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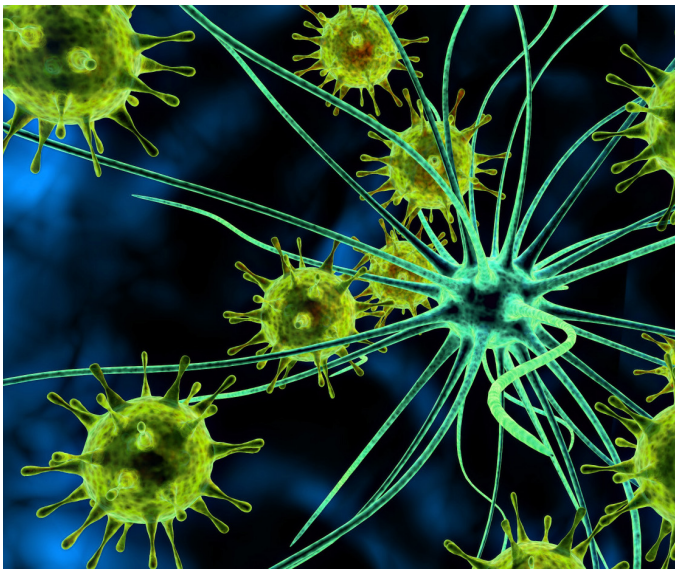
Assessing Neurovirulence and Neurotoxicity of Novel Viral Vector Platforms

INTRODUCTION



Viral vector delivery platforms are transforming the landscape of therapeutics and vaccines, including the burgeoning fields of gene therapy and personalized medicine. The path to adoption, though, has not been easy. Insufficient characterization of the biosafety of viral vector delivery platforms has had devastating effects—for example, a retrovirus-based treatment of children with X-linked severe combined immunodeficiency (SCID-X1) resulted in insertion of a therapeutic gene into proto-oncogene regions of the genome, leading to leukemia in 4 patients.[1,2] Despite early setbacks, novel viral vector platforms, including those with greater payload capacity than now-traditional adeno-associated viruses (AAV), are now gaining momentum.

Due to technological advancements that have improved viral vector safety and specificity, enhanced efficiency, scaled manufacturing processes, and generated clinical success, viral vectored therapeutic development and manufacturing is increasing at a rapid pace.[3] The global viral vector production market, valued at $5.22 billion in 2022, is expected to accelerate with an impressive projected Compound Annual Growth Rate (CAGR) of 14.21% through 2028.[3]

Innovative technologies are expanding applications for viral vectored medicines. Current applications include cell and gene therapies that use viral vectors to deliver genetic payloads or therapeutic proteins to treat chronic diseases.[1] Attenuated live viruses and viral vector delivery of mRNA are now widely used in vaccines that can be rapidly generated to address emerging infectious diseases.[4] Cancer vaccines utilizing intra-tumoral injections of oncolytic viruses are being developed to treat locally invasive cancers.[5,6] Viral vectors are also delivering immunostimulatory genes, like cytokines and chemokines, to elicit the body's immune response to fight cancer.[1]

Some viral vectored drugs have gained approval for use in humans.[1] Therapies now on the market to treat cancer include an adenovirus-based drug, Gendicine®, targeting non-small cell lung cancer and an oncolytic herpes simplex virus T-VEC for melanoma.[1] Genetic modification using a lentivirus-based treatment for a rare, heritable adenosine deaminase enzyme deficiency (ADA-SCID) disease that leads to severe

immunodeficiency is now available.[7] A rhabdovirus-based live viral vaccine, known as ERVEBO®, has been approved to immunize humans against Zaire ebolavirus.[1,8]

As development of new viral vector platforms proliferates, there is a need for faster, more accurate, and innovative safety testing.[1] Patient confidence in therapies created using new technologies hinges on the accurate and reliable verification of their safety.[4] Regulatory guidelines require strict evaluation of viral-based medicines and requirements are changing with the advent of new safety testing methods.[4, 9-14]

As part of a comprehensive safety assessment, it is essential to ensure that live viruses used in medical applications are devoid of neurological effects—and to do so early in the drug development process—using neurovirulence and neurotoxicity testing.[4] To safely bring newly developed viral vectored vaccines and therapies to patients, drug developers must rely upon expertise from an experienced partner with a track record of success in conducting complex, preclinical neurovirulence and neurotoxicity studies in compliance with regulatory requirements. Southern Research has established considerable technical expertise at the intersection of virology and toxicology and has specialized facilities to handle new viruses, including biosafety level 2 and 3 live viruses. These capabilities and infrastructure uniquely position Southern Research as a partner to organizations developing new viral vectored therapies, vaccines, and viral vector platforms.

This review features case studies of preclinical neurovirulence testing of viral vectored therapeutics using regulatory compliant methods at Southern Research. We also introduce the development of innovative neurovirulence test methods with enhanced sensitivity that refine the use of traditional animal models. Finally, we explore the breadth of experience and unique capabilities at Southern Research moving viral vectored medicines toward clinical application.

WHY NEUROVIRULENCE TESTING OF VACCINES AND VIRAL-BASED MEDICINES IS NEEDED

Confirmation of attenuation of pathogenic traits is necessary for the safe use of live virus therapies. A variety of approaches are used to attenuate viral vector pathogenicity. A natural approach uses serial passage of wild-type virus through cell cultures or animals allowing the virus to accumulate genetic mutations that attenuate viral pathogenicity.[4] Alternatively, targeted genetic engineering can be used to design safer vectors. One of the newest methods creates chimeric viruses, a method that combines genes from different viruses to create an attenuated viral vector. All viral vectors must be screened, especially those designed using genes from a naturally neurotropic virus such as yellow fever, for residual neurovirulence *in vivo*, prior to use in humans.[4] In addition, new vaccine seed lots should be tested to make sure that there is no change in neurotropism during the manufacturing process.

NEUROVIRULENCE TESTING: CURRENT METHODS AND FUTURE DIRECTION

For over 80 years, neurovirulence testing has been conducted primarily in animal models. Animal models outperform current *in vitro* models because all central nervous system (CNS) cell types and immune cell types are present, and behavioral assessments can be conducted to gauge neurological impairment.[4] The current gold standard test for live viral vaccine evaluation of neurovirulence is the monkey neurovirulence test (MNVT). It involves the direct injection of live virus into the brain of animals to evaluate CNS neurotoxicity effects after 21 or up to 32 days.[12] Both clinical observation and examination of brain histopathology is conducted to evaluate changes in neurological function and morphology.[4]

The MNVT has limitations, however. The test is expensive, takes approximately 30 days to complete,

and the availability of non-human primate (NHP) test subjects is limited. Fewer test subjects lowers the statistical significance of results and the number of dose levels that can be evaluated. In addition, the MNVT has limitations in clinical translatability to human disease in that it may not capture all mechanisms of virulence.[4] It has also been noted that MNVT does not work well for some viruses that demonstrate a wide range of clinical signs or show variable time-to-effect. In addition, neurovirulence expression may not be measured in the same way among viruses considered to be neurovirulent, which makes it difficult to establish regulatory control standards for viral vectored medicines.[4]

Because of known limitations with the MNVT, alternative *in vitro* and non-NHP *in vivo* methods are needed. Currently, no *in vitro* cell culture-based methods are accepted by regulatory agencies.[4] An attractive alternative, however, is neurovirulence testing in a rodent model. Rodents have been genetically characterized and their molecular toxicology biomarkers are well-defined. Rodents also reproduce rapidly, increasing their suitability for studies with more statistical power. Rodents play a large role in toxicity testing and are the model most accepted by global health authorities for nonclinical safety evaluation.[15] The critical need for a rodent model of neurovirulence testing of live viral vaccines testing is well established in the industry.[4]

In addition to animal model alternatives, control data is needed to set endpoints for assessing neurovirulence for both individual viruses and viral families. Endpoints are critical for ensuring neuro-attenuation of viral-based products and these endpoints will inform the future of vaccine and viral vectored drug design and development. In addition to histopathological markers, molecular endpoints measured using novel assessment methods are desirable.[4]

Southern Research is developing solutions for improved neurovirulence testing, with current efforts centered on the development of a mouse model as an alternative to non-human primates—a practice in line with refining the use of animals by using lower species whenever possible, and one that has shown greater sensitivity than the MNVT.

LIVE VIRAL VACCINE TESTING AT SOUTHERN RESEARCH

To date, Southern Research has conducted 3 studies of live viral vaccines for neurovirulence, viscerotropism, and immunogenicity in NHPs. Staff veterinarians administered intracranial stereotaxic injections in NHPs following WHO TRS 978 Guidance, Annex 2 & 3.[9,10] Two studies examining the safety of four Dengue master seed viruses and a study focused on the safety of yellow fever 17D vaccine seed virus have been successfully performed.[16]

In the yellow fever virus safety study, six test groups, each with ten NHP subjects per group, were administered one of the following test articles: master seed virus, working seed virus, yellow fever 17D vaccine P-240 (final product vaccine), Stamaril™ commercial yellow fever vaccine (control vaccine), WHO reference virus 168-73, or placebo at concentrations of 5,000-50,000 IU ($10^{3.7}$-$10^{4.7}$ IU/0.25 mL) **(Table 1)**.[16]

Intracranial dosing of NHPs occurred over a 2-day period with test groups divided into 2 cohorts with staggered dosing for males and females. Following a single intra-cranial injection, all NHPs survived and clinical signs resolved quickly. Daily observation and clinical scoring began on day 0 and continued for 30 days to determine neurovirulence, measuring mortality/morbidity, body weight changes, and clinical evidence of neurological impairment. Blood samples were collected to measure the amount of circulating virus in serum by plaque assay on days 0, 2, 4, and 6 after inoculation

to determine viscerotropism. Serum samples were collected pre-dosing, and again on days 14 and 30 to measure neutralizing antibodies to yellow fever using the $PRNT_{50}$ assay, for immunogenicity determination.

On day 30, euthanasia and necropsy were performed to examine vital organs, including the brain, for lesions or other abnormalities associated with neurovirulence. **(Table 2)**.[16]

Table 1. Study design for neurovirulence testing of live attenuated yellow fever vaccine.[16]

Group	N per Group	Test Article	Dose
1	10 (5M/5F)	Master Seed Virus 17D (MSV)	5,000 to 50,000 IU/0.25 mL
2	10 (5M/5F)	Working Seed Virus 17D (WSV)	5,000 to 50,000 IU/0.25 mL
3	10 (5M/5F)	Yellow Fever Vaccine at P-240*	5,000 to 50,000 IU/0.25 mL
4	10 (5M/5F)	Stamaril Commercial YF vaccine	5,000 to 50,000 IU/0.25 mL
5	10 (5M/5F)	Reference Yellow Fever Vaccine -WHO 17D 168-73	5,000 to 50,000 IU/0.25 mL
6	10 (5M/5F)	Placebo	0/0.25 mL

*Diluent for yellow fever vaccine at P-240 was water for injection.

Table 2. Summary of study activities performed with a 30-day test period.[16]

Study Day: / Procedure	Sample Type	0*	1	2	3	4	6	7	14	30
Intracranial dosing (IC)		✓								
Daily Observations		All animals are monitored twice daily for morbidity (AM/PM)								
Clinical Scoring		All animals were evaluated once daily, and scoring was performed in the AM								
Body Weight		✓		✓		✓	✓		✓	✓
Euthanasia/Necropsy										✓
Blood/Tissue Collections:										
Viral Load/Plaque Assay	Serum	✓		✓		✓	✓			
PRNT Assay	Serum	✓							✓	✓
Tissue Viral Load/ Plaque Assay	Tissue									✓

*IC dosing was performed over a 2-day period. Groups were divided into Cohort 1 (Groups 1, 2, 4) and Cohort 2 (Groups 3, 5, 6) and one cohort was dosed per day.

Endpoints for neurovirulence included statistically significant (at the 5% significance level) clinical scores (WHO recommended scores of 0-4) showing evidence indicating encephalitis, tremor, and paresis compared to controls and histopathological evidence of lesions or other abnormalities in the brain, spinal cord, and vital organs per WHO protocols.[16] The viscerotropism endpoint was defined as not >500 IU/0.03 mL for all 10 sera and not >100 IU/0.03 mL in 1 of 10 sera. Immunogenicity was assessed with an endpoint of at least 90% of NHP subjects immune within 30 days.

Outcomes of this study showed that the yellow fever seed viruses and final vaccine were comparable to the WHO reference virus 168-73 for clinical and histological score. Detailed results of the yellow fever vaccine study are beyond the scope of this review but are published.[16]

This successful study demonstrates the ability of Southern Research to conduct complex, regulatory compliant neurovirulence, viscerotropism, and immunogenicity studies for live viral vaccine candidates requiring BSL-2 or BSL-3 containment.

Intrathalamic NHP injection protocol development

Southern Research has optimized stereotaxic surgical protocols for delivery of live viral vaccines to the thalamus of NHPs **(Figure 1)**, in accordance with European Pharmacopeia 11.0, 2.6.18, Test for Neurovirulence of Live Virus Vaccines.[12] The thalamus is a relay center for processing of sensory information, other than the sense of smell, and the regulation of motor functions. Therefore, lesions in this part of the brain would be expected to have effects on activity and alertness.[17]

To develop this procedure, we evaluated the published literature on stereotaxic coordinates for intra-thalamic injection in a closely related NHP species. Surgical trials were conducted by injecting trypan blue dye into the incision site were used to verify local delivery to both hemispheres of the thalamus. We determined the best x, y, and z coordinates on the skull for intra-thalamic drug delivery in cynomolgus monkeys. Gross necropsy examination confirmed successful delivery of the trypan blue dye to both thalamic hemispheres (data not shown).

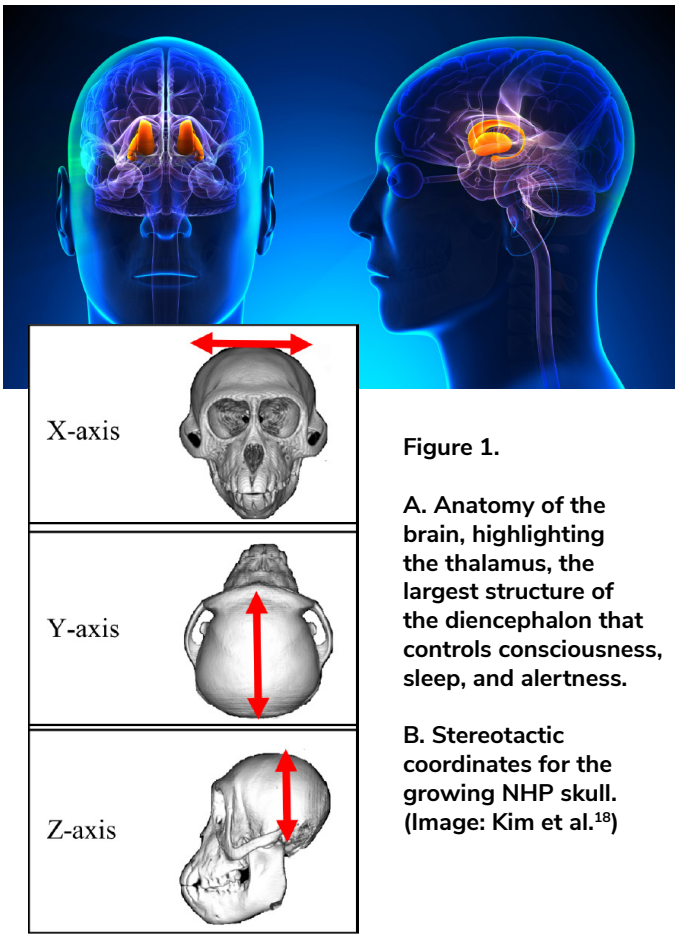


Figure 1.

A. Anatomy of the brain, highlighting the thalamus, the largest structure of the diencephalon that controls consciousness, sleep, and alertness.

B. Stereotactic coordinates for the growing NHP skull. (Image: Kim et al.[18])

NEUROVIRULENCE ASSAY DEVELOPMENT AT SOUTHERN RESEARCH

Southern Research scientists have implemented current regulatory neurovirulence testing methods and are working to develop new, more sensitive neurovirulence tests in suckling mice.

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Intracranial CD-1 suckling mice pilot study

Southern Research has conducted successful neurovirulence studies using a mouse model with the goal of reducing NHP testing. A pilot study in CD-1 suckling mice (pups) was performed using a single intracranial injection of 20 µL (50 PFU) of the WHO 168-73 live attenuated yellow fever vaccine on postnatal day 4 (PND4) **(Figure 2)**.

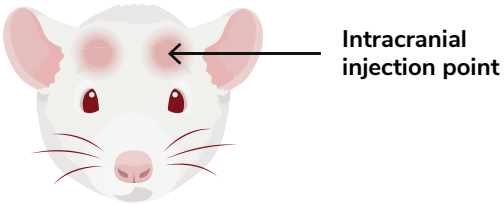

Figure 2. Diagram depicting location of intracranial injection site in suckling mice (pups).

The study was conducted across four test groups, with each receiving one of the following test articles: phosphate buffered saline (PBS), WHO 17D 168-73 vaccine, trypan blue dye, or no treatment (untreated controls) **(Table 3)**.

Pups were observed for neurological signs and survival for 7-10 days after intracranial injection **(Table 4)**. Body weight and clinical observation were performed daily. Euthanasia was scheduled on postnatal days 11-13. Endpoints for the study included greater than 80% survival in the PBS control group and a survival rate of less than 20% in positive controls (Group 2).

Activity changes were noted on PND 10 in the positive control mouse pups (WHO YF-17D vaccine), which led to early termination of Group 2 pups on PND11, on Day 7 after dosing. These results indicate that sucking mice are sensitive to WHO YF-17D neurovirulence at 50 PFU. The no-observed level for WHO YF-17D neurovirulence in mouse pups is reportedly 5-10 PFU (personal communication). The survival endpoints were met and only one of the control pups was lost after anesthesia on PND4. All other mouse pups in the PBS control, trypan blue, and satellite treatment groups survived until scheduled euthanasia. This model is being used by development partners to titrate the neurovirulence threshold for novel yellow fever vaccines in comparison to Stamaril™, a commercially available vaccine.

Table 3. Neurovirulence study design for intracranial administration of live virus vaccine in CD-1 suckling mice. Each of the F_0 females had an average of 6-8 pups per litter, with a minimum of 20 F1 pups treated in Groups 1, 2 and 4. Group 3 had only 1 F0 female and the pups had intracranial injections of trypan blue dye to confirm proper placement of the needle.

Group	N F0 females*	Test/Control Article	Dose Volume in F1 Pups	Scheduled Euthanasia
1	4	Phosphate Buffered Saline (PBS)	20 µL	PND 11-13
2	4	WHO YF-17D Vaccine	20 µL (50 PFU)	PND 11-13
3	1	Trypan Blue Dye	20 µL	PND 11-13
4	4	Untreated Control	-	PND 11-13

*Only F1 offspring from assigned pregnant dams received intracranial injections of test or control articles on postnatal (PND) Day 4. PFU refers to viral potency in plaque forming units.

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Table 4. Summary of daily activities in the neurovirulence study of suckling mice.

Study Day: / Procedure	GD 14 & GD 16*	PND 1	PND 4	PND 7	PND 10	PND 11-13
Body Weight – F_0 Dams	✓	✓	✓	✓	✓	✓
Terminal Body Weight -Pups						✓
Daily Observations	All animals were checked twice daily for morbidity (AM/PM)					
Pup body weight and clinical observations		Performed once daily				
IC dosing of mouse pups*			✓			
Necropsy and brain collection*						✓

* Intracranial dosing and brain collection at necropsy was only performed for F1 mouse pups.

Further, our development and use of a mouse model of neurovirulence demonstrates Southern Research's commitment to refine CNS safety methods and to replace, refine, or reduce the use of increasingly scarce NHPs for vaccine safety studies. This new mouse neurovirulence model is faster and more powerful for screening yellow fever neurovirulence. Other rodent models in development by industry include a rat pup model for mumps neurovirulence.

AN ADVANCED TOXICOLOGY PORTFOLIO

Southern Research leads the way in the development of modern neurovirulence and GLP compliant toxicity testing methods for confirmation of viral attenuation and comparison with approved vaccines on the market. Unique capabilities, such as working under stringent biosafety handling conditions, and experience collaborating with regulatory agencies and WHO reference vaccines, provide a strong foundation to support complex toxicological studies.

Southern Research has demonstrated the safety of oncolytic viruses for the treatment of locally invasive cancer and viral vectored vaccines with unique genetic payloads. In addition, we have evaluated the safety and efficacy of novel vaccines for SARS-Cov2 and vaccines for other emerging infectious diseases.[16] Our technical expertise includes the handling of adenovirus, adeno-associated virus, herpes simplex virus, flavivirus, measles virus, and poxvirus delivery vectors among others.[19] Scientists at Southern Research routinely conduct biodistribution, immunogenicity, and toxicological evaluation of mRNA and DNA vaccines in appropriate animal models.[19] Southern Research also has extensive experience measuring viral replication in target tissues using plaque forming assays.

As a full-service CRO with extensive molecular testing capabilities, Southern Research can conduct ELISAs to measure protein and cytokine levels and use PCR to assess gene expression levels to support studies on the safety and biodistribution of mRNA and DNA based therapeutics. Molecular biology and analytical services departments stand ready to develop novel assays in support of product development.

In addition to the studies featured in this review, Southern Research also has a proven track record in

reproductive toxicology. In fact, Southern Research recently completed a 10-year grant with the National Toxicology Program (NTP) to evaluate the reproductive toxicity of chemicals, drugs, and botanicals in rodents and rabbits. Industry partners are currently working with Southern Research on the evaluation of reproductive safety in first generation offspring of vaccine-treated pregnant dams using unique and proprietary rodent models.

By integrating reproductive safety testing into early vaccine development, developers can identify early signs of risk, expand clinical testing to include women of childbearing potential, and speed up vaccine development timelines.[20]

Southern Research is uniquely positioned to deliver reliable safety testing of vectored vaccines and gene therapy products aimed at solving today's most pressing public health challenges.

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