The Wistar Institute of Anatomy and Biology

Annual Progress Report: 2006 Nonformula Grant

Reporting Period

July 1, 2009 – June 30, 2010

Nonformula Grant Overview

The Wistar Institute of Anatomy and Biology received $4,206,096 in nonformula funds for the grant award period June 1, 2007 through May 31, 2011. Accomplishments for the reporting period are described below.

Research Project: Project Title and Purpose

Development of a Universal Influenza A Virus Vaccine - The purpose of this project is to generate a universal influenza A virus vaccine that induces protective immunity to all influenza A virus strains, including newly evolving pandemic strains. Current vaccines to influenza A virus induce protection mainly through neutralizing antibodies directed against surface proteins (antigens) of influenza virus. Since the viral surface antigens are constantly undergoing mutations, each year new vaccines have to be developed that are tailored to the newly evolved influenza A virus strains. A universal influenza A virus vaccine would work against all strains of the virus and hence reduce the need for annual vaccination campaigns. Furthermore, a universal influenza A virus vaccine would dramatically increase our overall preparedness for the next influenza A virus pandemic.

Anticipated Duration of Project

6/1/2007 - 5/31/2011

Project Overview

Influenza virus vaccines protect through induction of subtype specific antibodies against surface antigens of the virus. These antigens constantly mutate and vaccines thus have to be redesigned and reapplied annually. Vaccines against a new pandemic strain cannot be produced in advance, as the molecular composition of this strain cannot be predicted. The goal of this proposal is to generate a novel universal influenza virus vaccine that induces broad protection against all strains of the virus. We will test 5 hypotheses: 1) $CD8^+$ T cells to conserved internal proteins, such as the nucleoprotein (NP) and the matrix (M) protein, and antibodies to the ectodomain of the matrix 2 protein (M2e) of influenza A virus protect against diverse strains of the virus. 2) $CD8^+$ T cells and M2e-specific antibodies act synergistically and vaccines that induce both responses provide superior protection than vaccines that induce only one type of response. (3) Inactivated vaccines and infections with influenza A virus do not induce sufficiently potent cross-reactive immune responses to protect against a heterotypic infection. 4) Novel subunit

vaccines based on adenovirus vectors can induce protective cross-reactive T cell responses; and 5) adenovirus vectors expressing M2e can induce protective antibodies to M2e; such responses may be enhanced by a prime-boost regimen with M2e peptides. The proposal has 4 specific aims. We will develop vaccines based on replication-defective chimpanzee adenovirus vectors expressing NP, M or M2e and M2e peptides. Viral vector and peptide vaccines will be tested individually and then in combination for their ability to induce cross-reactive immune responses in aim 1 and for protection against challenge with different strains of influenza A virus in mice and ferrets in aim 2. We will test human subjects for cross-reactive immunity to influenza A virus strains in aim 3 to provide baseline data for future clinical trials with a universal influenza A virus vaccine. Cohorts will include subjects that are highly susceptible to influenza virus, that have been exposed to avian strains, and that are acutely infected or convalescent. Sera from volunteers will be tested for neutralizing antibodies and for antibodies to M2e. Peripheral blood mononuclear cells will be tested for T cells to NP and M. The analyses will show to what degree the magnitude and diversity of neutralizing antibodies to different strains of influenza A virus correlate with titers of antibodies to M2e and T cells to NP and M. Analyzing antibody titers to H5 and H7 will show if cohorts with reported exposure to birds have an increased prevalence of such antibodies. We will develop a cocktail of human monoclonal antibodies to M2e for treatment of acute influenza A virus infections in aim 4.

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Other Participating Researchers

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Michael R. Betts, PhD, Sherrill Davison, VMD, Sarah J. Ratcliffe, PhD, Guido Silvestri, PhD - employed by University of Pennsylvania
Susan Coffin, MD, MPH - employed by Children's Hospital of Philadelphia
Caroline C. Johnson, MD - employed by City of Philadelphia
Laszlo Otvos, PhD - employed by Temple University

Expected Research Outcomes and Benefits

We plan to develop a novel universal vaccine against influenza A viruses. A universal vaccine has to first show efficacy in animals and then safety and efficacy in humans. Once it is marketed, this novel vaccine will benefit all Pennsylvanians (and others) by reducing their vulnerability to influenza A virus infections including those caused by emerging pandemic strains. It will eliminate the routine need for annual vaccination against new variants of influenza A viruses and

it will remove the uncertainties associated with the selection of strains of influenza A virus that are incorporated each year into the vaccine. If the universal vaccine shows the expected efficacy in animals and humans, we would aim to license the technology to a Pennsylvania-based pharmaceutical company, which would benefit the Pennsylvania economy by providing new jobs and taxable sales revenues for the company. We will also develop a cocktail of monoclonal antibodies for early treatment of influenza A virus infections. This too would benefit Pennsylvanians, especially in the case of a pandemic where anti-viral drug supplies may rapidly become limited or circulating influenza A viruses may become drug-resistant. We will analyze different cohorts of Pennsylvanians for cross-reactive T and B-cell responses to influenza A virus, including those who are highly susceptible to influenza A virus infection, such as young children and those with demonstrated recent infections. This will facilitate the design of future clinical trials by providing baseline data about levels of responses that an efficacious universal vaccine will have to exceed. We will compare these to cohorts that experience frequent exposures to poultry, including those with documented exposures to birds that were infected with avian strains of influenza virus during previous outbreaks in Pennsylvania, to determine the likelihood of cross-over infections. This cohort will also serve as a valuable sentinel cohort in case a new avian strain of influenza A virus that can potentially infect humans reaches the birds population of Pennsylvania.

Summary of Research Completed

Project 1

Generation of AdC68 Vectors

Cloning of adenoviral vectors expressing three versions of M2e fusing with NP, AdC68 vector expressing NP or gD-NP fusion protein, and AdC68 hexon vector expressing M2e has been completed.

Generation of Reassortant Viruses and Other Reagents
The cloning steps for generating A/PR8 H5 and H7 reassortant viruses have been completed and these viruses have been rescued, expanded and transferred to Project 3. We finished generating lentivirus vectors that express the M2 sequences present in the AdC vector. By using lentiviruses, we generated stable cell 293T lines expressing M2e sequences and established cellular ELISAs (enzyme linked immunosorbent assays) to test antibodies to the M2e mutants.

AdC68 Vaccine-Induced Influenza A Virus-Specific Immune Responses and Protection Against Challenge
As reported last year, we primarily focused on AdC68-M2e(3)-NP/AdC6-M2e(3)-NP vectors used alone or in prime boost regimens and obtained the following results:

(1) M2e specific antibody responses. M2e specific antibody responses were measured in both C57BL/6 and ICR mice by a cellular ELISA that was modified from a previously published procedure (Fig.1a, b). We cloned the three full-length M2 sequences, from which the M2e sequences of the vaccines had originated, into lentivirus vectors. Lentivirus was rescued in 293T cells and used to infect HeLa cells to generate stable cell lines that express full length M2. A

control cell line was generated by infection of 293T cells with empty lentivirus. These cell lines were used as immunosorbents in an ELISA. The assay was standardized with a purified antibody that recognizes all of the 3 M2e sequences.

(2) NP-specific T cell responses. After priming with AdC68M2(e)3-NP, all of the mice developed detectable frequencies of NP-specific $CD8^+$ T cells in blood, which gradually declined. A booster immunization with AdC6M2(e)3-NP given 2 months after priming effected an increase in circulating NP-specific $CD8^+$ T cells. Mice were euthanized 4 months after priming; and frequencies of NP-specific $CD8^+$ T cells were determined from lymphocytes isolated from blood, spleens and lungs of individual mice (Fig. 2).

(3) Protective immunity. The AdC vaccine regimen provided protection to mice of different strains, including outbred mice, to challenge with two influenza A virus strains that show sequence differences within M2e. This work has been accepted for publication in Molecular Therapy. In the same manuscript, we describe the immune mechanisms that contribute to protection in vaccinated mice. Our results, based on experiments with genetically engineered mice and adoptive transfer studies, show that antibodies to M2e work in concert with T cells to NP.

(4) Protection induced by hexon-modified AdC vectors. Last year, we reported that the hexon-modified AdC vector expressing M2e can induce a modest reduction in viral titers compared to those in control mice. We then tested the survival of vaccinated mice after challenge to explore whether the reduction in viral titers resulted in a clinical benefit. Although there was a trend towards increased survival in vaccinated mice, this did not reach statistical significance.

Publications:

Zhou D, Wu T-L, Lasaro MO, Latimer BP, Parzych EM, Bian A, Li Y, Li H, Erikson J, Xiang Z, Ertl HC. (2010) A universal influenza A vaccine based on chimpanzee adenovirus expressing matrix 2 ectodomain and nucleoprotein protects mice from lethal influenza virus challenge. Mol Ther (accepted).

Additional Grant Support: None

Project 2

The Generation and Testing of Novel Peptide Vaccines

The influenza virus A matrix protein 2 ectodomain (M2e) sequence has remained highly conserved among various human influenza A strains and is, therefore, a promising target for a protective vaccine. Based on previous work using a synthetic M2e-based multi-antigenic peptide vaccine (Mozdzanowska at al., Vaccine 2003; Virology Journal 2007), we have generated novel peptide vaccines and investigated their efficacy in inducing anti-M2e antibody (Ab) responses and their ability to confer protection against viral challenge. In contrast to predecessor peptide, vaccination with the new peptides induced higher anti-M2e serum Ab titers against peptide and native M2e. We have tested the serum induced by vaccination with the K2 peptide for the ability to bind virus-infected cells using viruses with divergent M2e sequences and have demonstrated that the serum of vaccinated mice was cross-reactive against the influenza virus subtype A/FM

(H1N1) and induced significant protection against viral challenge with influenza A strains X31 (H3N2) and highly pathogenic PR/8 (H1N1).

We have extended these studies to determine the extent of cross-protective immunity with other influenza A viruses. To test for reactivity against the current H1N1 circulating strain reactivity of peptide-induced Abs to the H1N1 associated M2 was assessed by ELISA. For this, the M2e peptide from the current H1N1 was synthesized by Dr. Otvos and used as the target peptide. As shown in Figure 1, there is significant binding. Furthermore, serum induced by vaccination with the K2 peptide bound cells infected with additional variant viruses (Figure 2). In conclusion, our studies show promising results towards the further development of vaccination with M2e as a potential "universal" influenza vaccine.

Publications

Zhou D, Wu T-L, Lasaro MO, Latimer BP, Parzych EM, Bian A, Li Y, Li H, Erikson J, Xiang Z, Ertl HC. (2010) A universal influenza A vaccine based on chimpanzee adenovirus expressing matrix 2 ectodomain and nucleoprotein protects mice from lethal influenza virus challenge. Mol Ther (accepted).

Additional Grant Support

National Institutes of Health, 1 U19AI 083022-01 (Dr. J. Wherry PI.) Shaping Antiviral Immunity by the Inflammatory, Regulatory and Tissue Environment. (3/1/09-2/28/14)

Project 3

Influenza virus-specific T cell responses in different cohorts of human subjects

Healthy Adults: To date, we have analyzed 200+ young healthy adults for influenza virus NP or M specific T cell responses. Of these, ~26% had detectable responses in the initial ELISpot screen. These influenza virus-specific CD8 T cells are being analyzed by flow cytometry, and the functionality of the influenza virus-specific CD8 T cells is being determined by the ability to co-produce multiple cytokines and the expression of inhibitory molecules such as PD-1 and CD57.

Acutely infected adults: We have explored several approaches to obtain samples from adults during acute influenza virus infection. We have recently obtained samples from a prospective, live influenza virus challenge study in humans performed at Oxford University. A major advantage of obtaining samples from this study is that a time course is available; and in our first shipment we received samples from eight subjects on day 0, 7 and 28 following live influenza virus challenge. A total of 30 samples are available from this study, and we are expecting to receive additional frozen cells for analysis. One advantage of a longitudinal series is that, with multiple time points from the same person, the validity of any observations can be verified at additional time points and the kinetics of an immune response can be followed. Interestingly, only ~50% of the subjects analyzed to date had a detectable increase in their influenza virus-specific T cell responses in the PBMC by flow cytometry following infection. We are currently analyzing this issue in more detail with more subjects and also defining the changes in function and differentiation for the subjects that did have a response. Finally, we have a second potential source of samples from acute influenza infected subjects from Dr. Paul Goepfert, an infectious

disease clinician and researcher in Alabama. Dr. Goepfert has already cryopreserved PBMC samples from 3-5 adults acutely infected with influenza virus that he has agreed to send us. Dr. Goepfert is waiting to see if he can acquire a few more samples before shipment. Studies on this cohort are continuing in the final year of this project.

Acutely infected children: We have enrolled 217 pediatric subjects to date. As outlined last year, upon discussion with our scientific advisory board we decided use whole genome transcriptional profiling approaches in these pediatric samples. Since this change was made, we have recruited 64 subjects that are appropriate for microarray analysis. We now have samples for 22 influenza A infections, 15 RSV infections and 15 Rhinovirus infections. Additional samples include 6 metapneumovirus, 2 adenovirus, and 2 parainfluenza virus samples, but the lower number of samples for these infections might preclude detailed analysis. For influenza A and RSV infections, sufficient samples are also available in different age ranges (0-2yr n=8 Flu or 9 RSV; 2-5 yr n=4 Flu, 3 RSV; and >5yr n=10 Flu or 3 RSV) to allow an age dependent comparison of what is likely a primary response to these infections (<2yr old), what is likely a recall response (>5yr old), and an intermediate set to test predictions of the other two data sets. These samples are either frozen awaiting sorting or sorted awaiting RNA isolation. Sorts continue for purification of PBMC subsets for array analysis. In the upcoming year, we plan to try to increase our number of samples for some of these subsets of subjects and also to acquire PBMC from healthy control subjects for comparison. Finally, serological studies have been initiated to verify the status of previous influenza virus exposure in these pediatric subjects and to complement the microarray approaches.

Adults exposed to avian influenza virus strains: Human subjects from the poultry industry in Lancaster County and elsewhere in Pennsylvania where avian influenza outbreaks have occurred in birds have been identified. With the help of our colleagues Sherrill Davidson and Susan Casavant at the University of Pennsylvania School of Veterinary medicine, blood has been obtained from 36 subjects and T cell analysis performed. We have also recently collected samples from nine subjects in this cohort from Delaware. Samples from these subjects were stimulated with influenza virus-derived peptides and virus-specific CD8 and CD4 T cell responses analyzed and compared to responses from a healthy adult population with no obvious exposure to poultry from an ongoing study funded by different mechanisms. Preliminary studies suggested perhaps more consistent, and perhaps higher magnitude, T cell responses in the poultry industry subjects. These subjects have been re-bled for additional analysis and these studies are underway. In addition, serological studies in these subjects have been initiated to investigate the level and diversity of influenza virus-specific antibody responses in this cohort. Finally, subjects in live bird markets in Philadelphia are being identified; and we expect to obtain samples from this cohort in the near future.

Additional Grant Support

National Institutes of Health, 1 U19AI 083022-01 (Dr. J. Wherry, PI.) Shaping Antiviral Immunity by the Inflammatory, Regulatory and Tissue Environment. (3/1/09-2/28/14)

Project 4

Develop human monoclonal antibodies for passive protection against influenza virus infection

During the past year, we have continued to perform experiments aiming to obtain stable IgG-secreting B cells from putative influenza virus-immune individuals, and to optimize methods for their production. We have also had a change in personnel – Christina Mergenthaler who was carrying out these experiments has left the lab to go to graduate school, and has been replaced by Victoria Garcia. We have been purifying either $CD19^{+}IgM^{-}IgA^{-}IgD^{-}$ cells or $CD19^{+}$ cells from peripheral blood samples by fluorescence-activated cell sorting, plated these cells at various densities with CpG, irradiated allogeneic mononuclear cells and Epstein Barr Virus, and then used multiple cycles of expansion and restimulation to develop monoclonal B cell lines. We have used two sources of cells for these experiments; we were able to obtain one sample of blood from a CHOP patient that was confirmed as being influenza virus-positive, and we have also been using cells that were obtained from 11 different Pennsylvania poultry workers. Most of these experiments have given rise to stable cells lines, and we have been concentrating on stabilizing these cells lines and developing frozen stocks that we can maintain and then rethaw to assess specificity. To this end, we have been developing an ELISA-based screening assay that will allow us to determine whether each cell line is secreting an antibody (and determine its isotype), whether the antibody can react with antigens present in the 2008 Fluvirin influenza vaccine (containing H1N1, H3N2 and influenza B subunit components), whether it reacts with other influenza A viral components (such as nucleoprotein or matrix protein, based on reactivity with influenza virus A/PR/8/34), and whether it reacts with a synthetic M2e peptide. Initial studies have identified cell lines that are secreting antibodies, and samples from one of the poultry worker donors appeared in an initial screen to be generating antibodies that can react with Fluvirin antigens (this is currently in the process of being confirmed).

Publications: None

Additional Grant Support
National Institutes of Health, 1 U19AI 083022-01 (Dr. J. Wherry PI.) Shaping Antiviral Immunity by the Inflammatory, Regulatory and Tissue Environment. (3/1/09-2/28/14)

<u>2009/2010 School Year Annual Report on Summer Internship for Cheyney University of Pennsylvania Students at The Wistar Institute, Philadelphia, PA</u>

In the original proposal we requested funding for training of students from Lincoln University. We have not been able to set up a training program with Lincoln in spite of numerous attempts and therefore requested and obtained permission to use this funding for additional training of students from Cheyney.

Summer internships involving Cheyney students started in 2006. They continue to conduct very productive research activities in various laboratories under a wide range of biomedical research expertise that tackle different and interesting topics at The Wistar Institute. This year in particular included very talented students of which some had already completed the requirements for the Bachelor Degree in Chemistry and Biology Programs of the Department of Natural and Applied Sciences at Cheyney University. These students were distributed as follows based on the summer period:

Summer of 2010:

1. *Brian Manson:*
   PI name: Dr. Hildegund Ertl
   Research topic: Construction of HA-pseudotyped lentiviruses to allow for easier testing of neutralizing antibody responses to influenza virus.

2. *Tolani Adebanjo:*
   PI name: Dr. Luis Montaner
   Research topic: Tracking of plasmocytoid dendritic cells

   Mr. Adebanjo has graduated from Cheyney University with a Bachelor degree in Biology (3.91 GPA). He has already secured admission to two graduate programs (an MS in cell and molecular biology, and a Ph.D. in neuroscience). He decided to accept the Ph.D. program at Delaware State University in neuroscience with a full scholarship.

3. *Marium Adeyiga*
   PI name: Dr. John Wherry
   Research topic: ELISA (enzyme linked immunosorbent assay) to detect the antibodies against influenza virus.

4. *Adeyemi Adeyiga*
   PI name: Dr. Andrew Caton
   Research topic: Determination of Murine Antibody Immune Response; Cross-Reactivity to Two H1N1 Influenza Viruses.

5. *Denis Madente*
   PI name: Dr. Jan Erikson
   Research topic: Study vaccine-induced tertiary lymphoid organs within the lung and pulmonary pathology.

   He is an outstanding student who graduated from Cheyney with a bachelor degree in biology (4.0 GPA) and is planning to apply to medical school.

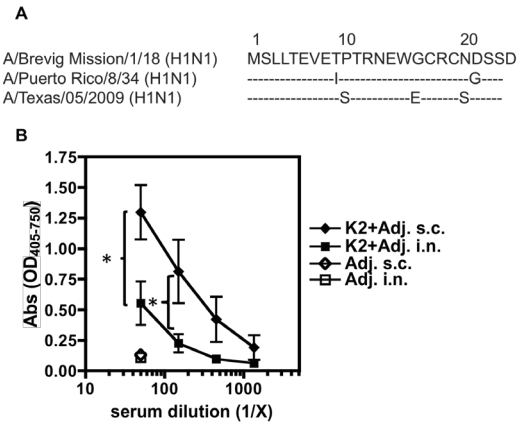

Figure 1: Serum from vaccinated mice bind H1N1 peptide.
A: M2e-sequences from influenza A H1N1 viruses 1918, 1934 and 2009
B: Sera from i.n. or s.c. immunized mice with Adjuvants alone or K2 peptide plus adjuvants were tested in an ELISA for binding to M2e-peptide from H1N1 circulating in 2009.

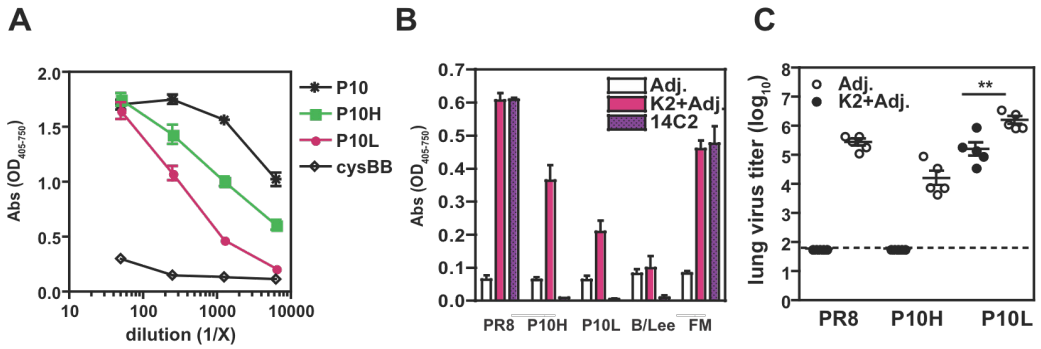

Figure 2: Vaccinated mice are protected against M2e-varient viruses.
A: Serum from n=3 mice after three i.n. immunizations with K2 peptide plus adjuvants were tested for their ability to bind M2e peptide with sequences from PR8 (P10) or mutations at amino acid position 10 from Proline to Histidine (P10H) or Leucine (P10L). As control cysteine-back-bone (cysBB) was used.
B: Pooled sera (1:250 dilution) from mice (n=5) immunized i.n. three times with adjuvants alone or K2 plus adjuvants were assayed in an ELISA for binding to MDCK cells infected with influenza virus A/PR/8, the M2e-escape mutants P10H and P10L, A/FM and B/Lee. Anti-M2e Ab 14C2 (1µg/ml) was used as a control.
C: Mice (n=5/group) were immunized i.n. with K2 plus adjuvants as described. Three weeks after 3rd vaccine administration, mice were challenged with 1000 $TCID_{50}$/50µl influenza virus A/PR/8, P10H and P10L. Five days p.i., viral titers in lungs were determined.