DESCRIPTION: State the application's broad, long-term objectives and specific aims, making reference to the health relatedness of the project. Describe concisely the research design and methods for achieving these goals. Avoid summaries of past accomplishments and the use of the first person. This abstract is meant to serve as a succinct and accurate description of the proposed work when separated from the application. . If the application is funded, this description, as is, will become public information. Therefore, do not include proprietary/confidential information. **DO NOT EXCEED THE SPACE PROVIDED.**

Cerebral ischemia offers a target for studying the action of biologically and therapeutically relevant cDNAs. The overall aim of this project is to employ an intra-arterially administered HSV amplicon vector for the delivery of the murine endothelial nitric oxide synthase (eNOS) cDNA into the cerebral endothelial cells of mice exposed to an ischemic insult. To accomplish this objective, further understanding is needed of the mechanisms that limit/impede efficient gene delivery *in vivo* and that produce unwanted viral vector toxicity. Because intra-arterially administered HSV vectors activate the complement cascade in mice and rats, initial vector infection of endothelial cells in cerebral vasculature is impeded and toxicity from subsequent inflammatory reactions occurs. To address these issues, our specific aims are to: **1) Determine the effect of inhibitors of complement activation on HSV amplicon infection of endothelial cells in mouse models of cerebral ischemia, 2) Determine if addition to the amplicon envelope of HSV's glycoprotein C, known to evade complement, will increase the efficiency and safety of gene transfer, 3) Determine if evasion of neutralizing immunity to the HSV amplicon will increase the efficiency and the safety of gene transfer and, 4) Determine if endothelial nitric oxide synthase (eNOS) gene transfer protects mice from cerebral ischemia.** We will employ an oversized and ICP27-deleted "helper" HSV cloned in a bacterial artificial chromosome to package the amplicon vector because, unlike other packaging methods, it does not regenerate cytotoxic replication-competent helper HSV. Amplicons expressing reporter genes will be delivered into the cerebral vasculature of mice before and after ischemia and transgene delivery into endothelial cells will be assayed in the presence or absence of transient complement depletion. Amplicons whose envelope possesses glycoproteins, shown to allow evasion of complement and immunoglobulins, will also be engineered and tested. Mice with genetic defects in complement function will further elucidate the contribution of these humoral responses in limiting amplicon-mediated transgene expression. These results will provide the basis for exploring whether eNOS cDNA delivery into mouse cerebral endothelium provides a neuroprotective effect in the presence of ischemia and whether eNOS gene transfer can be combined with other pharmacologic methods for raising eNOS activity to achieve additive or supra-additive neuroprotective effects. The significance of these studies is that they will increase our understanding of the factors responsible for limiting HSV amplicon delivery of transgenes into the brain, they will provide an avenue for reducing vector toxicity and will provide a significant advance in applying eNOS therapy for augmenting the microvascular circulation during cerebral ischemia.

PERFORMANCE SITE(S) (*organization, city, state)*

MASSACHUSETTS GENERAL HOSPITAL, BOSTON, MA

KEY PERSONNEL . See instructions on page 11. *Use continuation pages as needed* to provide the required information in the format shown below.

Name	Organization	Role on Project
E. Antonio Chiocca MD PhD	Massachusetts General Hospital	Principal Investigator
Yoshinaga Saeki, MD	"	Instructor Fellow
Hiroaki Wakimoto, MD PhD	"	Postdoctoral Fellow
Edyta Timinski, BS	"	Research technician
Michael M. Moskowitz, MD	"	Collaborator
Paul Huang, MD PhD	"	Collaborator
Michael C. Carroll, PhD	Harvard Medical School	Collaborator/Consultant

Type the name of the principal investigator/program director at the top of each printed page and each continuation page. (For type specifications, see **Specific Instructions** on page 6.)

RESEARCH GRANT
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*Type density and size must conform to limits provided in Specific Instructions on page 10.

Appendix *(Five collated sets. No page numbering necessary for Appendix)*

Number of publications and manuscripts accepted or submitted for publication *(Not to exceed 10)*: _____

Other items (list):

☐ Check if Appendix is Included

JUSTIFICATION (Use continuation pages if necessary):

From Budget for Initial Period: Describe the specific functions of the personnel, collaborators, and consultants and identify individuals with appointments that are less than full time for a specific period of the year, including VA appointments.

***For All Years*:** Explain and justify purchase of major equipment, unusual supplies requests, patient care costs, alterations and renovations, tuition remission, and donor/volunteer costs.

From Budget for Entire Period: Identify with an asterisk (*) on this page and justify any significant increase or decrease in any category over the initial budget period. Describe any change in effort of personnel.

For Competing Continuation Applications: Justify any significant increases or decreases in any category over the current level of support.

Personnel:

E.A. Chiocca will serve as the principal investigator. His research work in the last 3-5 years has focuse don generating selective and efficient viral vectors for the treatment of malignant glioma, an incurable form of brain tumor with an average life expectancy of one year. Recent work for his laboratory has shown that treatement efficacy for preclinical model of this disease is limited by the host antiviral response, the topic of this grant. He will spend 35% of his effort directing, supervising, and providing the overall research plan for this project. We thus ask of salary support for this effort.

Dr. Hiroaki Wakimoto is a neurosurgeon who has spent the last year (1999) in Dr. Chiocca's laboratory. IN this time, he has been critical to the implementation of the preliminary results of the current research project as evidenced by his co-authorship of Ikeda et al., Nature Med. 1999 and Ikeda et al., submitted. He has developed the neurosurgical skills needed to perform the intra-arterial catherization of rodents under the microscope and has been instrumental in data acquisition and analysis. He will continue to perform the numerous in vivo experiments and analyses for this project, on which he will spend 100% of his time. We thus request to cover his salary.

We are asking for additional postdoctoral support (to be named), in order to has a postgraduate scientist with immunological training join this effort. This person will be responsible for the performance of the numerous in vitro immunological assays and experiments, the analysis of results, their interpretation, and troubleshooting problems with immunological assays. We are asking for 100% effort and salary for this person.

Edyta Tyminski is a research technician in Dr. Chiocca's laboratory. Under the daily supervision of Drs. Wakimoto and the postdoctoral fellow to be named, she will be responsible for the growth of herpes simplex viral stocks, their purification and maintenance. This is a fairly labor intensive process since we use approximately 10^9 pfus per animal and the typical yield from one preparation (20 T175 flasks) is 5×10^9 - 10^{10} pfus. She will also be responsible for the Southern and PCR analyses of viral preparations to detect the presence of wild-type contaminants and the purity of the viral preparation. She will also provide assistance to Dr. Wakimoto in the performance of animal experiments: these require the aid of a person to help with the surgical exposure and injections. Finally, she will help Dr. Wakimoto with sectioning brains and histological and immunocytochemical analyses, another labor-intensive process.

Dr. Michael C. Carroll is an Associate Professor of Pathology at Harvard Medical School who has enthusiastically agreed to join our effort as a collaborator/consultant. He is an internationally renowned expert in the field of innate immunity and how this affects the survival of pathogens in hosts. He has published extensively on the topic and will provide invaluable expertise and aid to Drs. Chiocca, Wakimoto and the immunology postdoctoral fellow to be named related to the performance of the appropriate experiments, data analysis and interpretation as well as troubleshooting experimental failures. As a compensation for his invaluable effort, we would like to provide him with the sum of $ 140/ hour multiplied for 50 annual hours (Total: $ 7000).

Dr. Peter J.A. Davies will provide collaborative/ consultant interactions related to the quantitative PCR methodologies to detect viral genomes and expression in tissues. He is the director of the quantitative PCR Core at the University of Texas Medical School in Houston. We will provide him with samples that he will process for quantitative PCR analysis. For his effort, we are asking for 25 hour per year at $140/hour, as a consultantship (total: $ 3500).

Supplies: This interdisciplinary effort will require immunochemical reagents such as antibodies, purified complement column chromatography. We are asking for $ 15,000/year for these supplies. It will also require extensive tissue culture and we are asking for $ 15,000/year for these supplies. Surgical supplies including instruments, microscope maintenance and light bulbs, infusion pumps are needed and we are asking for $ 5000/year. Molecular biology reagents for PCR, Southern blot analyses, restriction enzyme analyses of viral DNA will cost $ 8000/year. Finally general consumables (gloves, pipettes, pipetmans, gel boxes, supplies for columns, quantitative PCR and RT-PCR) will cost $ 10,000/year.

Animal purchase expenses are as follows: Athymic rats (200/year) at $ 28 per rat = $ 5600. Btk-deficient mice (100 mice/year; Jackson labs) at $38 per mouse = $3800. C3, C4 and sIgM-deficient mice are available through Dr. Carroll. We will purchase them for a fee of $ 25 per mouse x 300/year = $ 7500. Finally, RAG2-deficient mice (50 /year) cost $ 50/mouse for a total of $ 2500. The sum total of purchase of animals is thuis $ 19,400/year.

Travel: We have also budgeted $ 3000 in travel expenses for Drs. Chiocca, Wakimoto and the postdoctoral fellow to be named to attaint the annual meeting of the American Society for Gene Therapy and the International Herpes Virus Workshop.

Other expenses: The cost of color pictures, reprints, and page charges for journals has been estimated to be about $ 3000/year. We have budgeted an additional $3500 for maintenance fees and service charges on our current equipment (hoods, centrifuges, freezers, spectrophotometers, microscopes). Animal per diem charges were calculated as follows. For rats, the charge is $ 1.35 per day per cage (2 rats per cage). Therefore this amounts to: 100 cages x 1.35 x 30 days = $3960. The acquisition/disposal fee is 2 x 100 x 1.32 = $ 264. So the total per diem charge for rats is $ 4224 per year. For mice (immunodeficient) the charge per cage (4 mice per cage) is 113 cages x $ 1.25 x 30 days= $ 4237. The acq/disp fee is 2 x 113 x 1.25 = $ 283. So the total for mice is $ 4520.

a. Specific Aims

Ischemic cerebral stroke is one of the leading causes of death and morbidity in humans. Even mild strokes can lead to devastating effects on individuals, their families as well as society in terms of loss of quality of life, employment, and expenditures of medical and financial resources[1]. Although some treatments are available, there is a need for exploring additional and novel therapeutic avenues[2]. One such avenue revolves around endothelial nitric oxide synthase (eNOS)[3,4]. In animal models of ischemia, up-regulation of eNOS mRNA or eNOS activity leads to a reduction in cerebral infarct size and an improvement in neurologic deficits due to eNOS-mediated increases in cerebral blood flow (CBF)[5-7]. This up-regulation can been achieved with chronic prophylactic administration of HMG-CoA reductase inhibitors that function by stabilizing eNOS mRNA and/or infusion of nitric oxide substrates, such as L-arginine. The combination of these two treatments possesses enhancing effects in terms of therapeutic outcome[8]. Since neither of these treatments functions by modulation of eNOS gene transcription, we hypothesize that increases in eNOS transcriptional units by gene transfer into endothelial cells would in itself be beneficial and, in combination with the described pharmacologic treatments, could provide an even greater therapeutic benefit.

Transient transcriptional elevations in gene expression can be achieved with gene transfer technologies[9,10]. Recent events in clinical trials of gene therapy have underscored the relevance of avoiding side effects from the viral vector construct[11-15]. Such side-effects can occur because of: **a)** low levels of expression of endogenous viral genes from the replication-defective vector[16,17], **b)** the presence of contaminating replication-competent viruses[18,19], and **c)** the host innate and elicited immune response against injected viral proteins that, while protective in removal of the perceived pathogen, can produce tissue/organ damage through excessive inflammatory reactions[20,21]. To overcome these limitations, we and others have been incrementally developing a "gutless" herpes simplex virus (HSV) vector system (amplicon)[22-26]. Ultimately, the main characteristics of this vector will be that: **a)** there will be no HSV gene sequences in its DNA, **b)** it will be essentially void of replication-competent contaminants, and **c)** its outside envelope will possess glycoproteins known to evade innate and elicited immune and inflammatory responses against it. In published and preliminary studies, we can show that we have achieved the first two objectives[23]. However for the purposes of this proposal, before we proceed with a vector that overexpresses the eNOS cDNA, it is important that we also achieve the third objective. We thus plan to test the hypotheses that reduction of innate and elicited immune responses against the vector leads to increased efficiency of transgene expression within cerebral vasculature and diminished toxic side-effects, that transgene expression can be maintained under conditions of cerebral ischemic injury, and that endothelial nitric oxide synthetase (eNOS) gene transfer into endothelial cells will reduce infarct volume in mice brains. Since eNOS expression in brain neurons could lead to the opposite effect, due to the known toxicities of nitric oxide on neuronal function[92-96], we plan to maximize endothelial cell eNOS gene delivery by intra-arterial administration of the gene transfer vector. Therefore, our specific aims will be to:

1) **Determine the effect of inhibitors of complement activation on HSV amplicon infection of endothelial cells in mouse models of cerebral ischemia.**
2) **Determine if addition to the amplicon envelope of HSV's glycoprotein C, known to evade complement, will increase the efficiency and safety of gene transfer.**
3) **Determine if evasion of neutralizing immunity to HSV will increase the efficiency and safety of gene transfer.**
4) **Determine if endothelial nitric oxide synthase (eNOS) gene transfer into cerebral endothelial cells protects mice from cerebral ischemia.**

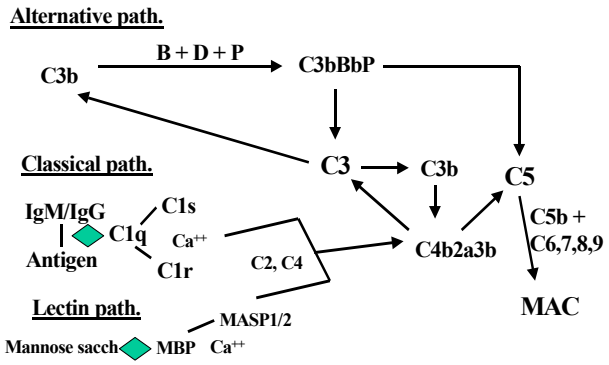
b. Background and Significance

B.1.HSV amplicons as vectors for gene transfer. A multitude of gene transfer vectors has been employed for the delivery of cDNAs with biologic and/or therapeutic potential into the central nervous system[27,28]. These include adenoviruses, adeno-associated viruses, and herpes simplex viruses (HSV) as well as several others. Some of the advantages of HSV include its efficient infection of cells and its ability to shuttle into nuclei large fragments of DNA or multiple copies of cDNAs due to its high packaging capacity (up to 150-160 kilobases)[29-31]. Two general strategies have been employed to engineer replication-defective HSV vectors: 1) **Recombinant vectors** are mutant HSV with deletions in several of the essential genes required for efficient viral replication, such as ICP4, ICP27, ICP22 and/or ICP0, together with the transgene of interest[29,32], and 2) **Amplicon vectors** are prokaryotic plasmids that contain two cis-acting sequences from HSV (*ori*, the origin of DNA replication and *pac*, the signal that permits packaging of the plasmid into virion capsid), the transgene of interest, and the customary prokaryotic DNA sequences, such as antibiotic resistance gene(s) and origin of bacterial DNA replication, needed for growing the plasmid in *E. coli* [33,34]. In order to package either type of HSV vector, the missing viral functions have to be provided in *trans.* For **recombinant vectors**, these functions are usually provided by transfecting the recombinant vector's DNA into cell lines stably transfected with the missing ICP4, ICP0, ICP22 and/or ICP27 function (packaging cells). The resulting cell lysate is composed of the multiply deleted HSV recombinant, but the possibility exists for regeneration of attenuated forms of replication-competent virus, albeit at a low frequency. For **amplicon vectors**, cotransfection of helper virus HSV and the amplicon generates a mixture of helper virus HSV and amplicon HSV. The presence of helper virus HSV (or replication-competent HSV) can provide an impediment to non-toxic and efficient gene transfer and expression in cells of the CNS.

To circumvent the problem of helper virus packaging in amplicon preparations, Fraefel et al. (1996) used a set of five overlapping cosmids containing the entire HSV genome but lacking the *pac* signals needed for packaging. Co-transfection of these cosmids together with the desired amplicon into appropriate mammalian cells (Vero or BHK) produced lysates containing packaged amplicon without helper virus. Therefore, for the first time a strategy was published that appeared to eliminate the problem of packaging helper virus together with amplicon. However, as experience accumulates with this methodology, two limitations have become apparent: 1) Titers of amplicon vector appear to be generally slightly reduced compared to titers that one could achieve using helper virus (routinely 10^6 transducing units/ml for the former compared to 5 x 10^6 tu/ml for the latter packaging method), and 2) re-generation of attenuated, but replication-competent, HSV could still occur (and does occur, as shown in the preliminary experiments of this application) due to homologous recombinatorial events between the common *ori* sequences present in both the amplicon and cosmid-based HSVs.

In order to provide a solution for the former limitation, we and others thus reasoned that reduced titers were likely due to the partition of helper HSV sequences into five separate cosmids[23,24]. Inefficiencies in the transfection of all five cosmids together with the amplicon and inefficiencies in HSV gene expression in transfected cells were possible culprits for the observed decrease in titers. To circumvent this, a relatively novel strategy was employed that consisted of subcloning the entire HSV genome, lacking *pac* sequences, into a bacterial artificial chromosome (BAC) or F-plasmid[35,36]. This HSV-BAC was then co-transfected into cells together with the amplicon. Small increases in vector titer were obtained when compared to the cosmid-based method (8.6 x 10^6 +/- 4 x 10^6 tu/ml for the HSV-BAC compared to 10^6 tu/ml for the cosmid), thus providing strong suggestive evidence that maintenance of HSV helper virus functions as a single genome in the BAC provided more efficient packaging than use of helper virus functions in 5 distinct cosmid subclones[23]. However, as shown in the preliminary results, re-generation of attenuated, but replication-competent HSV still occurs with HSV-BAC, even if at relatively low frequency. There is thus a need for further improvement in the HSV-BAC to completely eliminate replication-competent helper virus production. As we will show in our preliminary results, we have now achieved this objective and are confident that our newest amplicon stocks retain high titer with no helper virus regeneration.

B.2.Innate mammalian responses against viral vectors. It is becoming increasingly evident that innate and elicited humoral immune responses can modulate both the efficacy and the safety of viral vectors[37]. In general, innate factors, such as complement and natural antibodies, provide a major initial and innate host defense against infection of normal tissues by noxious viruses and thus would be expected to affect replication-defective viral vectors as well[38-40]. Complement activation can proceed through the classical, alternative[41], or lectin-pathway[42,43] (see diagram). In the classical pathway, binding of multiple molecules of IgG or a single molecule of IgM to an antigen will expose the antibody's Fc region to attachment by the C1q complement component. This leads to a sequential cascade of events that finally lead to the formation of the membrane attack complex (MAC) that lyses the pathogen and/or pathogen-infected cells. In the alternative pathway, C3 cleavage is maximized by encounter with the surface of a pathogen, activating down-stream effectors that generate the MAC, which lyses the pathogen. Finally, in the lectin-pathway, mannose-binding protein (MBP) (structurally related to C1q), serine proteases-MASP-1 and MASP-2- (structurally related to C1r and C1s), or C-reactive protein recognize high-mannose linked carbohydrates on the surface of pathogens, thus leading to complement activation[43,44]. Although HSV envelope glycoproteins have been shown to possess high-mannose-type O- and N-linked oligosaccharides[45], it is not known if complement becomes activated against HSV through the lectin pathway.



Complement activation produces a variety of anaphylotoxins, opsonins, and chemoattractive factors that stimulate inflammatory reactions[41]. Although these reactions are meant to be protective by destruction of the pathogen, they also cause additional damage to infected tissues[20]. In fact, recent work has shown that pharmacologic attenuation of complement activation can by itself provide neuronal protection in stroke by minimizing complement-induced inflammatory events[21]. In the context of vector delivery of transgenes to organs, complement activation could thus be deleterious not only in terms of efficiency of gene transfer but also in terms of its safety. Therefore, understanding how complement becomes activated upon vector delivery of transgenes is of paramount significance.

Complement could become activated by interaction with immunoglobulin binding to the vector surface. This would be important to determine because the percentage of humans with neutralizing immunity to HSV varies between 40 to 90%, with populations from underdeveloped countries and older individuals exhibiting most seropositivity[46]. The remainder of humans is seronegative and thus would be expected to exhibit only natural immunity upon encounter with the vector. However, the role of both innate natural immunoglobulins and elicited neutralizing antibodies in modulating the efficacy and safety of HSV as a gene transfer vector has not been thoroughly explored. We believe that this knowledge would be essential for an understanding of the limitations on efficacy and the potential for toxicity with regards to HSV amplicon-mediated gene transfer in the central nervous system.

B.4. Herpes simplex viral vectors: mechanisms of immune evasion. In order to efficiently replicate and propagate infections in mammalian cells, lytic viruses such as herpes simplex virus (HSV), have to employ several methods to escape the immune system. In a series of elegant studies, Friedman and colleagues and Spear and colleagues have provided evidence for the escape of clinical virulent isolates of HSV from complement through its interaction with one of the viral envelope glycoproteins (gC)[47-52]. This occurs through binding of C3b by gC, inhibiting the complement cascade, as well as by blocking the binding of properdin and C5 to C3b. The end-result is profound interference of both the classical and alternative pathways of complement activation, allowing for increased survival of both virus and virally infected cells. In addition, glycoproteins E and I from the viral envelope have been shown to possess Fc receptor activity for IgG[53-58]. The binding of IgG to the viral glycoproteins has been shown to lead to a significant decrease in antibody-dependent cellular cytotoxicity as well as complement-dependent lysis of virions and infected cells. A third important mechanism that facilitates viral escape from cytolytic T cells relies on blockage of human TAP-mediated transport into the endoplasmic reticulum of MHC-I class polypeptide products by the viral immediate-early gene product, ICP47[59]. Taken in combination, envelope and capsid proteins of virulent clinical

isolates of HSV can permit evasion by HSV of complement's action, of elicited humoral responses, and of cell-mediated antiviral responses.

B.5.Endothelial NOS and cerebral ischemia. Ischemic stroke is one of the leading causes of death in the USA and, if survived, it can be associated with significant neurologic morbidity and its related complications. Some therapeutic options are available in a prophylactic setting, such as antiplatelet agents, anticoagulants, or surgical/neurointerventional correction of stenotic deformities in the carotid circulation. In the acute phase of a stroke, thrombolytic agents can also be employed. In spite of these medical/surgical advances, both prophylactic and acute-phase treatment would clearly benefit from additional therapeutic options. Furthermore, any novel treatment whose effect minimizes the size of an evolving cerebral infarct is likely to positively affect its neurologic morbidity and mortality. Recent evidence points to a significant role for endothelial nitric oxide (eNOs) (type III) in enhancing cerebral blood flow and subsequently protecting ischemic cerebral tissue from injury[5-7,60-62]. Endothelial nitric oxide (type III) is synthesized by endothelial nitric oxide synthase (eNOS). Loss of eNOS activity has been associated with vasoconstriction, platelet aggregation, smooth muscle cell proliferation, and leukocyte adhesion because NO relaxes vascular smooth muscle, abrogates platelet aggregation and leukocyte adhesion. Mice with a genetic defect in eNOS exhibit larger cerebral infarctions after occlusion of the middle cerebral artery[63]. Pharmacologic-mediated increases in eNOS mRNA result in increased cerebral blood flow and smaller infarcts[6], while administration of the eNOS substrate, L-arginine, relaxes vascular smooth muscle and reduces infarct size after an ischemic event[8].

Additional confirmation of eNOS role in stroke protection was provided by the recent experiments of our collaborator on this grant proposal, Dr. M. Moskowitz. Treatment of mice for 14 days with pharmacologic inhibitors of 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase directly up-regulated eNOS gene expression as shown by mRNA and protein levels. Additional studies have shown that at least more than three days of drug treatment are required to achieve a significant neuroprotective effect (Dr. M. Moskowitz, personal communication). This effect was post-transcriptional and probably related to enhanced mRNA stability. Mice, with up-regulated eNOS and challenged with a two hour occlusion of their MCA, exhibited a 46% decrease in infarct size at 24 hours and a 37% decrease at 72 hours when compared with mice that possessed normal levels of eNOS. Clinically, this resulted in less significant neurologic deficits. Mice with a genetic defect in eNOS and that thus could not up-regulate its expression did not show stroke protection upon treatment with HMG-CoA reductase inhibitors. Although studies had suggested that cholesterol reduction could improve relaxation of vasculature[64], in the Endres et al. study the neuroprotective effects of the drug(s) were not related to their ability to modify cholesterol production or to alterations in physiologic parameters, but were related to enhancement of cerebral blood flow[6]. Additionally, when elevations in eNOS levels by increasing the stability of its mRNA[5] were coupled with increases in eNOS activity by supplementation of its substrate, even more significant therapeutic results were observed[8].

At least two questions remain unanswered by these studies: 1) Can more rapid up-regulation of eNOS gene expression provide more immediate neuroprotection? and 2) Can the observed enhancement of therapeutic effect (provided by the combination of HMG-CoA reductase-mediated increase in eNOS mRNA stability and L-arginine-mediated increase in eNOS activity) be further augmented by amplifying the number of eNOS gene transcriptional units in endothelila cells?

Although we are keenly aware that NO also possesses anti-platelet and anti-leukocyte aggregation properties that are of paramount importance for its action, we have chosen to focus on the mechanism of NO's enhancement of cerebral blood flow in this particular grant application.

NO generation within neurons in the parenchyma may possess deleterious side effects[92-97]. Therefore, another issue that arises from these studies relates to the differential effect of NO generated within endothelial cells compared to NO generated within brain parenchyma: in the former case, a favorable biologic effect occurs from enhancement of blood flow in the microvasculature, while in the latter case, toxicity from inhibition of mitochondrial metabolism in neurons could occur.

C. Preliminary Results/Pilot studies

C.1.Engineering of novel BAC: deletion of ICP27. We first hypothesized that deletion of ICP27, a gene essential for HSV replication, in the HSV-BAC would minimize the likelihood of regeneration of replication-competent HSV. This event occurs at a certain frequency with both the cosmid and the HSV-BAC method of packaging HSV amplicons as shown in Table 1. Three different amplicons of varying sizes were packaged using either helper virus. In all instances regeneration of replication-competent helper virus at titers that ranged from 6.7 up to 150 plaque-forming units (PFU)/ml was observed. This indicated that infection with a replicating HSV couldn't be excluded by using currently available vector stocks.

Table 1 – Replication-competent HSV helper virus regenerates upon amplicon packaging with current methodology.

Helper virus/amplicon	Amplicon titer (tu/ml)[B]	Replication-competent helper titer (pfu/ml)[C]	Amplicon/helper ratio
Cosmid/ Ampl. 1[A]	3.2×10^6	50	6.4×10^4
Cosmid/ Ampl. 2	2.1×10^6	28	7.5×10^4
Cosmid/ Ampl. 3	2×10^6	9.3	2.2×10^5
HSV-BAC/ Ampl.1	5×10^6	150	3.3×10^4
HSV-BAC/ Ampl.2	4×10^6	61	6.6×10^4
HSV-BAC/ Ampl.3	3.5×10^6	6.7	5.2×10^5

[A] The size of amplicons 1, 2, and 3 were 5.6, 9.4, and 14.3 Kb, respectively, and each contained a reporter transgene (lacZ or GFP), [B] Amplicon titers were assayed by enumerating reporter transgene expression on dishes of infected target cells, [c] Helper virus titers were assayed by enumerating plaque-forming units on Vero cell monolayers.

To circumvent the regeneration of replication-competent HSV in amplicon preparation, we reasoned that deletion of the ICP27 gene from the BAC (a gene essential for HSV replication) as well as addition of stuffer sequences in the BAC to increase it to a size that cannot be packaged into the capsid of the virions would eliminate the problem of re-generation of replication competent contaminants. We first proceeded to delete ICP27 from the HSV-BAC, but proceeded to do so in *E.coli* rather than in mammalian cells as currently and routinely done. By using *RecA*-mediated recombination, ICP27 was deleted from the HSV-BAC in bacteria, using published methods[65]. The engineering strategy is described in Figure 1. Briefly, a shuttle vector was engineered that possessed the *E. coli recA* gene, the tetracycline resistance gene (*Tc^r*), a temperature sensitive origin of bacterial replication, and the homologous regions (HRS) in HSV that flank the ICP27 gene with a kanamycin resistance gene located in the middle. Bacteria containing the old HSV-BAC (designated fHSVΔpacΔPacI) were transformed with this plasmid. Homologous recombination between the HSV sequences located 5' to ICP27 (designated as A) would occur at a certain frequency thus inserting the entire shuttle vector into the BAC. Bacterial clones containing this co-integrate can be selected by concomitant acquisition of chloramphenicol resistance (from the HSV-BAC), kanamycin and tetracycline resistance (from the shuttle vector) and permissivity for DNA replication at 43 C. In a certain percentage of these clones that contain the co-integrate intramolecular homologous recombination will occur between the regions that are located 3' to the ICP27 gene. This removes the entire ICP27 gene, the *recA* gene, the tetracyline resistance gene, and the temperature sensitive origin of DNA replication. This new recombinant BAC can then be resolved by selection in chloramphenicol, kanamycin, and fusaric acid (for tetracyline

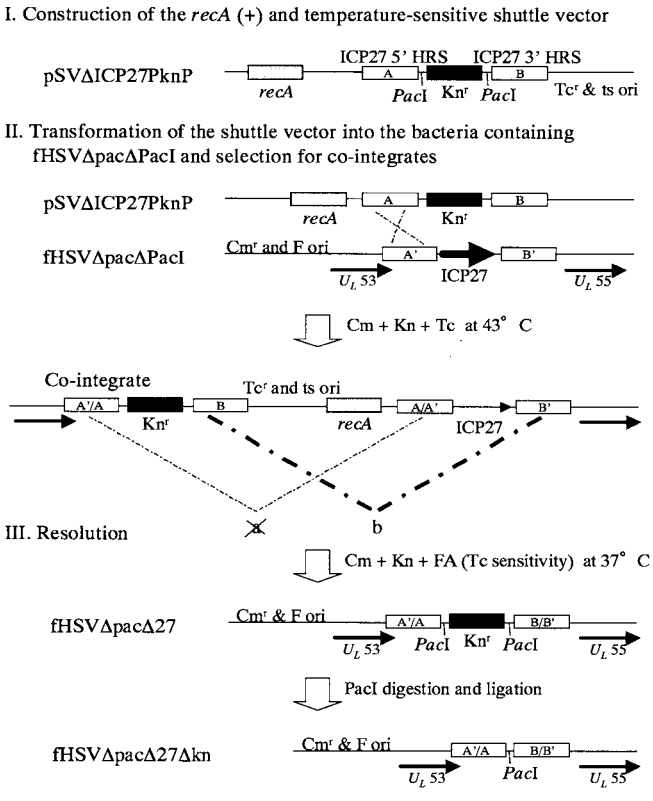


Figure 1 – Engineering strategy for *recA*-mediated deletion of ICP27 from HSV-BAC in *E.coli*

sensitivity) at 37 C. Finally, the kanamycin resistance gene can be spliced out by using the unique PacI restriction sites. Through this method, we have thus generated a novel HSV-BAC that contains a deleted ICP27 gene and that was designated HSV-BAC-Δ27 or fHSVΔpacΔ27Δkn. Restriction endonuclease and Southern blot analyses of the new BAC (HSV-BAC-Δ27) verified its correct genetic identity (data not shown).

To show that functional deletion of ICP27 in HSV-BAC -Δ27 had occurred, we tried to package amplicon 1 (expressing GFP) in Vero cells by cotransfection together with HSV-BAC -Δ27. Table 2 shows that neither amplicons nor recombinant HSV were recovered in these lysates indicating the absence of functional ICP27 expression in the HSV-BAC-Δ27.

Table 2 – Elimination of helper virus in amplicon preparations packaged with HSV-BAC-Δ27

Helper virus/amplicon/ cells	Amplicon titer (tu/ml)	Replication-competent helper titer (pfu/ml)	Amplicon/helper ratio
HSV-BAC-Δ27/ Ampl. 1/Vero	0	0	0
HSV-BAC-Δ27/ Ampl. 1/ 2-2	10^4	0	-
HSV-BAC-Δ27/ Ampl. 1/ 2-2 + transfected ICP27	10^6	0	-

However, if Vero cells were used that were stably transfected with ICP27 (2-2 cells) and/or were transiently transfected with a third plasmid containing an ICP27 expression cassette, then GFP-expressing amplicon 1 was packaged to titers of 10^4 and 10^6 tu/ml, respectively. Importantly, replication-competent HSV was not regenerated in these amplicon stocks even when the concentrated mixture (containing 10^6 – 10^7 tu/ml of amplicon) was added undiluted onto 2-2 or Vero cells. These results thus showed that deletion of ICP27 in the HSV-BAC resulted in the generation of an amplicon preparation devoid of replication-competent HSV (rc HSV).

C.2.Engineering of novel BAC: addition of ICP0 "stuffer". Although ICP27 deletion by itself appeared to eliminate the problem of rc HSV, an additional safety measure was engineered into the BAC. We reasoned that increasing the BAC-HSV to a size larger than the maximum size that could be packaged in virion capsids (160-165 Kb) would further ensure that replication-competent virus could not be re-generated. Using *Cre*-mediated recombination in *E. coli*, we proceeded to recombine in HSV-BAC-Δ27, a "stuffer" segment of DNA that contained the HSV ICP0 gene and its promoter. This was accomplished using published methodology[35]. An ICP0 insertional shuttle plasmid was engineered that contained the entire ICP0 gene, a kanamycin resistance gene, and a loxP site. Another loxP site was engineered into HSV-BAC-Δ27 and cotransfection of the shuttle plasmid, the BAC and another plasmid that expresses *Cre* recombinase produced a novel BAC (HSV-BAC-Δ27-0$^+$) that now measured 168 Kb. When this BAC was used to package the GFP-amplicon 1, no replication-competent virus was detected (>1 replication-competent virus per 10^8 packaged amplicons). Interestingly, addition of the extra ICP0 sequence also significantly increased titers of packaged amplicon to 10^7 tu/ml in supernatants, presumably through the enhancement of HSV immediate-early viral gene expression provided by additional ICP0 in the packaging cells. After supernatant collection and concentration by centrifugation, amplicon titers of 5 x 10^8 – 10^9 tu/ml without rc HSV are now routinely achieved in our laboratory. Taken together, use of HSV-BAC-Δ27-0$^+$ in transfection of ICP27-expressing packaging cells provides a lysate that contains high titers of amplicons without re-generating replication-competent virus, a concern that has afflicted current methods of amplicon preparation.

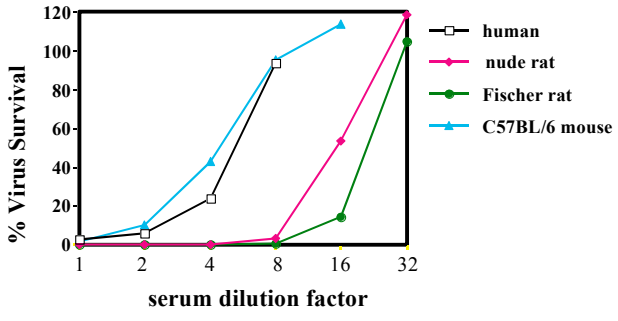


Figure 2 – Human, rat and mouse serum are potent inactivators of HSV vector. Serial dilutions of serum were employed in the study.

C.3. Presence of natural antibody activities against HSV vectors. *In vitro* exposure of HSV vectors to mouse, rat, or human plasma (from humans that do not possess neutralizing HSV antibodies) leads to rapid viral inactivation (figure 2). There has been a recent re-appreciation of the importance of the role that natural antibodies (of both IgG and IgM classes) play as an initial host defense against viral infection[38,40,66,67]. We thus reasoned that the inactivation observed in figure 2 could be an effect of natural Ig. ELISA was thus performed using an HSV amplicon vector as the antigen, serial dilutions of human (void of neutralizing HSV antibodies by Western blot, ELISA, and plaque-reduction assays), rat or mouse sera as the primary antiserum, followed by detection of the antigen/antibody complex with the appropriate secondary antibody. Table 3 shows that all three species possess natural antibody activities against HSV vectors, albeit with some variation in observed titers since rats and humans possessed relatively elevated levels of HSV binding by natural Ig when compared to mice.

Table 3 – Presence of natural IgM and IgG activities against HSV in sera

Species	Antibody titer (ELISA)	
	IgM	IgG
Human	4-16	4-8
Fischer rat (immunocompetent)	16-32	4
Athymic rat	16-32	<2
C57BL/6 mouse	2	<2

Additionally, Western blots using human, mouse, or rat serum as the antibody against electrophoresed, denatured HSV proteins demonstrate recognition of several HSV capsid and envelope antigenic determinants (results not shown). These results thus show that serum antibodies can bind and inactivate HSV amplicons, thus limiting transduction of cells and efficient transgene delivery. Clearly, if neutralizing and protective antibodies to HSV were present in serum, this inactivation would be expected to be even more limiting to efficient transduction of target cells.

C.4 Complement becomes activated against HSV vector through the classical cascade. In published experiments, we have found that the observed viral inactivation could be reduced by mild heat inactivation, known to eliminate complement. Therefore, as expected, natural antibody binding to the HSV vector was activating complement through the classical cascade. Several additional published and unpublished findings confirmed the veracity of this hypothesis: 1) Vector inactivation depended on physiological concentrations of calcium, eliminating the alternative pathway of complement activation as a likely contributor to the observed results[37], 2) Pharmacologic, humoral, or

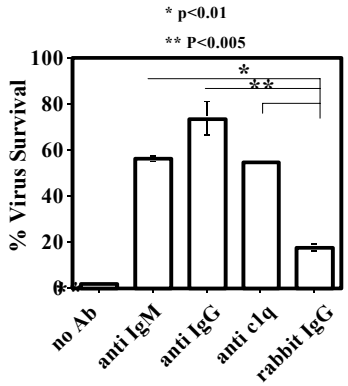


Figure 3 – Antibody neutralization using rabbit IgG raised against human IgM, IgG, or C1q reverses the antiviral activity of human plasma.

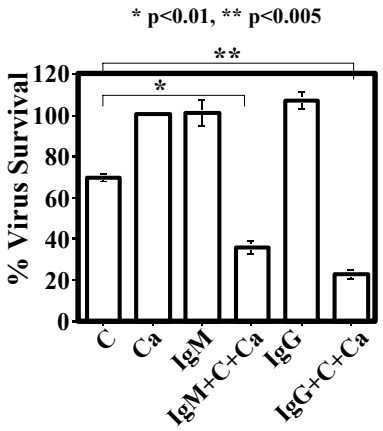


Figure 4 – Purified rat IgG or IgM, complement (C) and calcium (Ca) inactivate HSV vector.

biochemical depletion of Ig in serum reduced vector inactivation, showing that Ig facilitated the observed inactivation[37,68], 3) *In vivo* depletion of complement with cobra venom factor facilitated the initial infection of tumors by oncolytic HSV[68], 4) Antibody-neutralization of human serum with anti-human C1q or anti-human IgM or IgG also reversed the observed vector inactivation (figure 3), 5) Incubation of HSV vector with purified rat IgM or IgG, rat complement, and calcium also inactivated vector transduction (figure 4).

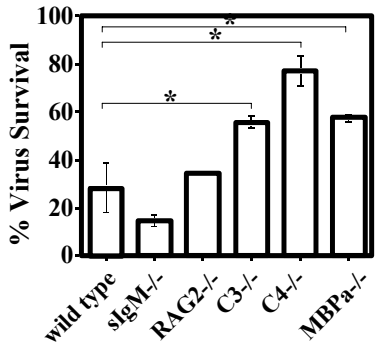


Figure 5 – Sera from C3, C4, and MBP, but not Ig "knock-out" mice neutralizes HSV vector more weakly than sera from sv129 or C57BL/6 wild-type mice. * p < 0.02

Strain-specific effects in the ability of serum to inactivate HSV exist. For example, three different vectors (hrR3, MGH1, and a lacZ-amplicon) derived from three different laboratory strains of HSV (strain KOS, F, and 17^{+}, respectively) were shown to be inactivated by rat serum[68]. However, clinical isolates of HSV, such as strain NS or strain MP, have been shown to be only mildly affected by serum and complement[51]. The unusual sensitivity of laboratory strains of HSV to complement activation provides strong suggestive evidence that their glycoprotein C (gC) is not as active in complement evasion and their gE/gI is not as strong in immunoglobulin evasion as the respective glycoproteins from the NS or MP clinical isolates of HSV. Therefore, we hypothesize that substitution of gC and gE/gI from strain NS into the envelope of HSV vectors could render the latter less susceptible to inactivation and more effective in transducing target cells.

C.5.In mouse, complement activation against HSV vectors occurs also through the Mannose Binding Protein (MBP) or lectin pathway. Further evidence for a role in complement's activation as limiting HSV vector transduction of target cells was provided by analyses of effects using sera from mice with genetic defects in complement function. Sera were collected from "knock-out" mice with homozygous defects in secreted IgM (sIgM -/-)[67,69], in immunoglobulin maturation (RAG2 -/-) (Jackson Laboratories), in C3 (C3 -/-), in C4 (C4 -/-) [70], and in Mannose binding protein (MBPa -/-) (available through Dr. A. Ezekowitz, MGH). Vector neutralization studies showed that 1: 2 diluted sera from mice with complement deficiencies (C3 and C4) were not as active in neutralizing HSV vector as sera from wild-type mice (strain sv129 or C57/BL6) (figure 5). Interestingly, sera from mice with defects in immunoglobulin production (sIgM and RAG2) were as active as control sera in antiviral activity. This indicated that mouse natural IgM or IgG binding to vector was not likely to contribute to the observed antiviral activity. This result thus showed that the mechanism of complement activation against HSV in humans and rats (i.e., complement activation through natural immunoglobulin(s)) was not operative in mice. Further support for MBP's role as an activator of complement against HSV vectors was provided by using mannan (100 μg/ml) to saturate MBP's capacity to bind to HSV. Figure 6 shows that mannan pre-incubation of mouse or rat serum reversed antiviral activity, while it had no effect on the ability of human serum to inactivate HSV vector.

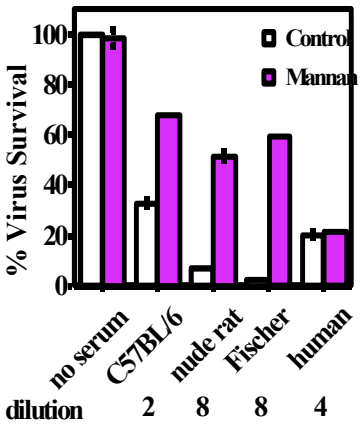


Figure 6 – Mannan pre-incubation reverses the antivector effect of mouse and rat, but not human, plasma. P values for mice and rats were < 0.0001

In summary, although sera from humans, rats and mice inactivate HSV vectors by activation of complement, important differences in the mode of complement activation are evident. In humans, activation occurs primarily through the classical pathway by binding of a "natural" IgM and/or IgG to the virion. In rats, activation occurs both through the classical pathway by binding of a natural Ig to the virion and through the MBP-pathway. In mice, activation appears to occur primarily through activation of the MBP-pathway. The activation of complement in rats through two different mechanisms also explains the relative strength of rat serum in inactivating the HSV vector when compared to mouse or human serum (Figure 1).

C.6.In *vivo* results after complement depletion. Although the aforementioned data showed that complement activation inhibited vector transduction *in vitro*, the significance of this result in an *in vivo* setting was uncertain. Our results suggested that C3 depletion would be expected to improve the transduction of cells in an organ such as brain by an intravascularly administered HSV vector. The above results thus showed that both in rats and mice depletion of complement levels resulted in increased transduction of a reporter gene by an HSV vector. We thus asked if complement depletion would result in increased transduction of cerebral endothelial cells by intravascular HSV vector. The advantage of intravascular administration, particularly as an intra-arterial bolus consists of: 1) delivery to large areas of the brain, 2)delivery to endothelial cells rather than neurons or astrocytes, an important factor for delivery of enzyme isoforms that could have markedly different effects dependent on the cell in which they are expressed[92], and 3) delivery of a neuroprotective transgene into the same arterial circulation affected by ischemia. Rats and mice were thus pretreated with either vehicle or CVF before administration of intravascular HSV amplicon. Animals were then sacrificed and their brain analyzed for lacZ gene expression. Figure 7 shows that endothelial cells in both rats (panels A and B) and mice (panels C and D) were found to express lacZ after CVF administration. Table 4 provides a comparative quantitative analysis of lacZ endothelial cells enumerated over ten high-powered randomly selected sections through the right hemisphere in animals treated with CVF or vehicle. With this method, we did not find evidence for transduction of neurons or astrocytes. This is not surprising since the large size of the HSV amplicon (150 nanometers) impedes its efficient passage across endothelial tight junctions.

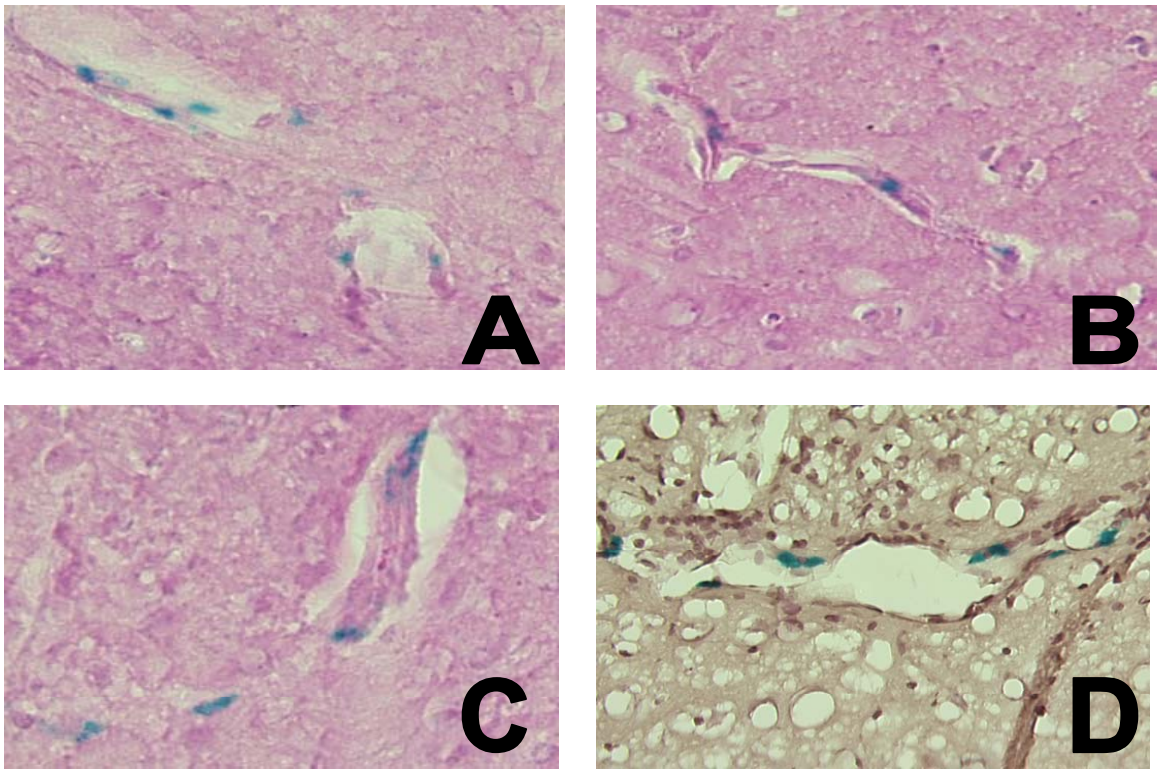


Figure 7 - Rats (panels A and B) and mice (panel C and D) were pretreated with CVF, followed by intra-arterial administration of a LacZ-expressing HSV amplicon. Twelve hours later, animals were sacrificed and cryosections (20 μm thick) were stained for lacZ expression and then counterstained with neutral red. The section in panel D was not counterstained. Shown are representative high-power microphotographs illustrating lacZ gene expression in endothelial cells lining cerebral blood vessels. Quantitative enumeration is provided in Table 4.

Table 4 – Average number of lacZ-positive cerebral endothelial cells per high-power field after amplicon administration into the carotid artery.

CVF	Vehicle
8 +/- 2	0.2 +/-0.1

These findings thus confirm that complement depletion facilitates the infection of endothelial cells in the brain after intra-arterial administration of an HSV vector.

C.7. Personnel involved in preliminary experiments.

Name	Role	Time period

E. A. Chiocca, MD PhD	Principal Investigator	1996-present
Y. Saeki, MD PhD	Postdoctoral Fellow/ Instructor	1997-present
H. Wakimoto, MD	Postdoctoral Fellow	1999-present
K. Ikeda, MD	Postdoctoral Fellow	1996-1999
E. Timynski, BS	Research Technician	1999-present

C.8. Relevant prior publications by PI

1. Pakzaban P. and **Chiocca E.A:** Nerve growth factor protects against herpes simplex virus type 1 neurotoxicity in the rat striatum. **NeuroReport 5**, 993-996, 1994.

2. Muldoon, L.L., Nilaver, G., Kroll, R.A., Pagel, M.A., Breakefield, X.O., **Chiocca, E.A.,** Davidson, B.L., Weissleder, R., Neuwelt, E.A.: Use of monocrystalline iron oxide nanocompound to monitor the distribution of adenovirus and herpesvirus in normal rat brain: comparison of delivery by direct inoculation and osmotic blood-brain barrier disruption. **Am. J. Pathol. 147**: 1840-1851, 1995.

3. Herrlinger, U., Kramm, C.M., Aboody-Gutterman, K.S., Johnston, K., Ikeda, K., Chase, M., Burwyck, J., Barth, R., Finkelstein, D., **Chiocca, E.A.,** Louis, D., Breakefield, X.O.: Pre-existing herpes simplex virus-1 (HSV-1) immunity decreases but does not abolish gene transfer by a HSV-1 mutant vector. **Gene Therapy 5:** 809-819, 1998.

4. Saeki, Y., Ichikawa, T., Saeki, A., **Chiocca, E.A.,** Tobler, K., Ackermann, M., Breakefield, X.O., Fraefel, C.: HSV-1 DNA amplified as a bacterial artificial chromosome in E.coli: Rescue of replication-competent virus progeny and packaging of amplicon vectors. **Human Gene Therapy 9:** 2787-2794, 1998.

5. Neuwelt, E.A., Abbott, N.J., Drewes, L., Smith, Q.R., Couraud, P.O., **Chiocca, E.A.,** Audus K.L., Greig, N.H., Doolittle, N.D.: Cerebrovascular Biology and the various neural barriers: challenges and future directions. **Neurosurgery 44**:604-8, 1999.

6. Ikeda, K., Ichikawa, T., Wakimoto, H., Silver, J.S., Deisboeck, T.S., Finkelstein, D., Harsh G.R. IV, Louis, D.N., Bartus, R.T., Hochberg, F.H., **Chiocca, E.A**.: Oncolytic virus therapy of multiple tumors in the brain requires suppression of innate and elicited antiviral responses. **Nature Medicine 5:** 881-888, 1999.

7. Ikeda, K., Ichikawa, T., Wakimoto, H., Jhung, S., Louis, D.N., **Chiocca, E.A.:** Complement depletion facilitates the infection of multiple brain tumors by intravascular, replication-conditional herpes simplex viral mutant. **J. Virology 74:** 4765-4775, 2000.

D. Research Design and Methods

D.1.Specific Aim 1: Determine the effect of inhibitors of complement activation on HSV amplicon infection of endothelial cells in mouse models of cerebral ischemia (Year 1-3).

D.1.2.Hypothesis: Transient reduction in complement activation increases intravascular HSV-amplicon delivery of a reporter transgene in cerebral endothelial cells in the presence of an ischemic insult and reduces toxic inflammatory reactions in brain and other tissues.

D.1.3.Rationale and Plan: Our published[37,68]and preliminary experiments have determined that complement activates against HSV vectors through the classical pathway in humans and rats and through the MBP pathway in mice and rats. This limits efficient transduction of cells by an HSV vector *in vitro* and *in vivo* and the resulting inflammatory events can be toxic to tissues. However, we do not know if the complement effect is also limiting to HSV vector infection in the presence of an ischemic insult. To maximize infection of cerebral endothelial cells rather than that of other neural cells, the vector will be administered through the carotid artery. We will plan to: 1) Assay reporter transgene delivery into cerebral endothelial cells of mice before and after the induction of an ischemic event, 2) Compare these results with those obtained when complement is reduced in mice, and 3) Confirm the importance of complement in limiting HSV amplicon infection of endothelium in mice using both pharmacologic inhibitors of complement pathways as well as testing mice with genetic defects in C3 and MBP.

D.1.4. Experimental Methods:

D.1.4.1. Determine reporter gene expression in mouse cerebral endothelial cells before and after the induction of an ischemic event (Year 1-2). Note: The methods described in d.1.4.1.1 – d.1.4.1.2 will be employed throughout the course of the project.

D.1.4.1.1. Preparation of HSV amplicons. HSV amplicons expressing the lacZ reporter gene will be prepared to high titer (10^9 tu/ml) by using HSV-BAC-$\Delta 27{-}0^+$ as the helper virus in the co-transfection. The lysate will then be concentrated by ultracentrifugation to achieve the desired titer. Lack of replication-competent virus in the preparation will be assayed by: 1) plaque assays on Vero cells, 2) plaque assays on 2-2 cells (Vero cells stably transfected with an ICP27 cDNA), and 3) failure to detect viral immediate-early (ICP27) and early (ICP6) genes by PCR and Southern blot analyses of PCR amplification products. Purified amplicon preparations will be titered on both Vero and Gli36ΔEGFR cells and titers expressed as transducing units/ml. As an additional vector for our studies, we will also prepare adenoviral vectors will be prepared that possess deletions in both the E1 and E3 genes and a lacZ transgene expressed from a CMV promoter. An additional control vector will consist of a recombinant HSV (Gal4) that possesses a deletion in the ICP4 immediate-early gene, thus rendering it incapable of replication[71]. A lacZ cDNA has been inserted within this region under control of the endogenous ICP4 promoter. This HSV vector is grown in Vero cells stably transfected with ICP4.

D.1.4.1.2. Animal methodologies. C57BL/6 and sv/129 mice will then be purchased through a commercial vendor (Taconic Farms, Germantown, NY). Surgical procedures will be performed in collaboration with Dr. M. Moskowitz's laboratory. Briefly, mice will anesthetized with 2-2.5% halothane and then maintained with 1% halothane/ 70% N_2O/30% O_2. After transoral intubation, mice will be artificially ventilated (SAR-830/P, CWE, Ardmore, PA). End-tidal CO_2 will be monitored using a microcapnometer (Columbus Instruments, Columbus, OH). The femoral artery and vein will be cannulated with a polyethylene catheter (PE-10, Intramedic, Becton Dickinson) for continuous arterial blood pressure and heart rate monitoring. Arterial blood gas and pH will be analyzed at baseline and after each infusion of substances. Rectal temperature will be maintained at approximately 37 C with a thermostatically controlled mat (temperature control, FHC, Brunswick, ME).

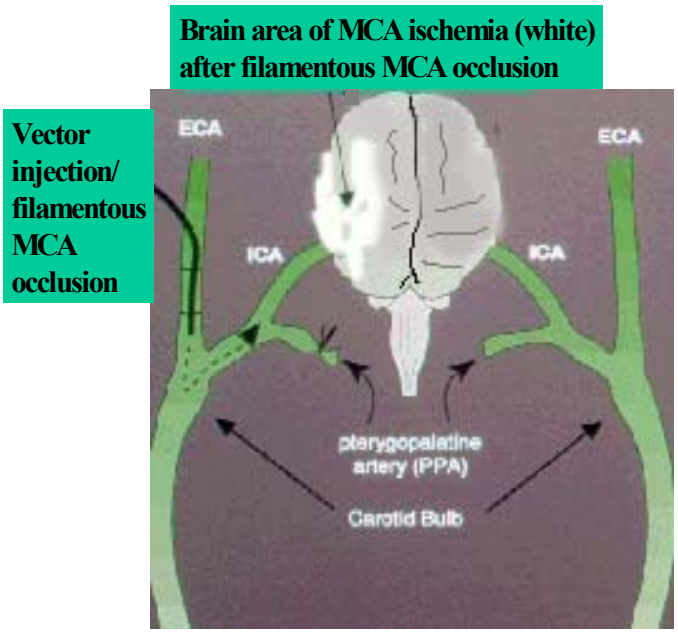


Figure 8 – Description of surgical technique. For both vector and filamentous MCA occlusion the external carotid artery is cannulated. White area of cerebral ischemia is shown if MCA occlusion had occurred.

To produce ischemia in the middle cerebral artery (MCA) distribution, the intraluminal filament technique will be used. Briefly, through a ventral midline incision, the right common and external carotid arteries will be isolated. A microvascular clip will be temporarily placed on the common carotid artery while the pterygopalatine artery will be isolated and ligated (figure 8). The external carotid artery will then be isolated and 8-0 nylon monofilament coated with silicone will be introduced into the internal carotid via the external circulation and advanced 10 mm distal to the carotid bifurcation so as to occlude the MCA. The common carotid artery clip will then be removed. Occlusion will be performed for 2 hours and then 22 hours (or sometimes 70 hours) of reperfusion will be allowed. For injection of amplicon vectors, the cannulated external carotid will be infused with a 100 microliter bolus of amplicon at 10^9 tu/ml. Adenovirus and recombinant HSV will also be injected as a 100 microliter bolus at 10^9 pfu/ml. After infusion, the clip on the common carotid artery is removed to restore blood flow. For most experiments the ECA will be cannulated at two different time periods. We will thus tie off the ECA stump first and on repeat surgical exposure the stump will be sectioned and recannulated, before final ligature.

D.1.4.1.3. Characterization of reporter transgene expression before and after ischemic insult. We will first determine the time course of reporter lacZ transgene expression before and after an ischemic event. Mice will be cannulated and lacZ-amplicons or mock administered at different time periods before and after an ischemic challenge. These time points are listed in Table 5.

Table 5 – Description of animal cohorts treated with intra-arterial vectors before, during and after ischemia.

	Time[A]								
Vector	72 hrs pre	48 hrs pre	24 hrs pre	Same Time	30 min post	1 hr post	4 hr post	24 hrs post	No ische[B]
Amp-LacZ	5[c]	5	5	5	5	5	5	5	5
Mock preparat.	5	5	5	5	5	5	5	5	5
Saline	5	5	5	5	5	5	5	5	5
Adeno-LacZ	5	5	5	5	5	5	5	5	5
HSV Recombin.	5	5	5	5	5	5	5	5	5

[A] Time of vector inoculation pre, post or during the ischemic challenge, [B] Animal cohorts not exposed to ischemic insults. Animals in these cohorts will be treated in parallel with vectors but will not undergo filamentous MCA occlusion, [c] Number of animals in cohort.

Additional experiments and controls will include the use of saline, adenovirus-LacZ, and Gal4, a replication-defective recombinant HSV. The inclusion of these vectors will allow for comparisons between different gene transfer paradigms. The rationale for the inclusion of adenovirus is that our published experiments have shown that complement also inactivates adenovirus vectors as well as infect endothelial

cells[68]. We thus believe that this vector could provide a useful alternative to the HSV amplicon. It is also useful to compare different gene delivery strategies to increase the possibility of experimental success.

After 2 hours of ischemia, reperfusion will be conducted for 22 hours, after which animals will be sacrificed. A list of proposed analyses that will be performed routinely on experimental animals and tissues is summarized in Figure 9. Briefly, cerebral infarct volumes will be determined by computer image analysis of brain sections (2 mm thick) stained with 2,3,5-triphenyltetrazolium chloride (TTC) as well as brain cryosections (20 μm thick) stained with hematoxylin and eosin. Paraffin-embedded sections will also be used to visualize infiltration of lymphocytes, plasma cells, neutrophils, and macrophages. If immune infiltrates appear prominent, then immunohistochemical staining for lymphocytic markers will also be performed to characterize involved populations. To assay for gene transfer, lacZ gene expression histochemistry will be performed on sections by using X-gal as described. Counterstaining with an antibody for rodent and murine endothelial cell antigen can confirm that lacZ expression occurs within endothelium and not adjacent cells. This would be important to determine because NO generation within endothelial cells would be expected to produce a different biologic outcome from NO generation iwthin brain parenchyma[92-97]. Because X-gal histochemistry can underestimate the extent of lacZ gene transfer, immunocytochemistry using an anti-β-galactosidase antibody will also be performed. Computer-assisted image analysis will also be used to quantitate the number of lacZ-positive endothelial cells per high-powered field in both the infarcted and penumbral ischemic zone. These analyses will be performed in the territory of MCA and collateral branches perfusing the brain. Additionally, evidence for LacZ gene transfer will be sought out in normal brain (both ipsilateral and contralateral to the ischemic insult). However, it should be noted that, in our preliminary studies, lacZ gene expression was exclusively observed in endothelial cells when the HSV amplicon was delivered intra-arterially. Additional studies will be performed to detect presence of vector antigens by immunocytochemistry against HSV capsid proteins or the adenoviral penton base. We will also determine presence of lacZ mRNA by semiquantitative RT-PCR. Cerebral hemispheres and aortas will be isolated and frozen, mRNA extracted, and RT-PCR performed using primers that would amplify the lacZ gene product. Internal control primers will be provided by use of primers that would amplify glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Western blot analysis

Figure 9 – A list of analyses performed on tissues and animals undergoing proposed experiments.

1) Infarct volume →	**a) TTC staining** **b) H and E staining**
2) Immune infiltration →	**a) H/E** **b) Immunohistochemistry**
3) Transgene/vector delivery →	**a) Xgal histochemistry** **b) βgal immunohistochem** **c) HSV capsid immuno.** **d) RT-PCR for reporter/viral transgenes** **e) PCR for viral genomes** **f) Western blot for reporter/viral genes** **g) eNOS activity**
4) Therapeutic outcome →	**a) Neurologic grading**
5) Physiological variables →	**a) MAP** **b) Heart rate** **c) pH** **d) $p0_2$/ $paCO_2$** **e) Absolute and relative CBF** **f) Temperature**
6) Toxicity analysis →	**a) Histology of other organs (brain, liver, lung, spleen)** **b) Immuno for lacz, viral antigens, immune cells** **c) PCR and RT PCR viral genes, reporter transgene** **d) Cytokine profile**

for β-galactosidase expression will provide a semi-quantitative estimate of reporter gene expression in ischemic/infarcted brain, ipsilateral normal brain, and contralateral ischemic brain. To further measure NOS activity, we will also assay the conversion of ^{3}H-L-arginine to ^{3}H-citrulline in lysates from treated and untreated brains in the presence of calcium[99]. Although the majority of the NO activity is likely to derive from nNOS rather than eNOS, we may be able to detect even a small difference after gene transfer. This assay is routinely performed in the laboratory of our collaborator (Dr. M.A. Moskowitz) and we will avail ourselves of his expertise for its performance. Cytokine profiles (IL6, 10, 2, 4 and TNFα and IFNs) in blood will be conducted as part of a toxicity screen. Taken in conjunction, these experiments will provide a semiquantitative estimate of reporter transgene expression by measuring mRNA and protein levels as well as a quantitative estimate of anatomic expression of the transgene within cerebral areas affected by the ischemic insult as well as control areas of brain. These studies will thus show if there are qualitative and quantitative differences in the levels and anatomic areas of reporter gene transfer before and after ischemia.

D.1.4.1.4.Limitations and alternatives. The rationale for vascular delivery is to target multiple cerebral endothelial cells located in the brain areas affected by ischemia. . This would be our first approach because our pilot data shows that intra-arterial delivery allows for infection of endothelial cells almost exclusively, while intracerebral delivery might lead to neuronal eNOS expression. However, it is possible that the aforementioned proposed studies will not show evidence of reporter gene transfer in endothelium subjected to an ischemic injury although it may show evidence of gene transfer in normal endothelium, as shown in our preliminary data. Studies have suggested the existence of residual blood flow (15% of baseline) after focal MCA occlusion and even greater blood flow (15-40%) during reperfusion (Dr. M. Moskowitz, private communication), but it is still possible that vector delivery through ischemic vasculature could be reduced. The alternative approach would consist of employing direct intracerebral stereotactic inoculation of vectors whose transgene(s) is under control of an endothelial-specific promoter, such as the eNOS[72,73], the Von Willebrand Factor[74], or the tie[100] promoters. An amplicon HSV or adenovirus vector with lacZ cDNA expression being driven by the promoter would likely target endothelial cells for reporter gene expression in cerebral areas affected by ischemia. This alternative approach could thus be easily implemented if intravascular delivery does not appear to efficiently infect ischemic cerebral endothelial vasculature. Another limitation may consist of the 24-hour time point that we selected as the end-point assay of our study. It is possible that reporter gene expression could change at later time periods because of physiologic and molecular alterations associated with an evolving ischemic infarct. We thus would plan to repeat the experiment described in Table 5 and perform the end-point assays described in d.1.4.1.3. on animals sacrificed 48 and 72 hours after the onset of the ischemic challenge.

D.1.4.2. Determine the effect of complement inhibition on reporter gene expression in mouse cerebral endothelial cells, before and after the induction of an ischemic event (Year 2-3).

D.1.4.2.1. Effect of CVF on reporter gene expression. Complement can be depleted *in vivo* with a variety of pharmacologic and peptide inhibitors such as cobra venom factor (CVF), soluble complement receptor 1 (sCR1) and derivatives, and C1 Inhibitor (C1 INH). Each of these factors may have different side effects in mice. We thus plan to test the effect of each inhibitor on the time course of reporter gene expression in endothelial cells in the context of cerebral ischemia. For the purposes of grant writing, we will assume that the experiments described in D.1.4.1.3. will show convincing evidence of endothelial gene transfer after intra-arterial delivery, but clearly we are prepared to employ direct stereotactic delivery into the brain as an alternative approach as outlined in D.1.4.1.4. We will first determine the effect of complement inhibition, mediated by CVF, on the efficiency and anatomic extent of reporter transgene delivery after intra-arterial HSV amplicon injection. The experiment depicted in table 5 will be repeated with animals that have been pre-treated with intraperitoneal CVF on day –1 (60 units/kg, i.p.) and day 0 (20 units/kg, i.p.) of virus

injection. Tissue, gene transfer, neurologic outcomes, and physiologic analyses, performed as described in Figure 9, will provide us with a detailed comparison of the efficiency of gene transfer in the presence or absence of CVF. Monitoring of physiologic variables will also ensure that observed differences are not due to CVF effects on blood pressure, cerebral blood flow, or heart rate.

D.1.4.2.2 Limitations and alternatives to CVF. Although our pilot studies reveal a significant increase in endothelial reporter gene expression after CVF treatment, CVF possesses systemic side effects that are not tolerable in larger vertebrates and humans. Therefore, if the CVF experiment does show a significant difference in ischemic endothelial cells, additional peptide agents that possess less systemic side-effects

Table 6 - Description of experiment to determine effect of CVF on transgene delivery.

	Time[A]								
Vector	72 hrs pre	48 hrs pre	24 hrs pre	Same Time	30 min post	1 hr post	4 hr post	24 hrs post	No ische[B]
Amp-LacZ	5[c]	5	5	5	5	5	5	5	5
Mock preparat.	5	5	5	5	5	5	5	5	5
Saline	5	5	5	5	5	5	5	5	5
Adeno-LacZ	5	5	5	5	5	5	5	5	5
HSV Recombin.	5	5	5	5	5	5	5	5	5

[A] Time of vector inoculation pre, post or during the ischemic challenge, [B] Animal cohorts not exposed to ischemic insults, [c]Number of animals in cohort.

could also be tried. These would include sCR1 and its derivatives[75,76]. sCR1 has been made available to us by Avant Pharmaceuticals (Needham, MA).While CVF provides generalized inhibition of complement function in treated animals as evidenced by its ability to decrease total complement hemolytic activity, questions may arise related to side-effects of this factor on animal physiologic parameters (blood pressure, blood vessel permeability, or heart rate). Therefore, more specific inhibitors of the complement system can be used first to confirm the veracity of the experiments described above and then to eliminate the function of the classical, alternative, or lectin pathways in a relatively selective manner. Soluble complement receptor 1 (sCR1) binds C3b, the activated form of C3, and promotes its inactivation by factor I. It has been shown to not affect pulmonary and cardiac physiologic parameters[20,77,78]. We will thus repeat the set of experiments described above using an infusion of sCR1 at 15 mg/kg instead of CVF. These studies will thus provide confirmation of selective interference of the complement system leading to enhanced viral infection of cerebral endothelial cells.

D.1.4.2.3. In vivo pharmacologic confirmation of MBP-pathway as major activator of complement in mice. While our pilot results described in section c. showed that in mice the MBP-pathway is the major activator of complement against HSV vectors, we plan to confirm these results *in vivo*. First, we can use selective inhibitors of different components of the complement cascade. To selectively inhibit the classical component of the complement cascade, we will use purified mouse C1 inhibitor protein (C1 INH), available though our collaborator, Dr. Michael Carroll (Harvard Medical School). Upon binding of antibody to antigen, the classical pathway becomes activated by binding of the C1 complex to the Fc region of IgG or IgM, which in turn activates C2 and C4 forming the C3 convertase enzyme (C2aC4b) that then activates the central component C3. The serine protease inhibitor, C1 INH functions by irreversibly binding C1, thus shutting off further activation of the classical cascade, tightly regulates the levels of the C1 complex. Previous studies have employed two doses of 100 μg of C1 INH administered to animals two and five hours after the time of challenge with pathogens. We will first measure serum C1 INH levels in mice at the time points shown in Table 6 by using a commercially available kit (Immunochrom C1 INH, Immuno AG, Vienna, Austria). Activation of the classical cascade leads to depletion of C1 INH due to its irreversible

binding to activated C1 complexes. We will then administer purified C1 INH and expect to see increases in the levels of C1-INH. We will then ask if there is an increase in the level of intracerebral endothelial cell transduction in mice treated with C1 INH and the viral vectors described in Table 6, using assays described in previous sections. Quantitative comparisons with results obtained with CVF and sCR1 should provide an estimate of the contribution of the classical pathway of complement to viral inactivation.

We will then attempt to determine if the alternative pathway of complement activation contributes to these events. A sCR1 deleted in the C4b binding region (designated as sCR1desLHR-A) has been described with properties that allow for relatively selective in vitro and in vivo inhibition of the alternative complement pathway[79-81]. This compound is available through Avant Pharmaceuticals (Needham, MA). We will thus compare the effect of inhibition of the alternative pathway of complement activation with inhibition of the classical pathway as described above by performing the experiment described in Table 6 in the presence of sCR1desLHR-A.

Finally, we will characterize contributions of the lectin or mannan pathway of complement activation. Mannan-binding protein (MBP) has been shown to possess calcium- and complement-dependent activities against influenza, HIV as well as other pathogens[43,82]. MBP binding to mannose-rich polysaccharides on the surface of pathogens, allows for calcium-dependent interactions with MASP-1 and –2, two MBP-associated serine proteases with structural and functional similarities to C1q and C1s (see diagram in section b), leading to activation of C2 and C4 and subsequent C3 convertase activity. A monoclonal antibody against mouse MBP is available through Dr. Alan Ezekowitz (Massachusetts General Hospital). We will determine if injection of this antibody depletes MBP in vivo and if MBP depletion augments the infection of cerebral endothelial cells by intravascular HSV. Alternatively, *in vivo* injection with mannan can be performed to saturate MBP and then determine whether injected vectors are more efficient in their transduction of cerebral endothelial cells. Comparative analyses with results obtained for depletion of classical and alternative pathways of complement activation should thus permit us to characterize the *in vivo* contribution of each towards limiting gene transfer.

D.1.4.2.4. In vivo genetic confirmation of MBP as the major complement activator in mice. To provide further confirmatory evidence related to complement's role in limiting oncolytic virus infection, mouse "knock-out" models will be used. Mice with homozygous genetic defects in the C3 or C4 component of complement will be employed for these studies. These mice are available through Dr. Michael C. Carroll (Harvard Medical School). In vitro experiments, we have tested the antiviral activity of wild-type mice (strain C57BL/6 and 129). Figure 2 shows that this activity is present in mice and figures 5 and 6 show that *in vitro* this activity is primarily due to MBP. We will thus employ C3 and MBP knock-out mice and ask if endothelial cell transduction as well as the end-point correlates described in Figure 9 differ in these mice when compared to wild-type mice.

D.2. Specific Aim 2: Determine if addition to the amplicon envelope of HSV's glycoprotein C, known to evade complement, will increase the efficiency and safety of gene transfer (Year 2-3).

D.2.1. Hypothesis: Addition of wild-type glycoprotein C to the envelope of the HSV amplicon allows for complement evasion and increased transgene expression.

D.2.2. Rationale: Fresh clinical isolates of wild-type HSV1 have been shown to effectively evade complement's ability to destroy the virion and the infected cell through the binding of gC to C3[51]. However, the findings described in the pilot data and in published experiments[68] show that complement evasion mediated by HSV vectors derived from laboratory strains has become attenuated through their long-

term passages in culture. The effect of tissue culture passage on gC's ability to evade complement has been noted in the past[48]. This suggests that gC in our vectors is likely to be mutated. We thus would plan to test the hypothesis that substitution of the gC from the HSV amplicon (strain 17^+) with the gC from a stock of a recent clinical isolate of HSV (strain NS) will increase the HSV amplicon's ability to evade complement and thus increase transduction of cerebral endothelial cells.

D.2.3. Experimental Methods-

D.2.3.1. Substituting gC from strain NS into the gC from HSV-BAC-Δ27-0$^+$. HSV 1 strain NS (low passage clinical isolate of HSV1) viral DNA will be purified by established methods. PCR primers spanning the gC region will be designed and used to amplify gC from MP. After subcloning into plasmid pBluescript II, gC will be sequenced. By *recA*-mediated recombination, we will delete the gC gene (strain 17) from HSV-BAC-Δ27-0$^+$ and insert a *loxP* site into the deleted gC, using the methods described in figure 1. After ensuring the correct genetic identity of this novel BAC, we will then design a novel targeting plasmid that bears the NS gC and then by *Cre*-mediated recombination, we will proceed to subclone the NS-gC into HSV-BAC-Δ27-0$^+$. This new BAC will be designated HSV-NS-BAC. To ensure that secondary unwanted mutations are not placed into this BAC, we will also derive HSV-BAC-Δ27-0$^+$ back from HSV-NS-BAC and designate this revertant BAC as HSV-BAC-Δ27-0$^+$ REV. Separate LacZ-expressing amplicons will then be packaged using all three BACs (HSV-BAC-Δ27-0$^+$, HSV-NS-BAC, and HSV-BAC-Δ27-0$^+$REV). These amplicons will be designated 17-amplicon, NS amplicon, and 17rev amplicon, respectively. Therefore, amplicons packaged using HSV-NS-BAC as the helper would be expected to possess the complementing-evading gC from NS strain into their envelope, while those packaged using HSV-BAC-Δ27-0$^+$ and HSV-BAC-Δ27-0$^+$ REV are expected to possess the gC from 17^+ strain.

D.2.3.2. Evaluating the complementing-evading properties of amplicon preparations. We will then proceed to test if the three amplicon preparations possess different complement-evading properties by assaying *in vitro* viral neutralization. Each of the 3 amplicon preparations will be pre-incubated with different dilutions (undiluted, 1:2, 1:4, 1:8, 1:16) of mouse, rat or human plasma (lacking HSV neutralizing antibodies) for one hour before adding onto Vero cells. Sixteen hours later lacZ-expressing cells will be counted. We expect to observe that amplicons packaged by HSV-NS-BAC will not be neutralized or will be neutralized much less than amplicons packaged by HSV-BAC-Δ27-0$^+$. No differences in antiviral activity of plasma against amplicons packaged by HSV-BAC-Δ27-0$^+$ and HSV-BAC-Δ27-0$^+$ REV are expected. This would provide strong evidence that the substituted gC from NS strain is completely responsible for the complement-evading ability of the amplicon packaged with HSV-NS-BAC.

D.2.3.3.Determine if the amplicon with gC from NS is able to infect and express endothelial cerebral cells upon intra-arterial administration, in the absence of complement depletion. We next will seek to determine if substitution of the gC from the NS strain into the amplicon envelope provides for increased infection and transgene expression even in the absence of pharmacologic or genetic depletion of complement. We will thus administer NS-amplicon, 17-amplicon, or rev17-amplicon into the carotid circulation of mice and then assay reporter gene expression in endothelium 12, 24 and 72 hours after injection. We expect to find that animals injected with the NS-amplicon will exhibit a significant increase in the number of endothelial cells expressing lacZ as well as in the anatomic area of transgene expression. As an additional control, CVF will be given to some animals to show that 17-amplicon and 17rev-amplicon treated animals exhibit an expected increase in the number of transduced endothelial cells. Finally, we will repeat the same experiment using the C3-/- and the MBP-/- knock-out mice to show that, while 17-amplicon and 17rev-amplicon exhibit an expected increase in transduction efficiency, the NS-amplicon does not.

Table 7 – *In vivo* testing of the transduction efficiency of amplicon possessing gC from NS versus that of amplicon possessing gC from 17 strain.

	Time		
	12 hours	**24 hrs**	**72 hrs**
NS-amplicon	5 mice	5	5
17-amplicon	5	5	5
17rev-amplicon	5	5	5
Mock	5	5	5

Taken in conjunction, these experiments should provide convincing evidence that the amplicon with NS gC in its envelope allows for more effective complement evasion *in vivo*, thereby producing increased transduction of endothelial cells in the brain.

We will then determine if the expected increase in expression of the reporter transgene with the NS-amplicon occurs also in the presence of an ischemic insult. We thus will inject the amplicons before and after MCA occlusion and then sacrifice mice 24 hours after the ischemic event to ask if there is evidence of reporter transgene expression. Additionally, a set of animals will be treated as described in Table 8 and sacrificed at 72 rather than 24 hours after MCA occlusion to determine how long transgene expression is sustained.

Table 8 - *In vivo* testing of the transduction efficiency of amplicon possessing gC from NS versus that of amplicon possessing gC from 17 strain before, during, and after MCA occlusion

	Time[A]								
Vector	72 hrs pre	48 hrs pre	24 hrs pre	Same Time	30 min post	1 hr post	4 hr post	24 hrs post	No ische[B]
NS-Amplicon	5[c]	5	5	5	5	5	5	5	5
17-Amplicon	5	5	5	5	5	5	5	5	5
NSrev-Amplicon	5	5	5	5	5	5	5	5	5
Mock	5	5	5	5	5	5	5	5	5

[A] Time of vector inoculation pre, post or during the ischemic challenge, [B] Animal cohorts not exposed to ischemic insults, [c]Number of animals in cohort.

We thus expect that the sum of the described experiments will determine with a high degree of certainty if inclusion of the gC from NS strain in the amplicon envelope provides increased endothelial cell transduction. This knowledge would significantly increase our ability to engineer HSV amplicons with increased effectiveness not only for cerebral models of ischemia, but also for other disorders of the central nervous system. Histopathologic analyses of other tissues (liver, spleen, lung, kidneys), as described in figure 9, will also provide a determination of possible toxic effects to other organs and help determine potential toxicities with this novel amplicon.

D.2.3.4. Limitations and alternatives. Failure to find a significant increase in transduction efficiency with the described NS-amplicon strategy might be due to several factors. One possible problem might relate to the relatively low level of gene transduction efficiency upon intravascular delivery. As discussed in previous sections, stereotactic delivery of the vectors could thus be used as an alternative delivery strategy. It is also possible that evasion of complement may not be sufficient to increase by itself to increase cerebral vasculature transduction. For instance, MBP binding to the vector could limit efficient infection. This could be addressed by saturating MBP *in vivo* with mannan. Finally, osmotic agents, such as mannitol, have been given by some investigators as a means to open the blood-brain-barrier and increase delivery of small molecules[83,84]. Although agents, such as viral vectors, possess a diameter that may be too large for passage even across endothelial tight junctions (for instance, HSV virions measure approximately 140-150 nm in

diameter), shrinkage of endothelial cells by the extracellular osmotic agent could expose a larger surface area to the intravascular virus, thereby enhancing infection efficiency. However, this could increase the number of transduced neurons and astrocytes[83-84], an undesirable outcome for delivery of eNOS. We thus could evaluate if an increase in endothelial cell transduction by administering mannitol at the time of HSV-amplicon intra-arterial injection is offset by an undesirable increase in transduction of neurons. Experiments described in Tables 7 and 8 will thus be repeated in the presence of mannitol to determine if gene transfer efficiency can be improved.

D.3.Specific Aim 3 Determine if evasion of neutralizing immunity to HSV will increase the efficiency and safety of gene transfer. (Year 2-4).

D.3.1. Hypothesis: Neutralizing antibodies to HSV vectors limit the infection of cerebral endothelial cells and reduction of HSV neutralizing antibody activity permits for increased transgene expression.

D.3.2. Rationale: Although studying the effect of innate immune molecules such as complement is useful for an understanding of the mechanisms involved in limiting gene transfer and is relevant to humans that do not possess neutralizing immunity to HSV, a majority of humans do possess neutralizing antibodies against HSV. In specific aim 1, we tested the hypothesis that activation of complement through the MBP pathway is a primary limiting factor in infection of cerebral endothelial cells in mice by intravascular vector in the absence of pre-existing HSV humoral immunity. In aim 2, we propose to generate a novel amplicon with the complement-evading properties of gC-NS. In this aim, we will expand the work described in aims 1 and 2 by considering the effect of pre-existing HSV neutralizing antibodies in limiting amplicon vector infection of cerebral endothelial cells and then pharmacologically reducing the antibody response to HSV. We will also engineer an amplicon that expresses the gE/gI envelope glycoproteins from strain NS, shown to evade immunoglobulin by the process of antibody bipolar bridging[85]. In this process, the Fab portion of the neutralizing antibody binds to HSV epitopes, while the Fc portion binds to gE/gI. Bipolar bridging on HSV's surface inhibits antibody-dependent cellular cytotoxicity[55], complement-enhanced antibody neutralization[86], and attachment of granulocytes to the Fc domain[87]. This Ig-evading amplicon will be tested *in vitro*, but not *in vivo* because gE/gI function as Fc receptors for human but not murine IgG[88].

D.3.3 Experimental Methods-

D.3.3.1. Determination of neutralizing antibody class in mice (Year 1). We first will seek to determine an effective vaccination paradigm in mice that will produce neutralizing immunity to HSV and then we will determine the Ig class of these antibodies. By ELISA, we will first quantitate the amount of HSV antibodies in the serum of C57/BL and sv 129 immunocompetent mice, and, as a control, immunodeficient RAG2 -/- mice. The availability of mice with genetic defects in Ig production will also provide confirmatory evidence for the effects of neutralizing humoral responses against the virus. Mice with homozygous genetic defects in recombinant activating gene- 2 (RAG-2) are deficient in total serum Ig and B cells and can be purchased through the Jackson Laboratories. Mice will undergo subcutaneous injection of the replication-conditional HSV, hrR3, (shown in our ref. 4 of section C.8 to elicit the production of HSV antibodies) or a mock preparation. This injection will be repeated a week later. Serum samples will be collected the day before, the day of and then on days 2, 4, 7, 10, 14, 21, and 30 after the last vaccination. To quantitate the antibody response, a standard curve will have to be generated for known concentrations of mouse antiHSV reacted to goat anti-rat Ig and then measured by ELISA. Then, wells of Immulon II plates (Nunc, Denmark) will be incubated with 10^{10} pfus of HSV. After the virus has adsorbed, wells will be blocked with 1% BSA and then 100 µl aliquots of various dilutions of immunocompetent or athymic rat serum will be added to the wells containing HSV. After overnight incubation, the plates will be rinsed with PBS and 0.05% Tween-20, before incubation with goat anti-rat immunoglobulin conjugated to horseradish peroxidase. After rinsing

the plates, substrate is added and then read on a microplate reader. Comparing the obtained value with the standard curve will provide a quantitative value of the amount of anti-HSV antibodies elicited by subcutaneous injection of hrR3. Previous experiments with neutralizing serum titers suggested that elicited humoral responses against HSV were relatively rapid and quantitatively large by day 7-14 after subcutaneous injection (see ref. 4 in section C.8). We will then determine the class of elicited humoral responses. A Western blot of HSV proteins will be probed with serum from immunocompetent and with serum from RAG2-/- mice collected at different time points after HSV injections. These blots will then be probed with a secondary antibody that consists of rabbit anti-mouse IgM or anti-mouse IgG conjugated to alkaline phosphatase. Detection of specific binding to HSV glycoproteins that are major antigenic determinants of HSV's envelope and reaction to antiIgM or antiIgG will thus determine the humoral antibody class elicited in mouse serum at different time points after HSV administration.

d.3.3.2. Effect of pre-existing immunity on infection of cerebral endothelial cells by intravascular HSV amplicon (YEAR 2-4). We then will seek to determine the impact of pre-existing humoral immunity to HSV in our experimental paradigm. We will assume that in specific aim 2 the NS-amplicon did show increased efficiency of gene transfer compared to the 17-amplicons. Mice with known neutralizing antibodies to HSV will undergo injections of the NS-amplicon or mock and then they will be sacrificed 12, 24, and 72 hours later to assay for gene transfer.

Table 9 – Strategy to determine the effect of neutralizing antibodies on vector infection

Mouse strains used for experiment	Time of sacrifice after intra-arterial NS-amplicon or mock		
	12 hrs	24 hrs	72 hrs
C57/BL6 mice	10[A]	10	10
Sv129	10	10	10
RAG2 -/-	10	10	10

[A] **Number of treated mice (5 with NS amplicon and 5 with mock).**

Analyses for reporter transgene expression will thus provide a quantitative estimate of the effect of neutralizing immunity on amplicon-mediated transduction of cerebral endothelial cells. We expect that a limiting effect will be observed. The use of RAG2 -/- mice should confirm that inhibition of Ig synthesis leads to increased efficiency of transduction by the NS-amplicon. If this is the case, then additional interventions will have to be considered to restore the ability of the NS-amplicon to efficiently infect the cerebral vasculature.

D.3.3.3. Pharmacologic inhibition of immunoglobulin synthesis to improve gene transfer. If a limiting effect is observed, we will then try to determine if pharmacologic inhibition of immunoglobulin synthesis can be used as a means to restore transduction of endothelial cells. Cyclophosphamide is a potent inhibitor of B cell maturation and Ig synthesis. We have previously shown that it can be used in rodents to eliminate the rise in HSV neutralizing antibodies and in a model of intracerebral tumor formation it has been used to increase HSV-mediated infection and lysis of the neoplasm[37]. We first will ensure that CPA injection (200 mg/kg, i.p.) reduces the titer of neutralizing antibodies in mice vaccinated with hrR3. ELISA will be performed on the serum of mice 2, 4, and 8 days after CPA treatment. If needed, an additional treatment with CPA can be performed to reduce even further the level of HSV antibodies. We expect this to be effective based on our previous results, but, if needed, additional anti-humoral agents can be tried. We then will repeat the experiment described in Table 9 to determine if pre-treatment with CPA can suppress the effect of neutralizing immunity. Mice with reduced levels of antiHSV will receive an intra-arterial administration of the NS-amplicon and then sacrificed at 12, 24, and 72 hours to assay for extent and efficiency of reporter transgene delivery. These experiments thus will clarify the role of neutralizing

antibodies in limiting amplicon infection and reporter gene delivery in cerebral endothelial cells and, if this role is found to be limiting, will provide a pharmacologic means to circumvent this.

D.3.3.4.Effect of pre-existing immunity on infection of cerebral endothelial cells after MCA occlusion. We will then repeat the experiment described in Table 9 in the context of an ischemic insult. If neutralizing immunity is found to not significantly alter vector infection as described in section d.3.3.2, then mice with neutralizing immunity to HSV will be injected with the amplicon in the context of an MCA occlusion followed by reperfusion for 22 hours or 70 hours. Animals will then be sacrificed and reporter transgene expression in endothelial cells assayed. If neutralizing immunity is found to be limiting to transgene expression, then pre-treatment with CPA will be performed to reduce the levels of neutralizing antibodies before repeating the experiment described in Table 10.

Table 10 - Description of cohorts and number of mice with neutralizing immunity to HSV treated with intravascular NS-amplicon in the context of MCA occlusion.

	Time[A]								
Mouse strain	72 hrs pre	48 hrs pre	24 hrs pre	Same Time	30 min post	1 hr post	4 hr post	24 hrs post	No ische[B]
C57/BL6	10[c]	10	10	10	10	10	10	10	10
Sv129	10	10	10	10	10	10	10	10	10
RAG2 -/-	10	10	10	10	10	10	10	10	10

[A] Time of vector inoculation pre, post or during the ischemic challenge, [B] Animal cohorts not exposed to ischemic insults, [c] Number of animals in cohort.

Taken in conjunction, these studies should provide a clear indication of the effect of neutralizing immunity on gene transfer in the context of cerebral ischemia. They also will provide potential pharmacologic solutions to improve the efficiency of gene transfer, if neutralizing immunity is found to be limiting.

D*.3.3.5. Re-engineering the NS-amplicon for effective evasion from immunoglobulins.* The finding that pharmacologic reduction of immunoglobulin can augment amplicon-mediated expression of a reporter transgene would suggest that further re-engineering of the NS-amplicon might be a strategy to more effectively evade natural and elicited immunoglobulins. In fact, clinical isolates of HSV have been shown to bind to the Fc region of human IgG through an interaction with the viral glycoproteins E/I (gE/gI) and by a process known as "bipolar bridging" this interaction reduces the Ig's antiviral effects manifested as antibody-dependent cellular toxicity, complement recognition and activation, and opsonization[85]. Therefore, we would plan to engineer an amplicon whose envelope possesses NS gE/gI in its envelope. By PCR, we would proceed to subclone gE/gI from NS strain and then subclone it into pBlueScript II. By *recA*-mediated recombination, we would then substitute the gE/gI genes in HSV-BAC-Δ27-0^{+}-NSgC with the ones subcloned from NS. The new BAC (and its control revertant) will then be used to package a novel amplicon whose envelope possesses gE/gI from NS strain, shown to evade Ig in a relatively effective manner. We would then test the ability of this novel amplicon (NSgE/gI-amplicon) to evade human Ig *in vitro,* by adding human antiHSV IgG (100 μg/ml) together with 10^4-10^5 tu of the NSgE/gI amplicon, NS-amplicon, or 17-amplicon. Purified human complement or heat-inactivated complement will then be added and neutralization of amplicon infectivity measured. We expect that NSgE/gI amplicon's infectivity should be minimally reduced by antiHSV, while that of NS- and 17-amplicon will be greatly inhibited by several logarithmic units. Because gE/gI provide an effective receptor for the Fc portion of human, but not mouse, IgG, these experiments will produce an amplicon (NsgE/gI-amplicon) whose activity can be tested *in vitro,* but not in mouse models of cerebral ischemia[88].

D.4.Specific aim 4: Determine if endothelial nitric oxide synthase (eNOS) gene transfer protects mice from cerebral ischemia (Year 3-5).

D.4.1. Hypothesis: HSV amplicon-mediated delivery of eNOS into cerebral vasculature protects mice from cerebral ischemia.

D.4.2. Rationale: The experiments depicted in specific aims 1 through 3 will elucidate the role of complement and neutralizing HSV immunity in limiting the efficacy of intravascular amplicon in transducing cerebral endothelial cells and in producing undesired inflammatory reactions. Potential solutions will be sought out. These will include use of pharmacologic inhibitors of complement and immunoglobulin function as well as the design of novel amplicons whose envelopes will possess glycoproteins from virulent clinical isolates of HSV, known to effectively evade the antiviral activity of both complement and immunoglobulins. Reporter transgene expression and transfer will be evaluated in wild-type mice, in mice with genetic defects in complement and Ig function, and in mice exposed to MCA occlusion to determine the best strategy for efficient transfer into the cerebral vasculature. The sum of these findings will provide the basis for testing the hypothesis that transfer of the eNOS cDNA into endothelial cells reduces the cerebral volume of infarcts and improves animal neurologic outcomes. This hypothesis is based on published data by our collaborator, Dr. Michael A. Moskowitz (MGH), and others who have shown that elevation of eNOS gene expression by chronic and prophylactic administration of HMG-CoA reductase inhibitors or provision of eNOS substrates correlates with neuroprotective effects in mouse stroke models[6,8,7]. Since neither drug functions at the level of transcription, rather increases eNOS activity by post-transcriptional and post-translational mechanisms[5], we will also test the hypothesis that the combination of eNOS gene transfer, HMG-CoA reductase administration, and eNOS substrate infusion exhibits additive or supra-additive therapeutic benefit in ischemia.

D.4.3.Experimental methods:

D.4.3.1. Generation of eNOS amplicons. We will assume that the NS-amplicon, described in specific aim 2, provided the most efficient transduction of cerebral endothelial cells. We will thus subclone into it the murine eNOS cDNA under control of the CMV promoter. After packaging the amplicon, sequence analysis will be performed to ensure the correct genetic identity of the subcloned insert. We will then determine if *in vitro* infection of endothelial cells results in eNOS overexpression. Human vein endothelial cells (HUVEC) and bovine aortic endothelial cells will be infected with the eNOS amplicon or a control amplicon that expresses GFP. Since both amplicons possess lacZ, efficiency of infection can be assayed by Xgal staining. RT-PCR and Western blot analyses will be carried out in infected cells to evaluate eNOS gene expression. Taken in conjunction, these studies should provide an indication of the ability of amplicons to efficiently deliver the therapeutic transgene in endothelial cells. If needed, additional amplicon constructs (or adenoviral vectors) could be generated using endothelial-specific promoters to target eNOS gene expression more specifically to endothelial cells.

D.4.3.2. Determine if eNOS gene transfer into cerebral endothelial cells reduces the size of cerebral infarcts and improves neurologic deficits. We then will optimize eNOS gene transfer to augment CBF. This will allow us to determine if *in vivo* transfer of the eNOS cDNA results in a reduced infarct volume upon MCA occlusion. The experiments described in aims 1-3 will tell us if an amplicon engineered with glycoproteins that evade the immune response also leads to more efficient infection of cerebral endothelial cells. The time courses described in Tables 5-10 also will provide us with a quantitative estimate of the levels and anatomic extent of reporter transgene expression in control mice versus mice that are undergoing a cerebral ischemic event. We will thus repeat the same experiment using the amplicon that expresses

eNOS. As controls, we will also inject an amplicon that expresses lacZ, or mock. To determine if eNOS cDNA delivery by other vectors is also effective, we will also test an adenovirus-eNOS construct.

Table 11 - Description of cohorts and number of treated animals per experiment.

	Time[A]								
Vector	72 hrs pre	48 hrs pre	24 hrs pre	Same Time	30 min post	1 hr post	4 hr post	24 hrs post	No ische[B]
Amplicon-eNOS	10[c]	10	10	10	10	10	10	10	10
Amplicon-lacZ	10	10	10	10	10	10	10	10	10
Adenovirus-eNOS	10	10	10	10	10	10	10	10	10
Adenovirus-lacZ	10	10	10	10	10	10	10	10	10
Mock	10	10	10	10	10	10	10	10	10

[A] Time of vector inoculation pre, post or during the ischemic challenge, [B] Animal cohorts not exposed to ischemic insults, [c] Number of animals in cohort.

Animals will then be sacrificed 24 hours after MCA occlusion and 22 hours after reperfusion. We will then determine the area of cerebral infarct by using TTZ staining and hematoxylin and eosin staining. This will tell us if eNOS gene transfer reduces infarct size in mice 24 hours after MCA occlusion. Additional analyses will be carried out as described in Figure 9. We will further determine if a similar result occurs 72 hours after MCA occlusion, by repeating the experiment shown in Table 11. Results from this experiment will determine if any reduction in infarct size observed at 24 hours is also present at 72 hours and thus show if effects of eNOS gene transfer are sustained for a relatively long-term.

We will also determine if eNOS gene transfer results in improved neurologic function in mice after MCA occlusion, when compared to mice treated with control lacZ gene transfer. A grading scale will be employed in which deficits are evaluated in a blind fashion by an observer. This scale ranges from 0 (no deficit), 2 (animals turns towards the ipsilateral side), 3(animal spins longitudinally) and 4 (unresponsive to noxious stimulus)[89]. This grading thus will be performed on the animals shown in Table 11 before sacrifice.

D.4.3.3. Does eNOS gene transfer alter physiologic parameters? To determine if eNOS gene transfer is associated with alterations in physiologic parameters, we will measure mean arterial blood pressure (MABP), heart rate, blood pH, blood PaO_2, and blood $paCO_2$ before, during, and after infusion of vector and/or production of MCA occlusion (Figure 9). These values will also be measured in animals immediately before sacrifice at the 24 and 72-hour time point. Measurements of these variables will help us evaluate if the procedures that we perform and if eNOS gene transfer produce systemic effects in treated animals.

Previous results have shown that chronic administration of HMG-CoA reductase (statins) elevated eNOS gene expression and produced an increase in absolute and regional cerebral blood flow (CBF)[6]. Furthermore, infusion of L-arginine (a substrate for eNOS) at 450 mg/kg over 15 minutes was also shown to produce increases in CBF, presumably by increasing NO generation within vessels[8]. We will thus determine if CBF is altered after eNOS gene transfer. Laser Doppler flowmetry can be used to measure changes in blood flow before and after eNOS gene transfer in the experimental groups described in Table 12. This procedure is routinely used in the laboratory of our collaborator (Dr. M. Moskowitz, MGH). This will thus permit to evaluate relative flow changes in CBF as a function of time after eNOS gene transfer. Absolute CBF in animals will also be measured using an indicator fractionation technique. Mice will be treated as described in Table 12. The jugular vein will also be cannulated and arterial blood will be continuously withdrawn form the femoral artery at a rate of 0.3 ml/ min using a pump (Stoelting, Wood

Dale, IL). One microcurie of N-isopropyl-[methyl 1,3-^{14}C]-p-iodoamphetamine (American Radiolabeled Chemicals Inc., St. Louis, MO) dissolved in 0.1 ml of saline will be injected as a bolus (less than 1 second). Twenty seconds after injection, the animal will be decapitated and blood withdrawal terminated. The removed brain will be frozen in isopentane chilled in dry ice. After the addition of scintigest (Fisher) to the brain and to blood samples and incubation at 50C overnight, scintillation fluid and H_20_2 will be added and radioactivity in both brain and blood measured by liquid scintillation spectrometry. Absolute CBF was then calculated using published mathematical formulas. To measure regional cerebral blood flow (rCBF) in the MCA territory, we will employ the ^{14}C-iodoantipyrine method. After halothane anesthesia, mice will be infused with ^{14}C-iodoantipyrine (5 μCi in 100 μl of saline) through the left femoral vein over 1 minute using a pump. During this time arterial blood samples will also be collected every 5 seconds (total 50-100 μl) and spotted onto preweighted filter paper discs. Animals will then be decapitated immediately and the head will be submersed in isopentane chilled over dry ice (-45 C), before storing on dry ice. Blood radioactivity will be measured by liquid scintillation counting, while the frozen heads/brains will be cut into coronal sections (20 μm thick) using a cryostat. After mounting the sections on glass coverslips they will be dried and then exposed to autoradiographic film for 3 days along with a set of ^{14}C-polymer standards from Amersham (Arligton Heights, IL). After selecting sections that correspond to areas supplied by the MCA (+1.54, +0.14, -1.94, and –3.88 from the bregma), the density of the autoradiogram over 4 to 5 brain structures will be measured with a computerized image analyzer and then the optical density values converted to radioactive content and CBF using the ^{14}C-polymer standards and the ^{14}C-iodoantipyrine blood curve. Therefore, rCBF will be measured in a separate experiment in animals before and after MCA occlusion and before and after vector injection (table 12).

Table 12 - Description of cohorts and number of treated animals for CBF measurements.

	Time[A]								
Vector	72 hrs pre	48 hrs pre	24 hrs pre	Same Time	30 min post	1 hr post	4 hr post	24 hrs post	No ische[B]
Amplicon-eNOS	5[c]	5	5	5	5	5	5	5	5
Amplicon-lacZ	5	5	5	5	5	5	5	5	5
Adenovirus-eNOS	5	5	5	5	5	5	5	5	5
Adenovirus-lacZ	5	5	5	5	5	5	5	5	5
Mock	5	5	5	5	5	5	5	5	5

[A] Time of vector inoculation pre, post or during the ischemic challenge,[B] Animal cohorts not exposed to ischemic insults, [c] Number of animals in cohort.

These experiments should clarify the role of eNOS gene expression in CBF modulation and provide confirmation of the role that this enzyme plays in increasing cerebral perfusion. Although we are keenly aware that NO also possesses anti-platelet and anti-leukocyte aggregation properties that are of paramount importance for its action, we have chosen to focus on the mechanism of NO's enhancement of cerebral blood flow in this particular grant application.

D.4.3.4. Characterization of eNOS gene transfer in mice with a genetic defect in eNOS. Repeating the aforementioned studies in an eNOS model of genetic deficiency will generate further evidence for a role for eNOS in cerebral protection during stroke. These "knock-out" mice are available though our collaborators, Dr. Paul L.Huang and Dr. Mike Moskowitz, MGH[63]. Delivery of an eNOS cDNA into mouse cerebral vasculature would be expected to also reduce cerebral infarct size, improve neurologic deficits, and increase CBF. Based on the experiments described in Table 11 and 12, we will thus know the time point during which the most significant protection occurs. We thus will employ this time point for our experiments, in order to minimize the number of "knock-out" mice that will have to be employed. In eNOS "knock-out"

mice, we would expect that delivery of the eNOS-amplicon (or the adenovirus-eNOS) would significantly improve the deleterious effects of cerebral ischemia when compared to the mice treated with the lacZ-amplicon (or adenovirus-lacZ). These mice experience much larger infarcts than wild-type mice in the MCA distribution upon challenge with an ischemic insult (Drs. Paul L. Huang and M.A. Moskowitz, personal communication). We thus expect that a therapeutic outcome in these animals would be even more significant. This result would provide fairly convincing evidence for cerebral protective effects mediated by eNOS gene expression.

D.4.3.5. Determination of eNOS gene expression. We also will plan to study eNOS gene expression in mice by RT-PCR and Western blot analyses. For RT-PCR, mRNA isolation and reverse transcription will be performed using commercially available kits. Semiquantitative RT-PCR will then be performed using primers that amplify a 340-bp fragment of mouse eNOS. As a control, amplification of GAPDH will also be performed. Messenger RNA also will be prepared from the eNOS "knock-out" mice treated with the lacZ-vectors as a negative control for reaction specificity and from the same mice treated with the eNOS-vectors. As additional controls, we will assay expression of both neuronal and inducible forms of NOS. These studies should thus provide a semiquantitative estimate of the time course of eNOS mRNA expression before and after vector administration. We will then quantitate expression of eNOS by Western blot analyses. A mouse mAb to eNOS (Transduction Laboratories, Lexington, KY) will be employed and immunoblots performed as described, using protein lysates from animal brains collected in the course of the experiments depicted in Tables 11 and 12. Taken in conjunction, these studies will thus show that mRNA and protein levels increase after eNOS gene transfer and will correlate this temporal increase with observed changes in cerebral blood flow, cerebral infarct volumes, and reduction in neurologic deficits.

D.4.3.6. Ennanced therapeutic effects by combining pharmacologic and genetic-mediated increases in eNOS expression. Pharmacologic means exist that allow for increases in eNOS gene expression and increases in eNOS activity. Chronic administration of HMG-CoA Reductase inhibitors has been shown to increase eNOS levels by stabilizing its mRNA[5], rather than by an increase in eNOS transcription (or transcription units). Infusions of nitric oxide donors or the amino acid L-arginine, also increase cerebral blood flow and the mechanism underlying this effect may be related to eNOS, since inhibitors of NOS attenuate the effect of L-arginine and this effect is not evident in mice with genetic defects in eNOS. Furthermore, the combination of chronic statin treatment and infusion of the NO donor, L-arginine, was shown to produce an increase in absolute blood flow of 38% compared to controls and this increase lasted longer than that seen in mice treated only with statins. The biologic outcome consisted in a significant reduction of cerebral infarct size and neurologic deficits in mice treated both with statins and with L-arginine compared to all other groups[8]. Since chronic statin treatment elevates eNOS by increasing mRNA stability and L-arginine increases eNOS activity presumably by provision of additional enzymatic substrate, the finding of enhanced interactions is not surprising. In this context, we thus hypothesize that additional elevations in the levels of eNOS by an increase in transcriptional units (i.e. gene transfer) would interact with the increases in eNOS mRNA stability and eNOS enzymatic activity to produce an even greater therapeutic response.

We thus would plan to treat mice with Simvastatin (Sim) for 14 days at 2 mg/kg s.c. daily followed by eNOS gene transfer and L-arginine (450 mg/kg) infusion. Alternatively, eNOS gene transfer could be performed in the course of Sim treatment to take further advantage of the positive effect of the drug on mRNA stability. Additionally, the doses of Sim and L-arginine were selected to yield a submaximal therapeutic effect on infarct size so that biologic effcets mediated by eNOS gene transfer could be readily detected. Control groups will consist of mice treated with each single agent alone or with combinations of two agents. Results from previous experiments will provide us with a determination of the best vector

(amplicon or adenovirus) and best time and route of administration for maximum therapeutic efficacy. The experimental groups thus will consist of:

a)Sim alone, b) ENOS amplicon alone, c)L-arginine alone, d) Sim + L-arginine, e) Sim + eNOS amplicon, f) Sim + lacZ amplicon, g) ENOS amplicon + L-arginine, h) LacZ amplicon + L-arginine, i) Sim + lacZ amplicon + L-arginine, and j) Sim + eNOS amplicon + L-arginine.

Animals will be sacrificed 24 hours after cerebral ischemia to determine cerebral infarct size. The assays described in Figure 9 will be carried out. To determine if supra-additive interactions are present between the three modes of elevating eNOS action, comparative analyses using the method of Chou-Talalay[90] or Greco[91] can be performed. These results thus will show that combinations of three different methods can produce elevated levels of eNOS and elevated blood flow, thereby producing longer and more effective protection of cerebral tissue upon an ischemic insult.

D.4.3.7. Limitations and alternatives. We realize that the success of the proposed project impinges on delivery of eNOS to endothelial cells in the microcirculation and not to neurons because NO generation would possess different effects in different cell types[6-8, 92-97]. We are confident that this would not occur, because our preliminary work shows that intravascular administration of a large molecule such as the HSV amplicon leads to infection of endothelial cells and not neurons. Furthermore, since transgene expression mediated by vectors is transient (in our hands, we can detect lacZ or GFP expression in brains injected with amplicons for 7-14 days), eNOS effects would be relatively short-term and thus long-term effects of NO on neurons would be unlikely. In spite of this, if we found that eNOS gene delivery was biologically deleterious because of NO effects in brain parenchyma, rather than in the microcirculation, we can easily pursue solutions, such as: a) using endothelial-specific promoters in the amplicon construct to drive eNOS expression in endothelial cells[72-74, 100], b) using regulatable promoters, such as the tetracycline regulatable elements, to express eNOS for only a short period of time (1-2 days) with concomitant administration of doxycycline to animals[98].

D.4.4. Conclusions. In summary, the proposed project will provide novel knowledge related to the engineering of efficient and safe HSV amplicons that can evade complement and neutralizing immunity. This provides the basis for testing the novel concepts that eNOS gene transfer can be neuroprotective in cerebral ischemia and can be coupled with eNOS mRNA stabilization and eNOS activity elevation to provide an augmented beneficial outcome. This possesses important therapeutic applications for cerebral ischemia.

Time Line

	Year 1	Year 2	Year 3	Year 4	Year 5
Aim 1	**X**	**X**	**X**		
Aim 2		**X**	**X**		
Aim 3		**X**	**X**	**X**	
Aim 4			**X**	**X**	**X**

E. Human Subjects

None

F. Vertebrate Animals

Before each procedure, adult C57/BL6 and sv129 mice (Taconic) and immunocompromised mice (Jackson Laboratories or collaborators) mice are anesthetized as described in section D.1. For intra-arterial injections, the procedure described in Rainov et al. (1996) is followed. Briefly, anesthetized mice are secured supine and an incision is performed in his neck. Under surgical microscopic guidance, the common carotid, external carotid, internal carotid, and pterygopalatine arteries are identified. After suturing the pterygopalatine artery, the common carotid is clamped using a microvascular cerebral aneurysm clip. The external carotid is then ligated distally, while the proximal portion is cannulated with a saline-filled angiocath. The procedure is performed in the absence of heparin, a known inhibitor of HSV infection. The common carotid artery clamp is released. Amplicon virus is infused as a bolus injection (10^8 pfus in 100 μl). The cannulation of the external carotid artery will result in retrograde flow up to the bifurcation and then anterograde flow into the internal carotid artery due to flow from the common carotid artery. At the end of the infusion, the proximal stump of the external carotid artery is ligated and the neck is sutured. For implantation of vectors in the brain, the animals are placed in a stereotactic apparatus (Kopf). Usually 5 animals per experimental group are needed for histologic analyses. A linear skin incision is placed above the skull, the bregma is identified, and a burhole is drilled at stereotactic coordinates 2 mm right lateral and 1 mm anterior to the bregma. A sterile needle is used to incise the dura and usually amplicon vectors (2- 5 microliters containing amplicon at a titer of 10^9 pfu/ml) are inoculated into the brain, employing the appropriate stereotactic coordinates. For histopathologic analysis, rats are monitored twice daily, so that brains can be removed within 12 hours after death. . When frozen tissue is needed, animals are rapidly sacrificed, their brains are harvested and frozen. Brain fixation was performed by immersion in a solution containing 4% paraformaldehyde in 0.9% sodium chloride and 10 mM sodium phosphate, pH 7.4, for 24 hours. Brain cryoprotection was performed by subsequent immersion in a solution containing 30% sucrose in 10 mM sodium phosphate, pH = 7.4, for an additional 24 hours. Brains are cut on a sledge microtome into sections that are 20 micron thick. After mounting on gelatin- coated microscope slides, sections are stained with the appropriate stains. Surviving animals undergoing defined end-point analyses of stroke status are anesthetized by administration of 1.2 mls of the ethanol/saline/pentobarbital solution described above and then sacrificed by in vivo perfusion through the left ventricle with 100 ml of 10 mM sodium phosphate, pH = 7.3 in 0.9% sodium chloride followed by 150-200 mls of 4% paraformaldehyde and 10 mM sodium phosphate, pH = 7.4 in 0.9% sodium chloride. Brains are dissected, placed for 24 hours in the above fixative, and then placed for an additional 24 hours in a buffer containing 0.2 M sodium phosphate (pH=7.4) and 30% sucrose. Brain sectioning is performed on a sledge microtome (50 micron sections) by serial sectioning throughout the extent of the tumor. This procedure usually involved sectioning brains in a rostral to caudal direction from the frontal lobes up to (but excluding) cerebellum and brainstem. Drugs (CPA, CVF, and sCR1) are usually administered as described in the text of the main grant. Throughout the studies animals that are judged to be in pain, moribund, comatose, unable to feed, drink or move will be euthanized. This judgment will be made by the MGH veterinarians (described below) and/or by the researchers. The Director of the Office of Laboratory Animal Resources administers the Animal Resources Program at the Massachusetts General Hospital. The MGH employs three full-time Veterinarians (two are Board-certified by the American College of Laboratory Animal Medicine (ACLAM), including the Director) and more than thirty Animal Technologists/Technicians to provide daily care and oversight. The hospital has an active and functioning Subcommittee on Research Animal Care (SRAC) which serves as the Institutional Animal Care and Use Committee (ECHOIC) as required by the Public Health Service Policy on Humane Care and Use of Laboratory Animals and the U.S.D.A. Animal Welfare Regulations. The program and facilities are fully accredited by the American Association for the Accreditation of Laboratory Animal Care. The animal facilities are specified-pathogen free (SPF) facilities. Within the hospital complex, approximately 55,000 sq.ft. of space are devoted to the housing and servicing of various species used in biomedical research consisting of facilities on the Main hospital grounds and the Charlestown Navy Yard (the Lawrence E. Martin Laboratories). There are five (5) fully equipped animal survival surgical suites within the MGH research facilities. These areas are designed to support a variety of surgical procedures. The majority of the animal resource space (85%) is located in newly constructed and/or recently renovated buildings. The facilities are designed to house convention, Specific Pathogen-Free, Viral Antibody-Free and Immuno-deficient rodents. Facilities also exist for providing Biological Safety Level 2 Containment. All experiments involving vectors will be conducted in the latter facilities. Conventional housing facilities for large animals (dogs, cats, nonhuman primates, swine, etc.) exist at both the Main Campus and Charlestown Navy Yard. The three veterinarians serve as members of the SRAC. Other members of the Subcommittee include: the Chairperson; 15 investigators who use animals in their research; two members of the

research administration of the hospital; three facility managers; and two members of the Boston community not otherwise associated with the hospital. The Hospital complies with the provisions of the Animal Welfare Regulations and is registered with the U.S. Department of Agriculture as an approved research facility (Registration No. 14-R-014). The animal care areas are inspected at least three times a year by the U.S.D.A. Representatives of the Massachusetts Department of Public Health also inspect the facilities frequently. The Hospital has an assurance statement on file with the Office for Protection from Research Risks at the N.I.H. The Institutional Assurance number is A3596-01.

All surgical procedures are carried out after anesthetizing animals as described above. Euthanasia is performed on animals that are judged by the veterinarian or investigators to be comatose, unable to move, feed, or drink.

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H. Consortium/Contractual Arrangements

None

i. Consultants:

Dr. Michael C. Carroll will provide his consultative expertise on innate immunity and immunology. He will aid us in the design and interpretation of the experiments described in this grant. He will also provide us with the mice that possess genetic defects in complement function. We expect to consult with him approximately 50 hours per year and thus ask for a fee of $ 140/ hour.