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
Michael M. Moskowitz, MD	"	Collaborator
Paul Huang, MD PhD	"	Collaborator
Michael C. Carroll, PhD	Harvard Medical School	Collaborator/Consultant
Alan B. Ezekowitz, MD	Massachusetts General Hospital	Collaborator
John D. Lambris, PhD	University of Pennsylvania Medical School	Collaborator

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
TABLE OF CONTENTS

*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.

The following consists of a revised version (revised portions are underlined in the text) of R01 NS 41571-01, with specific responses to critiques as follows:

Reviewer 1, Critique "However one needs to be cautious in that the studies that are proposed and those like it in the past have demonstrated very minimal impact on the evolving infarct when vectors have been delivered subsequent to the stroke."

Response: The reviewer is correct in his/her assessment of the importance of time in the success of the proposed strategy. **However, there are numerous clinical scenarios where the proposed strategy could be beneficial in a prophylactic setting.** The number of patients suffering a stroke during cardiac bypass operations is estimated to be 50,000/year worldwide[1,2]. Similarly, 5-10% of patients suffer ischemic damage to cerebral tissue during cerebral aneurysm surgery or embolization procedures. The percentage of patients who suffer a stroke during carotid endarterectomy procedures or neurointerventional carotid stenting is estimated at 2-5%[3,4]. Finally, patients with multiple transient ischemic attacks ("in crescendo"), with Moya-Moya disease, with dissections of cerebral arteries are at risk for strokes and could thus benefit from our proposed studies in a prophylactic setting.

Reviewer 1, Critique " However, the inclusion of the adenovirus expressing LacZ seems to be poorly defended and appears an add on to an otherwise good justification…"

Response: We have deleted all adenoviral vector experiments.

Reviewer 1, Critique "It is quite likely that the applicant's ability to target the intravascular compartment following stroke will be compromised"

Response: As a pilot study to determine if the cerebral endothelial compartment would be exclusively transduced by the intra-arterial HSV amplicon, we performed the experiment illustrated in figure A (figure 13 in main application). Mice were subjected to an occlusive event in their carotid circulation. Approximately 4 hours after this event, the lacZ-HSV vector was injected. Mice brains were harvested and stained for lacZ cDNA expression. **Selective expression of the transgene was still noted in the cerebral endothelial compartment, even after the stroke.** The large diameter of the virion (150 nm) is likely to impede its passage even across a disrupted blood-brain-barrier, where pore opening is estimated to be about 20 nm[5-8].

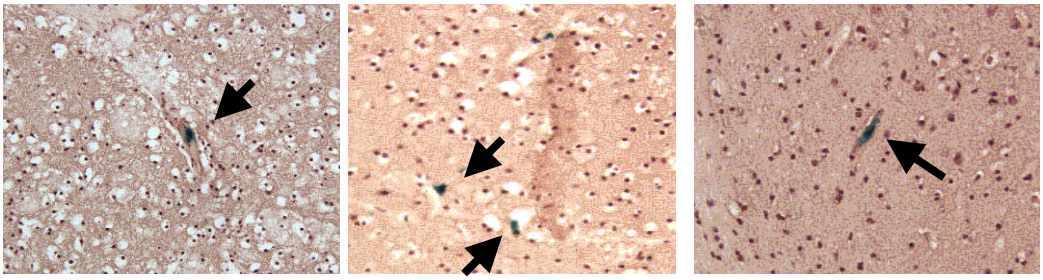


Figure A – Four hours after the onset of cerebral ischemia (left and middle panel) complement-depleted mice underwent carotid artery injection of the lacZ-HSV vector. Approximately 24 hours later, brains were harvested and stained for lacZ. Complement-depleted mice that were not subjected to ischemia were included as controls (right panel). LacZ expression in cerebral endothelial cells (arrows) is still observed without expression in the disrupted brain parenchyma (left and middle panel). As expected, lacZ expression in cerebral endothelium is observed in control mice as well (right panel)

Reviewer 1, Critique "Finally the investigator proposes yet another strategy in which he plans to utilize an antibody directed against MBP to deplete its activity in vivo. It is unclear whether this strategy has been employed previously and whether the antibody itself can saturate MBP in vivo to produce the desired effect. In many of these studies it is imperative that the investigator examine the pharmacologic disruption of the pathway that he is targeting. In the case of C1 INH he proposes experiments to do so. This is not the case for the C4b-deleted scR or for the MBP antibody studies"

Response: We agree with the reviewer's concern related to the need to test depletion of elements of the complement pathway not only by humoral means but also through pharmacologic depletion, which may be relevant in a therapeutic setting. For the alternative pathway, though, we can now show that it is unlikely to be involved in serum inactivation of virus because: i) calcium depletion eliminates serum's inactivation (the alternative pathway is independent of calcium, unlike the classical and lectin pathways) (see figure 17) and ii) no mutations in the gC domain responsible for evading alternative pathway components were found although mutations in the domains responsible for evading classsical/lectin components were observed (see figure 10). On the contrary, MBP does provide a relevant pathway of complement inactivation in mice. Therefore, we have expanded the section of the

proposed studies to include pharmacologic inhibition of MBP. Our pilot data suggests that this is easily accomplished in vitro with mannan (see figure 8) and have thus added a section describing these experiments (D.1.4.6).

Reviewer 1, Critique "We do not know much about these animals; in particular whether they have any compensatory changes associated with the loss of MBP, whether the cerebral vasculature is normal and therefore it is very difficult to interpret the consequences of gene transfer in this animal model"

Response: We have now included a paragraph describing these mice in more detail (section D.1.4.7). Because we plan to use both MBPa and MBPc knockout models, which were generated, in the laboratory of Dr. Alan Ezekowitz (MGH), we have included him as a collaborator for the proposed experiments (see attached letter).

Reviewer 1, Critique "The rationale underlying these experiments is that the laboratory strains contain gC that no longer possess the capability of perturbing the complement system, whereas the naturally occurring isolates appear to have gC active in this function. This is an interesting hypothesis although it is not well supported by experimental data."

Response: We have experimental data regarding the inhibition of amplicon infection by human serum compared to the lack of inhibition by a virulent clinical isolate of HSV, designated as NS (see fig. 9). Amplicon inhibition by serum is similar to that observed with HSV recombinant vectors (hrR3 and MGH1) derived from other laboratory strains. Comparison of the nucleotide sequence of gC from virulent strain NS (known to evade complement well) to that of amplicon's (from strain 17^+) shows no mutations in the 5' (amino terminus) that encodes the heparan sulfate binding domain (relevant for viral attachment to cells) and the C5/properdin binding domains (relevant for blocking the alternative pathway of complement activation)[9]. Instead, we have found nonconserved amino-acid substitutions in the C3b binding domains (figure 10). We thus believe that these amino-acid substitutions are responsible for altering evasion of amplicon from complement activation mediated through the classical or lectin pathways. In aim 2, this will be tested directly by substituting gC from one strain with that of another. Glycoprotein C mutations are occurring because of the relative lack of replicative fidelity of the HSV DNA polymerase in the absence of selective pressure to evade complement within the context of long-term tissue culture[10,11].

Reviewer 1, Critique "The experimental approach is cumbersome in that data could be obtained far more readily using a different approach. The investigator proposes to create in a rather complex series of molecular biologic steps a HSV BAC in which the endogenous gC is replaced with that from the naturally occurring isolate NS. It would be easier for the investigator to make the observation using available gC-deleted mutants and providing the NS gC in trans. That said, the applicant' strategy although convoluted can work…"

Response: We are grateful to the reviewer for his confidence in our strategy. We can now use either the "HSVquik" system or recA-mediated recombination[48] in BACs to modify any gene within the viral genome in a matter of weeks rather than months. Although unpublished, we have appended a copy of a recent poster to demonstrate our technical facility with the proposed strategy[12].

Reviewer 1 Critique, " It is quite uncertain as to whether complement inhibition utilizing this NS-derived gC will alter gene transfer efficiency…There is some concern that the gC substitution could change the tropism of the vector…While unlikely I think it is something that bears consideration"

Response: The amino terminal domain of gC (prior to the first cysteine – aa# 33-123) is important for binding to heparan sulfate on cell surfaces[9]. The four complement (C3b) binding domains of gC are instead located in separate domains of the polypeptide (domain 1: 124-137, domain 2: 223-246, domain 3: 276-292, and domain 4: 339-366)[9]. Therefore, the presence of single aminoacid alterations in any of these four C3b binding domains is unlikely to alter vector tropism.

Reviewer 1, Critique, "He proposes a series of experiments …to determine how best to elicit neutralizing immunity…He will employ a replication-conditional recombinant herpes virus, hrR3…It is not at all clear that this is the most sensible strategy to elicit anti-HSV immunity."

Response: We have employed this strategy in published experiments[13]. Clearly, if this method fails to elicit protective humoral immunity, we are prepared to use other viral mutants and schedules of immunization.

Reviewer 1, Critique "The outcomes of these experiments with respect to the efficiency of gene transfer are so critically contingent upon the observation that he will make in Specific Aim 1"

Response: We hope that the experiments of figure A and B can convince the reviewer that gene transfer in endothelium is observed before and after a stroke.

Reviewer 1, Critique "…the data underlying this particular observation (i.e. gE/gI's ability to evade antibody) are not available"
Response: There is an extensive literature on this subject[14-19]. To summarize it, we have included a paragraph in the background section (B4) as well as figure 18 (section D.3.2).

Reviewer 1, Critique: "…treatment…with a HMG-CoA reductase inhibitor is able to up-regulate the levels of eNOS through a post-transcriptional mechanism. The details of this are unavailable. The sequences within the transcript that presumably are responsive to the HMG CoA reductase inhibition are not described.
Response: We have expanded the background section to discuss these issues (Figure 1-3, B5).

Reviewer 1, Critique: "One of the central questions that is not addressed…and is among the most important is whether the vector…can express enough protein…Given the time requirement for HSV vectors to express…"
Response: The experiment shown in figure A shows that the HSV vector can transduce endothelium, express a transgene, such as LacZ, whose enzyme product (Beta-galactosidase) is catalytically active by Xgal staining. However, we agree with the reviewer that delivery after the stroke may not provide sufficient time to result in a therapeutically relevant effect. We thus have performed another experiment to determine whether delivery four hours before a stroke can also result in generation of catalytically active enzyme. Figure B (repeated as figure 12, C6) shows that endothelium is transduced and that catalytically active beta-galactosidase is generated in this time frame. The transgene is under control of the HSV IE promoter, which is turned on immediately after infection. It is estimated that HSV proteins under control of this promoter are present in the cell within a few hours of infection[20].

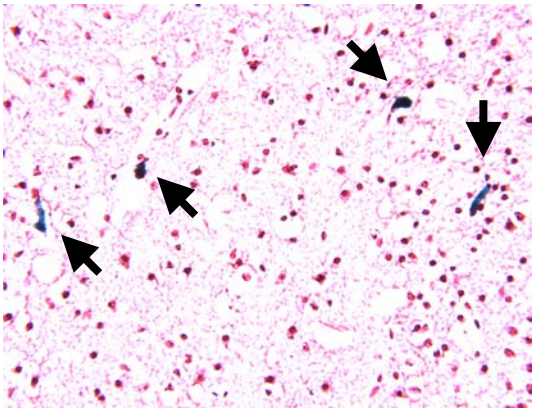


Figure B – Four hours before a middle cerebral artery occlusion, the LacZ-HSV vector was injected into complement-depleted or control mice. Brains were then harvested immediately after the ischemic insult. Shown is a representative microphotograph from the cortex of a complement-depleted mouse. Arrows point to lacZ-positive endothelial cells.

Reviewer 1, Critique: "…during the stroke, the blood brain barrier is not intact…and that the investigator may be delivering some vector beyond the endothelial compartment"
Response: please see above responses.

Reviewer 1, Critique: "Given adenovirus produces a robust immune response, this seems poorly considered."
Response: We have eliminated this section.

Reviewer 2, Critique: "…will the inclusion of adeno-lacZ and Gal4 add to the stated goal? Can the reporter experiments of D.1.4.1 be combined with… D.1.4.2?"
Response: We agree with the reviewer's recommendation and have eliminated adeno-lacZ and Gal4 experiments and combined the two sections.

Reviewer 2, Critique: "…it may be reasonable to sequence the two gC regions to verify that they are different"
Response: We have done so and shown differences between NS strain (wild-type) and the amplicon strain (see preliminary data in section C and fig. 10).

Reviewer 2, Critique: "…can HSV vectors also target red blood cells…"
Response: We believe that the reviewer's suggestion is interesting. Since it would require additional maneuvers to test (hemodilution?) that would be outside the immediate scope of the application (complement and antibody-based inhibition), we are not proposing experiments for this.

Reviewer 2, Critique: "…this vector construct can only be tested in vitro with human IgG…and, as such, is not entirely relevant…"
Response: We still believe that this will provide useful information, because over 50% of the population possess neutralizing antibody activity against viral antigens. Even if the gE/gI substitution cannot be tested in animal models, the in vitro results would provide us with a useful avenue to circumvent the immune response against the vector in seropositive humans.

Reviewer 2, Critique: "…the inclusion of adenoviral-lacZ may add little…"
Response: We have eliminated these studies.

Reviewer 2, Critique: "…budget for 3 years, instead of requested 5". **Response:** By eliminating time points and adenoviral experiments, we have revised our time frame to 3 years, as requested.

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[21]. Although a variety of experimental treatments have focused on events that occur after the stroke, **prophylactic** therapies could also be beneficial. for patients undergoing surgeries or neurointerventional procedures on brain, spine, neck and/or cardiac arteries as well as patients at risk for stroke development. There is thus a clear need for exploring additional and novel therapeutic avenues even in a prophylactic setting[22]. One such avenue revolves around endothelial nitric oxide synthase (eNOS)[23,24]. 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)[25-28]. This up-regulation can been achieved with chronic prophylactic administration of HMG-CoA reductase inhibitors that function by stabilizing eNOS mRNA[25,27] 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[29]. **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[30,31]. Recent events in clinical trials of gene therapy have underscored the relevance of avoiding side effects from the viral vector construct[32-36]. Such side-effects can occur because of: **a)** low levels of expression of endogenous viral genes from the replication-defective vector[37,38], **b)** the presence of contaminating replication-competent viruses[39,40], 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[41,42]. To overcome these limitations, we and others have been incrementally developing a "gutless" herpes simplex virus (HSV) vector system (amplicon)[12,43-49]. 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[44,48]. 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[50-55], 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.**

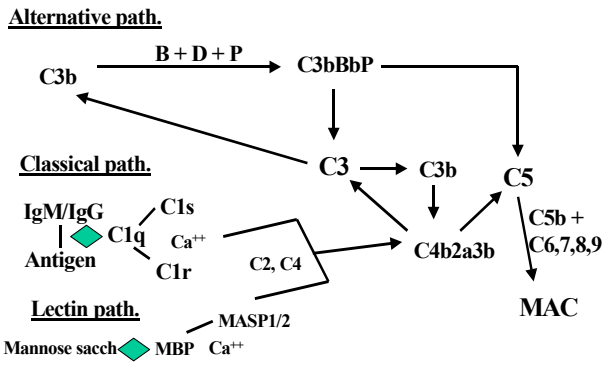
a. Background and Significance

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[56,57]. 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 in the CNS[31,58-60] 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)[61-63]. 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[61,64], 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* [65,66]. In order to package either type of HSV vector, the missing viral functions have to be provided in *trans.* 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 eliminate the use of helper virus and thus generate a truly "gutless", "helper-free" system, several strategies have been employed over time [67,68.44.49]. We and others have been employing bacterial artificial chromosomes (BACs) to provide "helper" functions. Since our last grant application, we have been able to show further improvement in the HSV-BAC to completely eliminate replication-competent helper virus production. Our newest amplicon stocks retain high titer with no helper virus regeneration (see reprint by Saeki et al. in appendix)[48].

As a gene therapy vector for the cerebral vasculature, HSV amplicons possess one advantage compared to other vectors. The diameter of the virion (150 nm) renders its passage across tight junctions of the blood-brain-barrier very difficult. Even when such junctions are disrupted, previous studies have failed to find replication-defective HSV vector passage across the blood-brain-barrier[8]. Therefore, an intra-arterial mode of delivery will expose primarily cells of the BBB (such as endothelium) to the vector.

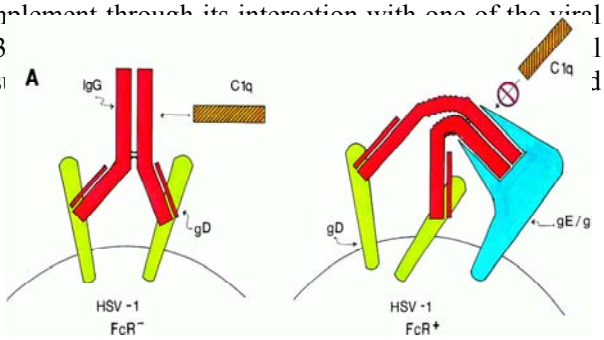
B.2.Innate mammalian responses against viral vectors. It is becoming increasingly evident that innate and elicited humoral immune responses can modulate both the efficacy and the safety of viral vectors[69]. In general, innate factors, such as complement and natural antibodies, provide a major initial 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[70-72]. Complement activation can proceed through the classical, alternative[73], or lectin-pathway[74,75] (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[75,76]. Although HSV envelope glycoproteins have been shown to possess high-mannose-type O- and N-linked oligosaccharides[77], 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[73]. Although these reactions are meant to be protective by destruction of the pathogen, they also cause additional damage to infected tissues[41]. 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[42]. 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[78]. 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)[79-84]. This occurs through binding of C3 as by blocking the binding of properdin and C5 to C3b. The end-res alternative pathways of complement activation, allowing for increased



In addition, glycoproteins E and I (shown in blue in the diagram) from the viral envelope have been shown to possess Fc receptor activity for IgG[15,85-89]. Through a mechanism of antibody bipolar bridging, gE/gI binding to the Fc portion of IgG (shown in red) neutralizes antiHSV IgG action even with concomitant binding of IgG Fab portion to HSV antigenic epitopes (such as gD, shown in green). The binding of IgG Fc to gE/gI has been shown to lead to a significant decrease in antibody-dependent cellular cytotoxicity as well as complement-dependent lysis (shown in orange) of virions and infected cells. **It should be noted that gC-mediated and gE/gI-mediated HSV evasion of complement and IgG action, respectively, has been described for virulent clinical isolates of HSV. Evasion by laboratory strains of HSV has not been studied in great detail.**

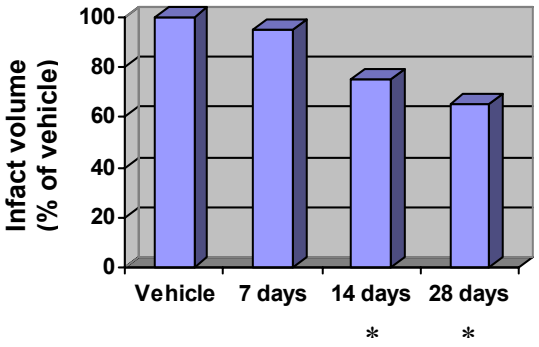


Figure 1 – (Adapted from ref. 28) Infarct volume showing the effects of 20 mg/kg/day of mevastatin administered 7, 14, or 28 days before a 2 hour occlusion+ 22 hour reperfusion of MCA in mice. * p <0.05.

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[25-27,90-92]. 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[93]. Pharmacologic-mediated increases in eNOS mRNA result in increased cerebral blood flow and smaller infarcts[26], while

administration of the eNOS substrate, L-arginine, relaxes vascular smooth muscle and reduces infarct size after an ischemic event[29].

Additional confirmation of eNOS's role in stroke protection was provided by the recent experiments of our collaborator on this grant proposal, Dr. M. Moskowitz. Treatment of mice for 7 to 28 days with pharmacologic inhibitors of 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase directly up-regulated eNOS gene expression as shown by mRNA (figure 2) and protein levels.[28] Clinically, this resulted in less significant neurologic deficits. Mice with a genetic defect in eNOS did not show stroke protection upon treatment with HMG-CoA reductase inhibitors. Additional studies have shown that at least three or more days of drug treatment are required to achieve a significant neuroprotective effect (Dr. M. Moskowitz, personal communication) and the best outcome is observed with 28 days of treatment (figure 1). This effect was post-transcriptional and probably related to enhanced mRNA stability (figure 2). At this juncture, the mechanism of enhanced mRNA stability is unknown, although studies by Dr. Moskowitz and collaborators have shown that changes in the endothelial actin cytoskeleton, which could be involved in the anchoring of mRNAs and co-localization of ribosomes and RNA-binding proteins, are likely involved[94]. Although studies had suggested that cholesterol reduction could improve relaxation of vasculature[95], 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[26]. Additionally, when elevations in eNOS levels by increasing the stability of its mRNA[25] were coupled with increases in eNOS activity by supplementation of its substrate, even more significant biologic effects were observed[29] (Fig 3).

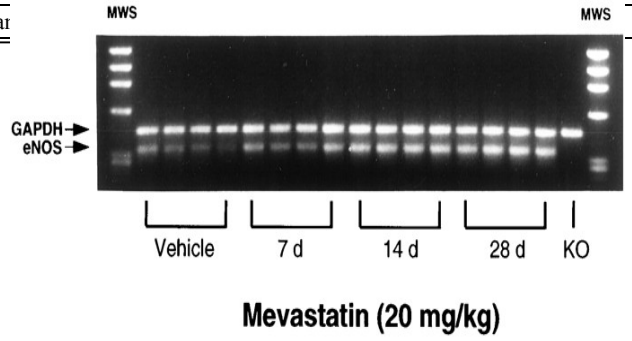


Figure 2 – (Adapted from ref. 28) Semiquantitative RT-PCR for eNOS mRNA showing the time-dependence of mevastatin treatment on eNOS mRNA up-regulation. Specificity of the reaction was shown by the absence of a band in the eNOS knock-out mouse (KO). GAPDH was used as internal control

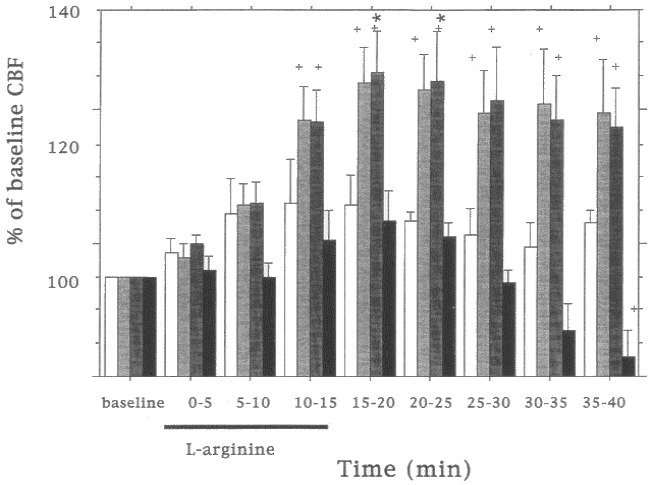


FIG. 3. Bar graph showing rCBF changes in simvastatin-treated mice after L-arginine or saline infusion. rCBF values for 2 mg/kg-treated animals infused with saline are represented by white (n = 3), L-arginine infusion in 2 mg/kg (n = 5) and 20 mg/kg (n = 6) treated mice are shown as light and dark shades, and 2 mg/kg-treated eNOS-null mice are shown as black bars (n = 2). There was no response to L-arginine in eNOS-null mice, whereas the response to L-arginine was robust and prolonged in mice treated with 2 mg/kg or 20 mg/kg simvastatin. Error bars denote SEM. *P < 0.05 compared with saline infused simvastatin (2 mg) treated mice; †P < 0.05 compared with baseline. *From:* Yamada et al. (2000) , ref. 29.

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 endothelial cells? (figure 14).** 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. 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. Novel amplicon packaging system. In our previous proposal, we had described an HSV-amplicon packaging method that essentially eliminated the generation of contaminant "helper virus". In this revised application, we will refer the reader to our published report (Saeki et al.) for details[12].

C.2. 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 4). 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[70,72,96,97]. We thus reasoned that the inactivation observed in figure 4 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 1 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.

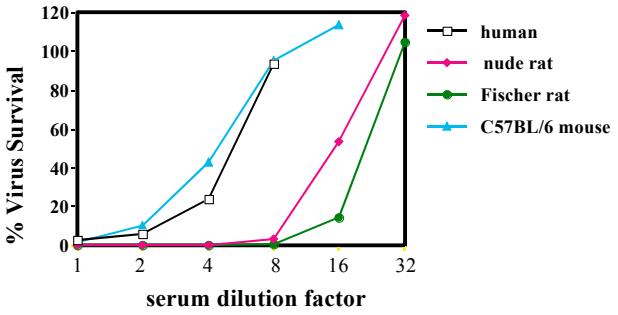


Figure 4 – Human, rat and mouse serum are potent inactivators of HSV vector. Serial dilutions of serum were employed in the study.

Table 1 – 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

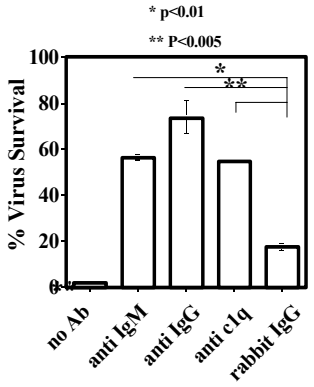


Figure 5 – Antibody neutralization using rabbit IgG raised against human IgM, IgG, or C1q reverses the antiviral activity of human plasma.

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.3 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[69](see figure 17), 2) Pharmacologic, humoral, or biochemical depletion of

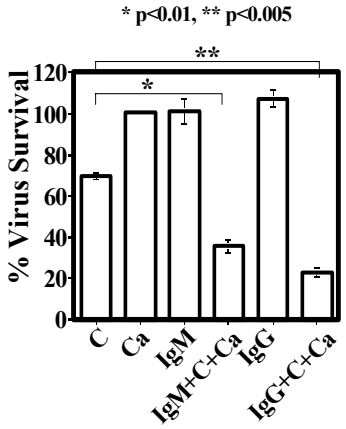


Figure 6 – Purified rat IgG or IgM, complement (C) and calcium (Ca) inactivate HSV vector.

Ig in serum reduced vector inactivation, showing that Ig facilitated the observed inactivation[69,98], 3) *In vivo* depletion of complement with cobra venom factor facilitated the initial infection of tumors by oncolytic HSV[98], 4) Antibody-neutralization of human serum with anti-human C1q or anti-human IgM or IgG reversed the observed vector inactivation (figure 5), 5) Incubation of HSV vector with purified rat IgM or IgG, rat complement, and calcium inactivated vector transduction (figure 6).

C.4. 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 inhibition of 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 -/-)[97,99], in immunoglobulin maturation (RAG2 -/-) (Jackson Laboratories), in C3 (C3 -/-), in C4 (C4 -/-)[71, 100], 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 7). 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 8 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.

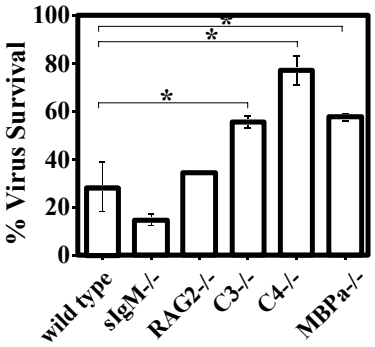


Figure 7 – Sera from C3, C4, and MBP, but not Ig "knock-out" mice neutralizes HSV vector more weakly than sera from sv129 or C57BL/6 wild-type mice. * p < 0.02

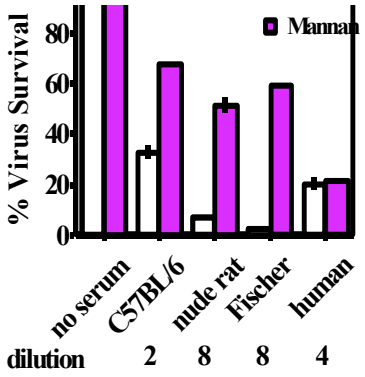


Figure 8 – Mannan pre-incubation reverses the antivector effect of mouse and rat, but not human, plasma. P values for mice and rats were < 0.0001

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 4).

C5. Nucleotide polymorphisms in the sequence of gC from NS strain compared to that of amplicon. 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[98]. However, clinical isolates of HSV, such as strain NS or strain MP, have been shown to be only mildly affected by serum and complement[83]. 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.

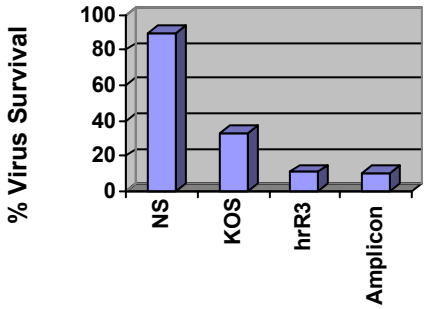


Figure 9 – Human serum differentially inactivates HSV strains and vectors

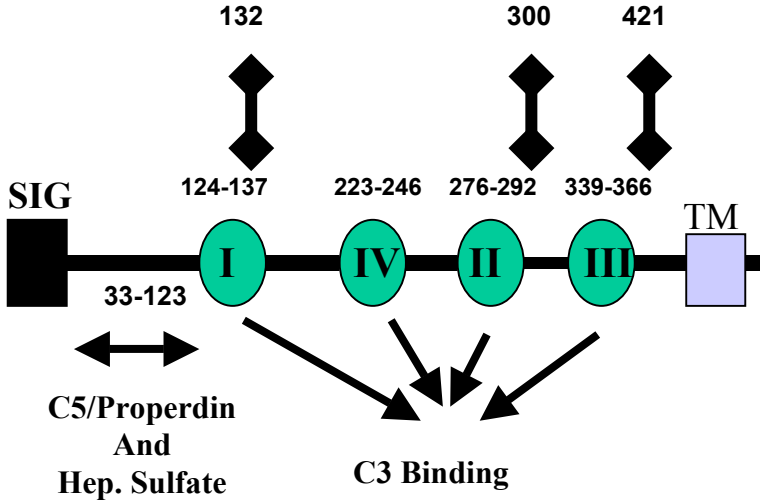


Figure 10 – Stick diagram of gC. SIG designates the signal sequence. C5/Properdin (alternative path.) and heparan sulfate domains (viral attachment) are located at amino acids 33-123. The four C3 binding domains (Classical and lectin path.) are at aa. 124-137, 223-246, 276-292, and 339-366. Nonconserved Amino acid changes between virulent strain NS and amplicon are at aa 132, 300, and 421 (Adapeted from ref.9).

The above findings do suggest that the aminoacid sequence of gC from different viral strains may differ, particularly in their complement binding domains. In fact, sequence analysis of gC from NS strain compared to that of amplicon (strain 17^+) reveals a total of five nucleotide polymorphisms that result in three non-conserved amino acid changes in complement binding domain 1 as well as two regions before and after domain 3 (figure 10). **These findings thus immediately suggest a molecular explanation for the observed differences in vector evasion from complement**. Experiments described in aims 2 (for gC) and aim3 (for gE/gI) will thus directly test the hypotheses that the observed polymorphisms in amino acid sequence result in alterations in complement and Ig evasion that impact directly on amplicon transduction efficiency. Substitution of gC and/or gE/gI from strain NS into the envelope of HSV vectors should render the latter less susceptible to inactivation and more effective in transducing target cells, if our hypothesis is correct.

C.5. 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. Furthermore, *in vitro* use of rat or mouse serum depleted of complement 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 *in vivo*. 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[54], 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 11 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 2 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. The large size of the HSV amplicon (150 nanometers) impedes its efficient passage across endothelial tight junctions[8]. These findings thus confirm that complement depletion facilitates the infection of endothelial cells in the brain after intra-arterial administration of an HSV vector.

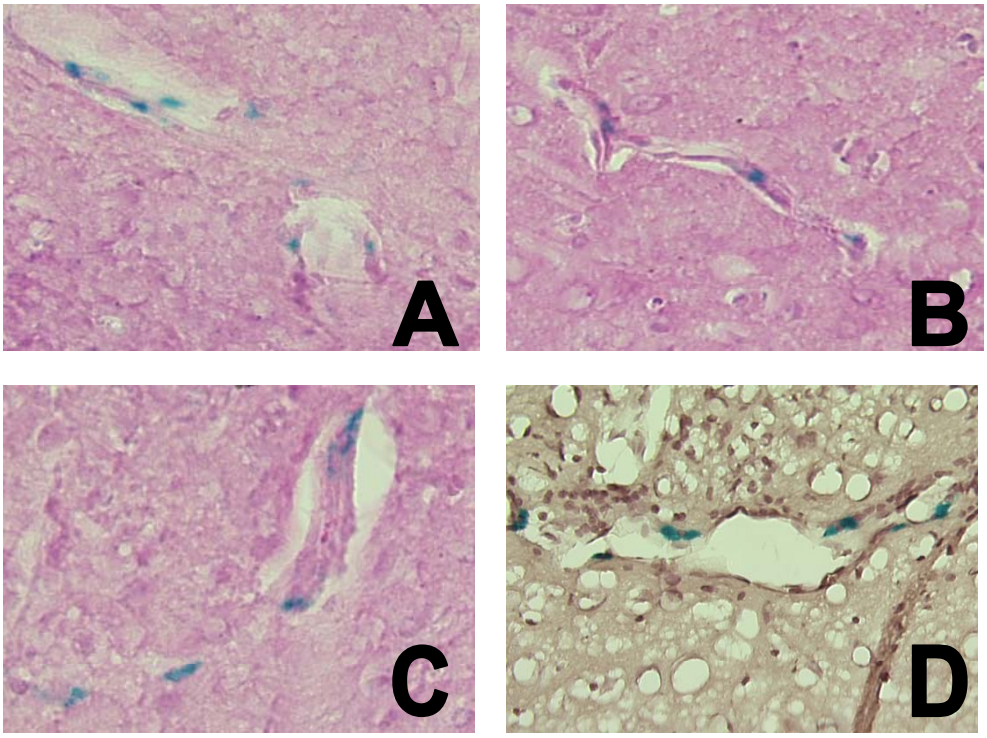


Figure 11 - Rats (panels A and B) and mice (panel C and D) were pretreated with CVF, followed by intra-arterial administration of a LacZ-expressing HSV amplicon. Twelve hours later, animals were sacrificed and cryosections (20 μm thick) were stained for lacZ expression and then counterstained with neutral 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 2.

Table 2 – 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

C6. Catalytically functional transgene is delivered by HSV vector 4 hours before MCA occlusion. Because eNOS gene delivery and expression by the HSV amplicon would have to occur fairly rapidly, we tested the time course of expression of lacZ. Four hours before MCA occlusion, the vector was delivered intra-arterially in both CVF (Complement-depleted) and control mice. Mice were then sacrificed four hours after MCA occlusion and presence of catalytically active lacZ gene product (β-galactosidase) was assessed by enzymatic histochemical staining. LacZ-positive endothelial cells could be found in endothelial cells in the cortex of the CVF-treated mice (Figure 12), while rare positive cells were present in control (table 3). These results thus suggested that catalytically active enzyme could be delivered by the vector within the endothelial compartment four hours before a stroke.

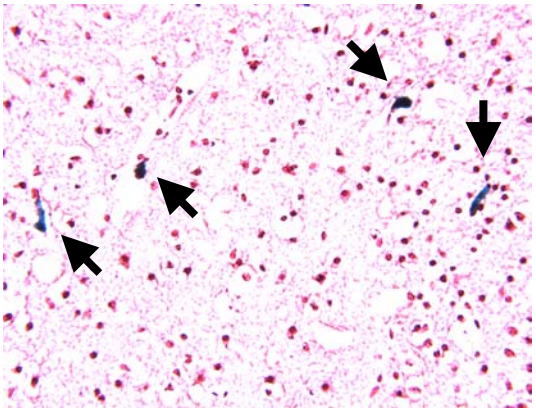


Figure 12 – Four hours before a middle cerebral artery occlusion, the LacZ-HSV vector was injected into complement-depleted or control mice. Brains were then harvested immediately after the ischemic insult. Shown is a representative microphotograph from the cortex of a complement-depleted mouse. Arrows point to lacZ-positive endothelial cells.

Table 3 – Average number of lacZ-positive endothelial cells per high-power field after intra-arterial amplicon in mice, four before MCA occlusion.

CVF	Vehicle
6 +/-0.1	>1

C7. Disruption of the blood-brain-barrier by the infarct does not alter the selectivity of vector transduction to the endothelial compartment. Since stroke disrupts the blood-brain-barrier, the possibility exists that intra-arterial HSV vector delivery could result in passage across the disrupted tight junctions and infection/transduction of astrocytes/neurons. We thus tested this possibility by producing an ischemic insult and then delivering the vector four hours later. Animals were then sacrificed 12 hours after vector administration. Figure 13 shows that lacZ expression remained confined to the endothelial compartment of the brain. The failure of passage of the vector should not be too surprising considering that the diameter of HSV virion is estimated to be 150 nm. while the diameter of disrupted tight junctions has been estimated to measure approximately 20 nm[5-7].

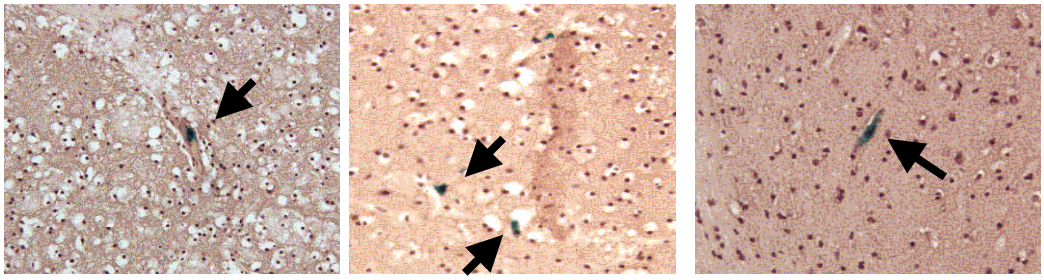


Figure 13 – Four hours after the onset of cerebral ischemia (left and middle panel) complement-depleted mice underwent carotid artery injection of the lacZ-HSV vector. Approximately 24 hours later, brains were harvested and stained for lacZ. Complement-depleted mice that were not subjected to ischemia were included as controls (right panel). LacZ expression in cerebral endothelial cells (arrows) is still observed without expression in the disrupted brain parenchyma (left and middle panel). As expected, lacZ expression in cerebral endothelium is observed in control mice as well (right panel)

The experiments of figure 12-13 thus provide supportive evidence that a catalytically active gene product can be expressed selectively in endothelium in the time that precedes and as well as in the time that follows(four hours before and four hours after) an ischemic brain insult. This suggests that eNOS cDNA transfer can result in intravascular NO production during the time period of the stroke.

C8. Why eNOS cDNA transfer? The experiments published by Dr. Moskowitz and collaborators have shown that biologic maneuvers which increase eNOS activity (such as mRNA stabilization by HMG-CoA reductase inhibitors or provision of substrate by L-arginine infusion) result in therapeutically significant effects in mouse models of brain ischemia[26,28,29,94]. These effects consist of a reduction in infarct size and an increase in cerebral blood flow. The combination of the two maneuvers appears to be even more efficient in providing a biologic effect (figure 3). One limitation of each maneuver is in its time-dependence. HMG-CoA reductase inhibitor effects require at least 3 days of treatment and its effectiveness appears to increase with length of treatment (see figures 1 and 2). L-Arginine infusion is effective for a very short time period immediately before stroke induction (see figure 3).

Vector-mediated eNOS cDNA transfer should instead provide a more rapid avenue to up-regulate eNOS expression when compared to stabilization of its mRNA. Furthermore, increase in eNOS transcriptional units would be expected to synergize with increase in eNOS mRNA stability and with increase in eNOA activity (figure 14). Delivery of eNOS into the endothelial cell compartment can occur selectively and rapidly with our vectors and mode of administration (figure 11-13).

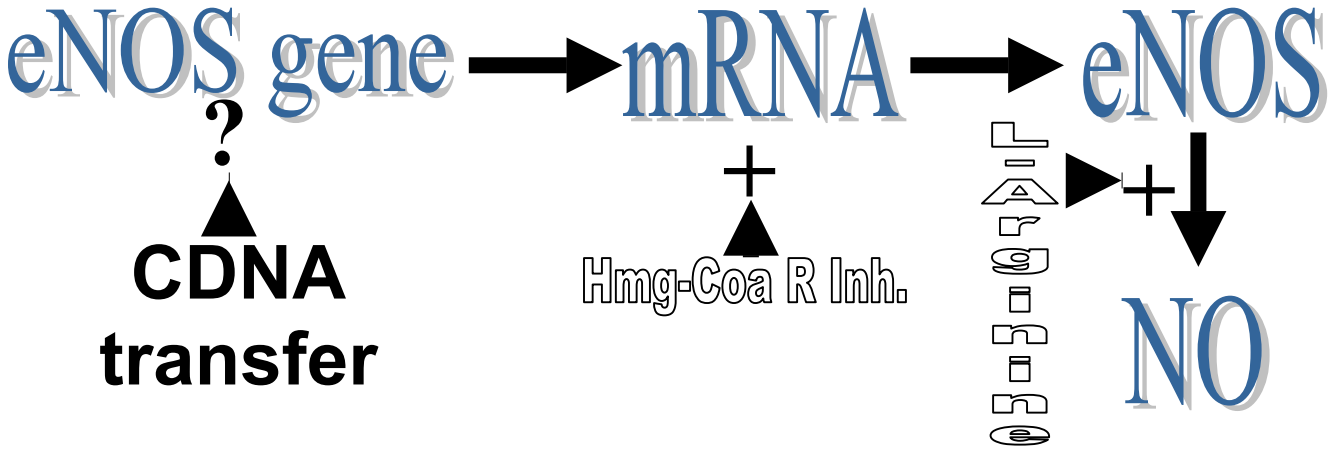


Figure 14 – One of the hypotheses of this grant proposal is that eNOS cDNA transfer will increase eNOS gene transcriptional units in endothelial cells. Coupled with Hmg-Coa Reductase inhibitor-mediated stabilization of eNOS mRNA as well as with L-arginine-mediated provision of substrate, this will result in a supra-additive increase in NO production, increase in cerebral blood flow, and neuroprotective effects in ischemia models.

C.9. 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.10. Relevant prior publications by PI and by Dr. Moskowitz (collaborator)

1. 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.

2. 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.

3. Endres, M., Laufs, U., Huang, Z., Nakamura, T., Huang, P., **Moskowitz, M.A.,** Liao, J.K.: Stroke protection by 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase inhibitors mediated by endothelial nitric oxide synthase **Proc. Natl. Acd. Sci. USA 95:** 8880-8885, 1998.

4. 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.

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8. Saeki, Y., Fraefel, C., Ichikawa, T., Breakefield, X.O., **Chiocca, E.A.:** Improved helper virus-free packaging system for HSV amplicon vectors using an ICP27-deleted, oversized HSV-1 DNA in a bacterial artificial chromosome. **Molecular Therapy 3:** 591-601, 2001. **(INCLUDED IN APPENDIX)**

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10. **Wakimoto, H., Ikeda, H.,** Ichikawa, T., Pasternack, M.S., **Chiocca, E.A.:** The complement response against an oncolytic herpes simplex viral vector is species-specific in its activation pathways. (In preparation) **(INCLUDED IN APPENDIX)**

11. **Saeki, Y., Terada, K**., Oshima, K., **Tyminski, E.,** Wade-Martins, R., Kaneda, Y., **Chiocca, E.A.:** Development of a rapid method for the generation of recombinant HSV vectors, (Manuscript in preparation) (Abstract/ Poster presented at ASGT 2001, *Molecular Therapy* 3: 122, 2001. **(INCLUDED IN APPENDIX).**

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).

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[69,98]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. Our new preliminary results show that complement activation is also limiting to HSV vector infection, at least for the four hours preceding and four hours following an ischemic insult. We would like to first expand this time course. To recapitulate our strategy, we can maximize infection of cerebral endothelial cells rather than that of other neural cells, by administration of the vector 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:
Determine CVF-mediated changes in gene expression in mouse cerebral endothelial cells before and after the induction of an ischemic event in CVF-treated and control mice). 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. 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-Δ27–0^+ as the helper virus in the co-transfection[48]. 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.

D.1.4.2. 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). 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 15). 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. 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.

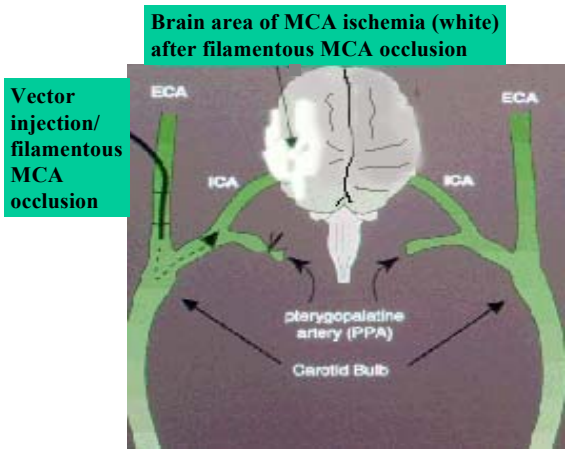


Figure 15 – Description of surgical technique. For both vector and filamentous MCA occlusion the external carotid artery is cannulated. White area of cerebral ischemia is shown if MCA occlusion had occurred.

D.1.4.3. Characterization of reporter transgene expression before and after ischemic insult in CVF-treated and control mice. We will then expand our pilot data by determining additional time course points for reporter lacZ transgene expression before and after an ischemic event in CVF-treated or control mice. Since our last proposal, w ehave simplified experimental parameters by removing the 48 hour pre and 24 hour post time points as well as adenoviral-lacZ experiments. 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 6 for control and table 7 for CVF-treated mice. For the complement-depleted cohort, animals will be pre-treated with intraperitoneal CVF on day –1 (60 units/kg, i.p.) and day 0 (20 units/kg, i.p.) of virus injection (Table 5).

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

Table 4– Description of animal cohorts treated with intra-arterial vectors before, during and after ischemia.

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

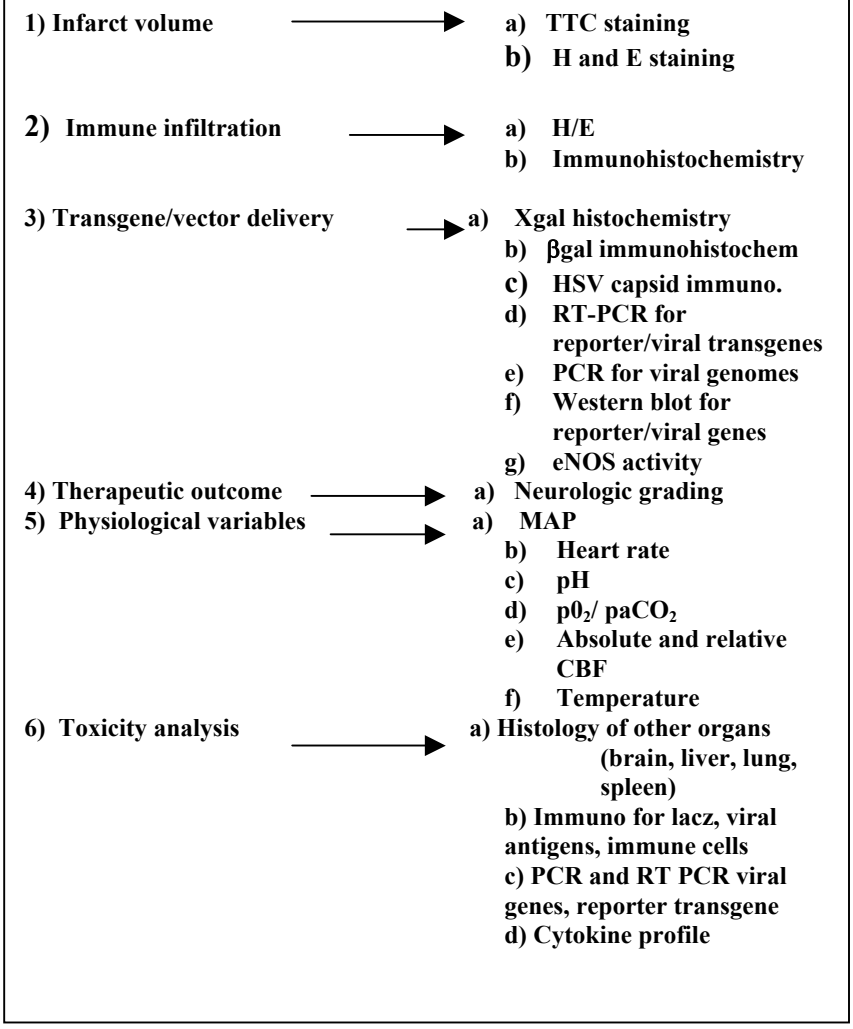
Table 5 - Description of experiment to determine effect of CVF on transgene delivery.

[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.

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 16. 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 within brain parenchyma[54]. 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, <u>even after the ischemic event had disrupted the blood-brain-barrier.</u>** Additional studies will be performed to detect presence of vector antigens by immunocytochemistry against HSV capsid proteins. 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 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[101]. 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

Figure 16 – A list of analyses performed on tissues and animals undergoing proposed experiments.



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.1.4.4.Limitations and alternatives to routes of delivery. 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. Although the aforementioned proposed studies may not show evidence of reporter gene transfer in endothelium subjected to an ischemic injury, our preliminary resulsts do show evidence of gene transfer in endothelium, even after the stroke. This would be in agreement with studies that 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. 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[102,103], the Von Willebrand Factor[104], or the tie[105] promoters. We have obtained the eNOS promoter from Dr. William Sessa (Yale University). An amplicon HSV with lacZ cDNA expression being driven by the eNOS 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. In our laboratory, we have generated a multitude of amplicon vectors with different promoters and thus we remain confident of our ability to easily switch to these alternative experiments.

Another limitation may consist of the fact that gene expression after the stroke may not provide benefit. However, we do believe that **prophylactic** gene expression (as demonstrated by Dr. Moskowitz's studies on eNOS) will still be a useful therapeutic modality for those patients at increased risk for stroke (i.e. undergoing surgical or neurointerventional procedures).

D.1.4.5. 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 could also be tried. These would include sCR1 and its derivatives[106,107]. 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[41,108,109]. 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. Finally, we have begun a collaboration with Dr. John Lambris (U. of Pennsylvania) who has synthesized a 13 aminoacid cyclical peptide (compstatin) that binds to the C3c component of complement and blocks its C3-convertase activity. This peptide has been tested in baboons and pigs and has shown to be safe and effective in complement inhibition[110-113]. We would thus plan to test this inhibitor in our mouse model of ischemia as described above.

D.1.4.6. 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 4, 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 not attempt to determine if the alternative pathway of complement activation contributes to these events because we recently have shown that this pathway is unlikely to be involved. Calcium-depleted serum from rats, humans or mice was no longer able to inactivate vector (Figure 17). This would be consistent with the nucleotide sequence of gC from different viral strains. We have not found mutations/polymorphisms in the sequence of gC in the amino terminus (aa 33-123) which contains domains relevant to binding and inactivation of C5a and properdin (figure 10, page 30). This region of gC is also involved in viral tropism because it contains the heparan sulfate binding domain[9]. In contrast, the domains involved in binding C3b (classical and lectin pathways) are in the middle of gC (after aa 123).

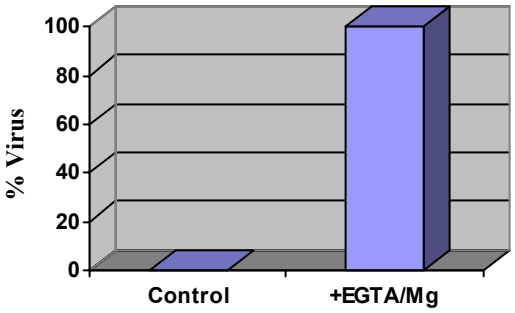


Figure 17- Calcium depletion eliminates serum inactivation of HSV vector, suggesting that the calcium-independent alternative pathway of complement activation is not relevant.

However, we still plan to 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[75,114]. 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 (figure 8). A dose-escalating experiment will be conducted whereby increasing concentrations of mannan would be administered to mice. We would then harvest serum from these animals to determine a range of mannan doses which revert serum's inactivation of vector. These doses would the be employed to pharmacologically inhibit the MBP (lectin) pathway. Finally, our newest collaborator (Dr. John Lambris, University of Pennsylvania) has synthesized peptide inhibitors of MBP[115] and has made these available to us (please see attached letters).

Comparative analyses with results obtained for depletion of classical pathways of complement activation should thus permit us to characterize the *in vivo* contribution of each towards limiting gene transfer.

D.1.4.7. In vivo genetic confirmation of complement and 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 4 shows that this activity is present in mice and figures 7 and 8 show that *in vitro* this activity is primarily due to MBP. In mice, there are two isoforms of MBP (MBPa and MBPc), which are both involved in activation of complement against a variety of viral pathogens (personal communication, Dr.A. Ezekowitz). Our collaborator, Dr. Ezekowitz, has made mice with homozygous genetic defects in MBPa (used in the experiment of figure 7, page 29) as well as double-mutants in both MBPa and MBPc. These mice are viable and do not appear to possess gross anatomic abnormalities of brain or other organ systems. We will thus employ C3 and both MBP knock-out mice and ask if endothelial cell transduction as well as the end-point correlates described in Figure 16 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 1-2).

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[83]. However, the findings described in the pilot data and in published experiments[98] 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[80]. Sequence analysis of gC in our amplicon vectors have revealed the presence of three non-conserved amino acid changes (compared to complement-evading clinical isolates, such as NS), one of which is in the C3 binding domain (figure 10). 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.

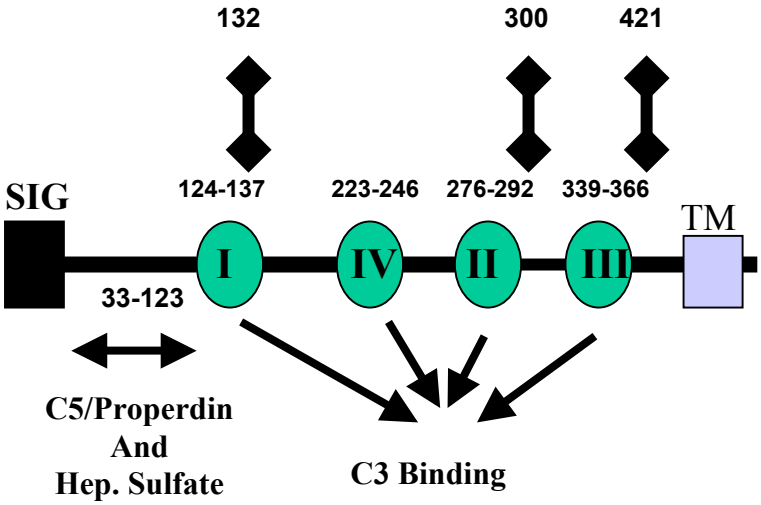


Figure 10 – Stick diagram of gC. SIG designates the signal sequence. C5/Properdin (alternative path.) and heparan sulfate domains (viral attachment) are located at amino acids 33-123. The four C3 binding domains (Classical and lectin path.) are at aa. 124-137, 223-246, 276-292, and 339-366. Nonconserved Amino acid changes between virulent strain NS and amplicon are at aa 132, 300, and 421.

D.2.3. Experimental Methods-

D.2.3.1. Substituting gC from strain NS into the gC from HSV-BAC-Δ27-0$^+$ (YEAR 1). 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[48] and/or by using the "HSVquik" system[12], 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 Saeki et al. (see appendix). 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 (YEAR 1). 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 (YEAR 2). 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.

	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

TABLE 6 – *In vivo* testing of the transduction efficiency of amplicon possessing gC from NS versus that of amplicon possessing gC from 17 strain.

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 7 and sacrificed at 72 rather than 24 hours after MCA occlusion to determine how long transgene expression is sustained.

	Time[A]

Table 7 - *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

Vector	72 hrs pre	24 hrs pre	Same Time	30 min post	1 hr post	4 hr post	No ische[B]
NS-Amplicon	5[c]	5	5	5	5	5	5
17-Amplicon	5	5	5	5	5	5	5
Nsrev-Amplicon	5	5	5	5	5	5	5
Mock	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 16, 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 cerebral vasculature transduction. For instance, MBP binding to the vector could limit efficient infection, even without complement activation. This could be addressed by saturating MBP *in vivo* with mannan.

D.3.Specific Aim 3 Determine if evasion of neutralizing immunity to HSV will increase the efficiency and safety of gene transfer. (Year 1-3).

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[9] (figure 18). In this process, the Fab portion of the neutralizing antibody binds to HSV epitopes(such as gD), while the Fc portion binds to gE/gI. Bipolar bridging on HSV's surface inhibits antibody-dependent cellular cytotoxicity[15], complement-enhanced antibody neutralization[14], and attachment of granulocytes to the Fc domain[118]. 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[119].

In spite of inability to test this construct *in vivo*, the *in vitro* experiments will still prove extremely useful. Because 50-80% of the human population possesses neutralizing IgG to HSV1, it is likely that a vector that evades the C3 portion of complement (and thus complement-mediated lysis) but cannot evade IgG may still be

hampered in its transduction efficiency, once it is being tested in human trials. At this juncture, the capacity to have an Ig-evading vector will be an extremely valuable alternative.

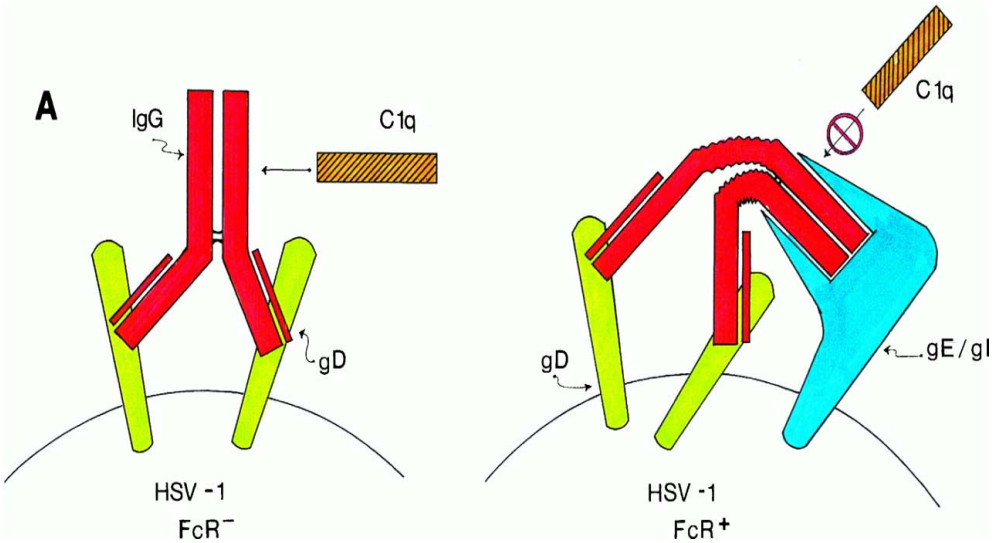


FIG. 18 (A) Model showing the HSV-1 FcγR blocking complement-enhanced antibody neutralization. On the left, an antibody molecule (red) binds to its target antigen (shown in green as HSV-1 glycoprotein gD) by its Fab domain. The absence of an FcγR enables C1q (brown) to bind to the antibody Fc domain, leading to activation of complement and complement-enhanced antibody neutralization. On the right is shown an example of antibody bipolar bridging in which an antibody molecule (red) binds to its target antigen (green) by the Fab domain while the Fc domain of the same antibody molecule binds to the HSV-1 FcR (blue), which blocks the interaction of C1q (brown) with the IgG Fc domain. Adapted from Nagashunmugam et al., 1998(ref. 8).

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. [13] 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 with hrR3 [13]. 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). 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.

	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).

Table 8 – Strategy to determine the effect of neutralizing antibodies on vector infection

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 (YEAR 2). 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[69]. 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 8 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 (YEAR 2). We will then repeat the experiment described in Table 8 in the context of an ischemic insult. <u>If neutralizing immunity is **not** found to significantly alter vector infection</u> 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. <u>If neutralizing immunity is found to be limiting to transgene expression</u>, then pre-treatment with CPA will be performed to reduce the levels of neutralizing antibodies before repeating the experiment described in Table 9.

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

Table 9 - Description of cohorts and number of mice with neutralizing immunity to HSV treated with intravascular NS-amplicon in the context of MCA occlusion.

RAG2 -/-	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 (YEAR 3).* 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[9] (figure 18). 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[119].

D.4.Specific aim 4: Determine if endothelial nitric oxide synthase (eNOS) gene transfer protects mice from cerebral ischemia (Year 2-3).

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[26,29,27,28]. Since neither drug functions at the level of transcription, rather increases eNOS activity by post-transcriptional and post-translational mechanisms[25], 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 (figure 14).

D.4.3.Experimental methods:

D.4.3.1. Generation of eNOS amplicons (YEAR 2). 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 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 (YEAR 3). 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 4-9 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..

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

Table 10 - Description of cohorts and number of treated animals per experiment.

[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 16. We will further determine if a similar result occurs 72 hours after MCA occlusion, by repeating the experiment shown in Table 10. 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)[120]. 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? (YEAR 3) 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 16). 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)[26]. 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[29]. 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 11. 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 11. 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 11).

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

Table 11- Description of cohorts and number of treated animals for CBF

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 (YEAR 3). 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[93]. 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 Tables 10 and 11, 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 would significantly improve the deleterious effects of cerebral ischemia when compared to the mice treated with the lacZ-amplicon. 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 (YEAR 3). 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 10 and 11. 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. Enhanced therapeutic effects by combining pharmacologic and genetic-mediated increases in eNOS expression (YEAR 3). 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[25], 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[29]. 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 (figure 14).

We thus would plan to treat mice with Simvastatin (Sim) (or Mevastatin – see figure 1-2) for 14 or 28 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 16 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[121] or Greco[122] 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[50-55,101]. 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[50-55], 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[102], 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[123].

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
Aim 1	**X**		
Aim 2	**X**	**X**	
Aim 3	**X**	**X**	**X**
Aim 4		**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.