Published in final edited form as:

Vaccine. 2011 October 13; 29(44): 7659–7669. doi:10.1016/j.vaccine.2011.07.140.

The NYCBH vaccinia virus deleted for the innate immune evasion gene, E3L, protects rabbits against lethal challenge by rabbitpox virus

Karen L Denzler^a, Amanda D Rice^b, Amy L MacNeill^c, Nobuko Fukushima^a, Scott F Lindsey^b, Greg Wallace^b, Andrew M Burrage^b, Andrew J Smith^b, Brandi R Manning^b, Daniele M Swetnam^b, Stacey A Gray^b, RW Moyer^b, and Bertram L Jacobs^{a,d}

^aBiodesign Institute, Arizona State University, Tempe, Arizona 85287-5401, USA

^bDepartment of Molecular Genetics and Microbiology, University of Florida, Gainesville, Florida 32610

^cDepartment of Pathobiology, University of Illinois, Urbana, Illinois 61802

Abstract

Vaccinia virus deleted for the innate immune evasion gene, E3L, has been shown to be highly attenuated and yet induces a protective immune response against challenge by homologous virus in a mouse model. In this manuscript the NYCBH vaccinia virus vaccine strain was compared to NYCBH vaccinia virus deleted for E3L (NYCBHΔE3L) in a rabbitpox virus (RPV) challenge model. Upon scarification, both vaccines produced a desired skin lesion, although the lesion produced by NYCBHΔE3L was smaller. Both vaccines fully protected rabbits against lethal challenge by escalating doses of RPV, from 10 LD₅₀ to 1,000 LD₅₀. A single dose of NYCBHΔE3L protected rabbits from weight loss, fever, and clinical symptoms following the lowest dose challenge of 10 LD₅₀, however it allowed a moderate level of RPV replication at the challenge site, some spread to external skin and mucosal surfaces, and increased numbers of secondary lesions as compared to vaccination with NYCBH. Alternately, two doses of NYCBHΔE3L fully protected rabbits from weight loss, fever, and clinical symptoms, following challenge with 100 to 1,000 LD₅₀ RPV, and it prevented development of secondary lesions similar to protection seen with NYCBH. Finally, vaccination with either one or two doses of NYCBHΔE3L resulted in similar neutralizing antibody titers following RPV challenge as compared to titers obtained by vaccination with NYCBH. These results support the efficacy of the attenuated NYCBHΔE3L in protection against an orthologous poxvirus challenge.

Introduction

The New York City Board of Health (NYCBH) vaccinia virus (VACV) is a first generation smallpox vaccine that was used with success during the campaign to eradicate smallpox [1]. Vaccination using VACV was effective against variola virus infection due to the cross protection that occurs between orthopoxviruses, and the NYCBH strain caused the lowest

© 2011 Elsevier Ltd. All rights reserved.

^dCorresponding author: Bertram L Jacobs, bjacobs@asu.edu, School of Life Sciences, CIDV/Biodesign, Arizona State University, Box 875401, Tempe, AZ 85287-5401, Phone 480 965-4684, FAX 480 727-7615.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

rate of adverse side effects as compared to other VACV strains such as Lister or Copenhagen [2]. However, low rates of adverse side effects such as postvaccinial encephalitis, progressive vaccinia, vaccinia eczematum, generalized vaccinia, and myopericarditis have been observed with this vaccine. The development of second generation vaccines by using tissue culture growth systems rather than live animals has been employed to improve quality control in vaccine preparation and potentially reduce side effects. A tissue culture plaque isolate that was derived from the NYCBH vaccinia virus strain, ACAM2000™, was tested clinically in 2007 [3]. Vaccination resulted in equivalent neutralizing antibody and T cell responses as the NYCBH vaccine, however similar adverse reactions were also observed [3]. Thus a safer yet immunologically effective vaccine is needed to protect against a possible reintroduction of variola virus or from zoonotic spread of monkeypox into the human population.

An attenuated vaccine derived from NYCBH (ACAM2000™) was constructed by deletion of the innate immune evasion gene, E3L (NYCBHΔE3L) [4]. The E3L gene encodes proteins that contain a C-terminal double-stranded RNA (dsRNA) binding domain and an N-terminal Z form nucleic acid (Zα) binding domain [5,6]. The dsRNA binding domain binds and sequesters dsRNA, a byproduct of viral transcription, and prevents proinflammatory signal transduction and activation of type I IFN-induced antiviral proteins [7,8]. The Zα domain of E3L shows homology to Zα domains present in human ADAR1 and murine DAI proteins [9]. Mutations that decrease the affinity of the Zα domain of ADAR1 for Z DNA have been made in the E3L Zα domain, and these mutations result in decreased pathogenicity of VACV [9]. Both domains of E3L have been shown to be necessary for pathogenicity in an animal model [10], and previous studies have shown that deletion of the entire E3L gene from VACV results in a virus that is nonpathogenic, replicates to titers three logs lower in skin than wild type VACV, and provides complete protection against a homologous lethal challenge in mice [4].

NYCBHΔE3L was used to vaccinate rabbits and tested for the ability to provide cross-protection against lethal challenge by rabbitpox virus (RPV). RPV is an orthopoxvirus that is closely related to VACV, and infection of rabbits with RPV produces a systemic disease that mimics human variola infection [11,12]. This study shows that the NYCBHΔE3L strain fully protects rabbits against challenge with escalating doses of RPV. While a single dose protected rabbits from mortality, weight loss, fever, and clinical symptoms following the lowest dose challenge of 10 LD₅₀, it allowed RPV replication at the challenge site and increased numbers of secondary lesions in the skin as compared to vaccination with NYCBH. However a single dose of NYCBHΔE3L prevented spread to internal organs and reduced the number of secondary lesions as compared to mock-vaccinated rabbits. Alternately, two doses of NYCBHΔE3L fully protected rabbits from mortality, weight loss, fever, and clinical symptoms, following challenge with 100 to 1,000 LD₅₀ RPV, and it prevented development of secondary lesions similar to protection seen with NYCBH.

Materials and Methods

Cells lines and virus stocks

BHK-21 and RK-13 cells were maintained in Minimal Essential Medium (MEM) (Cellgro) supplemented with 50mg/ml gentamycin and 5% fetal bovine serum (FBS, Hyclone). CV-1 cells were maintained in MEM with Earle's Salts (Gibco, Grand Island, NY) supplemented with 2mM glutamine 50U/ml penicillin G, 50mg/ml streptomycin, 1mM sodium pyruvate, 0.1mM nonessential amino acids, and 10% FBS.

VACV WR (Western Reserve), NYCBH (ACAM2000™, kindly provided by Acambis), and NYCBHΔE3L were propagated in BHK cells and RPV was propagated in CV-1 cells using

standard methods [13,14]. All virus stocks were partially purified by centrifugation through a 36% sucrose pad and resuspended in TBS (WR, NYCBH) or PBS (RPV). Viral titers were determined on RK-E3L (RK-13 cells stably expressing the VACV E3L gene, Wong, Denzler, and Jacobs, unpublished results) or CV-1 cells, respectively.

Housing, vaccination, and challenge of rabbits

Nine-week old New Zealand White rabbits from Myrtyles Rabbitry (Thompson Station, Tennessee) were housed in solid side rabbit cages and fed standard rabbit pellets *ad libitum*. Each rabbit was microchipped at the initiation of the study to transmit body temperature and identification number to a DAS-5007 reader (Bio Medic Data Systems, Seaford, DE).

Vaccination was performed on the left flank by scarification. After shaving, the virus was administered into the skin via bifurcated needle until a drop of blood was visible (approximately 20 jabs). Challenge was performed six weeks after vaccination on the right flank by intradermal injection using a 27-gauge needle.

Clinical monitoring of rabbits

Clinical scores were determined by monitoring weight, temperature, heart rate, respiration rate, intake and output, and overall well being of the animal, and these were recorded for 10 days following vaccination and for 14 days following challenge. Criteria for euthanizing the rabbits included respiratory distress (open mouth breathing, depressed respiration rates), or weight loss of greater than 10% of the initial body weight. All animal procedures were approved by the University of Florida Institutional Animal Care and Use Committee.

Tissue titration

Rabbits were euthanized on day 6 post-challenge followed by resection of tissues. Tissues (lung, liver, spleen) were processed using a mini beadbeater and resuspended in 10% wt/vol. Skin samples were digested in 2 volumes/wt of 1mg/ml Collagenase D at 37°C for 2 hours followed by dilution to 10% wt/vol. Supernatants were assayed for viral load. All samples were resuspended and diluted in 1XMEM, 2% FBS and viral load was titrated on RK-E3L cells.

Plaque reduction neutralization assay (PRNT)

Blood was drawn by venipuncture from the ear vein at various days post-vaccination or post-challenge and allowed to clot. After centrifugation the serum was removed and stored at -80°C until ready to assay. Endogenous complement was inactivated by heating at 56°C for 30 minutes followed by serial dilution in 1XMEM. Serial dilutions were made in 1XMEM containing 10% mouse complement (Innovative Research, Novi, Michigan). A known amount of VACV (WR) was added to each dilution tube and incubated at 37°C for 2 hours followed by titration in duplicate on RK-E3L cells. The neutralizing antibody titer was measured as the inverse of the serum dilution at which a 50% reduction in plaquing efficiency occurred and was calculated by linear regression.

Histology and immunohistochemistry

Rabbits were euthanized on day 6 post-challenge followed by resection of tissues and storage in 10% normal buffered formalin (NBF). After 18 hours NBF was replaced with PBS and later followed by embedding in paraffin and sectioning at a 3 µm thickness. Some slides were treated with xylene to remove paraffin, rehydrated, and stained with hematoxylin and eosin (H&E). The rest of the slides were used for immunohistochemical (IHC) staining. After removing paraffin and rehydrating, slides were incubated in Tris/EDTA at 95°C for 20 minutes for antigen retrieval. Blocking was done with Background Sniper (Biocare

Medical), and staining was done using vaccinia virus mouse monoclonal antibody (Genway Biotech) at a 1:100 dilution followed by anti-mouse HRP polymer (Biocare Medical). The HRP polymer was detected using DAB (Vector Labs), and hematoxylin was used to counterstain tissue sections.

Statistical analysis

Statistical analyses were performed using GraphPad Prism 5, GraphPad Software, Inc. For temperatures, weights, and clinical scores, two way ANOVA tests followed by Bonferroni posttests were performed to determine differences between vaccination groups.

Results

Vaccination with NYCBHΔE3L protects against challenge with a low dose (10 LD₅₀) of RPV

Three groups of eight rabbits were vaccinated by scarification (day 0) with 1×10^6 plaque-forming units (pfu) NYCBH, NYCBHΔE3L, or were mock vaccinated with PBS (Fig. 1A). At 6 days post vaccination the size of the pocks at the vaccination sites for NYCBHΔE3L were visibly smaller than those for NYCBH (Fig. 1B), although distinct lesions were seen in all 8 rabbits vaccinated with NYCBHΔE3L. Six weeks after vaccination (day 42) the rabbits were challenged with 100 pfu RPV by the intradermal route (Fig. 1A). All eight mock-vaccinated rabbits died at 10–11 days post challenge while all NYCBH- and NYCBHΔE3L-vaccinated rabbits survived RPV challenge (Fig. 2A). Temperatures, weights, and clinical scores of the rabbits were monitored both during vaccination and challenge phases as seen in Fig. 2B, C, and D. Temperatures and clinical scores of mock-vaccinated rabbits differed statistically from both vaccinated groups at 5 days post challenge until death, and weights differed significantly from both vaccinated groups at 7 days post challenge until death. Vaccination with the attenuated NYCBHΔE3L vaccine protected rabbits equally as well as NYCBH from a lethal low dose challenge with RPV.

Vaccination with NYCBHΔE3L does not prevent RPV replication in skin and mucosal surfaces, but does prevent spread to visceral organs

Primary lesions from the rabbits that were vaccinated and challenged with 10 LD₅₀ RPV as described above were monitored following RPV challenge. At day 5 post-challenge the RPV lesions were photographed. Fig. 3A and B show that during the course of the challenge, mock-vaccinated rabbits developed a red swelling with a dark necrotic center which reached a maximum average diameter of 4.6 ± 0.69 stdev ± 0.25 SEM cm. Rabbits vaccinated with NYCBH developed a less severe red swelling with a lack of necrosis, and the lesions reached a maximum diameter of 1.5 ± 0.23 stdev ± 0.08 SEM cm. Rabbits vaccinated with NYCBHΔE3L developed a red swelling with a necrotic center similar to that seen in mock-vaccinated rabbits, however the maximum average diameter of the lesions was 2.5 ± 1.35 stdev ± 0.48 SEM cm. Therefore, rabbits vaccinated with NYCBHΔE3L showed a similar gross lesion at the site of challenge as mock-vaccinated animals, but the lesions were smaller on average.

The development of secondary lesions was monitored during the course of RPV challenge. Rabbits that were mock-vaccinated developed 29 ± 16 stdev ± 5.7 SEM inflammatory skin lesions at sites distant from the area of challenge, suggestive of systemic RPV dissemination. In contrast, rabbits vaccinated with either NYCBH (6.9 ± 2.8 stdev ± 1.0 SEM) or NYCBHΔE3L (18.1 ± 22.9 stdev ± 8.1 SEM) showed reduced numbers of secondary lesions following challenge, although NYCBHΔE3L-vaccinated rabbits developed more lesions on average than NYCBH-vaccinated rabbits.

Three groups of two rabbits were similarly vaccinated and challenged with 10 LD₅₀ of RPV as shown in Fig. 1A, and tissues were resected for further analysis. At 6 days post challenge, RPV titers were present in skin from mock-vaccinated rabbits while rabbits vaccinated with NYCBHΔE3L showed 1.5 log reduced titers at the challenge site as compared to mock-vaccinated rabbits (Fig. 3C). In comparison, no measurable RPV was recovered from the challenge site in rabbits vaccinated with NYCBH. Histological analysis of the skin at the site of RPV challenge at day 6 showed classic deep ulcers in samples from rabbits either mock-vaccinated or vaccinated with NYCBHΔE3L, but not in rabbits vaccinated with NYCBH (Fig. 3D). Specifically, the lesions had marked mononuclear inflammation surrounding an area of epithelial and dermal necrosis and marked suppurative inflammation within the necrotic area, the deep dermis, and the subcutaneous tissue. One of the two NYCBH-inoculated rabbits had a marked mononuclear infiltrate in the dermis without epithelial damage, whereas no lesions were observed in the second NYCBH-vaccinated animal. Poxvirus antigen was detected at the site of challenge in one of the mock-vaccinated rabbits, whereas poxvirus antigen was not detected in rabbits vaccinated with NYCBH or NYCBHΔE3L (Fig. 3D).

No pathologic changes following challenge were detected in grossly healthy skin from any of the rabbits. External skin harvested from the gonad area, secondary lesion sites, or flank areas of both of the mock-vaccinated rabbits had measurable RPV titers 6 days post-challenge (Fig. 4 A, B, and C). Skin sections from rabbits vaccinated with NYCBH had no measurable titers of RPV. Whereas one rabbit vaccinated with NYCBHΔE3L had measurable titers at all three sites, and one rabbit had measurable titers only at the site of a secondary lesion.

RPV titers were detectable in the spleen, lung, and liver of mock-vaccinated rabbits only (Fig. 4 D, E, and F). Rabbits vaccinated with either NYCBH or NYCBHΔE3L had no detectable titers of RPV indicating a lack of spread from the skin and mucosal surfaces to the visceral organs. IHC staining of tissues showed that poxvirus antigen was detectable in the skin, spleen, and lung of one of the two mock-vaccinated rabbits but was not detected in NYCBH- or NYCBHΔE3L-vaccinated rabbits (data not shown). There were increased numbers of pyknotic cells in the spleen of the mock-vaccinated rabbit that had positive staining for poxvirus antigen, but otherwise splenic samples were unremarkable. Small focal areas of necrosis were observed in the liver of both mock-vaccinated rabbits and in the thymus of one NYCBHΔE3L-vaccinated rabbit, but poxvirus antigen was not detected in these organs.

Inflammation was noted in lung tissue from all experimental groups; this was most severe in NYCBHΔE3L-vaccinated rabbits, mild to moderate in mock-vaccinated rabbits, and very minimal or absent in NYCBH-vaccinated animals (Fig. 5). None of the inflammation observed in the rabbits was associated with IHC staining for poxvirus antigen. However, small multifocal aggregates of mononuclear cells stained positive for poxvirus antigen in the lungs of one mock-vaccinated rabbit (data not shown).

Endpoint plaque reduction neutralizing antibody titers (PRNTs) are similar in rabbits vaccinated with NYCBH or NYCBHΔE3L after challenge with 10 LD₅₀ of RPV

Endpoint sera from the three groups of eight rabbits were obtained from mock-vaccinated rabbits at days 10–11 post challenge or from NYCBH- and NYCBHΔE3L-vaccinated rabbits at day 14 post challenge. Heat inactivation of complement prior to PRNT assays resulted in similar antibody titers in all three groups (data not shown). However, the addition of complement back into the assay resulted in higher measurable titers in both NYCBH- and NYCBHΔE3L-vaccinated groups. In this assay mock-vaccinated rabbits showed neutralizing antibody titers at the time of death, but titers were lower than those seen in

rabbits vaccinated with NYCBH (185) or NYCBHΔE3L (207) (Fig. 6). Additionally, antibody titers were similar in rabbits vaccinated with NYCBHΔE3L as compared to rabbits vaccinated with NYCBH. Less variation in antibody titers, as seen by the standard error of the mean, was noted in the NYCBHΔE3L-vaccinated group (see discussion).

Vaccination with NYCBHΔE3L fully protects against challenge with escalating doses of RPV

Three groups of four rabbits were mock vaccinated or vaccinated with NYCBH and NYCBHΔE3L vaccine strains as shown in Fig. 7. Two doses of 1×10^6 pfu NYCBHΔE3L were used in these experiments since a single dose resulted in some replication of RPV in skin and mucosal surfaces during the lower dose challenge, and because two doses of VACV deleted for the E3L gene result in an increase in neutralizing antibody titers in mice [3]. The first dose of NYCBHΔE3L was given at day -14, and the second dose was given at day 0. The other groups were vaccinated with a single dose of 1×10^6 pfu NYCBH or were mock vaccinated at day 0. Six weeks after vaccination (day 42) rabbits were challenged with 100 LD₅₀ or 1,000 LD₅₀ RPV by the intradermal route (Fig. 7). Figure 8A shows that 3 out of 4 mock-vaccinated rabbits died 9–10 days after challenge with 100 LD₅₀ RPV while all of the rabbits in the vaccinated groups survived. The sole mock-vaccinated rabbit that survived challenge displayed an increase in temperature, weight loss, and an increased clinical score typical of RPV infection, but these values were at the less severe end of the range of scores (data not shown). Figure 9A shows that 100% of the mock-vaccinated rabbits died upon challenge at day 8 with 1,000 LD₅₀ RPV while all the vaccinated rabbits survived when vaccinated with either a single dose of NYCBH or two doses of NYCBHΔE3L.

Temperatures, weights, and clinical scores of the rabbits were monitored during vaccination and challenge phases. Figures 8B and 9B show that temperatures did not vary between the NYCBH- and NYCBHΔE3L-vaccinated groups with the exception of one time point at day 7 of vaccination (Fig. 8B). Weight change did not differ between vaccinated groups as shown in Fig. 8C, while in Fig. 9C the NYCBHΔE3L-vaccinated rabbits showed a slight increase in weight gain during the second vaccination phase, but then showed a reduced weight gain during the challenge phase as compared to NYCBH-vaccinated rabbits. However, after RPV challenge the NYCBHΔE3L-vaccinated rabbits steadily gained weight. In both Figures 8D and 9D, mild clinical scores (5 or lower) were recorded for the first NYCBHΔE3L vaccination, while scores from the second vaccination with NYCBHΔE3L did not differ from the mock-vaccinated group. Clinical scores for the NYCBH group (7 or less) were slightly higher than those seen in the NYCBHΔE3L-vaccinated group as would be expected for a replication competent vaccine and showed significant differences at days 5–8 (Fig. 8D and 9D). Upon challenge, the mock-vaccinated group receiving 100 LD₅₀ RPV showed significant differences in body temperature, weight loss, and clinical scores at day 4 until death as compared to both vaccinated groups (Fig. 8 B, C, and D). Additionally, the mock-vaccinated group challenged with the highest dose of 1,000 LD₅₀ RPV showed significant differences in body temperature, weight loss, and clinical scores as compared to both vaccinated groups, and these occurred at day 3 post challenge and continued until death (Fig. 9 B, C, and D). Therefore, vaccination with two doses of NYCBHΔE3L was as effective as one dose of NYCBH at protecting rabbits from lethal challenge with 100–1,000 LD₅₀ of RPV.

The maximum size of the primary lesions and the development of secondary lesions was monitored following challenge with escalating doses of RPV. At a challenge dose of 100 LD₅₀, the primary lesions in mock-vaccinated rabbits reached a maximum diameter of 5.75 $\pm$ 0.87 stdev $\pm$ 0.43 SEM cm while NYCBH- and NYCBHΔE3L—vaccinated rabbits reached 1.48 $\pm$ 0.22 stdev $\pm$ 0.11 SEM cm and 1.55 $\pm$ 0.17 stdev $\pm$ 0.09 SEM cm,

respectively. Similarly, at a challenge dose of 1,000 LD₅₀ mock-vaccinated rabbits showed primary lesions reaching 6.8 $\pm$ 1.15 stdev $\pm$ 0.58 SEM cm while NYCBH- and NYCBH Δ E3L-vaccinated rabbits reached 2.48 $\pm$ 1.69 stdev $\pm$ 0.84 SEM cm and 2.78 $\pm$ 2.34 stdev $\pm$ 1.17 SEM cm, respectively. In the same way, the number of secondary lesions was monitored during the challenge phase. At a challenge dose of 100 LD₅₀ mock-vaccinated rabbits developed 25.5 $\pm$ 17.93 stdev $\pm$ 8.97 SEM secondary lesions while both NYCBH- and NYCBH Δ E3L-vaccinated rabbits developed 0.25 $\pm$ 0.5 stdev $\pm$ 0.25 SEM and 1.75 $\pm$ 1.71 stdev $\pm$ 0.85 SEM lesions, respectively. Similarly, at a challenge dose of 1,000 LD₅₀ RPV, mock-vaccinated rabbits which succumbed to infection 1–2 days earlier than at the challenge dose of 100 LD₅₀ developed 14.75 $\pm$ 11.47 stdev $\pm$ 5.74 SEM lesions while both NYCBH- and NYCBH Δ E3L-vaccinated rabbits developed 0.5 $\pm$ 0.58 stdev $\pm$ 0.29 SEM and 1.75 $\pm$ 0.96 stdev $\pm$ 0.48 SEM lesions, respectively. Therefore, two doses of NYCBH Δ E3L was as effective as one dose of NYCBH at protecting rabbits at the primary site of challenge and from development of secondary lesions at high dose challenges with RPV.

Neutralizing antibody titers are similar in rabbits vaccinated with a single dose of NYCBH or with two doses of NYCBH Δ E3L both after vaccination and after challenge with escalating doses of RPV

Sera from the three groups of eight rabbits that were mock-vaccinated, vaccinated with a single dose of NYCBH, or vaccinated with two doses of NYCBH Δ E3L were assayed for PRNT antibody titers at day 41 post vaccination, one day prior to challenge with RPV. Mock-vaccinated rabbits had no detectable neutralizing antibodies in their sera, while PRNTs were similar in rabbits vaccinated with NYCBH (78) or NYCBH Δ E3L (81) (Fig. 10A). Individual PRNTs are graphed in Fig. 10B and show the large variation in titers observed with NYCBH as compared to NYCBH Δ E3L. A greater standard error of the mean was observed in rabbits vaccinated with a single dose of NYCBH than with two doses of NYCBH Δ E3L suggesting that the two doses of NYCBH Δ E3L produced more consistent levels of neutralizing antibodies. Vaccinated animals were divided into three groups of four rabbits and then challenged with 100 or 1,000 LD₅₀ of RPV as described in Figures 8 and 9. Endpoint sera was collected on the day of death for the mock-vaccinated rabbits or on day 13 post challenge for the vaccinated rabbits. In rabbits challenged with 100 LD₅₀ RPV, similar neutralizing antibody titers were seen in all groups of rabbits whether they were mock-vaccinated or vaccinated with NYCBH or NYCBH Δ E3L (Fig. 10B). Therefore, challenge with RPV mediates a primary antibody response in the mock-vaccinated rabbits at day 9–10 post challenge, but it is not sufficient for protection. Similarly, all three groups of rabbits challenged with the highest dose of 1,000 LD₅₀ RPV had measurable PRNTs, but titers were lower for the mock-vaccinated rabbits which succumbed to infection and died 1–2 days earlier than mock-vaccinated rabbits challenged with 100 LD₅₀ RPV. Rabbits vaccinated with either NYCBH or NYCBH Δ E3L had similar antibody titers after challenge with either 100 or 1,000 LD₅₀ RPV (Fig. 10B and 10C, respectively).

Discussion

Vaccination with NYCBH Δ E3L fully protected rabbits from challenge with escalating doses (10–1000 LD₅₀) of RPV. The size of the pock produced by vaccination with NYCBH Δ E3L was noticeably smaller than that produced by the wild type NYCBH vaccine strain, indicating that there is less virus-induced tissue damage at the vaccination site. This has been observed in mice scarified with VACV deleted for the E3L gene in which viral replication is 3 logs lower in skin than that seen for wild type VACV [4]. However the clinical measurements that included temperature, weight loss, and an overall clinical score

showed no differences between NYCBH- or NYCBHΔE3L-vaccinated rabbits during challenge with all three doses of RPV.

Full protection in VACVΔE3L-vaccinated mice has been observed after lethal challenge with VACV when scarifying with a dose of 1×10^6 pfu [4]. The same dose of 1×10^6 pfu was used in this study since it produces a 100% “take” rate in mice that coincides with full protection. While a single vaccination with NYCBHΔE3L fully protected rabbits against challenge with 10 LD₅₀s of RPV, it did allow some replication and spread of RPV to distal sites. Titers of RPV at the site of challenge in NYCBHΔE3L were observed in both vaccinated rabbits and were 1.5 logs lower than titers in mock-vaccinated rabbits. One NYCBHΔE3L-vaccinated rabbit had RPV titers in skin harvested from the gonad, a secondary lesion site, and from the flank area of the rabbit, and these titers were similar to those seen in both mock-vaccinated rabbits. The second NYCBHΔE3L-vaccinated rabbit had titers only at a site where a secondary lesion developed. Vaccination with NYCBHΔE3L protected both rabbits from RPV spread to spleen, liver, and lung, while both mock-vaccinated rabbits had RPV titers in spleen and liver, and one mock-vaccinated rabbit had titers in the lung. One of the mock-vaccinated rabbits had 2–4 logs higher RPV titers than the second mock-vaccinated rabbit in flank skin, spleen, and liver as well as detectable titers in the blood (data not shown). This rabbit had positive IHC staining for poxvirus antigen at the primary vaccination site and in the spleen and lung tissues. Additionally, this rabbit had a higher morbidity as seen in its clinical scores at day 6 post challenge than the second mock-vaccinated rabbit (data not shown), and it is likely that the second mock-vaccinated rabbit would display similar morbidity, tissue titers, and IHC staining of tissues given 1–2 more days.

The presence of neutralizing antibody titers has been correlated with protection against smallpox in humans [15,16]. Similarly in this study, neutralizing antibodies can be correlated with protection of rabbits from RPV both after vaccination and challenge phases. In the experiment using the lowest challenge of 10 LD₅₀ of RPV, PRNTs were measured at endpoint times. While the mock-vaccinated rabbits displayed lower titers of neutralizing antibodies against VACV, both NYCBH- and single dose NYCBHΔE3L-vaccinated rabbits had similar, higher titers in the sera. Interestingly, the NYCBH-vaccinated rabbits showed a larger standard error of the mean in titers indicating a broad range of very low to very high titers measured in sera after challenge, while the NYCBHΔE3L-vaccinated rabbits produced more uniform levels of neutralizing antibodies. This trend was also observed in the post vaccination measurements of Fig. 10A and B where rabbits that received two doses of NYCBHΔE3L showed more uniform antibody titers than rabbits vaccinated with a single dose of NYCBH. However, endpoint titers measured after challenge with higher doses of RPV (100–1000 LD₅₀) resulted in similar titers in both NYCBH- and NYCBHΔE3L-vaccinated rabbits, and so challenge with higher doses of RPV appeared to normalize the antibody response between vaccine groups. It is of interest that the mock-vaccinated rabbits were able to mount a neutralizing antibody response to RPV. This response is likely a primary, IgM mediated response, and although titers in the 100 LD₅₀ challenge experiment were as high as those seen in vaccinated rabbits at endpoint (day 10–11), the response was not sufficient to protect rabbits from death. It is likely that the primary response in mock-vaccinated rabbits is mounted at too late a time for protection, since rabbits in the 1000 LD₅₀ challenge experiment showed 10-fold lower neutralizing antibody titers at the endpoint measurement of day 8. The detection of neutralizing antibodies in this study is in contrast to a study by Garza et al. in which no PRNTs were detected in Dryvax-vaccinated rabbits [17]. A 5-fold higher vaccine dose and the addition of complement to the PRNT assays in this study may explain the observed difference.

To measure neutralizing antibody titers, we chose a PRNT assay which used VACV WR that was prepared by standard methods resulting in a majority of virus particles being of the intracellular mature virion (MV) form rather than the extracellular enveloped virion (EV) form [20]. Therefore the neutralizing antibodies measured in this study were specific for MV. It was surprising that heat-inactivated sera from vaccinated rabbits had lower neutralizing antibody titers. These apparently lower neutralizing titers suggested that either a small portion of serum antibodies were specific for the MV form of VACV typically used for neutralization assays or that MV neutralization involved antibody isotypes that bind complement well [18,19]. We found that the addition of complement to the assay resulted in measurable neutralizing antibody titers. Therefore the neutralizing antibodies measured in this study were directed in part against MV. It has recently been shown that neutralization of the EV form of VACV *in vivo* depends on the presence of complement [23], so it is possible that antibodies specific for MV also require complement. However, it must be recognized that the sera also likely contain antibodies against the EV form that is thought to be involved in dissemination of the virus since EV, though minimal in the inoculum would be formed during infection of the animal [21,22]. In fact, a single dose of NYCBHΔE3L reduced the number of secondary lesions and prevented dissemination of RPV to visceral tissues in two rabbits and to mucosal surfaces in one rabbit suggesting EV-neutralizing antibodies were indeed present.

Histological examination of lungs from rabbits vaccinated with a single dose of NYCBHΔE3L and then challenged with 10LD₅₀ of RPV showed a higher level of inflammation as compared to rabbits that were vaccinated with NYCBH. The increased inflammation did not appear to be associated with clinical pathology since both groups of vaccinated rabbits displayed similar low grade respiratory symptoms after challenge (data not shown), and neither group had detectable titers of RPV in the lungs indicating the lack of virus dissemination to the lungs. However, the NYCBHΔE3L-vaccinated rabbits displayed a high level of peribronchial inflammation in spite of the attenuated replication observed after challenge with RPV. This is likely due to the fact that deletion of the E3L gene from VACV, which allows strong proinflammatory gene expression *in vitro*, similarly allowed a strong proinflammatory response *in vivo* during vaccination, and may ultimately have allowed a stronger memory response upon challenge [7]. In fact, Rice et al. have recently shown that direct intratracheal inoculation of mice with VACVΔE3L leads to an increased influx of immune cells into the lungs, even in the absence of detectable virus replication [24]. This influx is not observed in mice infected with the same dose of wild type VACV and suggests that the presence of E3L limits the immune response.

The NYCBH strain of VACV causes the lowest rate of adverse side effects of all well characterized vaccine strains. We had hoped that deletion of the E3L gene which farther attenuates the replicative capacity of the vaccine and reduces pathogenicity in a homologous virus challenge mouse model would show efficacy in heterologous virus challenge models. We found that a single dose of NYCBHΔE3L protected mice following challenge with ectromelia virus (Denzler et al., submitted). However, a single dose of NYCBHΔE3L as reported in this study allowed some “breakthrough” of viral replication in the skin as well as the development of secondary skin lesions. Two doses of NYCBHΔE3L prevented the development of secondary skin lesions following high dose RPV challenge, so two doses were similarly used in a monkeypox virus challenge model using cynomolgus macaques. While two doses of NYCBHΔE3L protected 75% of the animals from death, they did not protect animals from viremia or the development of secondary lesions (Denzler et al., submitted). This suggests the need for a higher dose or a greater replicative capacity during a vaccination regimen with NYCBHΔE3L and further supports the need for multiple animal models to assess the efficacy of candidate vaccines.

Highlights

>NYCBHΔE3L vaccine candidate protects rabbits from rabbitpox virus challenge.
 >Potential safer smallpox vaccine. >Induces similar titers of neutralizing antibodies as current smallpox vaccine.

Bibliography

1. Rosenthal SR, Merchlinsky M, Kleppinger C, Goldenthal KL. Developing new smallpox vaccines. *Emerg Infect Dis*. 2001 Nov–Dec; 7(6):920–926. [PubMed: 11747717]
2. Kretzschmar M, Wallinga J, Teunis P, Xing S, Mikolajczyk R. Frequency of adverse events after vaccination with different vaccinia strains. *PLoS Med*. 2006 Aug;3(8):e272. [PubMed: 16933957]
3. Greenberg RN, Kennedy JS. ACAM2000: a newly licensed cell culture-based live vaccinia smallpox vaccine. *Expert Opin Investig Drugs*. 2008 Apr; 17(4):555–564.
4. Jentarra GM, Heck MC, Youn JW, Kibler K, Langland JO, Baskin CR, et al. Vaccinia viruses with mutations in the E3L gene as potential replication-competent, attenuated vaccines: scarification vaccination. *Vaccine*. 2008 Jun 2; 26(23):2860–2872. [PubMed: 18455281]
5. Chang HW, Jacobs BL. Identification of a conserved motif that is necessary for binding of the vaccinia virus E3L gene products to double-stranded RNA. *Virology*. 1993 Jun; 194(2):537–547. [PubMed: 8099244]
6. Kim YG, Lowenhaupt K, Oh DB, Kim KK, Rich A. Evidence that vaccinia virulence factor E3L binds to Z-DNA in vivo: Implications for development of a therapy for poxvirus infection. *Proc Natl Acad Sci U S A*. 2004 Feb 10; 101(6):1514–1518. [PubMed: 14757814]
7. Langland JO, Kash JC, Carter V, Thomas MJ, Katze MG, Jacobs BL. Suppression of proinflammatory signal transduction and gene expression by the dual nucleic acid binding domains of the vaccinia virus E3L proteins. *J Virol*. 2006 Oct; 80(20):10083–10095. [PubMed: 17005686]
8. Pestka S, Langer JA, Zoon KC, Samuel CE. Interferons and their actions. *Annu Rev Biochem*. 1987; 56:727–777. [PubMed: 2441659]
9. Kim YG, Muralinath M, Brandt T, Percy M, Hauns K, Lowenhaupt K, et al. A role for Z-DNA binding in vaccinia virus pathogenesis. *Proc Natl Acad Sci U S A*. 2003 Jun 10; 100(12):6974–6979. [PubMed: 12777633]
10. Brandt TA, Jacobs BL. Both carboxy- and amino-terminal domains of the vaccinia virus interferon resistance gene, E3L, are required for pathogenesis in a mouse model. *J Virol*. 2001 Jan; 75(2):850–856. [PubMed: 11134298]
11. Li G, Chen N, Roper RL, Feng Z, Hunter A, Danila M, et al. Complete coding sequences of the rabbitpox virus genome. *J Gen Virol*. 2005 Nov; 86(Pt 11):2969–2977. [PubMed: 16227218]
12. Adams MM, Rice AD, Moyer RW. Rabbitpox virus and vaccinia virus infection of rabbits as a model for human smallpox. *J Virol*. 2007 Oct; 81(20):11084–11095. [PubMed: 17686856]
13. Condit RC, Motyczka A. Isolation and preliminary characterization of temperature-sensitive mutants of vaccinia virus. *Virology*. 1981 Aug; 113(1):224–241. [PubMed: 7269240]
14. Condit RC, Motyczka A, Spizz G. Isolation, characterization, and physical mapping of temperature-sensitive mutants of vaccinia virus. *Virology*. 1983 Jul 30; 128(2):429–443. [PubMed: 6577746]
15. Downie AW, K M. The antibody response in man following infection with viruses of the pox group. III. Antibody response in smallpox. *J Hyg (Lond)*. 1958 Dec; 56(4):479–487. [PubMed: 13611244]
16. Mack TM, Noble J Jr, Thomas DB. A prospective study of serum antibody and protection against smallpox. *Am J Trop Med Hyg*. 1972 Mar; 21(2):214–218. [PubMed: 5061278]
17. Garza NL, Hatkin JM, Livingston V, Nichols DK, Chaplin PJ, Volkmann A, et al. Evaluation of the efficacy of modified vaccinia Ankara (MVA)/IMVAMUNE against aerosolized rabbitpox virus in a rabbit model. *Vaccine*. 2009 Sep 4; 27(40):5496–5504. [PubMed: 19632316]

18. Leatherbarrow RJ, Dwek RA. Binding of complement subcomponent C1q to mouse IgG1, IgG2a and IgG2b: a novel C1q binding assay. *Mol Immunol.* 1984 Apr; 21(4):321–327. [PubMed: 6610105]
19. Mosmann TR, Coffman RL. TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. *Annu Rev Immunol.* 1989; 7:145–173. [PubMed: 2523712]
20. Moss, B., editor. *Poxviridae: the viruses and their replication.* Philadelphia: Lippincott Williams & Wilkins; 2007.
21. Payne LG. Significance of extracellular enveloped virus in the in vitro and in vivo dissemination of vaccinia. *J Gen Virol.* 1980 Sep; 50(1):89–100. [PubMed: 7441216]
22. Smith GL, Vanderplasschen A. Extracellular enveloped vaccinia virus. Entry, egress, and evasion. *Adv Exp Med Biol.* 1998; 440:395–414. [PubMed: 9782308]
23. Benhnia MR, McCausland MM, Moyron J, Laudenslager J, Granger S, Rickert S, et al. Vaccinia virus extracellular enveloped virion neutralization in vitro and protection in vivo depend on complement. *J Virol.* 2009 Feb; 83(3):1201–1215. [PubMed: 19019965]
23. Rice AD, Turner PC, Embury JE, Moldawer LL, Baker HV, Moyer RW. The Role of vaccinia virus genes E3L and K3L and the host genes PKR and RNaseL during intratracheal infection of C57BL/6 mice. *J Virol.* in press.

Fig. 1. Vaccination and low dose challenge timeline. (A) Groups of 8 rabbits were mock-vaccinated or vaccinated with 1×10^6 pfu NYCBH or NYCBH Δ E3L via scarification at day 0. At day 42 rabbits were challenged with 10LD₅₀ RPV and were monitored for 2 weeks. (B) Representative photographs of the pocks were taken at 6 days post vaccination. Black lines correspond to 1 cm.

Fig. 2.

Challenge of vaccinated rabbits with 10 LD₅₀ RPV. Groups of 8 rabbits were mock-vaccinated or vaccinated with NYCBH or NYCBH Δ E3L via scarification and monitored for 10 days. Six weeks later (day 42) rabbits were challenged with 100 pfu RPV and monitored for 14 days. (A) Percent survival of rabbits following challenge with RPV. (B) Temperature, (C) percent weight change, and (D) clinical scores of rabbits were monitored during vaccination and challenge phases. Lines end at the death of an animal. On the day of challenge (day 42) weights were reset to 100%. Standard error of the mean (SEM) is indicated by error bars. An * indicates a significant difference of $p=0.001$ between both mock and NYCBH or mock and NYCBH Δ E3L.

RPV challenge site

A

B

C

Fig. 3.

Characterization of the challenge site. Groups of rabbits were mock-vaccinated or vaccinated with NYCBH or NYCBH Δ E3L. Six weeks later rabbits were challenged with 10 LD₅₀s RPV. (A) Representative photographs of the challenge site from groups of 8 were taken at 5 days post challenge. Black lines correspond to 1 cm. (B) Maximum diameter of the primary lesions (cm) over the course of the entire experiment from groups of 8 rabbits represented in box and whisker plots. Box represents the median and 50th quartile, whiskers represent the range, and shadow represents the 95% confidence interval. (C) Tissue titers from groups of 2 rabbits were measured at the site of RPV challenge. (D) Histological analysis of skin from groups of 2 rabbits at the site of RPV challenge. Top two rows of panels are samples stained with H&E and bottom panels are stained using monoclonal antibody specific for a late poxvirus protein. The black box on the $\times 50$ magnification photomicrographs designates the area shown in the $\times 200$ magnification photomicrographs. Skin from the second mock-vaccinated animal is designated by a white box.

RPV tissue titers – day 6 post challenge

Fig. 4.

Replication and spread of RPV in challenged animals. RPV tissue titers from groups of 2 rabbits that were mock-vaccinated (1 and 2) or vaccinated with NYCBH (3 and 4) or NYCBHΔE3L (5 and 6) were measured in skin from gonad (A), secondary lesions (B), or flank areas (C) at 6 days post challenge. RPV titers were similarly measured from spleen (D), liver (E), or lung (F) tissues at 6 days post challenge.

Fig. 5.

Histological sections of lungs from challenged rabbits. Top and bottom rows of panels are samples stained with H&E and middle panels are stained using monoclonal antibody specific for a late poxvirus protein. The black box on the $\times 200$ magnification photomicrograph designates the area shown in the $\times 500$ magnification photomicrographs.

Fig. 6.

Endpoint plaque reduction neutralizing antibody titers are similar in rabbits vaccinated with NYCBH or NYCBHΔE3L. The geometric means of PRNTs from groups of 8 rabbits that were mock-vaccinated or vaccinated with NYCBH or NYCBHΔE3L followed by challenge with 10 LD₅₀ of RPV were determined. Serum was harvested post challenge on day 10–11 from mock-vaccinated rabbits or on day 14 from rabbits vaccinated with NYCBH or NYCBHΔE3L. Titers from individual rabbits were determined as the inverse of the serum dilution at which a 50% reduction in plaquing efficiency occurred. Standard error of the mean (SEM) is indicated by error bars.

Fig. 7.

Vaccination and high dose challenge timeline. (A) Groups of 4 rabbits were vaccinated with 1×10^6 pfu NYCBHΔE3L on days -14 and 0 or were mock-vaccinated or vaccinated with 1×10^6 pfu NYCBH on day 0. At day 42 rabbits were challenged with 100LD₅₀ or 1,000 LD₅₀ RPV and were monitored for 2 weeks.

Fig. 8.

Challenge of vaccinated rabbits with 100 LD₅₀ RPV. Groups of 4 rabbits were vaccinated with NYCBH Δ E3L at days -14 and 0, with NYCBH at day 0, or were mock-vaccinated at day 0 via scarification and monitored for 10 days. Six weeks later rabbits were challenged with 1,000 pfu RPV and monitored for 14 days. (A) Percent survival of rabbits following challenge with RPV. (B) Temperature, (C) percent weight change, and (D) clinical scores of rabbits were monitored during vaccination and challenge phases. Lines end at the death of an animal. On the day of challenge (day 42) weights were reset to 100%. Standard error of the mean (SEM) is indicated by error bars. An * indicates a significant difference of $p=0.001$ between both mock and NYCBH or mock and NYCBH Δ E3L. A + indicates a significant difference of $p=0.001$ between NYCBH and NYCBH Δ E3L.

Fig. 9.

Challenge of vaccinated rabbits with 1000 LD₅₀ RPV. Groups of 4 rabbits were vaccinated with NYCBH Δ E3L at days -14 and 0, with NYCBH at day 0, or were mock-vaccinated at day 0 via scarification and monitored for 10 days. Six weeks later rabbits were challenged with 10,000 pfu RPV and monitored for 14 days. (A) Percent survival of rabbits following challenge with RPV. (B) Temperature, (C) percent weight change, and (D) clinical scores of rabbits were monitored during vaccination and challenge phases. Lines end at the death of an animal. On the day of challenge (day 42) weights were reset to 100%. Standard error of the mean (SEM) is indicated by error bars. An * indicates a significant difference of $p=0.001$ between both mock and NYCBH or mock and NYCBH Δ E3L. A + indicates a significant difference of $p=0.001$ between NYCBH and NYCBH Δ E3L.

Fig. 10.

Plaque reduction neutralizing antibody titers (PRNT) are similar in rabbits vaccinated with NYCBH or NYCBHΔE3L. The geometric means of PRNTs from groups of 8 rabbits that were mock-vaccinated, vaccinated with a single dose of NYCBH, or vaccinated with two doses of NYCBHΔE3L were determined from serum harvested at day 41, one day prior to challenge (A). Individual PRNTs from (A) are shown as dots and the geometric means are shown as lines (B). Endpoint serum was harvested from groups of 4 on day 9–10 from mock-vaccinated rabbits or on day 13 from vaccinated rabbits, and PRNTs were measured following challenge with 100 LD₅₀ RPV (C). Endpoint serum was harvested on day 8 from mock-vaccinated rabbits or on day 13 from vaccinated rabbits, and PRNTs were measured following challenge with 1000 LD₅₀ RPV (D). Titers from individual rabbits were determined as the inverse of the serum dilution at which a 50% reduction in plaquing efficiency occurred. Standard error of the mean (SEM) is indicated by error bars.