, recent Mexican epizootics were shown to originate from a virulent form of subtype IE VEEV. Equine pathogenesis studies were performed to determine whether the virulence of subtype IE VEEVs correlate with the establishment of infection and/or modulation of the immune response in the natural host. Groups of 2 or 3 horses were inoculated subcutaneously with 2,000 plaque forming units (PFUs) of one of three VEEVs: 1) MX01-32, a virulent subtype IE VEEV that was isolated in 2001 from sentinel hamsters in Chiapas, Mexico, and determined to be genetically related to the 1996 Mexican epizootic, 2) 68U201, an avirulent subtype IE VEEV (enzootic control), and 3) 3908, a virulent subtype IC VEEV (epizootic control). Horses were monitored carefully for illness and fever throughout the study and were humanely euthanized at 48 hr (early stage) or 14 days (late stage) after infection or when the animals became paralyzed or anorexic. Interferon (IFN) bioassays showed that MX01-32 elicited a diminished and delayed IFN response when compared to 68U201 and 3908. These results were noticeably similar in pattern to body temperature elevations during early infection. In contrast, MX01-32-infected horses developed viremia titers that were comparable to and earlier than 3908-infected horses. Tissues were also collected immediately after euthanasia and processed for histopathology and immunohistochemistry. At day 2 after infection, there were no histopathological lesions observed in the VEEV-infected equine cerebral tissues, but severe encephalitis and extensive perivascular cuffs of lymphocytes were evident in the brain by day 10 after infection. Independent of VEEV subtype, neurons and Purkinje cells in the equine brain were infected by day 2 after infection; however, MX01-32 and 3908 were less established in the draining lymph nodes at day 2 after infection than 68U201. To determine which amino acid residue changes potentially facilitated the emergence of virulent subtype IE VEEV, the complete genome of MX01-32 was sequenced and compared to several enzootic and epizootic VEEV strains. Sequencing analysis of MX01-32 found 23 amino acid substitutions when compared to avirulent subtype IE VEEV 68U201. Six of the 23 substitutions were found in epizootic strains of VEEV, suggesting one or more of these substitutions may be responsible for the acquisition of equine virulence. In summary, the findings of this study reveal important information on the differential impact of virulent and avirulent strains of subtype IE VEEV on the equine immune system, providing new insight for the future development of improved vaccines and diagnostics.

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Transposon-Based Approach For Generating Chimeric Viral Genes.

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Alphaviruses are widely used systems for expression of heterologous genes and development of recombinant vaccines. We developed a new approach which can be applied for insertion of large molecules, such as GFP, into alphavirus-specific proteins. To insert the entire GFP sequence into Sindbis virus (SINV) nsP2, we used Tn-based random insertion approach. We identified two sites in SINV nsP2 that can tolerate presence of the GFP sequence without deleterious effect on virus replication. One of these sites is located in amino terminus of SINV nsP2 after a.a. 8, 9 or 11, and another site is in a short peptide, predicted to form no regular structure, that is located between RNA helicase and proteinase domains. These two sites can be used for cloning the additional sequences without alterations of protein functioning in RNA replication. Viruses with GFP insertions in nsP2 were tested for their replication, stability of insertions and RNA synthesis. GFP was not only stably expressed during virus passaging in tissue culture, but also attenuated virus, and this can be advantageous for development of vaccines for more encephalitogenic alphaviruses.

The developed method of random insertions can be applied 1) for cloning of antigens into the structural and nonstructural proteins of the viruses having RNA-positive genome; 2) for efficient presentation of the antigens and 3) studying the domain structure of the virus-specific proteins.

Airway epithelial cell response to human metapneumovirus infection.

Bao X, Liu T, Spetch L, Kolli, D, Garofalo R.P and Casola A.

Human metapneumovirus (hMPV), first identified from children with respiratory symptoms in 2001, is a significant respiratory pathogen worldwide. Here, we investigated the cellular responses to hMPV in airway epithelial cells, the major target of hMPV infection and also the first defense barrier against respiratory infections. Human alveolar type II-like epithelial cells (A549) were infected with hMPV at multiplicity of infection (MOI) of 3. Cells were harvested at different time post-infection to collect cell supernatants and prepare either total RNA or cytoplasmic and nuclear proteins. Chemokines and cytokines were investigated by ELISA and Bioplex assay, as well as by ribonuclease protection assays (RPA). Our results show that hMPV infection of alveolar epithelial cells induced a significant production of chemokines and cytokines, as well as type I interferons. HMPV-induced chemokine expression required viral replication, since it could not be induced by the addition of UV-inactivated hMPV. When we compared chemokine and cytokines secretion between hMPV and respiratory syncytial virus (RSV), we found that hMPV is a weaker inducer of pro-inflammatory mediators, but a stronger inducer of type I interferons. hMPV-induced secretion of interferon-β was higher than the one induced by RSV infection. Furthermore, hMPB, but not RSV, induced IFN- α secretion in A549 cells. We also found that hMPV infection induced activation of Nuclear Factor (NF)-κB and interferon regulatory factors (IRFs) transcription factors, which have been shown to play a fundamental role in controlling the expression of these chemokines following viral infections. The signaling pathway leading to the expression of immune and antiviral genes in response to hMPV infection, as well as the role of hMPV viral proteins in cellular signaling, are currently being investigated. (Supported by NIH/NIAID T32 AI 07536)

Induced Gene Expression Revealed By High-Density Microarrays in Human Metapnemovirus-Infected Epithelial Cells.

Bao X, Sinha M, Luxon B, Liu T, Spetch L, Garofalo R.P and Casola A

Human metapneumovirus (hMPV) is a recently identified respiratory paramyxovirus. Little is known about the immune and cellular responses to hMPV infection. Here, we infected human alveolar type II-like epithelial cell line (A549) with hMPV at MOI of 1 for various time at 37 °C. RNA from control and infected cells were extracted using RNAqueous®-Midi Kit (Ambion, Austin, TX). Biotinylated cRNA target was then synthesized and probed using GeneChip Human Genome HGU133 plus 2.0 Array containing 47,400 well-characterized human genes (Affymetrix, Santa Clara, CA). The purpose of this study was to 1) investigate the spectrum of chemokines, cytokines and interferons elicited by hMPV infection in airway epithelial cells; 2) identify signaling molecules associated with hMPV infection for further investigation of the intracellular signaling networks controlling the expression of immune mediators. Expression of chemokines, cytokines and signaling molecules, following hMPV infection, was confirmed by real-time PCR, Western blot, or Bioplex aasay. Our results show that hMPV is a strong inducer of both CC and CXC chemokines, as well as cytokines and type I interferons. While the induction of IFN-α was transient, with a peak at 48 h, two other types of IFNs, β and λ, were constantly induced. In term of signaling molecules, we found that various pattern recognition receptors (PRRs) were induced by hMPV infection, including TLR-3 and the RNA helicases RIG-I and MDA-5. The role of RIG-I in mediating hMPV-induced signaling was confirmed by further investigations of other members of our laboratory. RIG-I downstream signaling molecules, such as MAVS, IKKε, and the transcription factors NF-kB and IRFs, were also induced by hMPV infection. (Supported by NIH/NIAID T32 AI 07536)

Host Astrocyte Cells Interactions with Virulent and Avirulent Venezuelan equine encephalitis virus

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The pathogenic arthropod-borne alphavirus, Venezuelan equine encephalitis virus (VEEV) is a zoonotic pathogen that is endemic in northern South America, Trinidad, Central America, Mexico, and Florida and which has been responsible for significant morbidity and mortality during epizootic outbreaks in both equines and humans. VEEV was weaponized by the United States and the former Soviet Union in the 1950's and 1960's and other countries have been or are suspected to have developed VEEV as a potential bioterrorist agent. The overall mortality rate in humans infected with VEEV is 0.5–1%, with up to 20% in patients who develop encephalitis. The live attenuated TC-83 vaccine strain of VEEV was developed in the 1960s using a traditional approach of serial passaging in tissue culture of the virulent Trinidad donkey (TrD) strain. This vaccine causes adverse, sometimes severe reactions in human vaccines. The TC-83 vaccine has also been used for immunization of equines and can lead to febrile clinical illness with viremia levels that are potentially sufficient to infect mosquitoes and initiate a transmission cycle. These findings indicate significant residual virulence of TC-83 strain in both humans and horses. The TC-83 strain also retains residual murine virulence and is lethal for suckling mice after intracerebral (i.c.) or subcutaneous (s.c.) inoculation. The need to develop a more effective vaccine remains. Inflammation of CNS tissues is an important pathological feature of viral infection and is characterized by glial activation (microglial and astrocytic cells), leucocyte recruitment, and the expression of proinflammatory mediators such as cytokines and chemokines. Many receptors and chemokines are expressed in astrocytes, suggesting that chemokines may serve as communication signals between microglia and astrocytes. Primary cultures of adult mouse astrocytes were inoculated with one VEEV strain (TC-83) or with the recombinant vaccine virus and the cytokine/chemokine profiles were determined using the BioRad Bio-Plex system. Large fold differences were observed as compared to mock inoculated with both strains. Briefly significant large fold increases were observed in levels of TNFalpha, RANTES and eotaxin. Greater than two fold increases, considered biologically relevant, changes were observed in levels of IL-6, IL-10 and MIP-1beta. Differences in cytokine/chemokine profiles between the two VEEV strains may be informative in the developing new more effective vaccines capable of eliciting a protective immune response against VEEV infection.

Screening complete molecular libraries of Schu S4 components for T cell selected immunogens, and protection selected protective-antigens.

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Our objective is to obtain new molecular vaccine candidates for F. tularensis. This is being done by genomic-scale molecular synthesis of Schu S4 components followed by their functional screening. A murine pulmonary model for primary infection with Biovar A virulent F. tularensis has been developed. This provides a relevant model of disease for us to carry out a two-pronged approach. In one project we are synthetically generating all the proteins of tularemia and screening these in T-cell assays. Those that are immunogenic will be tested as vaccines. In our second project we are screening all protein-coding sequences directly in challenge-protection assays. Both projects rely on the production of a library of open-reading frames corresponding to the complete genome, in optimized expression systems. In particular, we are building all coding sequences of Schu S4 as 3,484 synthetic gene fragments, and manipulating them into two different high throughput expression systems. Each will be placed in efficient in vitro expression constructs optimized for high-specificity yields and easy purification. The in vitro translated products will matrix-arrayed into designated pools for stimulation of T cells from tularemia-infected animals. The ORFs will also be placed in linear expression constructs for direct animal immunization. An advanced version of expression library immunization (ELI) will be conducted to identify the genome's protective antigens. The output from these screens will be compared, affording an assessment of their utility in applying to vaccine discovery for other intracellular bacteria.

Development of an OspC-based tetravalent, recombinant, chimeric vaccinogen that elicits bactericidal antibody against diverse Lyme disease spirochete strains

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Lyme disease is caused by the spirochetes Borrelia burgdorferi, B. garinii and B. afzelii and is transmitted to humans by infected Ixodes ticks. It is the most common vector-borne disease in the North America and Europe, and remains an emerging disease of considerable importance due to its potentially serious cardiac, neurological, and arthritic sequelae. There is no commercially available human Lyme disease vaccine, despite panel recommendations and pharmacoeconomic studies indicating a clear need. The outer surface protein C (OspC) of the Lyme disease spirochetes is expressed during tick feeding and early mammalian infection, making it an attractive candidate vaccinogen; however, significant sequence heterogeneity has been an impediment to its development. There are approximately 28 dominant phyletic clusters or types of OspC associated with human infection. Sequence identity within types is generally >97%, while between types it is 55-80%. OspC immunization is protective, but only against strains bearing similar or identical OspC protein. To better understand the antigenic characteristics of OspC, linear epitopes presented during murine and human infection were mapped, and primarily localized to the loop 5 and alpha helix 5 regions. Using that information, a recombinant, chimeric, tetravalent vaccine was constructed using epitope-containing regions from four OspC types (A, B, K, and D) associated with invasive human infection. The chimeric construct was highly immunogenic, and elicited an IgG response against all of the included type-specific epitopes. These antibodies bound the native epitope on the Borrelia cell surface, and mediated complement-dependent bacterial killing against isolates bearing each of the included OspC types. Initial variability between epitope-specific titers was equalized by modification of the chimeric vaccinogen construction. These modifications also altered the epitope-specific isotype profiles, an effect that may be advantageous in directing the immune response to elicit the greatest in vivo protection. The results of this initial vaccine construct suggest that an effective and broadly protective polyvalent OspC vaccine can be produced as a recombinant, chimeric protein. This is the first report of a rationally designed, epitope-based polyvalent chimeric Lyme disease vaccinogen, and is the first OspC-based vaccinogen shown to elicit bactericidal antibodies against diverse isolates expressing different OspC types.

Expression of lipidated Coxiella burnetii antigens: A new vaccine approach.

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Coxiella burnetii, the causative agent of Q-fever in humans and domestic animals, is an obligate intracellular pathogen that replicates in the acidic phagolysosomal compartments of macrophages. C. burnetii is considered a bio-threat agent and hence there is a need for developing novel vaccine approaches to stimulate appropriate innate and cell mediated immune response required to control intracellular replication of C. burnetii. We modified an immunodominant B cell antigen of C. burnetii to facilitate interactions with Toll-like receptor-2 (TLR-2) in order to induce a Th1 type T cell response against C. burnetii. We cloned the sequence corresponding to the first 24 amino acids of the outer surface protein A (OspA) of Borrelia burgdorferi upstream of the mature sequence of Coxiella burnetii macrophage infectivity potentiator (CbMip). This will convert the soluble form of CbMip (without the leader sequence) to a lipoprotein due to the presence of OspA lipidation tag. We further cloned a borrelial promoter PflgB upstream of ospA-cbmip and a sequence corresponding to 6X-Histidine tag at its 3'end. The sequence corresponding to PflaB-ospA-cbmip-6XHis was cloned into pBSV2 to generate a plasmid designated pLE26 which will facilitate expression of CbMip under the control of a borrelial promoter as a lipidated protein in a non-infectious strain of B. burgdorferi. The expression of modified CbMip in B. burgdorferi will facilitate post-translational modification of cysteine at position 17 of the OspA lipidation tag. Moreover, the presence of the 6X-Hisidine tag at the C-terminus of modified CbMip will facilitate purification of lipidated CbMip from other borrelial proteins. Immunoblot analysis of E. coli Top 10 cells carrying pLE26 with the anti-CbMip and anti-His tag mabs revealed expression of CbMip with intact 6X Histidine tags. Consistent with previous observations, we had expression of 24 kDA, 15.5 and 15 kDA CbMip analogs expressed indicating that the expression profile of CbMip analogs under the control of a borrelial promoter was similar to the expression under the control of its native promoter or other heterologous promoters. Furthermore, analysis of detergent soluble fractions of E. coli expressing pLE26 revealed partitioning of 24kDA CbMip analog to the detergent phase while the 15.5 and 15 kDA analogs partitioned to the aqueous phase. Flow cytometric analysis of E. coli expressing pLE26 with anti- CbMip monospecific sera reveled expression of CbMip on the surface indicating that the fusion of OspA lipidation signal allows for localization of CbMip with the membranes. These foregoing studies indicate that pLE26 has all the characteristics that will facilitate expression of the 24 kDa CbMip as a lipoprotein in B. burgdorferi. We are in the process of determining the levels of cytokine and antibody response in BALB/c mice with the purified LipCbMip-His antigen and determine if the nature of the immune response induced will be capable of providing protection against challenge with virulent C. burnetii. These studies will help to develop novel vaccine design strategies for the prevention of Q fever.

Differential Interaction Of Dendritic Cells With Rickettsia: Impact On Host Resistance And Susceptibility To Murine Spotted Fever Rickettsiosis

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Spotted fever group rickettsioses could develop to be severe or even life-threatening diseases. To the same inoculum of Rickettsia conorii, C57BL/6 (B6) mice are resistant while C3H/HeN (C3H) mice are highly susceptible to fatal disease. In the present study, we examined the role of dendritic cells (DCs) in resistance or susceptibility to severe and fatal rickettsiosis. We found that bone marrow-derived dendritic cells (BMDCs) from resistant mice harbored a greater quantity of intracellular R. conorii than DCs from susceptible mice at early time points after infection. While rickettsiae were detected only within the cytosol of target endothelial cells, rickettsiae were localized in phagosomes as well as the cytosol of C3H and B6 DCs. Rickettsial infection drove DCs from both mouse strains to maturation status with up-regulated expression of CD80, CD86, CD40, MHC class I, and MHC class II comparing to respective untreated DCs. However, DCs from resistant mice infected in vitro with R. conorii exhibited slightly higher expression of OX40L, less increased expression of CD40 and greater IL-12 production than C3H DCs. In vitro DC-T cell co-culture revealed that R. conorii-infected DCs from resistant mice initiated earlier activation of naïve CD4 T cells, more Rickettsia-specific Th1 dominant polarization, less antigen dependent Foxp3+ CD4+T reg cells and a greater expansion of cytotoxic T cells than those from susceptible mice. Overall, these data suggested an important role of DCs initiated suppressive T cell response in determining host susceptibility to rickettsial disease.

Stem loop structure present in the capsid gene of WNV is required for efficient replication of the WNV genome; importance in vaccine generation.

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West Nile virus (WNV) is a member of the genus Flavivirus that includes many important human pathogens such as dengue, yellow fever, and tick-borne encephalitis viruses. Since its introduction into the Western hemisphere, WNV has spread all over the United States and caused hundreds of documented fatal infections. To date there is no approved vaccine to prevent WNV encephalitis, although a promising vaccine candidate consisting of a pseudo-infectious flavivirus (RepliVAX) lacking most of the C gene has been reported recently (Mason et al, Virology, 2006;351:432).
Like other members of the genus, WNV genome is a single stranded RNA of positive polarity approximately 11.2 kb in length that is translated as a single polyprotein that is subsequently cleaved into 10 proteins: 3 structural (C, prM, E) that incorporated in virions and 7 non-structural that essential for replication. It was shown that flavivirus RNA circularizes through pairing of complementary conserved sequences (cyclization sequence or CS) on 5' and 3' end of the genome. Sequences downstream of the CS (encoding C, prM, and E) can be deleted from the genome, producing WNV replicons. Replicons can replicate when introduced in cells but are unable to spread unless trans-complemented with the missing structural proteins. Previously, we have reported that replicons that have complete C coding sequence form larger foci on packaging cells (Fayzulin et al, Virology, 2006;351:196); suggesting that C-encoding sequences might play a role in WNV genome replication.
RNA on the 5' and 3' ends of the flavivirus genome can form stem loop (SL) structures that are likely to function in replication. mFold 3.2 predicts the presence of several stem loop structures on the 5' end of WNV genome, extending from the untranslated region through part of the C gene. We studied the effect of SL5 overlapping codons 35 to 56 of C on replication of WNV replicons and found that SL5 is required for efficient replication of West Nile replicons. Deleting or destabilizing SL5 by silent mutations delayed replication of WNV replicons by several hours whereas deletion of sequences downstream of SL5 had no effect on replication when compared to wt replicons.
Understanding the role of SL5 in efficient WNV genome replication will allow us to improve the RepliVAX vaccine candidates that are under development in our laboratory.

Subversion Of Lung Dendritic Cell Functions By Paramyxoviruses Infection

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Respiratory tract dendritic cells (DCs) act as a portal for virus invasion as well as potent antigen-presenting cells involved in the antiviral host response. At least three subsets of pulmonary DCs have been described in mice: the CD11c+MHCII+ myeloid or conventional DCs (cDCs), CD11cintB220+Ly6C+ plasmacytoid DCs (pDCs), and the recently characterized CD11cintB220+CD49b+ NK interferon-producing DCs (IKDCs). Respiratory syncytial virus (RSV) and human metapneumovirus (hMPV) are paramyxoviruses with worldwide distribution that are the main cause of neumonia and bronchiolitis in young children and elderly people. The immune response induced by these two viruses is not protective and recurrent infections can occur throughout life. How RSV or hMPV infection affect the host immune response is not well understood. Therefore, in this study we evaluate the frequency and functions of lung DCs during RSV and hMPV infection. BALB/c mice were infected with RSV or hMPV, control animals were inoculated with PBS. Subpopulations of lung DCs were determined and isolated by using specific antibodies and analyzed by flow cytometry and cell sorting. IFN- production was determined by ELISA and additional cytokines were determined by Bio-Plex system. Proliferation of T cells activated by cDCs was assessed by thymidine incorporation. Our results show that lung DCs were differentially recruited by RSV and hMPV. cDC were recruited into the lungs of RSV- and hMPV-infected mice in a high frequency (7-fold over control mice) showing a peak between 8-10 days after infection and this recruitment was sustained at least 18 days after infection. IKDC were recruited into the lungs of infected mice in a lower frequency than cDC (2-fold over control) and they returned to basal levels by day 10 after infection. On the other hand, pDC in RSV- and hMPV-infected mice peaked at day 3 and 8 days after infection, respectively. Regarding the effect of these viral infections on the immune activity of lung DCs, we interestingly observed that cDC isolated from RSV-infected mice showed an impaired capacity to present OVA peptide to CD4+ T cells compared to cDC from control mice. Regarding the effect on pDCs, we also observed that lung pDC isolated from either RSV- or hMPV-infected mice showed a reduced capacity to produce IFN- in response to TLR9 agonist or to a second viral infection. In addition, the production of other cytokines including MIP-1 , MCP-1, eotaxin and RANTES was also affected. These findings provide novel evidence that the immune capacity of both cDC and pDC functions are impaired by paramyxoviruses infection having this effect an impact on the innate and adaptive immunity.

Respiratory Syncytial Virus (Rsv) Induces Cellular Oxidative Stress By Down Regulating The Expression Of Antioxidant Enzymes

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Respiratory syncytial virus (RSV) is one of the most important cause of lower respiratory tract infection in infants and young children. Oxidative stress has been shown to play an important role in the pathogenesis of acute and chronic lung inflammatory diseases such as asthma and chronic obstructive pulmonary disease (COPD). We have previously shown that RSV infection induces reactive oxygen species (ROS) in vitro, which are involved in the expression of important proinflammatory chemokines. We have also shown that RSV induces oxidative stress in lungs in vivo, using a mouse model of RSV infection. The mechanism of RSV-induced oxidative injury in the airways, however, was not investigated. Using a proteomics approach to detect changes in protein expression in the airways of infected mice, we identified a number of antioxidant enzymes that were down regulated following RSV infection. To further investigate the mechanism of RSV-induced oxidative injury, we measured expression and activity of superoxide dismutase 1 (SOD 1), SOD 2, SOD 3, catalase, glutathione peroxidase (GPx) and glutathione S-transferase (GST) in A549 cells, a type II-like alveolar epithelial cell line, and in small airway epithelial (SAE) cells, normal human cells derived from terminal bronchioli. RSV infection induced a significant increase of lipid peroxidation products such as malondialdehyde (MDA), 4-hydroxylnonenal (HNE) and F2-8 isoprostanes in infected cells compared to control cells. There was also a significant decrease in the levels of GSH/GSSG ratio in RSV infected cells compared to control cells. RSV infection induced a significant decrease in expression of SOD 1, SOD 3, catalase, GPx and GST with a concomitant increase in the expression of SOD 2. Enzymatic assay results showed that total SOD activity was increased but catalase, GPx and GST activities were decreased. Similar results were also found in SAE cells. These findings suggest that RSV-induced cellular oxidative damage is the result of an imbalance between ROS production and antioxidant cellular defenses. Modulation of oxidative stress represents a novel pharmacological approach to ameliorate RSV-induced acute lung inflammation.

Transcriptional terminations of Rift Valley fever virus

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Rift Valley fever virus (RVFV) (genus Phlebovirus, family Bunyaviridae) has a tripartite negative-strand genome (S, M and L-segment), and causes a mosquito-borne endemic disease in sub-Saharan African countries, as well as major epidemics among humans and livestock. The RVFV S-segment uses an ambi-sense strategy to express N mRNA and NSs mRNA from viral and antiviral-sense S-segments, respectively, while M and L mRNA are synthesized from the viral-sense RNA segments. Although it is known that Phlebovirus mRNAs lack the 3'-end poly(A) sequence, the mechanisms of bunyavirus transcriptional termination are poorly understood. In RVFV, the M mRNA termination site is reported, whereas the termination sites of N, NSs and L mRNA are unknown. In the present study, we first identified the 3'ends of all RVFV mRNAs by using RNase protection assays. Our analyses of viral RNA elements that regulate mRNA termination revealed that both a conserved pentanucleotide sequence, 3'-CGUCG-5', located in the intergenic region of the S-segment and the 5'untranslated region of the M-segment, and homopolymeric tracts of the G and C sequences, which were present upstream of the pentanucleotide sequence, were important for the transcriptional termination of N, NSs and M mRNAs. In addition, we found that L mRNA termination appears to require a stable stem-loop structure, which was present in the 5' untranslated region of the L-segment.

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Critical role of pulmonary macrophages in innate immunity to Respiratory syncytial virus

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Background: Respiratory syncytial virus (RSV) is the leading cause of upper and lower respiratory tract infections worldwide. Studies have shown that exuberant inflammatory events triggered by RSV infection are linked to the pathogenesis of lower respiratory tract infections and are associated with the release of cytokines, chemokines, and other potent inflammatory mediators in the respiratory mucosa. However, the cellular complexity of lung presents a significant challenge for assessing in vivo the specific cell contribution to the inflammatory and immune process. The main obstacle to develop an effective vaccine is our poor understanding of the interrelationship between viral replication in the airways, the mechanisms of protective vs pathogenic host immune responses and the severity of clinical disease. An unexplored facet of RSV vaccinology is the identity and role of antigen-presenting cells (APC) within the respiratory tract. Macrophages, a major cellular defense against early infections in the lung, represent ~ 95% of the cellular component of bronchoalveolar lavage (BAL) fluids in normal lung and an important APC in the lung. For that reason, here we investigated the potential interaction between RSV and macrophages either directly or indirectly and its impact on disease and cytokine response in a mouse model. Methods: Balb/c mice were depleted of macrophages by intra nasal instillation of dichloro methylene bisphosphonate (Cl2MBP) liposomes and after 48 hr were infected with RSV A2 (pfu 1x107). Viral titer was assessed by a plaque assay at day 5 and lung histopathology was determined on day 7 post infection. Cytokine responses were measured in bronchoalveolar lavage (BAL) using luminex-based bioplex system at different time points of RSV infection. Results: Macrophage depletion in mice caused a significant increase in viral replication ($P<0.01$), but no prolonged shedding was observed. Also a significant increase in the inflammation score as revealed by histopathological examination of the lung sections (14 ± 1.54 vs 40 ± 8.8, $P<0.05$). Flow cytometric analysis of BAL and lung revealed that macrophage depletion caused an increase in neutrophil (Gr1hi CD11cneg) and dendritic cell (CD11chiMHCIIhi) infiltration into the airways compared to undepleted mice. Macrophage depleted mice has a profound impairment in the innate immunity as indicated by the decreased levels of interferon (IFN) α (721.4 ± 79.86 vs 60.45 ± 11.55 pg/mL, $P<0.001$) and β (1385 ± 214.5 vs 15.14 ± 7.077, $P<0.001$) in BAL compared to undepleted infected mice. However no change in IFN γ level was observed. Depletion of macrophages causes suppression of the proinflammatory cytokines TNF-α and IL-6, chemokines RANTES and MCP-1, and increased secretion of IL-12 p40, IL-1α, IL-1β, G-CSF and IL-17. Conclusion: This study demonstrates that alveolar macrophages play an important role in immune and inflammatory response following viral infections in the lung.

Flavitrack, An Annotated Database To Compare Flavivirus Sequences

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Many human encephalitic and hemorrhagic diseases, including tick-borne encephalitis (TBE), dengue fever, West Nile and yellow fever, are caused by vector borne flaviviruses. Although a wealth of sequence data for flaviviruses has accumulated over the last two decades that has helped uncover the genome organization and replication mode of these viruses, there are currently no effective antiviral drugs and few vaccines against them. We have used stereophysicochemical variability plots (SVPs) to combine sequence and structural analyses in order to track viral evolution and to determine functional areas of proteins and conserved antigenic sites[1,2]. To further aid in this effort, we have established a database of flavivirus genomes to enable structure based sequence comparison and rapid phylogenetic evaluation of these diverse pathogens. Flavitrack (http://carnot.utmb.edu/flavitrack) contains over 475 complete genomic sequences from about 40 different flaviviruses, as well as related information on known mutations and literature references. In addition, each sequence has been assigned a unique identifier, a "license plate", which summarizes its date and place of isolation, phenotype, and lethality. This enables us to run very large sequence alignments and interpret the data with regard to vector and symptom specificity within viral subclasses and strain evolution.

Primary applications for Flavitrack include sequence retrieval, BLAST comparison of a sequence to others in the database, obtaining sequence alignments, and building cluster or phylogenetic trees. In addition, Flavitrack provides access to our in-house program PCPMer[1,2] to quantitatively evaluate and visualize, using SVPs, areas of the viral proteins affected by mutations. Principal components analysis of aligned sequences, coupled with their license plates, allows another way to visualize the evolution of sequence families and individual sequence groups. Flavitrack offers precalculated multiple sequence alignments integrated with the Jalview multiple alignment editor. The complete sequence alignment of the archived polyproteins shows conserved regions despite considerable diversity. For example, 8 of 12 cysteines (4 of 6 disulfide bonds) in the Envelope protein are absolutely conserved in even distantly related flaviviruses such as dengue, TBE, and the non-vector borne flaviviruses, such as cell fusing agent (CFA), and Tamana bat. Sequence identity between dengue type 1 virus (DV1) and other flaviviruses was 16% (Tamana bat), 23% (Kamiti river), 24% (CFA), 37% (Apoi), 39% (TBE), 44% (yellow fever), 51% West Nile), 68% (DV4), 72% (DV2), and 78% (DV3). Flavitrack will also eventually contain structures or 3-D models for all flavivirus proteins, allowing combined sequence/structure analysis to characterize common B- and T-cell epitopes.

[1]Schein et al., Virol. J. 2005 Apr 21;2(1):40.
[2]Negi et al., J. Mol. Model (Online) 2006 Sep;12(6):921-9.

MCP-1 PRODUCTION by INtestinal Myofibroblasts in response to staphylococcal enterotoxin A: relevence to staphylococcal enterotoxigenic disease.

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Background: Food poisoning due to Staphylococcal enterotoxins A and B (SEA and SEB) affects hundreds of thousands of people annually. SEA and SEB induces massive intestinal cytokine production, which is believed to be the key factor in staphylococcal enterotoxin (SE) enteropathy. MHC class II molecules are the major receptors for SEs. We recently demonstrated that normal human intestinal myofibroblasts (IMFs), which are located just beneath the epithelium, express MHC class II molecules. We hypothesized that IMFs are among the first cells to respond to SEs and contribute to the massive cytokine production associated with SE pathogenesis. Methods: We analyze the pattern and temporal sequence of cytokine/chemokines induced by SEA and SEB in cultured IMFs by using Real-Time PCR, ELISA, and Cytokine Bead Arrays. Results: In this study we demonstrated that primary IMFs bind SEs in a MHC class II-dependent fashion in vitro. We also demonstrated that SEs can cross the epithelium in co-culture of IMFs with an intestinal epithelial cell line, CaCo-2, and bind to the MHC class II molecules on IMFs. IMFs responded to SEA, but not SEB exposure with 3- to 20-fold increases in the production of proinflammatory chemokines (MCP-1, IL-8), cytokines (IL-6), and growth factors (GM-CSF and G-CSF). Their production was inhibited by 80-90% in the presence of anti-MHC class II blocking antibodies. The SEA induction of the proinflammatory mediators by IMFs resulted from the efficient crosslinking of MHC class II molecules, since crosslinking of class II MHC by biotinylated anti-HLA-DR antibodies induced similar cytokine patterns. The studies presented here show that MCP-1 is central to the production of other cytokines elicited by SEA in IMFs, since its neutralization with specific antibodies in the cultures also prevented the expression of IL-6 and IL-8. Conclusion: MCP-1 may play a leading role in initiation of inflammatory injury associated with staphylococcocal enterotoxigenic disease.

PD-L1/PD-1-Mediated Interaction Between Normal and Ulcerative Colitis-Derived Colonic Myofibroblasts and T cells

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BACKGROUND: Current theories regarding the pathogenesis of ulcerative colitis (UC) suggest that UC represents a disruption of tolerance to intestinal microflora, leading to dysregulation of mucosal CD4+T cell responses and chronic inflammation. Recent evidence indicates that regulation of this response involves, at least in part, PD-1/PD-L1 negative co-stimulator interactions between T cells and antigen presenting cells (APC). Moreover, strong upregulation of these molecules has been observed in the inflamed colon of with inflammatory bowel disease patients. However, the nature and spectrum of PD-L1-expressing APCs in normal and inflamed colon are poorly characterized. We recently reported that normal human colonic myofibroblasts (CMFs), which are located in the lamina propria just beneath the epithelial layer, are novel non professional APCs that constitutively express negative (PD-L1 and PD-L2) and positive (ICOSL and B7-H3) costimulators and can suppress proliferation of CD3-activated CD4+T cells. We thus hypothesize that PD-L1+ CMFs are novel, local negative regulator of T cells in the colon and that abnormalities in CMF-mediated immune inhibitory signals may contribute to UC pathogenesis. METHODS: We have analyzed expression of PD-L1 by using FACS analysis of acutely isolated and cultured CMFs and evaluated PD-L1 involvement in CMF mediated suppression of CD3-activated CD4+ T cells using lymphoproliferation assays and IL-2 ELISA. A combination of real time RT-PCR, Western Blot and FACS analysis was used to compare PD-L1 expression by CMFs from UC patients versus the control group. RESULTS: We demonstrated that ~ 15% of freshly isolated lamina propria mononuclear cells from normal mucosa constitutively express PD-L1. CMFs represented ~ 80 % of this PD-L1-expressing population. In vitro functional analyses demonstrated that CMFs suppress proliferation of CD3-activated CD45RA+CD4+T cells via cell-contact-dependent mechanisms. Blockade of PD-L1 on CMFs enhanced T cell proliferation and IL-2 production. When compared to normal colon, a significant increase in PD-L1, but not PD-L2, was observed on CMFs isolated from UC patients. CONCLUSIONS: These results are consistent with the hypothesis that CMFs expression of negative co-stimulators may favor their suppressive function on the response of activated CD4+ T cells in the colonic mucosa. Our data also indicate that PD-L1 contributes to the CMF-mediated suppressive capacity and suggest that abnormalities in expression of this molecule on CMFs may play a role in UC associated immunopathogenesis. Supported by NIDDK, GRIP and the Gulf Coast DDC, James W McLaughlin Endowment.

Use of preventive vaccination with Dryvax® to establish proof-of-concept for a novel animalmodel of human hemorrhagic smallpox.

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Smallpox was eradicated over 25 years ago, and yet remains a global public health threat, and a feared biowarfare and bioterrorism agent. The only countermeasure currently available against the disease is preventive vaccination with the vaccinia (smallpox) live virus vaccine. Recently, our research group developed an animal model of human hemorrhagic smallpox using monkeypox virus infection in ground squirrels. This model was developed to screen candidate antivirals and new preventive vaccines against smallpox. Proof of principle was established demonstrating efficacy of Dryvax® against lethal monkeypox virus challenge in our experimental animal model. Two groups of animals were used in our study: a vaccine group and a placebo group. All animals had their back shaved over an area covering approximately 1 square inch. Animals in the vaccine arm were then vaccinated by scarification with 106 pock forming unit of calf-lymph derived vaccinia (smallpox) live virus vaccine, NY State Department of Health strain (Dryvax®) with a bifurcated needle dipped into the reconstituted suspension, according to the vaccination protocol. Normal saline was used for the animals in the placebo group. All animals were monitored daily for 5 weeks to detect potential adverse events. After five weeks, all animals were challenged with a lethal dose of monkeypox virus inoculum, equivalent to 200 LD50, and observed each day for signs of illness. Morbidity, mortality, and weights were recorded daily. On day 7 p.i., animals were sacrificed to collect blood and tissue samples for analysis. Our findings show that the animals in the vaccine group did not develop any clinical disease evocative of monkeypox, while all of the animals in the placebo group became ill and showed typical severe monkeypox disease. One animal in the vaccine group died of sudden death, but cause of death remained undetermined. In contrast, eighty percent (80%) of the animals in the placebo group died, and the remaining 20% were severely ill when sacrificed. Clinical laboratory values remained in the normal range for the vaccinated animals, while the animals in the placebo group had elevated transaminases, leukocytosis, and prolonged clotting times. Microscopically, the animals in the vaccine group had a normal appearance, while those in the placebo group showed typical monkeypox-related pathologic changes in their liver, lung, and spleen sections. No virus was detected in any blood or tissue sample collected from the animals in the vaccine group; in contrast, all animals in the placebo group had high virus titers in their blood, liver, spleen, and lung samples. Tissue sections from the animals in the vaccine group were negative when tested for viral antigen by immunohistochemistry, while those from the animals in the placebo group showed positive staining in the lymph nodes, thymus, splee, lung, bronchial epithelium, trachea, and liver. The pathophysiology of monkeypox virus infection in ground squirrels, and its prevention by immunization with licensed smallpox vaccine, establishes this small animal model as appropriate for testing preventive and therapeutic strategies for the most lethal form of smallpox, hemorrhagic smallpox.

Characterization of Pulmonary Dendritic Cells from Vaccinated, Mycobacterium bovis-infected and Non-vaccinated, Mycobacterium bovis-infected Cattle

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The lung, though exceptionally adapted for efficient gas exchange, is a vulnerable interface between the environment and the internal tissues and organs of the host. A population of dendritic cells (DCs) are sentinels of the mucosal surface of the airway that engage primarily in immune surveillance, and antigen acquisition in the local environment for subsequent presentation to T cells in the regional lymph node. To date, there are no reports that have characterized the biology of specific DC subsets in lung and mediastinal lymph nodes of Mycobacterium bovis (M. bovis)-infected cattle. Therefore, we obtained postmortem samples of lung and lymph node from animals at 90 days for in situ analysis, either vaccinated or non-vaccinated, M.bovis-infected cattle. We collected granulomatous tissue from lung and mediastinal lymph nodes (MLN) of vaccinated and non-vaccinated cattle following infection with M. bovis and identified DC populations via FACs analysis and confocal microscopy. Interestingly, DCs isolated from the lung and MLN of these treatment groups expressed different combinations of various DC markers examined, e.g., MHCII, DEC-205, CD40, CD80, CD86. The pattern of expression of these DC markers also indicates that more than one subset of DC exists in the MLN and lung. Therefore, through the production of cytokines and ability to elicit a robust cell-mediated immune response, the various DC subsets isolated from vaccinated and non-vaccinated cattle are likely to dictate the nature of M. bovis infection in cattle.

Alphavirus-based vaccines against Rift Valley Hemorrhagic Fever Virus

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Rift Valley fever virus (RVFV) is a representative member of the Bunyaviridae family that is in continuous circulation in the livestock-raising regions of Africa. The introduction of sheep and cattle, which are not native to this continent, into intensive farming, has resulted in the emergence of massive mosquito-borne epidemics. Death rates are very high for domestic animals. Moreover, the fatality rate for fetuses in pregnant livestock is 100%, and the fatality rate for newborn lambs is 90%. The virus has spread to new geographic areas, as evidenced by an extensive epidemic in Egypt in 1977. Most recently, RVFV caused a massive epidemic in sub-Saharan Africa in 1997-98 and spread across the Red Sea to Saudi Arabia and Yemen, causing devastating disease outbreaks in sheep and cattle. RVFV is also a significant human pathogen with an approximate 1% mortality. There is no treatment for humans or vaccine for the livestock that are such important amplifiers of the virus. Our goal is to create a new type of preventive recombinant vaccine against RVFV infection. This vaccine will combine the safety of inactivated and subunit vaccines and the efficacy of live attenuated vaccines and will be based on Sindbis and Venezuelan equine encephalitis virus replicons and viruses expressing structural proteins of RVFV. They will efficiently protect against RVFV infection, and it will be possible to manufacture them on an industrial scale.
We are developing i) the optimal strategy for expression of structural proteins of RVFV by recombinant Sindbis and VEE viruses, ii) the system for delivery of recombinant genomes into antigen-presenting cells and optimal presentation of the RVFV-specific antigens, and iii) the manufacturing procedure for the large-scale production of the recombinant viruses. We believe that final constructs and procedures will be a basis for an alternative strategy of rapid response to emerging viral infections that will require development of vaccines and their large-scale production for immediate use.
Vaccine candidates that protect mice efficiently against the lethal RVFV infection will be presented.

Impact of selective lymphoid deficiencies on encephalitis and virus persistence in the murine brain (VEEV)

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ABSTRACT

Gamma-delta T cells displaying a surface phenotype consistent with memory cells can consistently be found in the central nervous system (CNS), but their role in protection against invading viruses and other microorganisms is not understood. We show here a definitive role for these cells in clearance of a pathogenic arthropod-borne alphavirus, Venezuelan equine encephalitis virus (VEEV), from the CNS in mice previously immunized with a chimeric, live-attenuated alphavirus. This study demonstrates a strict requirement for . TCR-bearing T cells but not for TCR-bearing T cells in protection from lethal encephalitis in response to a recombinant alphavirus vaccine. A significant finding is the observation that T cells are critical for complete viral clearance of the VEEV challenge strain from the brain of vaccinated mice after experimental exposure. In asymptomatic mice lacking T cell function, we consistently isolated persistent viral variants from the brains, reflecting the incomplete clearance of VEEV or alternatively, the selection for low virulence VEEV mutants.

PRINCIPAL INVESTIGATOR / STAFF

Interferons: Natural Defense Against Anthrax And Other Bacterial Infections In Vivo

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Background: Interferons (IFN) are believed to play a role in innate immunity during many viral, bacterial, and protozoal infections. During bacterial infections, production of interferons and protection by interferons has been reported for streptococci, staphylococci, Haemophilus, Shigella, tubercle bacilli, Pseudomonas, and Bacillus anthracis, in vitro and in vivo. In vitro IFN is reported to activate and protect macrophages against anthrax infection. We addressed the hypothesis that, in vivo, interferon also plays a defensive role against inhalation anthrax. We studied the protective effect of interferon and an interferon inducer during experimental anthrax in a murine model. In addition, we studied infection of IFN type I receptor KO mice (IFNAR KO).
Experimental Approach: Adult Swiss-Webster mice were pretreated with either recombinant murine interferon β (rMuIFNβ) injected by the i.p. route or with the double-stranded RNA IFN inducer Poly I:CLC injected i.m. and/or i.n. 24 hr prior to intranasal challenge with Bacillus anthracis Ames spores.
Results: Mice pre-treated with Poly I:CLC were more resistant to progression of lethal infection with B. anthracis than control animals, as shown by significant delay in time to death. To determine whether the protective action of the inducer was due to IFN, other groups of mice were treated with rMuIFNβ or rMuIFNγ prior to nasal instillation of anthrax spores. Protection occurred with the type 1 IFN rMuIFNβ, but, surprisingly, not with type 2 rMuIFNγ. These results suggested that Poly I:CLC conferred protection by inducing type 1 IFN. No synergy or additive effects were observed by combining Poly I:CLC and rMuIFNγ. The results suggest that the type 1 IFNs are more important than type 2 IFN in innate immunity to anthrax. Consistent with a natural defensive role for type I IFN is the preliminary finding that IFNAR KO mice develop an accelerated disease compared with wild type mice. Studies of IFN production during infection are ongoing.
Conclusions: It is likely that the IFN type 1 component of the innate immune response is responsible for stimulating a significant portion of the resistance to anthrax in vivo, as it does with other bacteria. The findings also support the possibility of clinical usefulness.

Genomes to Vaccines.
A high throughput approach to subunit vaccine discovery.

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We have developed a genome wide high throughput screening approach for subunit vaccine discovery. It is based on several novel technologies improving gene assembling, expression, genetic immunization and screening. SynBuild-Hi is our new gene design program. The program extracts all protein-encoding sequences from an entire pathogen genome; next, it recodes each gene so as to facilitate production and generate high levels of normalized expression. Once genes are recoded, SynBuild-Hi designs sets of overlapping oligonucleoties for synthetic gene assembly. Using our unique, high fidelity, cost, time and labor efficient block-building approach all genes are assembled with our linear expression element technology into highly potent and immunogenic expression cassettes and used for genetic immunization. Expression Library Immunization is a unique animal immunization and challenge scheme, which relies on protection as a screening readout. It allows quick and accurate evaluation of thousands antigens for their ability to raise protective immune respond using relatively small number of animals. The approach has been successfully used for identifying vaccine candidates for several bacterial, viral, and fungal pathogens. The sets of previously unknown protective antigens have been identified, confirmed and advanced into validation studies. We are continuing to perform genome-wide screens and expand our database of protective antigens. We believe it will allow us to delineate a pattern and develop highly efficient algorithms for prediction protective vaccine candidates.

Antibody-Mediated Protection against Genital Herpes Simplex Virus type 2 Infection in Mice by Fc gamma Receptor -Dependent and -Independent Mechanisms

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The mechanisms by which HSV-specific IgG antibodies protect against HSV disease are not well understood. The requirement for Ab interactions involving Fc receptors (Fc R) or virus neutralization in protection was examined in a murine model of genital HSV-2 infection. IgG antibodies isolated from the serum of HSV-immune mice and HSV glycoprotein D (gD) -specific mAb both possessed neutralizing activity and conferred protection against HSV-2 when administered prior to genital inoculation; however, the mechanisms involved in protection differed. Polyclonal HSV-specific serum IgG was approximately 50-fold less efficient at virus neutralization compared to the gD-specific mAb and administration of polyclonal Ab protected normal mice against HSV disease but not mice lacking the gamma chain subunit utilized in Fc RI, Fc RIII, Fc RIV, and FcepsilonRI receptors. Additionally, this protection was diminished in mice depleted of Gr-1+ immune cell populations known to express Fc R. By contrast, passive administration of IgG1, IgG2a, or IgG2b switch variants of the gD-specific mAb did not prevent initial infection of the genital tract but resulted in lower virus loads in the vaginal epithelium and provided significant protection against acute infection of the sensory ganglia and disease independently of host expression of the Fc R. Together, these data suggest that Ab exhibiting efficient virus neutralization activity may provide protection independent of biological activities associated with the Fc region. However, less efficient neutralizing Ab may protect via mechanisms requiring antibody-Fc R interactions. These data suggest that measurement of HSV neutralization activity in Ab elicited by candidate vaccines may not be a sufficient indicator of efficacy.

Aerosol Infection of BALB/c Mice with Brucella melitensis and Brucella abortus and an Evaluation of Protective Efficacy Against Challenge.

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Brucellosis is a zoonotic disease of worldwide distribution that can be transmitted via intentional or accidental aerosol exposure. Development of improved vaccine strains against Brucella species for use in animals as well as in humans, must consider the possibility of challenge infection via aerosol. The differences in immune response resulting from such exposure needs to be evaluated to properly determine vaccine efficacy. In this study, we have employed the use of a Madison aerosol chamber to infect deep lung tissue of mice to elicit systemic infections with either B. abortus or B. melitensis at varying doses. The results reveal that B. abortus causes a chronic infection of lung tissue in BALB/c mice and peripheral organs at low doses. In contrast, B. melitensis infection diminishes at a more rapid rate and requires higher infectious doses to obtain infection rates between animals similar to B. abortus. In either case persistence of the organism is prolonged compared to other routes of exposure and it is hypothesized that the lung may serve as a source of chronic infection that would seriously exacerbate human disease due to the absence of the clearing mechanisms documented in the murine model. However, despite these concerns unmarked deletion mutants BA∆asp24 and BM∆asp24 consistently confer superior protection in mice against homologous and heterologous aerosol challenge infection, and should be considered viable candidates as vaccine strains against brucellosis.

Essential Role Of RIG-I And MAVS In Human Metapneumovirus-Induced Cellular Signaling.

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Human metapneumovirus (hMPV) is a recently identified pathogen responsible for a significant portion of respiratory tract infections in young children, elderly, and immunocompromised patients, causing bronchiolitis, croup, asthma exacerbation, and even pneumonia. Due to its clinical prevalence, the need to develop an effective treatment and a safe vaccine for hMPV is very high, in terms of urgency and economical impact. While there is an emerging literature on the clinical and epidemiological reports of hMPV infections, very little is known regarding the cellular signaling elicited by this pathogen in airway epithelial cells, the major target of hMPV infection. In the present study, we show that RIG-I (retinoic acid inducible gene I), an RNA helicase binding double stranded viral RNA, is the main pattern recognition receptor (PRR) leading to interferon regulatory factors (IRF) activation and subsequent expression of important inflammatory and antiviral genes. hMPV infection readily induced the expression of RIG-I, both at the mRNA and protein level in A549 cells, a type II-like alveolar epithelial cell line.. Overexpression of dominant negative RIG-I significantly reduced hMPV-induced Interferon(IFN)-β and RANTES gene transcription, as well as activation of an IRF-driven reporter gene plasmid. SiRNA downregulation of RIG-I expression confirmed the important role of this molecule in hMPV-induced cellular responses. MAVS (mitochondrial antiviral signaling protein), a CARD-domain containing protein, was recently identified as the adaptor protein linking RIG-I to activation of downstream molecules such as IKKε and TBK-1, which are involved in IRF phosporylation. hMPV infection induces the mitochondrial expression of MAVS and overexpression of a protein lacking the N-terminal CARD domain significantly reduced IFN-β , RANTES and ISRE-driven promoter activation in response to hMPV infection. Together these results demonstrate that the RIG-I-MAVS signaling pathway plays a fundamental role in the initiation of epithelial cell responses leading to the production of important immune and antiviral molecules involved in the innate immune response to hMPV infection.

Interpretation of Information About Vaccines on the Internet

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Background: Most Internet users find health information through search engines. However, these list both good sites and misinformation sites. The National Network for Immunization Information (NNii) was created to provide science-based information about vaccines. NNii Web sites peer-reviewed content includes essays about immunization issues and synopses of articles from the refereed literature.
Objective: Learn how visitors to www.immunizationinfo.org locate the information they seek and how they establish whether the information is reliable or not.
Design/Methods: Visitors were tracked utilizing advanced Web analytics software (urchin.com) for the period 11/01/2004 - 10/31/2005. Click tracking was performed for the 3-month period 9/1 - 11/30/2005 (opentracker.net). Referral sources were identified and search engine queries and destinations were evaluated for some of the most commonly-sought information.
Results: For the 12-month period, NNii had 8.2 million hits, 120,453 visitors, 146,953 sessions and 494,115 page views. For the 3-month tracking period, 52% of 47,366 visitors came from a search engine, 26% by referral from other Web sites, and 22% were nonreferral visitors (repeat visitors who came by bookmark or typing the address).
Vaccines and the Diseases they Prevent is the most visited section, accessed by both nonreferral visitors and search engine. Essays such as Vaccines and Autism and Mercury and Vaccines were found by search engines 1,078 of 3,131 and 2,658 of 3,607 sessions, respectively.
In contrast, essays about evaluating online information were accessed mostly by visitors scanning related articles to that which they initially sought. For example, only 44 of 6,470 sessions for Vaccine Misinformation (the most-read article for 18 consecutive months) came from search engines (1,217 came through links from other sites). Similarly, Evaluating Information about Vaccines on the Web was found by search engine 89 of 5,077 times (894 times by links from other sites). Thus, readers of these essays located them by related topics links on other articles, either at NNii or elsewhere.
Conclusions: NNii visitors not only sought information about vaccines but also to understand how to evaluate the information that they found. In countering misinformation, providing guidance about assessing the reliability of health information may be as important as the information itself.

Subepithelial Myofibroblasts are Novel Non-Professional Antigen Presenting Cells in the Human Colonic Mucosa

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The human gastrointestinal mucosa is exposed to a diverse normal microflora and dietary antigens, and is a common site of entry for pathogens. The mucosal immune system must respond to these diverse signals with either the initiation of immunity or tolerance. Antigen presenting cells (APCs) are important accessory cells that modulate T cell responses which initiate and maintain adaptive immunity. The ability of APCs to communicate with CD4+ T cells is largely dependent on the expression of class II MHC molecules by the APCs. Using immunohistochemistry, confocal microscopy, and flow cytometry, we demonstrate that α-smooth muscle actin+, CD90+ subepithelial myofibroblasts (stromal cells) constitutively express class II MHC molecules in normal colonic mucosa and that they are distinct from professional APCs such as macrophages and dendritic cells. Primary isolates of human colonic myofibroblasts (CMFs) cultured in vitro were able to stimulate allogeneic CD4+ T cell proliferation. This process was dependent on class II MHC and CD80/86 co-stimulatory molecule expression by the myofibroblasts. We also demonstrate that CMFs, engineered to express a specific DR4 allele, can process and present human serum albumin (HSA) to an HSA-specific and DR4 allele-restricted T cell hybridoma. These studies characterize a novel cell phenotype which, due to its strategic location and class II MHC expression, may be involved in capture of antigens that cross the epithelial barrier and present them to lamina propria CD4+ T cells. Thus, human colonic myofibroblasts may be important in regulating local immunity in the colon.

Center for Biodefense and Emerging Infectious Diseases and The Western Regional Center of Excellence in Biodefense and Emerging Infectious Diseases Research

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The Center for Biodefense and Emerging Infectious Diseases (CBEID) is a multi-disciplinary umbrella organization that builds on the strengths and work of more than 60 researchers in UTMB's Center for Tropical Diseases and various departments on campus. The CBEID has two main objectives: 1) to reduce the vulnerability of the US and other nations to the use of biological weapons for warfare and terrorism; and 2) to alleviate suffering from emerging and tropical infectious diseases through application of basic, applied, and field research, and through education. Fields of research include basic molecular and structural biology, animal models of infectious disease pathogenesis, and aspects of vaccine and anti-viral drug development and evaluation. The culminating achievement of the CBEID is its selection in 2003 to lead the Region VI Center of Excellence in Biodefense and Emerging Infectious Diseases Research (RCE), now known as the Western RCE (WRCE). In collaboration with over forty other institutions in Texas, New Mexico, Oklahoma, Arkansas and Louisiana, the WRCE program is a response to the need for strong infrastructure and multifaceted research, training and development activities applying the best basic, translational and clinical science to the generation of new diagnostic, therapeutic and vaccine countermeasures in support the NIAID Biodefense Research Agenda. All scientific projects in the WRCE have as their ultimate aim the development of a product through programs of investigator-directed research. Training a new generation of science professionals to perform biodefense research activities is also central to the WRCE's strategic plan. The current scientific program of the WRCE includes:
1) the development of vaccines against brucellosis, tularemia, anthrax, glanders, typhus, Rocky Mountain spotted fever, Rift Valley fever, Ebola, Lassa fever, dengue virus, smallpox, and Venezuelan, eastern, and western equine encephalitis;
2) new therapeutic agents against Bacillus anthracis (including the spore), arenaviruses, filoviruses, alphaviruses, flaviviruses, bunyaviruses, Ebola, SARS coronavirus, and cryptosporidiosis;
3) advanced diagnostic methods for Q fever, typhus, glanders, melioidosis, Category A-C viruses, Norwalk virus, cryptosporidiosis, and botulinum toxin;
4) development of vaccines and antiviral therapies for potential human pandemic strains of influenza; and
5) the development of a small animal model for oral ingestion of ricin toxin.
To assist investigators in their research and product development, the WRCE has developed and maintains comprehensive core facilities that support the WRCE's research and training activities, as well as other investigators in the region conducting NIAID biodefense research. Seven regional cores and two trans-Center scientific cores are available to researchers. They include the Nonhuman Primate Core; Computational Biology Core; Small Animal Core; BSL4 Core; Pathogenesis Expression Core; Law, Policy, and Ethics Core; Center for Ultrastructural Studies of Infectious Pathogens; the trans-Center Nonhuman Primate Aerobiology Core; and the trans-Center Small Molecule Screening Laboratory. Information about these cores and the current WRCE scientific program can be found on the RCE web site at:
http://www.rcebiodefense.org/rce_pub/rce6/rce6pub.htm

Immunogenicity and safety of recombinant Sindbis/Eastern equine encephalitis virus in mice

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Eastern equine encephalitis virus (EEEV) causes severe encephalitis in North America with mortality rates between 30-80% in humans and up to 95% in equines, and survivors of the infection often have severe neurological and physical sequelae. Currently, there are no licensed vaccines for EEEV in humans, and only formalin-inactivated vaccines are available for veterinary use. These vaccines are poorly immunogenic, provide only short-term protection, require frequent boosters for the maintenance of antibody levels, and have the potential to contain live, virulent virus. EEEV is routinely isolated from eastern United States, but EEEV has also been isolated from Michigan, Wisconsin, and Ontario. Recently, there has been an increase of the number of human and equine EEE cases in the United States. Two recombinant Sindbis/EEEV viruses were developed and investigated for their potential use as vaccines. Both chimeric viruses replicated efficiently in cell cultures and were highly attenuated in outbred mice. Vaccinated mice did not develop detectable disease or viremia and elicited high titers of neutralizing antibodies. Upon challenge with a lethal dose of EEEV, the higher dose vaccinated mice (>104PFU/mouse) were completely protected from disease. These findings support the potential use of these recombinant viruses as effective vaccine candidates.

The Effects of Bacillus anthracis Edema Toxin on Murine Macrophage Function

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Background: Bacillus anthracis secretes two proteinacious toxins, lethal toxin (LeTx) and edema toxin (EdTx), which contribute to virulence. LeTx is a Zn++ metalloproteinase that nicks members of the MAPKK signaling proteins. EdTx is a Ca+2, calmodulin-dependent bacterial adenyate cyclase that converts ATP to cAMP. The increase in intracellular cAMP contributes to the gross edema observed in anthrax patients and disrupts the functions of immune cells.
Methods and Results: We investigated the effect of EdTx on macrophage activation by measuring LPS-induced cytokine production and phagocytic activity in RAW 264.7 cells and primary murine peritoneal macrophages. Cells were incubated with LPS and EdTx for 24 h, and then, the cell culture supernatants were assayed for 18 different murine cytokines using the Bio-Plex Mouse 18-Plex Assay (Bio-Rad, Hercules, CA). EdTx inhibited LPS-induced production of TNF-α and IL-12. Alternatively, levels of G-CSF and IL-6 were increased in EdTx-treated primary macrophages. To determine the effects of EdTx on macrophage phagocytic activity, RAW 264.7 cells were incubated with toxin for 1, 3, and 6 h then analyzed with the Vybrant™ Phagocytosis Assay Kit (Molecular Probes). There was no difference in phagocytosis compared to the negative control cells after 1 hour incubation with the toxin. However, at 3 and 6 hour, EdTx was as effective as the positive control, (cytocholasin B, 1 μg/ml) in inhibiting phagocytic activity of the treated macrophages. The effects of the toxin on the ability of macrophages to kill B. anthracis cells were also examined.
Conclusion: B. anthracis EdTx inhibits LPS-induced cytokine production and phagocytic activity of murine macrophages. These data suggest that EdTx, like LeTx, enables the bacteria to evade the host innate immune response.