

A Molecular and Immunological Investigation of Cellular
Responses to Dengue Virus:
Identification of Potentially Upregulated Host Genes and the
Construction of a Vaccinia Virus Expressing the Dengue 1 Hawaii
NS3 Protein

by

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ABSTRACT

The purpose of this thesis for the degree of Master of Science was to use molecular and immunological techniques to study cellular responses to dengue virus infection. In the initial study, Differential Display was used to compare mRNA expression in dengue-infected K562 cells and mock-infected cells. Cloning and sequencing were then used to identify cellular genes that were potentially up-regulated in response to Dengue virus infection. These genes included bleomycin hydrolase and a dystrophin homologue.

The goal of the later part of this research was to construct a recombinant vaccinia virus expressing the dengue 1 Hawaii NS3 protein. Cytotoxic T-lymphocyte assays and protein gel electrophoresis showed that the NS3 protein was being expressed. This construct was then used to study the cytotoxic T-cell response of a dengue 1 vaccine recipient. The results of this study showed that this individual has dengue 1 NS3 specific T-cells and also that this vaccinia virus can be used for subsequent T-cell studies.

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GENERAL BACKGROUND

Dengue Virology

The Dengue virus is a member of the family Flaviviridae. These viruses are single-stranded positive-sense RNA viruses. Dengue viruses exist as four serotypes 1, 2, 3, and 4. The genome encodes three structural proteins and seven non-structural proteins. The structural proteins are the Capsid (C), Membrane (M), and the Envelope (E). The non-structural proteins are designated as NS1, NS2a, NS2b, NS3, NS4a, NS4b, NS5. The genome is approximately 10.7 kilobases and is translated into a single 350 kDa polyprotein that is cleaved by host cell and viral encoded proteases (Fields *et al.*, 1996).

Dengue Diseases

Dengue viruses cause two syndromes: Dengue Fever (DF) and Dengue Hemorrhagic Fever/Dengue Shock Syndrome (DHF)/(DSS). Dengue Fever is a self-limited febrile disease, characterized by fever, muscle aches, bone pain and retro-orbital pain (Kurane and Ennis, 1992). Other symptoms and signs include severe arthralgias, headache, accompanying maculopapular rash, leukopenia, and mild thrombocytopenia (Rothman, 1997). These symptoms subside without complications within about a week.

In some patients, viral infection can cause plasma leakage into interstitial spaces. As a result, patients suffer hypovolemia and sometimes circulatory collapse. This syndrome is termed DHF because it is almost always accompanied by thrombocytopenia and hemorrhaging. When plasma leakage becomes profound, shock can occur. This is known as Dengue Shock Syndrome. DHF was categorized into four grades, 1 through 4, by the World Health Organization, where 1 is the least serious and 4 is the most serious. DHF grades 3 and 4 are called DSS (Technical Advisory Committee, 1980). The pathogenesis of DHF/DSS is not fully understood. Immediate medical treatment can abate the hypovolemia and usually prevent lasting organ dysfunction (Rothman, 1997). This condition is often life threatening, especially for children between the ages of 3 and 14, as shown in Figure 1.

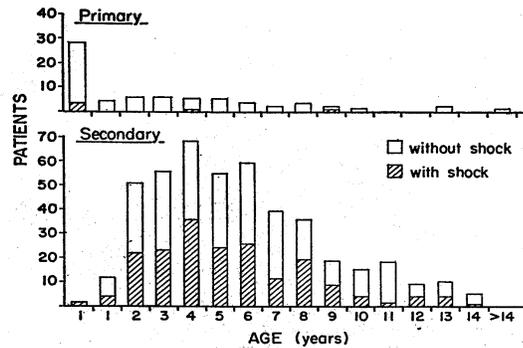


Figure 1: Age distribution and the occurrence of shock in DHF.
From Halstead *et al.*, 1970.

Dengue Hosts

The mosquito vector *Aedes aegypti* transmits the dengue virus (Halstead, 1981). These mosquitoes transmit the virus to humans, and it is thought that the virus is perpetuated by the human-mosquito-human transmission cycle. Lower primates can be infected with dengue but do not develop clinical symptoms of DHF or DSS (Gubler, 1994). The mosquito breeds in stagnant pools of water near human habitats, especially in warm climates such as in South America and Southeast Asia (Gubler, 1989). There have been many outbreaks in recent history, the most notable of which is the Cuban outbreak in 1981 (Guzman *et al.*, 1984). This outbreak claimed 158 people, while 350,000 were estimated to be infected. Although almost every country in the Western Hemisphere has attempted to eradicate the mosquito, only Cuba succeeded after the 1981 outbreak using military force and pesticides. The success was not permanent however, as Cuba experienced another dengue outbreak in 1997. According to the government, 838 people were infected but independent news agencies put the toll at 30,000. The death toll ranged from 3-20 (Taubes, 1997). Recently, the numbers of this mosquito species have been increasing on the North American continent, appearing in Florida, Texas, Alabama, and Mississippi. This increase may promote the emergence of dengue diseases, thus posing a potential health threat in the continental United States. Figure 2 shows the geographical representation of regions affected or potentially affected by dengue infection.

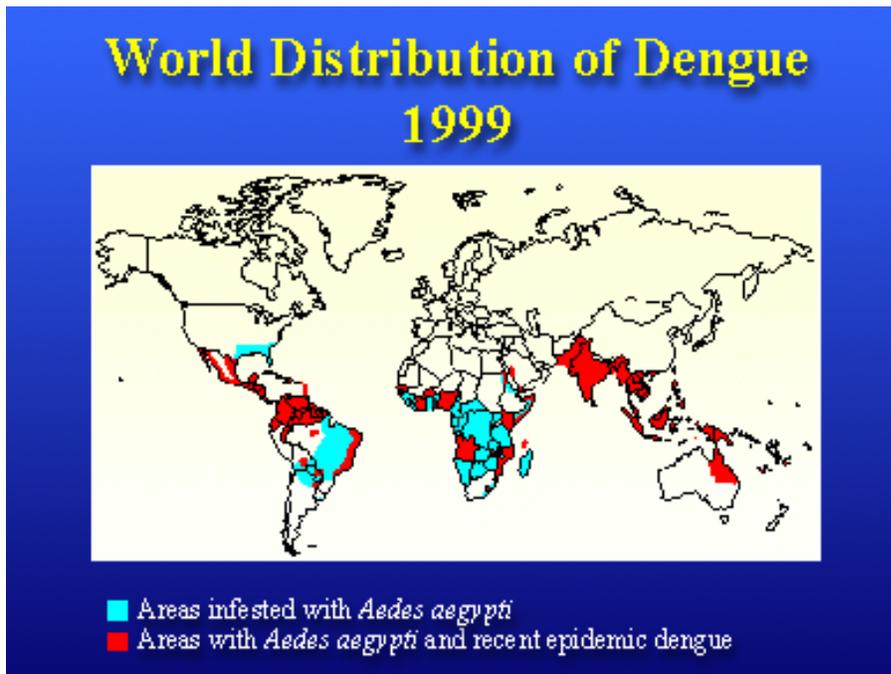


Figure 2: Geographic Regions of Recent Dengue Activity. From The Center for Disease Control Public Health Image Library. (<http://phil.cdc.gov/Phil/default.asp>)

Animal Models

Because the virus has only been shown naturally to infect mosquitoes, humans, and lower primates, the development of an economical model has been problematic. Baby mice that are challenged intracerebrally with dengue virus have been shown to produce subclinical infections, but symptoms are generally sporadic. Adult mice generally produce no symptoms (Boonpucknavig *et al.*, 1981). Therefore, work has been done to create a disease model using SCID (Severe Combined ImmunoDeficiency) mice (Bosma *et al.*, 1983). SCID mice that were reconstituted with adult peripheral blood lymphocytes have been shown to support dengue type 1 infection, but the frequency of such infection is low (Wu *et al.*, 1995). The infection frequency was shown to be improved with implantation of the human cell line K562 infected with dengue type 2 virus. The K562-SCID mice do not show the clinical symptoms of DHF/DSS, but the fact that they have extra cerebral infection sites make them a more suitable animal model than cerebrally-infected mice (Lin *et al.*, 1998). The lack of a proper animal model for DHF/DSS requires that we employ methods of epidemiology to study the disease in humans. Consequently, the molecular aspects of dengue virus and infection are currently limited to *in vitro* models.

Recent Developments in Therapy and Prevention

Currently, methods of attacking the virus are being researched. The development of antisense oligonucleotides that bind to the viral positive sense RNA genome to block its replication and translation *in vitro*, is one such advance. However targeting the oligos to the cytoplasm, where the virus replicates, and not to the nucleus has been a hindrance in the further development of this treatment (Raviprakash *et al.*, 1995). There has also been an attempt to infect *A. aegypti* mosquito with another virus carrying an antisense RNA to the dengue pre-membrane protein, which would block the replication of dengue in the mosquito salivary gland. Although this approach may work in theory and in the laboratory, field applications are not yet feasible, and the effect of the carrier virus has not been tested in humans (Olsen *et al.*, 1996). A live attenuated tetravalent dengue vaccine has been in development for several years. Phase I clinical trials with the vaccines are currently underway (Jirakanjankit *et al.*, 1999).

Dengue Pathogenesis

The cascade of dengue pathology begins with the primary infection of the host. Cytotoxic, helper and memory T-cells are activated and antibodies to the virus are secreted. The T-cell population is both serotype specific and serotype cross-reactive due to the high homology between the serotypes. Upon secondary infection by a different serotype, enhancing antibodies that are pre-existing from primary infection cause what is known as antibody dependent enhancement (ADE) of the viral infection.

ADE by dengue antibodies was first reported in 1977 by Halstead *et al.* Their research demonstrated that human sera from dengue-infected patients enhanced dengue infection of peripheral blood mononuclear cells (PBMC) from non-immune patients. ADE occurs when neutralizing antibodies at sub-neutralizing concentrations, or non-neutralizing antibodies, bind dengue virus (Henchal *et al.* 1985). IgG is the primary immunoglobulin associated with dengue ADE. Antibodies to the Fc γ receptor (Fc γ R) of antigen-presenting cells block antibody dependent enhancement of dengue. Thus, it has been elucidated that the dengue virus particle and the IgG antibody to the virus bind to create a virus-antibody complex. This complex binds to the Fc γ R via the Fc portion of the IgG, thus enhancing infection of the cell by bringing into closer proximity dengue particles with the putative heparan sulfate receptor or other receptors (Gollins and Porterfield, 1984; 1985).

Following cellular infection, serotype cross-reactive CD4⁺ and CD8⁺ memory T-cells are activated. The CD4⁺ T-cells produce lymphokines such as IFN γ and IL-2 in response to activation, causing an upregulation of Fc γ R, MHC-I, and MHC-II production in new monocytes (Kelley *et al.*, 1983). As a result of lymphokine production, it is thought that the complement cascade is activated, and in conjunction with the lymphokines, cause plasma leakage, hemorrhage, and shock.

Epidemiological observations have shown that there is a role for ADE in the pathogenesis of DHF/DSS. It has been observed that, in Thailand, 99% of DHF/DSS afflicted children over the age of 1 year have antibodies to dengue virus before the infection causing DHF/DSS (Halstead 1988). In patients under the age of one year, Kliks *et al.* (1988) have shown that there is a correlation between the age of infants who developed DHF/DSS upon primary infection the titer of existing antibodies in the sera of the mothers. However, there have been rare cases of patients with DHF/DSS who did not have pre-existing antibodies to dengue and who showed a primary immune response. These patients provided evidence that there may be other factors involved in the pathogenesis of DHF/DSS, including virulence of virus strains and host genetic factors (Scott *et al.* 1976).

Purpose of Thesis

The purpose of this thesis is to investigate the cellular and immunological response to dengue infection. The research in Part 1 was to prescreen for potential upregulation due to dengue infection. Differential Display was used to compare mRNA expression of Dengue-infected and mock-infected cells. Cloning and sequencing was used to identify genes that may be upregulated due to a stress response to infection. The goal of Part 2 was to construct and use a recombinant vaccinia virus expressing a dengue protein to examine the T-cell repertoire of an experimental dengue vaccine recipient.

BACKGROUND - PART 1

This research was conducted in conjunction with Dr. Tammy Spain-Santana and Cynthia Drainville ('99 WPI)

K562 Cell Line and Dengue Type 2

The *in vitro* system that was chosen to study the effects of dengue infection on cellular gene expression is the human K562 immortalized cell line. This line was isolated from a patient with chronic myelogenous leukemia and was first characterized as an erythroleukemic cell line. However, a recent study has shown that the cells are “multipotential, hematopoietic malignant cells that spontaneously differentiate into recognizable progenitors of the erythrocyte, granulocyte and monocytic series” (Lozzio *et al.*, 1981), and can therefore be characterized as a myelomonocytic cell line. Primary monocytes can be cultured *in vitro*, but the isolation of monocytes alone is difficult and the isolation can cause non-specific activation. In addition, primary monocytes have a low infection rate in culture. Other cell lines, such as U937 and THP-1, that have been used previously to model monocytes were found to not be suitable to the current study due to the fact that a high infection rate can only be achieved with pre-treatment to activate the cells (Spain-Santana, personal communication 1998). This pre-treatment with activators would complicate the current study, which is investigating the immediate reaction of the cells to dengue infection.

The dengue strain that was chosen was a strain of dengue serotype 2 isolated in Sri Lanka. Dengue 2 strains have been shown to more frequently cause severe dengue infection in Cuban, Caribbean and South American dengue outbreaks (Rico-Hesse *et al.*, 1997). Secondary infection by serotype 2 has been shown to be a risk factor in the development of DHF/DSS (Sangkawibha *et al.*, 1984). Also, dengue virus 2 strains (D2V) are the most efficient at infecting cells *in vitro*. It is the serotype most commonly used in laboratory studies. The Sri Lanka strain was found to be more infectious than the D2V New Guinea C strain in preliminary experiments (Spain-Santana, personal communication 1998).

Differential Display and its Uses

Differential Display (DD) is a widely used method for identifying differentially expressed genes. Subtractive hybridization and microchip arrays are other technologies used for the same purpose. Differential display has several advantages over hybridization assays. Hybridization assays often result in a loss of information about differential expression of highly homologous proteins within a gene family. Because DD is a non-hybridization technique, gene family differential expression can be detected. The identity of differentially expressed genes can be verified via secondary methods (Martin *et al.*, 1998).

Verification can be achieved using Northern Blot Analysis, but it requires time and can be very laborious when you have many genes. Most importantly, Northern blotting is not sensitive enough to detect many genes detectable by Differential Display. Martin *et al.* (1998) discuss strategies and techniques aimed at minimizing false positives and avoiding the use of impure Northern Blot probes that are generated from PCR-amplified DD bands. This research at Dana Farber centered around breast cancer from which they have developed a new strategy for Differential Display in order to identify and verify gene expression among a large number of genes. They identified the differentially expressed bands, excised, and eluted them. The bands were directly sequenced and were queried against GenBank by BLAST. They defined a match as "... $\geq 96\%$ identity of bases over a stretch of ≥ 30 bases" (Martin *et al.*, 1998). A gene specific primer (20mer) was designed so that it would hybridize to the DD fragment with the arbitrary primer site. The primer was also used to PCR amplify a homologous probe for Northern Blot Analysis.

Their streamlined strategy (Figure 3) has enabled them to identify tumor suppresser genes rapidly with a negligible rate of false positives (Martin *et al.*, 1998). There were two approaches regarding sequence information. If it was readable, it was used to design a gene specific primer. Those that were poor were cloned and screened for differential expression.

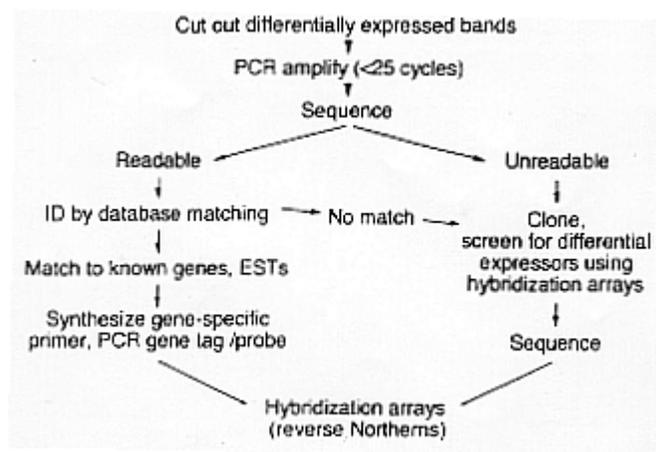


Figure 3: Strategy for Identifying and verifying differential expression of DD bands. From Martin *et al.*, 1998.

Martin *et al.* (1998) consider direct sequencing as the first useful step in identifying and verifying DD bands versus hybridization assays because it only takes into account populations that are best represented. Hybridization assays focus on what binds the best-not population representation.

MATERIALS AND METHODS - PART 1

Virus preparation and cell culture

Dengue type 2 virus Sri Lanka was cultured in C6/36 mosquito cells as previously described (Kurane *et al.*, 1984). The human cell line K562, a myelomonocytic cell line was grown in RPMI 1640 medium supplemented with L-Glutamine, penicillin, streptomycin, and 10% fetal bovine serum (FBS). The cultures were maintained at 37°C.

Infection of K562 cells

Twenty-four hours prior to infection, 10^7 cells were put into mid-log phase by adding 20% FBS RPMI and incubating for 24 hours at 37°C. Cells in mid log phase were then washed twice with 2% FBS RPMI. After removal of excess media, pellets were resuspended and D2V (MOI: 10) or a control of C6/36 cell culture supernatant was added. Virus-infected cells were designated KD and mock-infected cells were designated KC. Virus was adsorbed to cells for 2-3 hours at 37°C with shaking. Cells were then incubated in a total volume of 10 ml of 10% FBS RPMI and at 37°C for 24 hours. Cells were harvested by washing with PBS and then resuspended in 1 ml of PBS. 10 microliters of this suspension was dotted onto a slide for indirect immunofluorescence Assay (IFA) (see below). Cells were then resuspended in Buffer RLT plus β -mercaptoethanol according to the Qiagen RNeasy Kit and were stored at -70°C.

Indirect Immunofluorescence Assay (IFA)

Cells adhered to glass slides were fixed in EtOH at -20°C for 10 minutes. Slides were stored at 4°C until staining. Fixed cells were incubated with anti-D2V hyper-immune ascitic fluid in a humidifying tray at 37°C for 30 minutes. Slides were then washed with phosphate-buffered saline (PBS). Cells were incubated with fluorescein or rhodamine conjugated goat anti-mouse IgG at 37°C with humidity for 30 minutes. Cells were counted under a light microscope. Dengue antigen positive cells were visualized under a UV light microscope. Dengue antigen positive and negative cells were counted to determine the percentage of infected cells.

Total RNA extraction

The RNeasy Kit by Qiagen was used to prepare total RNA from D2V-infected cells and mock-infected cells. RNA was quantified spectrophotometrically by measuring absorbance of UV light at 260 nm and using the following equation: $OD (A_{260}) \times \text{Dilution Factor} \times 40 \text{ ng/ul} \times \text{total volume} = \text{total yield}$, where $OD (A_{260})$ is the optical density of the sample at a 260 nm wavelength. Total RNA was stored at -70°C until ready for use.

mRNA Isolation

Cellular mRNA was isolated from total RNA using the PolyATtract System mRNA isolation kit (Promega). 150 µg of total RNA was expected to yield approximately 5 µg mRNA. Prepared mRNA was stored at -70°C until Northern Blotting.

Differential Display (DD)

The Delta Differential Display kit (CLONTECH Laboratories, Inc.) was used to compare RNA from dengue-infected and mock-infected cells. The Kit comes with 10 P primers of arbitrary sequence of 25 nucleotides and 9 T primers of 30 nucleotides, which contain a poly-T anchor at the 3' end. The P primers were designed to recognize common sequence motifs found in coding regions of eukaryotic (especially mammalian) mRNAs (Differential Display Protocol). We chose to perform PCR with combinations 1 of each of P and T primers, starting with T9 and P1-P10, and T8 and P1-P10. Differential Display primer sequences are shown in Table 1.

Table 1: Differential Display Primer Sequences (CLONTECH Laboratories, Inc.)

Name	Length	Sequence
T8	30-mer	CATTATGCTGAGTGATATCTTTTTTTTTGC
T9	30-mer	CATTATGCTGAGTGATATCTTTTTTTTTGG
P1	25-mer	ATTAACCCTCACTAAATGCTGGGGA
P2	25-mer	ATTAACCCTCACTAAATCGGTCATAG
P3	25-mer	ATTAACCCTCACTAAATGCTGGTGG
P4	25-mer	ATTAACCCTCACTAAATGCTGGTAG
P5	25-mer	ATTAACCCTCACTAAAGATCTGACTG
P6	25-mer	ATTAACCCTCACTAAATGCTGGGTG
P7	25-mer	ATTAACCCTCACTAAATGCTGTATG
P8	25-mer	ATTAACCCTCACTAAATGGAGCTGG
P9	25-mer	ATTAACCCTCACTAAATGTGGCAGG
P10	25-mer	ATTAACCCTCACTAAAGCACCGTCC

Total RNA from mock-infected cells and dengue-infected cells was used to synthesize cDNA per the Differential Display protocol. PCR was performed in a Perkin-Elmer Thermal Cycler 480 with mock-infected cDNA, cell-infected cDNA, positive control total RNA, water controls and the appropriate primer combinations. PCR cycles were as follows:

1 Cycle:
94°C 5 min
40°C 5 min
68°C 5 min
2 Cycles:
94°C 2 min
40°C 5 min
68°C 5 min
25 Cycles:
94°C 1min
60°C 1min
68°C 2 min
68°C for 7 minutes

PCR samples were analyzed on a 5% polyacrylamide/8M urea denaturing gel, prepared and run as directed. After the run, the gel was transferred to Whatman paper and dried on a gel dryer at 80°C for at least 30 minutes. The DD PCR products were visualized by autoradiography. Bands that appeared in the dengue-infected sample but not in the mock-infected sample were excised out of the differential display gel and eluted in 40 ul TE buffer by heating for 5 minutes at 100°C and then incubating the sample at 60°C overnight. The eluted cDNA was re-amplified with PCR according to the Delta Differential Display Kit. PCR cycles were as follows:

30 Cycles:
94°C 1 min
60°C 1min
68°C 2 min

Prior to being sequenced, PCR products were desalted using a QIAquick PCR Purification Kit (QIAGEN). For the direct sequencing reactions, a 40:1 primer to template ratio was used. The T primer that was used to generate each product was used for sequencing. ABI dye-terminator sequencing was performed. Sequences were analyzed by the GCG program (Wisconsin package) and GenBank BLAST Search tool at the NCBI website.

Cloning

cDNA eluted from a differentially expressed band of the differential display gel was isolated and ligated into the pCR2.1-TOPO vector using a TOPO TA Cloning Kit (Invitrogen). The plasmid was transfected into competent cells which were then plated on LB agar plus ampicillin (50 µg/ml) and x-gal for blue/white selection. The plates were incubated 24 hours at 37°C. Ten white colonies from each plate were picked and grown overnight in 2 ml Luria Broth with ampicillin (100 µg/ml). Plasmid DNA was prepared using QIAprep Spin Miniprep columns (Qiagen). Plasmid DNA was digested with *EcoRI* to confirm the presence of an insert. 500 ng of PhiX174 DNA-Hae III Digest was used for a marker. Gels were stained with ethidium bromide. Plasmid DNA was sequenced with primers T7 and M13R.

Northern Blot analysis

mRNA samples were separated by gel electrophoresis on a 2% agarose gel. Using standard Northern Blotting protocols (Current Protocols in Molecular Biology), the mRNA was transferred to a nitrocellulose membrane. The membrane was then dried and RNA cross-linked to the membrane under UV light for 3 minutes. Northern Blots were first probed with Actin or GAPDH (Ambion). Probes for Northern Blots were generated from DD bands by PCR amplification of differentially expressed DD products

cloned into plasmids. PCR of plasmid DNA was performed with the following specifications with primers from the original differential display:

30 Cycles:
94°C 1 min
60°C 1min
68°C 2 min

10 µl of the PCR reaction was then gel purified by the QIAquick Gel Extraction kit. The gel purified DNA (25 ng to 100 ng) was used as template for probe using the StripEZ StripAble probe synthesis (Ambion). Unincorporated nucleotides were removed with MicroSpin G50 columns (Pharmacia Biotech). Specific Activity was measured using a scintillation counter. Probe was hybridized to the blot in Hybridization solution (5X SSC, 1% SDS; 42°C rolling overnight) and the Northern was washed according to standard methods: 2X with 2X SSC/0.1% SDS, 5 minutes at room temperature, 2X with 0.2X SSC/0.1% SDS, 5 minutes at room temperature. The Northern was visualized and analyzed with a PhosphorImager. Northern were stripped using Mild Stringency Strip Solution (5 mM Tris, pH 7.5, 2 uM EDTA, pH 8.0, 0.1X Denhardt's Solution) at 65°C for 2 hours and rinsed with 2X SSC, or using the StripEZ protocol for removal of StripAble Probe (with 1X Probe Degradation Buffer 68°C for 10 minutes followed by 1X Blot Reconstitution Buffer at 68°C for 10 minutes).

RESULTS - PART 1

Purpose

The purpose of Part 1 was identify potentially upregulated host genes of K562 cells infected with dengue virus

Differential Display

Differential Display was used to compare gene expression levels in two populations: dengue-infected K562 cells (KD) and mock-infected K562 cells (KC). Eighteen primer combinations were used for the display. Any bands that were present in the KD lanes but not in the KC lanes were excised and the DNA used for further analysis. From these gels, shown in Figure 4, bands 3, 6, 30, 64, and 57 (not shown) were isolated.

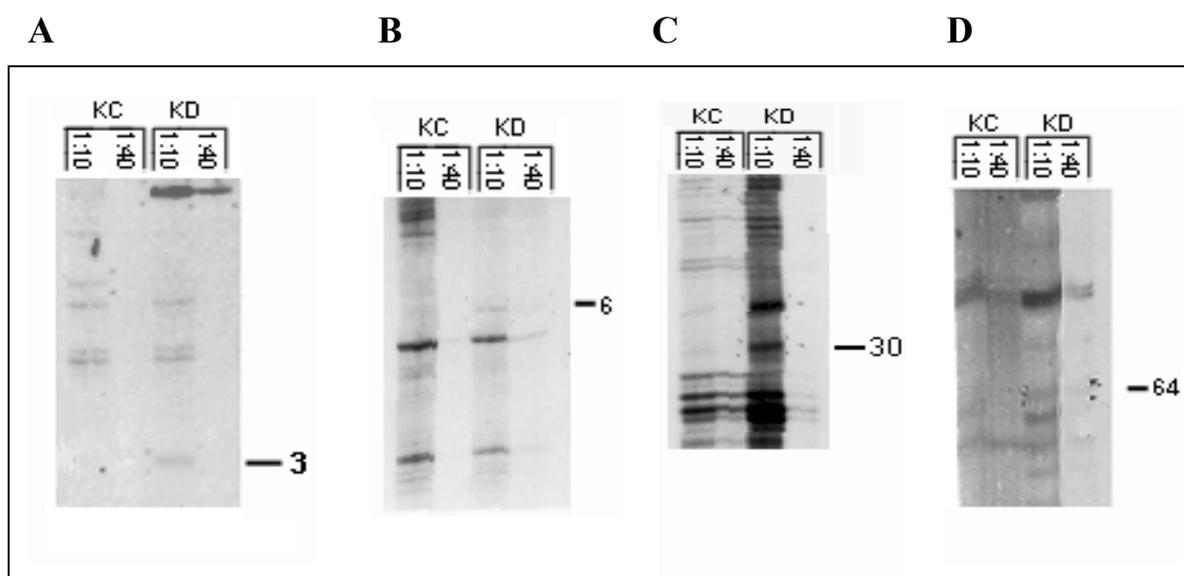


Figure 4: Differential Displays that identified bands 3, 6, 30, and 64. (Band 57 is not shown). Each display has four lanes: mock-infected (KC) 1:10 dilution, mock-infected 1:40 dilution, infected (KD) 1:10 dilution, and infected 1:40 dilution. Primers for each Differential Display reaction are as follows: Panel A: Band 3 P2+T9, Panel B: Band 6-P2+T9, Panel C: Band 30-P1+T9, Panel D: Band 64-P6+T9. See Materials and Methods for primer sequences.

Band Cloning

Once these bands had been identified as potentially upregulated mRNAs, PCR reamplification was performed on the isolated cDNA in order to determine fragment size, as shown in Figure 5. A Phi X 174/Hae III digest Marker was used. Table 2 summarizes the sizes of these bands of interest, which are band 3 at 240 base pairs, band 6 at 800 base pairs, band 30 at 200 base pairs, band 57 at 110 base pairs and band 64 at 450 base pairs. The reamplification products were then cloned into plasmid TOPO TA pCR2.1 and transformed into *E. coli*. Plasmid DNA was prepared from as many as 10 colonies per band. Restriction digests of the plasmids with *EcoRI* was performed to confirm size and presence of insert (Data not shown). Figure 5a shows the size of the insert of each clone of interest. The figure shows that each clone has an insert of the size of the original DD product.

Table 2: Sizes of DD Bands

DD Band	Size
3	240 bp
6	800 bp
30	200 bp
57	110 bp
64	450 bp

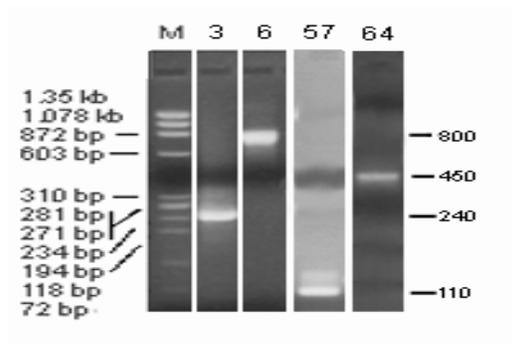


Figure 5: PCR Reamplification of bands 3, 6, 57, and 64 (30 not shown). PCR reactions were performed with original DD primers for each band, run on 2% agarose gels and stained with ethidium bromide.

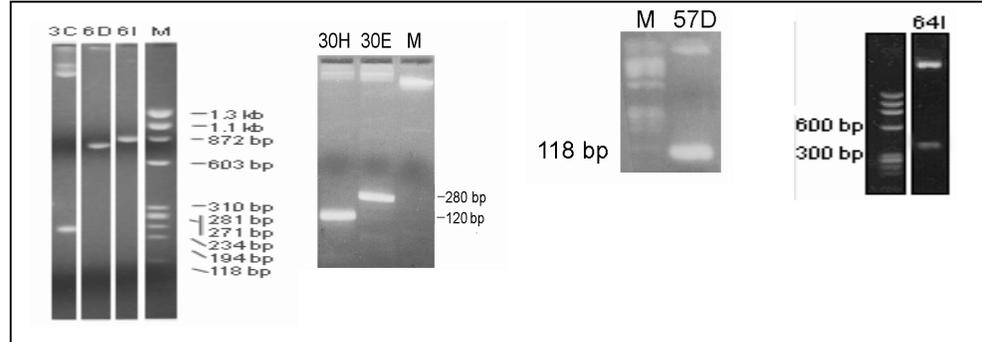


Figure 5a: PCR Reamplification of clone inserts by PCR

Attempted Confirmation of Upregulation by Northern Blot Analysis

The confirmation of differential expression was attempted initially for bands 30 and 57 using Northern blots. cDNA from clones 30B, 30E, 30H, 57B, 57F, 57G, 57H and 57J2 was used to generate radioactively-labeled DNA probes for Northern Blot analysis. Messenger RNA from mock-infected and dengue-infected K562 cells was isolated and separated on a denaturing agarose gel, and transferred to a nitrocellulose membrane. Data from two experiments with probes 30H and 57B can be found in Figures 6 and 7. After quantitative analysis of detected bands and standardization to Actin or GAPDH, confirmation of up-regulation of 30H and 57B could not be replicated. For example, in the first experiment using a probe for 30H, a value of 1.7 was calculated for the standardized ratio of dengue-infected to mock-infected signals. This would indicate that the mRNA that this probe is binding to is upregulated. However, upon a repeat experiment with the same probe, a value of 0.5 was calculated indicating that the mRNA is down-regulated. The results were similar with probe 57B. This could be due to technical errors including problems with probe preparation or varying amounts of RNA on the blots.

PhosphorImager Volumes and calculations

Blot	Probe	Actin KC	Actin KD	probe KC	probe KD	sKC	sKD	sKD/sKC
A93-2	30H	13192	6799	16230	14225	1.23	2.1	1.700589
A94-3	30H	4938	4880	20108	10877	4.07	2.2	0.547358
A93-3	57B	12031	3711	39068	13902	3.25	3.7	1.153631
A94-4	57B	5554	3851	227752	83342	41	22	0.527757

Calculations:

Probe KC/Actin KC = sKC

Probe KD/Actin KD = sKD

sKD/sKC = Standardized Ratio

Figure 6: Northern Blot Calculations of the Standardized Ratios for each experiment.

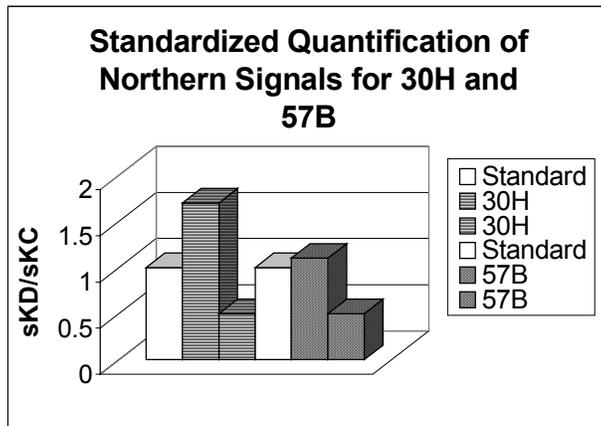


Figure 7: Standardized Quantification of Northern Signals for Probes 30H and 57B.

Band Identification by Sequencing

In spite of Northern verification analyses producing ambiguous results, clones for bands 3, 6, 30, 57, 63 and 64 that contained the correct size insert were sequenced. Analyses of the sequences were accomplished with GCG programs and were then queried in GenBank. The BLAST queries generated several possible gene candidates for the identities of several clones, which are summarized in Table 3. Only nonviral host genes that had some possible correlation with dengue infection or pathogenesis were chosen for further investigation. These genes are human bleomycin hydrolase (clones 6D/6I) and human dystrophin gene (clone 3C), Human activin (clone 3A), human topoisomerase II (clone 64I), and human D9 splice variant B (clone 6J). The sequence alignments as generated by BLAST have been previously reported (Brown and Drainville, 1999)

Table 3: List of Candidate Genes

Clone	Known Genes	BLAST Score
6D/6I	H. Sapiens Bleomycin hydrolase	561/428
3C	Human Dystrophin gene	42
64I	Human DNA topoisomerase II	577
3A	Activin beta C chain	184
6J	Human D9 splice variant B	841
30H	AML1 EAP translocation break point	200
30H	H. sapiens Acute myeloid leukemia associated	200
30H	Human mRNA for EBV small RNAs	200
57B	Human Ribosomal protein S25	121
Unknown Genes		
30E	Human DNA seq f1 PAC 265J14	186
57D	Hu DNA seq PAC 370M22 on chr 22q12-qter.	133

DISCUSSION - PART 1

The purpose of this study was to identify potentially upregulated cellular genes in K562 monocyte cells following dengue infection. This study is unique in that in the field of dengue research, little has been determined about the immediate cellular response to infection during the inflammatory response. T-cell responses are clearly important to the pathogenesis of dengue infections but immediate monocytic reactions involved in sending cellular signals to aid in viral clearance, could be involved in viral replication or disease pathogenesis.

Our observations during this study allow us to draw some general conclusions about the K562 infection system as a whole. First, we have noted that the dengue virus is not an obtrusive virus. K562 cells do not undergo morphological changes when infected with the virus. Differential Display reflects this notion. Over the span of this study, Spain-Santana calculated that only about 1.6% of cellular genes were affected by viral infection (personal communication, 1998). An unusual aspect of this is that most of the genes affected seem to be upregulated rather than down regulated. Generally, when a virus infects a cell, it is assumed that the virus shuts down cellular genes to concentrate on the replication, transcription, and translation of its own genes (Fields *et al.*, 1994). However, dengue seems to cause the cell to activate many cellular genes that are not otherwise activated.

Differential Display was used to perform a genome-wide screen for candidate genes that may be upregulated in K562 cells after dengue type 2 infection. The Differential Display gels were run in triplicate using the same cDNA samples, and the identified bands of interest were later cloned. DD is a PCR-based method which is not quantitative. To avoid false positives, bands that appeared in the KD lane and not in the KC lane were chosen for analysis because they may indicate a significant level of transcription in infected cells that is lacking in mock-infected cells. Clones were sequenced and then queried by BLAST to identify candidate genes. These clones include the following: 6D/6I, 6J, 64I, 3A, and 3C. A BLAST score of close to 200 bits or better (with the exception of 3C) was seen with each of these clones thereby meeting our criterion for probable gene identity. Our general goal was to identify host genes that appear to be upregulated but which were not known to be involved in the immunopathology of dengue infection.

Clones 6D/6I were identified as Bleomycin Hydrolase (Accession X92106). Clone 6I (score 428) showed 97% homology to the human BH gene in 231 out of 236 bases, spanning the region 879-1114. The homologous region was in the coding region of the BH gene. The fact that 2 clones out of 10 shared homology (94%) to each other and were each separately identified as BH is significant. Each DD band contains many same-sized messages and is it not immediately clear if there is one or more genes represented in the band that is upregulated. The fact that the clones were essentially identical indicates that the message is abundant in the band and could most probably be a gene that is upregulated. Bleomycin Hydrolase (BH) is a cysteine protease whose primary amino acid sequence was first deduced through the use of a rabbit liver cDNA library (Koldamova *et al.*, 1998). According to sequence analysis, the human BH lacks a signal sequence. This suggests that it has a cytosolic/nuclear location (Bromme *et al.*, 1996). Ferrando *et al.* (1997) performed further analysis of the 5' flanking region and found that the human BH gene did not contain consensus transcriptional sequences such as the TATA or CCAAT box. This is consistent with the belief that the absence of such elements signifies a house keeping gene, thereby supporting its widespread expression within human tissue. Additional analysis of the 5' flanking region identified an AP-1 consensus site (TGACTCA) upstream from the translation initiation site (Ferrando *et al.*, 1997). AP-1 is recognized by members of several families of transcription factors: c-Fos, c-Jun and ATF-1. AP-1 has also "...been found to mediate induction of different genes by a variety of tumor promoters, cytokines, and growth factors" (Angel *et al.*, 1991). It is not clear as to the types of pathological implications that the AP-1 sequence match may have, however, it may affect the role of the BH gene (Ferrando *et al.*, 1997).

Bleomycin is an anti-cancer drug that was isolated from *Streptomyces verticillus*. It is part of a family of DNA cleaving glycoproteins that is widely used in the treatment of human cancers. The therapeutic efficacy of Bleomycin, unfortunately, is limited by tumor resistance and the development of pulmonary fibrosis (Koldamova *et al.*, 1998). Bleomycin Hydrolase inactivates the anti-tumor glycopeptide bleomycin, which is one possible mechanism as to why there is tumor resistance (Bromme, 1996; Koldamova, 1998). It is plausible that the cellular upregulation of bleomycin hydrolase is a response to the stress of dengue infection by an unknown mechanism.

Clone 64I was identified as DNA Topoisomerase II (Accession J04088). Clone 64I (score 577) showed a 99% homology to the human Topoisomerase II gene in 294 out of 295 bases, spanning the region 3153-3447. This homologous region was the coding region of the top2 gene. The top2 gene is essential for eukaryotic cell survival, and is also fundamental in cell growth development (Larsen *et al.*, 1996; Sng *et al.*, 1999). Topoisomerase II is a component of the nuclear matrix and an enzyme that is required for DNA metabolism: chromosome segregation and condensation, replication, and transcription (Larsen, *et al.*, 1996; Matsuo *et al.*, 1993; Nitiss, 1998; Sng, *et al.*, 1999). There are also two DNA transport cycles that it catalyzes: DNA breakage/religation and ATP hydrolysis (Matsuo *et al.*, 1993; Morris *et al.*, 1999). Topoisomerase exists as two isoforms α and β that express different cell cycle and tissue expression patterns. In proliferating cells, the α isoform is upregulated (Sng *et al.*, 1999). Heck *et al.* (1986) considers topoisomerase to be "...a sensitive and specific marker for proliferating cells."

It is possible that the topoisomerase II gene is heat shock inducible (Matsuo *et al.*, 1993) and is a target for several important anti-cancer agents. The implications in anti-cancer drug resistance include the rearrangement and altered expression of these genes (Sng *et al.*, 1999). When cells become stressed either from physical (e.g. heat) or chemical (e.g. low pH) stressors, heat shock genes become induced and cause the release of Heat Shock Proteins (HSPs) (Macario, 1995). HSPs possess an intrinsic capacity to protect cells during harsh environmental conditions and in combat against pathogens (De Maio, 1999). According to Voellmy (1994), there is recent data that suggests a possible connection between cellular control mechanisms and stress response. Multhoff *et al.* (1998) believe that "HSPs act as immunological target structures either by themselves because of an unusual expression pattern, or they are carrier proteins for immunogenic peptides." Non-MHC restricted effector cells such as natural killer cells and γ/δ TcR positive T lymphocytes have been shown to be major contributors in recognizing HSP. According to Moseley (1998), there is data that further supports the role of HSPs in enhancing antigen presentation to T lymphocytes. Although it is not clear as to how Topoisomerase II may be induced as a heat-shock-inducible gene as a result of dengue infection, there may be some possible connection between dengue infection and an elicited T-cell response due to the HSPs that are released.

Both Bleomycin Hydrolase and Topoisomerase II may have some implication involving anti-cancer research, however, there is currently no evidence shown by anyone that there is a correlation between the dengue infection and cancer.

Clone 6J was identified as Human D9 Splice Variant B (Accession U95007). Clone 6J (scores 841, 161) showed homology within two regions. The first region showed a 95% identity to the human D9 Splice Variant gene in 544 out of 572 bases, spanning the region 91-659. The second region showed a 96% identity to the gene in 88 out of 91 bases, spanning the region 1-91. These homologous regions are part of the coding and 3' UTR regions of the D9 gene. The function of the D9 gene has not been characterized to a great extent. There is some indication, however, that it may be involved in hematopoietic suppression (Scott *et al.*, 1996). This could be potentially interesting in light of recent data indicating that dengue infection of cord blood mononuclear cells inhibits hematopoietic progenitor growth. The chemokine MIP-1 α (Macrophage inflammatory protein) is implicated in this suppression (Margue *et al.*, 1998). Dengue infection is often associated with hypocellular bone marrow and leukocytopenia, which could be indications of bone marrow suppression.

Clone 3A was identified as Activin beta-C chain (Accession X82540). Clone 3A (scores 184,149) also showed homology in two regions. The first region showed a 100% identity to the Activin gene in 93 out of 93 bases, spanning the region 2112-2204. The second region showed a 100% identity to the gene in 75 out of 75 bases, spanning the region 2014-2088. These homologous regions are found in the 3' untranslated region of the Activin gene, a region with possible regulatory roles (Tanimoto *et al.*, 1993). Activin, along with inhibin and follistatin (FS) are proteins that are well characterized in their ability to regulate follicle-stimulating hormone (FSH). Activin, a member of the transforming growth factor B superfamily, acts through binding of its Type I and Type II serine/threonine kinase receptors for signal transduction. Activin subunits and their receptors are widely expressed in a variety of fetal and adult tissues.

FS is known to be an activin-binding protein and was also shown to bind cell-surface heparan sulfate (HS) proteoglycans (Nakamura *et al.*, 1991). A proposed role for FS-activin binding was to inactivate the signal transducing capability of activin, as shown by Hashimoto *et al.* (1997), in rat pituitary

cells. Another study had hypothesized that the FS-activin complex, which binds HS, facilitates activin-receptor binding through local cell-surface interactions. In either case, FS and/or HS seem to aid in the regulation of activin activity (Nakamura *et al.*, 1991).

Heparan sulfate is a putative cellular receptor for dengue virus (Chen *et al.*, 1997) (Note that HS has not been described on monocyte cell membranes, but that doesn't preclude the possibility of it being present). One possible explanation for the role of activin may be to increase the viral accumulation on the surface of monocytes or other infected cells. Infected cells could secrete an increased level of activin, which could then bind FS and subsequently HS on other cells. This complex might initiate a conformational change that allows dengue viral particles to bind HS. HS could, as has been previously proposed, be a receptor or could simply aid in bringing the virus into closer proximity with the cell surface. The major problem with this hypothesis is that monocytic cell lines monocytes (U937, K562 and others) are not known to express or secrete follistatin, and thus it is not known how this intermediate would be present. Alternatively, activin may be acting as a signal-transducing molecule by binding its receptors (monocytes do express activin receptors). However, the nature of the signal, perhaps to increase proliferation of monocytes or other cells, is unclear.

Clone 3C was identified as Human Dystrophin (Accession U60822). Clone 3C (scores 42,36) showed homology in two regions. The first region showed a 89% alignment to the Dystrophin gene in 33 out of 37 bases, spanning the region 81417-81452. The second region showed a 85% alignment to the gene in 36 out of 42 bases, spanning the region 81514-81555. These homologous regions are found in a MER repeat region. Although the evidence that this gene is actually upregulated is not convincing, the role that this gene may play in dengue infection could be potentially interesting and deserves further investigation.

Our study identified the dystrophin gene as a candidate for up-regulation in monocytes as a result of dengue infection. Although dystrophin has never been shown to be expressed in monocytes, there exists the possibility that we have identified a dystrophin homologue. There are several possible explanations for the up-regulation of a dystrophin-like molecule. First, it has been shown that the α -dystroglycan (DG) unit of the Dystrophin-Associated Protein Complex (DAPC) is a receptor for the arenaviruses Lymphocytic choriomeningitis virus (LCMV) and Lassa Fever virus (Cao *et al.*, 1998). The α -DG unit is an extra-

cellular membrane protein that binds to the extra-cellular matrix. The Cao study indicates that the extreme N-terminus of the α -subunit, with possible post-translational modifications, is necessary for virus binding. Arenaviruses share several characteristics with flaviviruses, including a single-stranded positive sense RNA genome and an association with hemorrhagic fever (Fields *et al.*, 1994). There is then the possibility that dengue virus uses this subunit as a cellular receptor. A putative cellular receptor heparan sulfate (HS) has been described for dengue virus type 2 (Chen *et al.*, 1997), but this does not preclude the possibility of multiple receptors.

An alternative explanation for the upregulation of a dystrophin protein is rather more complex. Dystrophin, in skeletal muscle and neurons especially, binds the molecule Nitric Oxide Synthase (NOS) to the cytoskeleton. NOS synthesizes Nitric Oxide (NO), which is a well-characterized free radical that acts as a neurotransmitter and also functions in smooth muscle relaxation (Bredt, 1996). However, NO is integral in the understanding of several antiviral mechanisms. NO has been shown to have an antiviral effect on the flavivirus Japanese Encephalitis virus (JEV) (Lin *et al.*, 1997). Monocytes have what has been termed inducible NOS (iNOS) that is activated in response to high levels of Interferon- γ (Ding *et al.*, 1988). Lin *et al.* showed that NO synthesized by iNOS inhibits the replication of the viral RNA genome, accumulation of viral proteins and virus release (1997). NO production has also been implicated in the inflammatory response after resuscitated hemorrhagic shock, causing permanent organ damage (Hierholzer *et al.*, 1998). The similarities to dengue infection, which includes elevated IFN-gamma levels and liver involvement, indicate that the NO antiviral mechanism could be playing a role in dengue pathogenesis.

Thus we hypothesize that upon dengue infection, a dystrophin-like protein could be essential to aid in the cascade of cellular signals to initiate a NO antiviral response. The diffusion of NO could play a role in dengue fever and shock and liver damage. Due to the complexity of viruses, it is not an impossibility that the virus could be taking advantage of the cell's innate defense mechanism, and thus using the dystrophin molecule as a receptor to multiply-infect monocytes or to amass viral particles.

Preliminary experiments using Northern Blot Analyses were inconclusive in confirming the upregulation of the genes studied. The results of repeated experiments did not agree. This could be due to several reasons. One reason could be suboptimal probe preparation; leaving unlabeled (cold) strands of

our cDNA. This would compete with labeled probe and provide inaccurate results. Another possibility is that the message that we were probing for was rare. It is conceivable that this rarity in RNA can be represented unequally between Northern blots, thereby creating conflicting results.

The clones sequenced above represent candidate genes that need further confirmation of their upregulation. Techniques such as Northern Analysis, RNase Protection Assays, Reverse Northern Dot Blots or DNA microchip arrays could be used to verify the upregulation of these candidate genes.

Because this study was a genome-wide search for differentially expressed genes, there are literally limitless types of experiments that could be performed. Most importantly, the upregulation of the genes (especially of Bleomycin Hydrolase) that we have identified needs to be confirmed. This could be accomplished by using one or more of the techniques listed above. Once this has been done, assays such as ELISAs could be performed to confirm the translational upregulation of the gene and aid in the elucidation of the gene's potential role in immunopathology. The Delta Differential Display kit that was used in this study contained 9 T primers and 10 P primers. With a possible 90 PT combinations available, the primers could be used separately or used in several other pair combinations to offer a wide range of experimental opportunities to identify respective genes through Differential Display. Furthermore, our study used mRNA from the time point of 24 hours post infection. It would be opportune to perform time course differential displays or at other times post infection to compare banding patterns.

BACKGROUND - PART 2

The Dengue NS3 Protein: Structure and Functions

The dengue protein NS3 may be one of the most important proteins for viral pathology. Studies of the CD4+ and CD8+ cytotoxic T-lymphocyte (CTL) responses in patients immunized with candidate live monovalent dengue vaccines have shown that the NS3 protein is the most antigenic viral protein (Gagnon *et al.* 1996; Mathew *et al.* 1996). Many serotype-cross-reactive and serotype-specific epitopes on NS3 have been identified, which is contrary to the belief that most immunodominant viral proteins contain 1 or 2 T-cell epitopes. A more complete understanding of the functions of the NS3 protein in viral replication, protein cleavage, and immune response is highly warranted, especially as a target for future treatment and prevention of dengue diseases.

The serine protease domain of NS3 has been analyzed by x-ray diffraction by Murthy, Clum, and Padmanabhan (1999). They have shown that the molecule contains 2 alpha helices and 5 beta pleated sheets, as shown in Figure 8 (PDB ID 1BEF, 1999). This domain encompasses the N terminal 181 residues of the 618 amino-acid NS3 protein. An alignment of the first 180 amino acids from the NS3 proteins from dengue virus types 1-4, (not shown) shows that the catalytic triad (His-51 Asp-75 Ser-135) is completely conserved in all four types.

The remaining amino acids at the C terminal end of the NS3 protein have been characterized as having both NTPase and helicase activities. This region contains motifs associated with these two proposed functions, including the DEXH family and the putative DEAH helicase family (Fu *et al.* 1998). The helicase and NTPase activities may function in viral replication. The NTPase activity, specifically of ATP, may serve to provide free energy required for RNA binding, unwinding, and removal of secondary structures during negative-strand synthesis (Arai and Kornberg 1981; Deng and Shuman 1996; Hagler, Luo and Shuman 1994). In support of these proposed functions, Fu *et al.* (1998) have shown that the NS3 protein can complex with RNA at the 3' non-coding region, and that the NS5 protein stimulates ATPase activity. They speculate that the NS5 protein may catalyze negative-strand synthesis during viral RNA replication.

Figure 8: Serine Protease Domain of the NS3 Protein (Murthy, Clum, and Padmanabhan, 1999)



Summary of previously identified NS3 T-cell Epitopes

Several studies between 1995 and 1998 have uncovered approximately nine regions of the dengue NS3 protein that contain epitopes that stimulate T-cell clones from dengue-immune patients. A summary of the epitopes can be found in Table 4.

Region 1 was discovered by Kurane *et al.* (1995). Clones JK4 and JK43 were isolated from a D3V immune donor and were both cross-reactive for all four serotypes. The epitope recognized by these clones is a 9 aa epitope between 146 and 154 of the D3V NS3 protein. The last 7 amino acids of this epitope are completely conserved between all 4 types while the first and second positions vary. Dengue 2, 3, and 4 have a valine at amino acid 146 while dengue type 1 has an isoleucine. Conversely, Dengue 1, 2, and 3 have a valine at amino acid 147, while Dengue 4 has an isoleucine.

Region 3 was identified by Mathew *et al.* (1998) using CTL clones established from PBMC of a Thai child recovering from natural secondary infection. The clones from patient KPP94-024 were serotype 2, 3 and 4 cross-reactive, but dengue 1 NS3 was not tested due to a lack of a vaccinia virus construct. The epitope was mapped to amino acids 221-235 of the NS3 protein, which is identical in serotypes 2, 3, and 4. It is interesting to note that the epitope on Dengue type 1 NS3 differs by two amino acids: a serine for an aspartic acid at position 9 and a glutamic acid for aspartic acid at position 12. One could hypothesize that the clones isolated in this study may not be Dengue type 1 NS3 cross-reactive due to the two amino acid substitutions.

Table 4: Summary of Previously Identified Dengue NS3 T-cell Epitopes

<u>Source</u>	<u>Cell Line</u>	<u>Region</u>	<u>CD4/CD8</u>	<u>A.A. of gene</u>	<u>Epitope</u>
Kurane 1995	JK4,JK43	1	CD4+	146-154	VIGLYGNGV
Zeng 1996	JK44	2	CD4+	202-211	RKYLPAIVRE
Mathew 1998	KPP94-024 3C2	3	CD8+	221-235	LAPTRVVAEMEEAL
Zeng 1996	JK15	4	CD4+	241-249	IRYQTTATK
Okamoto 1998	JK10,JK34,JK39,JK28,JK26	5	CD4+	255-264	EIVDLMCHAT
Okamoto 1998	JK49	5	CD4+	257-266	V DLMCHATF
Zeng 1996	JK13, JK5	6	CD4+	351-361	WITDFVGKTVW
Livingston 1995	CB6.17, CB 2.8	7	CD8+	500-508	TPEGIIPTL
Zivny <i>et al.</i> , 1999			CD8+	235-243	AMKGLPIRY
Zivny <i>et al.</i> , 1999			CD8+	71-79	

Okamoto *et al.* (1998) identified Region 5 using clones from a D3V immune donor. All clones were cross-reactive for all four serotypes, which can be supported by the observation that this region is completely conserved in all four serotypes. Two overlapping epitopes between amino acid 255 and 266 of the NS3 D3V protein were defined in this study. Clones JK10, JK26, JK28, JK34, and JK 39 recognized the 10 amino acid sequence between amino acids 255 and 264 while JK 49 recognized the epitope between 257 and 266.

Region 7 was identified in a study of CD8+ HLA-B35 restricted T-cell clones. The donor from both Mathew *et al.* (1996) and Livingston *et al.* (1995) was immunized with dengue type 4. Mathew *et al.* identified T-cells that were cross-reactive for D2V, D3V, and D4V. Livingston *et al.* identified clone CB6.17 which is D4V serotype specific, and clone CB2.8 which is cross-reactive for D2V and D4V (subcomplex specific). The smallest core epitope was determined to be amino acids 500-508 of the D4V NS3 protein. This region of NS3 is rather well conserved between the four serotypes. Serotypes 1 and 3 are identical at this epitope, while 2 and 4 differ at position 8, substituting a serine or a threonine, respectively. Serotype 2 also has a methionine at position 9, which is a lysine in the other three serotypes.

HLA Restriction of T-cell Epitopes

Antigen presentation to T-cells is accomplished by a complex known as the Human Leukocyte Antigen (HLA). The genetic composition of the complex encodes three loci for Class II HLA molecules and 7 loci for Class I HLA molecules. Class I and II molecules are similar in that they are both glycoproteins and are involved in antigen presentation. Class I HLA present antigen to CD8+ T-cells and

are expressed on almost all cells of the human body, with the exception of red blood cells. HLA I molecules predominantly present antigens processed from bacteria or viruses that replicate within the cytosol of a cell. HLA II predominantly present antigens from invaders that replicate outside of the cell or within cellular vesicles. CD4+ cells are the recipients of antigen presentation by HLA II molecules. HLA Class II molecules are expressed predominantly on macrophages and other professional antigen-presenting cells.

The human genome contains two copies of each allele for each locus of the HLA complex. The genes involved with HLA class 1 molecules are B, C, E, A, H, G, and F. The genes encoding HLA class 2 molecules are DPB, DPA, DQB, DQA, DRB, and DRA. The one allele through which an epitope of an antigen is recognized is termed the HLA restriction of that epitope. This allele is necessary (but not sufficient) to have T-cell recognition of the epitope.

MATERIALS AND METHODS – PART 2

Cell lines and Viruses

Donor FS was immunized with an experimental live-attenuated dengue virus type 1 vaccine. Peripheral blood specimens were obtained from the donor 4 months after vaccination (Green *et al.*, 1993). Cells were cryopreserved for further use. Dengue virus type 1 Hawaii was provided by Walter E. Brandt, Walter Reed Army Institute of Research. The recombinant vaccinia virus expressing D3V NS3 protein was provided by Dr. M. Brinton, Georgia State University, Atlanta, GA. The recombinant virus expressing D4V NS3 protein was provided by Dr. C. J. Lai, National Institutes of Health, Bethesda, MD. CV-1 (ATCC CCL 70) are African Green monkey kidney cells used for virus propagation. Human Tk- cell line 143B (ATCC CRL 8303) is an osteocarcinoma cell line used for the selection of recombinant vaccinia virus. Various T-cell clones, BLCL and PBMC were provided by Dr. Francis Ennis, UMass Medical Center, Worcester, MA.

CDNA Synthesis

RNA from Dengue 1 Hawaii virus in C6/36 cell supernatant was prepared using the QIAamp Viral RNA preparation kit, and stored at -20°C . cDNA synthesis was performed for 10 μl of viral RNA with AMV-RT enzyme and 1 μl (0.1 U/ μl) random primer in a 50 μl reaction volume. The reaction was incubated at 72°C for 2 minutes, and 42°C for 5 minutes. The following were then added to the reaction tube: 20 μl 5X Reaction Buffer, 20 μl 2.5mM dNTP, 2.5 μl (40 U/ μl) Rnasin and 3 μl (10 U/ μl) AMV-RT. The reaction was further incubated at 42°C for 1 hour and then at 95°C for 10 minutes. The resulting cDNA was stored at -20°C .

PCR

PCR amplification of the D1V NS3 gene and truncated gene fragments was carried out using the XL GeneAmp kit from Perkin Elmer. Primer sequences are shown in Table 5.

Table 5: Sequence of PCR Primers

Primer Name	Length	Direction	Sequence	Restricti on site
D1VNS35'ATG	32 nt	Sense	TT <u>GTTCGACAT</u> GTCTGGAGTGTATGGGACACA	<i>Sall</i>
D1VNS33'-2	29 nt	Antisense	TTGGATCCTCTTCTTCCTGCTGCAA <u>ACTC</u>	<i>BamHI</i>
D1V1116	33 nt	Sense	TTT <u>GTTCGACAT</u> GGTGAAGAGTGAACACACAGG A	<i>Sall</i>
D1V884-H3	33 nt	Antisense	TTT <u>AAGCTTT</u> AATATGCTGGCTGGATCGGTAAA	<i>HindIII</i>

The primer pair D1VNS35'ATG/D1VNS33'-2 amplifies the 1.8 kb NS3 gene, with flanking *Sall* and *BamHI* sites (underlined in the sequences above). The primer pair D1VNS35'ATG/D1V884-H3 amplifies the first 885 nucleotides of the NS3 gene (designated "884"). The primer pair D1V1116/D1VNS3'-2 amplifies the last 1111 nucleotides of the NS3 gene (designated "1116"). There is a 144-nucleotide overlap of the PCR products of these two primer sets. The sense primer was also designed to contain an ATG (shown in bold in the sequence above) that will enable consequent translation in the Vaccinia Virus to begin properly and in frame for NS3 protein synthesis. The PCR conditions are shown in Table 6.

Table 6: PCR Cycles

Temperature	Time	Cycles
94°C	1 minute	1
94°C	30 seconds	35X
56°C	1 minute	
72°C	5 minutes	
72°C	10 minutes	1
4°C	End	

PCR was performed using the Perkin Elmer GeneAmp XL PCR Kit. A manual hot start PCR reaction was prepared with 12 µl of 3.3X XL Buffer II, 8 µl 2.5mM dNTP, 4 µl each of 20 µM primers (sense and antisense), and 4 µl of Mg(OAc)₂ 25mM in a reaction volume of 40 µl, and heated to 94°C. When the reaction was at temperature, the following were then added: 18 µl 3.3X XL Buffer II, 1 µl rTth polymerase, 1µl cDNA template, and 40 µl dH₂O. The reaction was covered with mineral oil and allowed to cycle as

above. The annealing temperature used (56°C) was calculated as a function of the % GC of the bases that actually anneal to the template and the number of bases that anneal to the template.

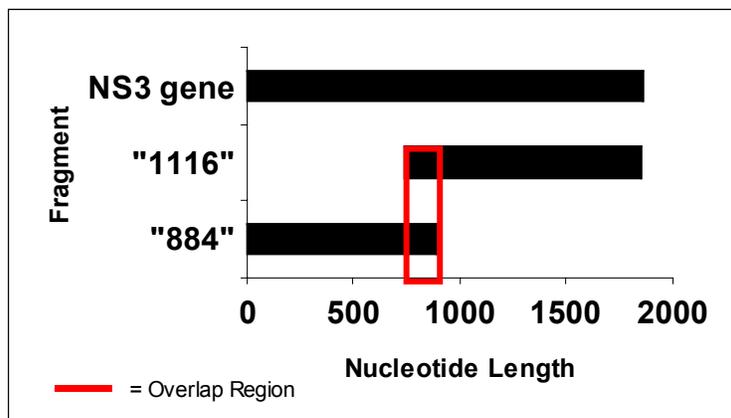


Figure 9: Diagram of Truncated Gene Fragments

Cloning of truncated gene fragments

Once PCR products were amplified, 3' single A overhang was added by the following method. 2.5 units of Taq polymerase was added to the PCR reaction and incubated at 72°C for 10 minutes. Then, the DNA was phenol-chloroform extracted and precipitated with 3M sodium acetate and 100% ethanol. DNA was pelleted by centrifugation, and washed with 70% ethanol and allowed to air dry. It was then resuspended in water or Tris-Cl (pH 8.0). The entire reaction was gel electrophoresed and gel purified using QIAGEN QIAquick. TOPO TA Cloning Kit (Invitrogen) was used to clone the product by the following method. Five nanograms of gel purified product was added to 1 µl plasmid pCR-2.1. Then it was incubated for 5 minutes at room temperature. One microliter of 6X Cloning Stop solution was added, mixed and kept on ice. Transformation into chemically competent TOP 10 cells was by the following method. Two microliters of cloning reaction was added to one vial of competent cells, thawed and put on ice. Cells were mixed gently and incubated on ice for 30 minutes. The cells were then heat shocked at 42°C for 30 seconds and 250 µl SOC medium was added. They were then shaken horizontally at 37°C for 30 minutes for ampicillin selection. Fifty to 100 µl were plated on prewarmed Luria Broth (LB) Agar with ampicillin (50 µg/ml) plates and incubated at 37°C overnight. Ten clones were picked and grown up overnight in 3 mL LB medium with ampicillin (50 mg/ml) (LBA) and a patch plate was prepared. Plasmid DNA was miniprepred with QIAGEN QIAprep Spin prep. Five hundred microliters was saved for a 50% glycerol stock. Clones were screened by *Eco RI* digestion.

Cloning of the full-length Dengue 1 Hawaii NS3 gene

Cloning of the full-length gene was accomplished by fusing the 5' half ("884") and the 3' half ("1116") using a unique restriction enzyme site (*BsmBI*) in the overlap region of these two fragments. See Figure 9. The 884 clone and the 1116 clone (in pCR2.1 plasmid) were digested with *Sall* and *BsmBI*. Digestion reactions were gel electrophoresed on 0.7% or 2% TBE gels. The 600 bp fragment released from the 884 digest and the 5kb fragment from the 1116 digest were excised and gel purified with QIAGEN QIAquick. Epicentre Fast-Link Ligation Kit was used to anneal and ligate cohesive ends using 2 µl of the 5kb fragment and 4 µl of the 600bp fragment. The ligated plasmid was then transformed into TOP10 chemically competent cells as above. Ten clones were picked and grown in 3 ml LBA medium. Plasmid DNA was miniprep and clones were screened by *EcoRI* digestion to release the full length (1.8kb) band. Once clones with full-length insert have been isolated, they were digested with *Sall* and *HindIII* as was the plasmid pMJ601. Digests were electrophoresed on a 0.7% gel, isolated and gel purified with Qiaquick Gel purification kit (pMJ601 at 7kb and NS3 at 1.8kb). The Epicenter Fast-Link kit was used to ligate cohesive ends using 2 µl plasmid and 4 µl insert. The ligated plasmid was then transformed into TOP10 chemically competent cells as above. Ten clones were picked and grown in 3 ml LBA medium. Plasmid DNA was miniprep and clones were screened by *Sall/Hind III* digestion to release the full length (1.8kb) band. Cultures of clones containing full-length gene were scaled up by a 1/500 dilution of overnight culture into 50 ml LBA. The cultures were grown overnight, shaking, at 37°C. Forty milliliters of the culture was maxiprep and the DNA was resuspended in 200 µl Tris-Cl overnight. Concentration was determined by spectrophotometer analysis. One microgram of plasmid in less than 150 µl buffer was used for transfection into Vaccinia Virus.

Sequencing of Dengue Type I NS3 Hawaii

The NS3 gene was sequenced using the truncated gene fragments 884 and 1116 cloned into the TA cloning vector pCR2.1. The TA cloning vector contains a M13 reverse primer site upstream of the cloning site, and a T7 primer downstream of the cloning site. Sequencing primers were designed to encompass the entire NS3 gene. Note that these primers were designed based on the sequence of Dengue Type 1 Singapore (Fu *et al.*, 1992).

Recombinant Vaccinia Virus Construction

A 25 cm² flask was seeded with 10⁶ CV-1 cells in MEM with penicillin, streptomycin and L-glutamine (complete) with 10% FBS and was grown to 50-80% confluency (overnight) at 37°C, with 5% CO₂. A vial of wild-type Vaccinia virus (wt VV) was thawed and 30 µl wt VV was mixed with 30 µl trypsin, vortexed and incubated for 30 minutes in a 37°C water bath. It was vortexed every 10 minutes and sonicated 2X for 30 seconds. Twenty microliters of the solution was added to 9.28 ml MEM with 2.5% Bromodeoxyuridine (BrdU). The media was aspirated off CV-1 cell layers and the cells were inoculated with 1 ml of wt VV solution. The cells were incubated for 2 hours at 37°C with 5% CO₂. One microgram of recombinant plasmid was diluted with 150 µl serum-free, antibiotic free MEM. QIAGEN Superfect Protocol was used for transient transfection of adherent cells, as follows. Thirty microliters of Superfect was added to the DNA solution and the tube was vortexed for 10 seconds and incubated at room temperature for 5-10 minutes. Medium was aspirated from CV-1 cells and they were then washed with 4 ml PBS. One ml of 10%FBS MEM complete was added to the transfection solution and the transferred to the flask of cells. The flask was incubated for 2-3 hours at 37°C with 5% CO₂. After incubation, media was aspirated and cells were washed with 4mL PBS. 5 mL of fresh growth medium (10%FBS MEM complete) was added to the flask and it was incubated for 24 hours.

Transfected cells were harvested by scraping cells from flask and they were transferred to a 15 ml tube. The tube was centrifuged at 2500 rpm for 5 minutes at 4°C. The media was aspirated off and the cell pellet was resuspended in 0.5 ml MEM 0.5% BrdU. The cells were then freeze-thawed 3X in ethanol/dry ice to lyse cells. The lysed cells were stored at -70°C. HuTK-143B cells were prepared from a continuous culture by seeding 5 X 10⁵ cells/well in a 6-well plate. Growth medium was complete MEM 10%FBS 0.5%BrdU. Two plates per plasmid were prepared. And were grown at 37°C with 5%CO₂ overnight. The CV-1 cell lysate was thawed and 210 µl of lysate and 210 µl trypsin were mixed and incubated for 30 minutes in a 37°C water bath. The tubes were vortexed every 10 minutes and sonicated 2X for 30 seconds. Dilutions at 10⁻¹, 10⁻² and 10⁻³ were prepared. Medium was aspirated from the HuTK-143B cells and 1 mL of the appropriate dilution was added. The plates were incubated for 2 hours at 37°C with 5% CO₂, and were shaken occasionally. Medium was aspirated and 2 mL /well complete MEM 10% FBS 0.5% BrdU was added. Plates were incubated for 2 days at 37°C with 5% CO₂. For the plaque agarose overlay, 2% Low Melt agarose was melted and incubated until 45°C. All reagents were warmed to 45°C. The recipe for the agarose overlay is shown in Table 7 (enough for 12 wells).

Table 7: Agarose Overlay Mixture

Reagent	Volume
2% LMP agarose	20mL
Basal Eagle Media	20mL
Neutral Red	600ul
X-Gal (4%)	330ul
BrdU (5mg/mL)	200ul

Medium was aspirated from infected HuTK-143B cells. Three ml of the agarose mixture was dripped slowly into each well. Agarose was allowed to set up at room temperature for 20 minutes and the plates were then incubated at 37°C with 5% CO₂ overnight. Recombinant Vaccinia Virus plaques appeared dark blue. 2-3 plaques were picked by inserting a Pasteur pipette into each plaque, scraping gently and taking up agarose plug. Plug was then placed in a cryovial and the picking was repeated 2X-3X per plaque. 0.5 mL complete MEM 0.5% BrdU was added to each vial. It was then vortexed, freeze/thawed as above 3X, and sonicated and freeze/thawed an additional cycle. Plaque Purification in HUTK-143B cells was repeated 2 more times.

Amplification and Titration of Recombinant Virus

HuTK-143B cells were prepared in a 12-well plate and grown to confluency. 250 µl of sonicated plaque isolate was added to the monolayer and incubated for 2 hours at 37°C with 5%CO₂, as was hand-rocked every 15 minutes. Medium was aspirated and 1 ml complete MEM 10% FBS with BrdU was added. Plated were incubated for 2 days until cell rounding was visible. HuTK-143B cells were prepared in a 25cm² flask and grown to near confluency. One half of the medium was aspirated from each well in 12-well plate. With cell scraper, monolayer was dislodged and transferred to a cryocentrifuge tube. Cells were centrifuged for 30 seconds at maximum speed and the supernatant was aspirated. Cell pellet was resuspended in 0.5 ml complete MEM 2.5%FBS with BrdU. Cells were freeze-thawed in an ethanol/dry ice bath 3 times. 250 µl of this lysate was diluted in 750 µl of complete MEM 2.5%FBS with BrdU. The entire mixture was added to the confluent monolayer in the 25cm² flask. The flask was incubated for 1 hour at 37°C with 5% CO₂. 4ml complete MEM 2.5%FBS with BrdU was overlaid and the flask was incubated for 2 days at 37°C with 5% CO₂. Cells were scraped and transferred to a 15 ml conical tube. The tube was centrifuged for 5 minutes at 2500 rpm at 4°C. The cell pellet was resuspended in 0.5mL complete MEM 2.5%FBS with BrdU, freeze-thawed 3 times, and sonicated 2 times. One day previously, 5 x 10⁷ cells in 25ml complete MEM 10%FBS were placed in a 175 cm² flask and were incubated at 37°C

with 5% CO₂. On day of infection, medium was aspirated from cells, and 250 µl lysate and 1.75 ml complete MEM 2.5% FBS were added to the cell layer. The cells were incubated for 1 hour at 37°C with 5% CO₂ and the flask was hand-rocked every 15 minutes. 25 ml complete MEM 2.5% FBS was overlaid on the cells and they were incubated for 3 days at 37°C with 5% CO₂. Cells were then detached by scraping and were transferred to a centrifuge tube and centrifuged for 5 minutes at 2500rpm at 4°C. The medium was aspirated and cell pellet was resuspended in 2ml of complete MEM 2.5% FBS and was then freeze-thawed 3 times and sonicated. 0.5 ml aliquots were prepared and stored at -70°C.

Titration:

One 6-well plate of HuTK-143B cells was grown to near confluency for each virus to be titered. 30 µl of previously prepared lysate and 30 µl trypsin were mixed and incubated for 30 minutes in a 37°C water bath. Dilutions 10⁻² through 10⁻⁸ were made. 1 ml of dilutions 10⁻⁶, 10⁻⁷, and 10⁻⁸ were added to cells in duplicate wells of the 6-well plate. The plate was incubated for 2 hours at 37°C with 5% CO₂ and was hand-rocked every 30 minutes. 2 ml per well of complete MEM 10% FBS was overlaid. The plate was incubated for 2 days at 37°C with 5% CO₂. Medium was then aspirated and 0.5 ml of 0.2% Crystal violet was added to each well. The plate was incubated at room temperature for 5 minutes. Dye was aspirated and the wells were allowed to air dry. Plaques were counted and multiplied by dilution factors, taking into account initial dilution with trypsin.

Short-term Pulse Labeling of Expressed Proteins with ³⁵S Methionine

10⁶ CV-1 cells were seeded in a 25 cm² flask and were allowed to reattach for 2 hours. Equal volumes of recombinant vaccinia virus and 0.25% trypsin were mixed and incubated at 37°C for 30 minutes. Growth medium was added and 1 ml of this virus solution was added to the cell monolayer. The flask was incubated for 1 hour at 37°C with 5% CO₂. Cells were then overlaid with 4 ml MEM 10% FBS and incubated for 24 or 48 hours. Media was aspirated from the flask and replaced with 0.5 ml methionine free MEM. The flask was incubated for 20 minutes at 37°C with 5% CO₂. 50 µCi of ³⁵S Methionine was added per flask and the flask was incubated for 30 minutes at 37°C. Media was removed and 2 ml ice-cold PBS was added. Cells were then scraped and transferred to an eppendorf tube. The cells were centrifuged at 13,000 rpm for 1 minute. Media was aspirated and discarded. The cell pellet was resuspended in 100 µl cell lysis buffer. The tube was vortexed, incubated on ice for 5 minutes, and centrifuged at 13,000 rpm at 4°C for 5 minutes. Supernatant was transferred to a new tube and stored at -20C until sample analyzation on SDS- PAGE gel. An 8% SDS- Discontinuous (Laemmli) protein gel was prepared. Cell lysate sample was prepared by mixing 25 µl lysate with 25 µl 2X gel loading buffer (1M DTT) and was heated at 95°C for 5 minutes before loading onto gel. The gel was run at 200-220 volts for about 2 hours. Gel was fixed

in freshly prepared glacial acetic acid:methanol:water 10:20:70 with 1/100 of glycerol for 20-30 minutes. The gel was dried on gel dryer at less than 80°C and analyzed by phosphorimaging.

Generation of bulk culture cytotoxic T-cells

Effector cells were prepared by rapidly thawing frozen PBMC from donor FS (FS PBMC). Cells were washed with 5ml RPMI 10%FBS and centrifuged at 1500 rpm 2 times. Cells were diluted to 2×10^6 in a volume of 750 μ l (per well) RPMI with HEPES, 10% Human AB serum, penicillin, streptomycin, and L-glutamine and placed in a 48-well plate. 250 μ l live dengue 1 Hawaii virus was added per well, at no less than 10^7 PFU/ml. Cells were incubated for 7 days at 37°C with 5% CO₂.

Cytotoxicity Assay

Target cells were prepared from BLCL from donor FS (FS BLCL). 2 to 5×10^5 of these cells were placed in a 15 conical tube and 5ml RPMI 10%FCS was added, spun at 1500 rpm for 5 minutes, and the supernatant was aspirated. To cell pellet, recombinant Vaccinia construct was added at a MOI of 5-10. The cell pellet was vortexed and incubated at 37°C for 1 to 1.5 hours and was vortexed every 15 minutes. The tube was centrifuged and the supernatant was aspirated. The cell pellet was resuspend in 1 ml RPMI 20% FCS and was incubated at 37°C with 5%CO₂ overnight. On the day of the assay, 1-2 $\times 10^5$ cells were removed to a 15 ml conical tube. Effector to Target (E/T) Ratio was set at 37.5: and 75:1. Cells were washed and the supernatants were aspirated. 25mCi of ⁵¹Cr in a 1:10 dilution with RPMI medium with 10% FBS was added to each cell pellet. Cells were incubated at 37°C for 1 hour and were vortexed every 15 minutes. Cells were washed 2X. 1 ml of medium was added to each cell pellet, vortexed, and 50 μ l was removed for a cell count. 4 ml of medium was added to the remaining cells and washed. Cell dilutions were determined and cells were resuspended to the appropriate cell concentrations. 0.1 ml of targets was added to a 96-well U bottom plate. For minimum release, 0.1 ml of growth medium was added to 6 wells and for maximum release 0.1 ml RENEX 1:20 dilution was added to 6 wells. A minimum and maximum release was prepared for each target used. Effectors were prepared by pooling like wells of effectors. 5 ml medium was added and the cells were washed. The supernatant was aspirated and effectors were resuspended in 1-2 ml media. Cell concentration was determined. 0.1 ml of effectors was added to the wells with targets The plate was incubated at 37°C with 5% CO₂ for 4 hours. The plate was carefully removed from the incubator. Supernatants were harvested with the Skatron Harvesting Systems. A gamma counter was used to count radioactivity. Specific lysis was determine by the formula: (experimental cpm-minimum cpm)/(maximum cpm-minimum cpm) X 100.

T-cell Cloning by Limiting Dilution

Bulk culture FS T-cells were diluted to 3, 1, and 0.3 cells per well with 10^5 gamma-irradiated PBMC feeders in a total volume of 100 μ l. One 96-well U-bottom plate was prepared per dilution. Cells were incubated in AIM-V media supplemented with 20% FBS, 50 units/ml rIL-2, and 1/1000 of 12F6, an anti-CD3 monoclonal antibody for 7 days at 37°C in 5% CO₂. On the seventh day, 100 μ l AIM-V 20% FBS with 100 units/ml rIL-2 per well was added. ½ of the media was changed every 3 days supplementing with 50 units/ml rIL-2. Cells were restimulated with anti-CD3 mAb and gamma-irradiated PBMC every two weeks. After about 1 month, clones that were growing well (large cell button) were transferred to a fresh 96-well plate. Clones that are growing well were screened by CTL using 50 μ l of each effector with 2×10^3 targets per well. Remaining cells were saved and only those that showed vD1vNS3 specificity were restimulated. These clones were transferred to a 48-well plate and were allowed to grow for 1 week. They were then retested by CTL.

CTL preparation of previously established T-cell clones

T-cell clones were removed from liquid nitrogen storage and were quickly thawed in a 37°C water bath. They were quickly transferred to 5ml of RPMI 10% FBS and were spun at 1500 rpm for 5 minutes. The media was aspirated. Gamma-irradiated feeders were resuspended in AIM-V with 20% FBS, 50 units/ml rIL-2 and 1/1000 12F6 mAb to about 2.7×10^6 cells/ml. Clone cell pellets were then resuspended in 1 ml of feeders and were then transferred to one well of a 48-well plate. They were incubated at 37°C with 5% CO₂ for 7 days. Cells were restimulated with anti-CD3 mAb and irradiated allogenic PBMC every two weeks.

Separation of PBMC from whole blood for use as Gamma Irradiated Feeders

Whole blood was diluted with an equal volume of serum free RPMI. 30 ml of the diluted blood was layered slowly over 15 ml Ficoll-Hypaque in a 50ml centrifuge tube. The tube was centrifuged at 1500 rpm for 35 minutes with no brake. The clear interface containing the PBMC was removed and pooled with another interface in a 50 ml tube. Serum free media was added to a final volume of 50 ml and then was spun at 1500 rpm for 5 minutes. The supernatant was removed and 10 ml serum free media was added to the pellets and vortexed. Pellets were pooled in one 50 ml tube and were irradiated for 40 minutes at 3000 to 3500 rads.

Precursor Frequency Assay

PBMC were quick-thawed and washed 2X in 5ml RPMI 10% FBS at 1500 rpm for 5 minutes media was aspirated. Cell pellet was resuspended in growth medium and cells were counted. 2×10^7 PBMC were irradiated. In two 96-well plates, prepare the following concentrations of non-irradiated PBMC in 24 replicate wells per concentration.: 2×10^6 cells/ml, 1×10^6 cells/ml, 6×10^5 cells/ml, 4×10^5 cells/ml, 2×10^5 cells/ml, 1×10^5 cells/ml, 5×10^4 cells/ml, 0 cells/ml. 0.1 ml was added to each well. 0.1 ml of 1×10^6 cells/ml gamma irradiated PBMC was added to all wells except 2×10^6 cells/ml. 0.02 ml Dengue type 1 Hawaii virus was added to each well and the plates were incubated for 9-10 days at 37°C with 5% CO_2 . On the day before the CTL, targets of autologous BLCL infected with vD1vNS3, vD3VN3, or Vac D (wt vaccinia) were prepared. 0.05ml effectors were tested with 2×10^4 targets/ml with appropriate minimum and maximum releases. Negative wells were defined as having less than 10% specific lysis for each target.

RESULTS - PART 2

Purpose of Part 2

The purpose of the second half of this thesis was to construct, evaluate, and use a vaccinia virus expressing the dengue virus serotype 1 NS3 protein in dengue virus specific cytotoxicity assays.

Cloning of Dengue 1 NS3 gene into Vaccinia Transfer Vector

The Dengue 1 NS3 gene is 1.8 kb in length. cDNA was amplified by RT-PCR from RNA isolated from the dengue 1 Hawaii virus. PCR was used to amplify the gene from the cDNA. The full-length gene product was divided into two segments by PCR and each fragment was cloned into the pCR2.1 vector (Invitrogen). The PCR incorporated two restriction enzyme sites into each gene segment. A *Sall* site was included at the 5' end of the 5' gene segment, and a *HindIII* site was added at the 3' end of the 3' gene segment. In addition a start codon was included at the 5' end of the PCR product.

Using a unique restriction enzyme site of *BsmBI* in the overlap region of the two gene segments, the whole gene product was fused into the pCR2.1 plasmid. Figure 10 shows the full-length product of 1.8 kb that was cloned into pCR2.1 (4kb) and then released from the vector by the *EcoRI* enzyme for analysis.

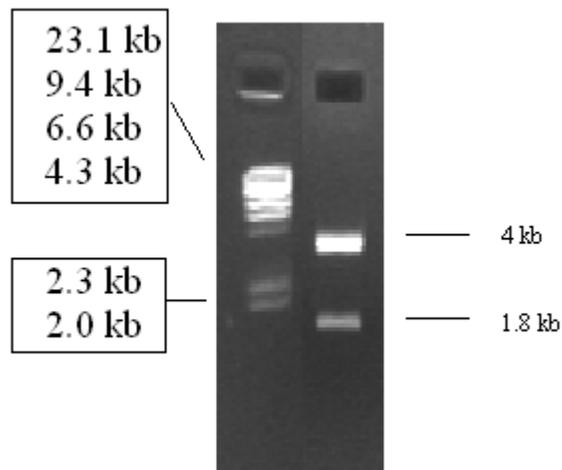


Figure 10: Verification of Cloning of the Dengue 1 NS3 gene. The NS3 gene (1.8kb) was released from the plasmid PCR2.1 (4kb) using *EcoRI*.

Recombinant Vaccinia Virus Isolation

Once fused into pCR2.1, the NS3 gene was released with *Sall* and *HindIII* and ligated into the 7kb vaccinia transfer vector pMJ601. Figure 11 shows the verification of this cloning. This plasmid, created by Davison and Moss 1990, has a synthetic late promoter and contains the Lac Z gene and TK- (thymidine kinase) gene under the control of a vaccinia virus promoter. These genes are used for selection of recombinant vaccinia from wild type vaccinia virus in the screening process. TK- phenotype cells were transfected with the pMJ601 gene construct and infected with wild type vaccinia. Homologous recombination between the TK gene of the plasmid and the vaccinia genome form recombinant vaccinia viruses containing the gene of interest. These were then screened out using X-gal, which in the presence of the Lac Z gene gives recombinant plaques a blue color, as shown in Figure 12. In addition, the presence of 5-bromodeoxyuridine (BrdU) kills any wild type virus expressing the TK protein because the TK protein phosphorylates BrdU and it is incorporated into DNA with a lethal effect. Recombinant viruses were screened 3 times by agarose overlay plaquing, and were then tested for expression of the protein of interest.

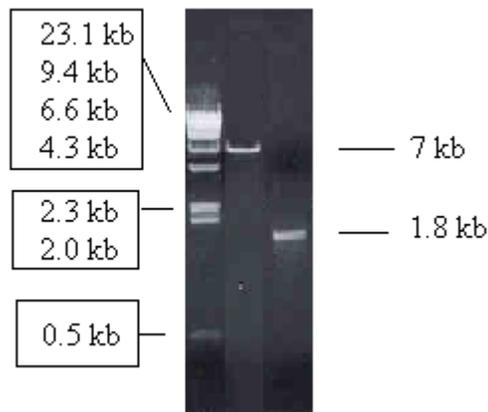


Figure 11: pMJ601 Vaccinia Transfer Vector (7kb) and NS3 gene (1.8kb). They were digested with *HindIII* and *Sall* and ligated together.

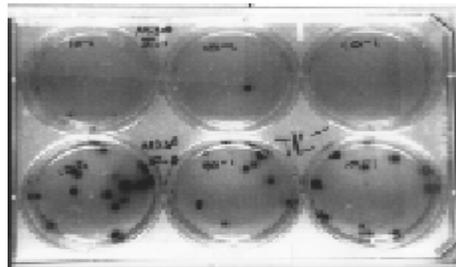


Figure 12: Virus Plaque selection by X-gal. Dark spots indicate a functional beta-galactosidase gene of the recombinant vaccinia viruses.

Dengue 1 Hawaii Sequence Analysis

The full-length cDNA sequence of DEN1 Singapore strain S275/90 has been described previously (Fu *et al.*, 1992). DEN1 Hawaii has not been fully sequenced, but sequence comparison between the Singapore and the West Pacific strain West Pac 74 (Puri *et al.*, 1998) shows that the NS3 gene of these two dengue type 1 strains are 94% homologous, lending validity to the assumption that the Hawaii strain will be very similar to the Singapore strain. A portion of the sequence has been published by Chow, Seah, and Chan, (1994) that comprises 426 nucleotides of the viral sequence. Using clones of the 5' and 3' segments of the Dengue 1 Hawaii NS3 gene, the complete sequence was determined by ABI sequencing at the Nucleic Acid Facility of the University of Massachusetts Medical School. This sequence is shown in Figure 13 and is aligned with the reference sequence Dengue 1 Singapore. A single dot indicates nucleotide identity. The Hawaii sequence was found to be 95% homologous to the Singapore sequence at the nucleotide level. The translation of the Hawaii gene is shown in Figure 14. It is again shown aligned with the translation of the Singapore NS3 gene. The Hawaii protein is 98% homologous to the Singapore protein, with 15 amino acid differences within the 619 amino acid protein.

```

1 tctggagtgttatgggacacacctagccctccagaagtggaaagagcagt 50
1 ..... 50

51 ccttgatgatggtatctatagaattatgcagagaggactggtgggcaggt 100
51 .....c....a..... 100

101 cccaagtaggagtgaggagttttccaagacggcggttccacacaatgtgg 150
101 ....g.....a.....a..... 150

151 cacgtcaccaggggagctgtccttatgtaccaagggagaggctggaacc 200
151 ..t.....c.....t....a..... 200

201 aagctgggccagtggtcaaaaaagacttgatctcatatggaggaggttggg 250
201 g..t..... 250

251 ggtttcaaggatcctggaacacgggagaagaagtgcaggtgattgctggt 300
251 ..... 300

301 gaaccaggaaaaaaccccaaaaatgtacagacagcgcgggtaccttcaa 350
301 .....g.....c.....c..... 350

351 gaccctgaaggtgaagttggagctattgcctagattttaaacccggca 400
351 .....c.....c..a..... 400

401 catctggatctcccatcgtagaacagagaaggaaaaatagtaggtctttat 450
401 .....t.....g..... 450

451 ggaaatggagtagtgacaacaagtggaacctacgtcagtgccatagccca 500
451 .....g.....t.. 500

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501 agccaaagcatcacaagaagggcccctaccagagattgaggacgaggtgt 550
501 ...t.....t..... 550

551 ttaggaaaagaaacttaacaataatggacctacatccaggatcggggaaa 600
551 600

601 acaagaagatatcttccagccatagtcctgaggccataagaaggaacgt 650
601t....a.....gc. 650

651 ggcacactaattttggctcccacaagggttgctcgcttccgaaatggcag 700
651 ...t..g.....c.....a.....t..... 700

701 aggcgctcaagggatgccaataaggtaccaacaacagcagtgaaagagt 750
701t..g..... 750

751 gaacacacagggaaaagagatagttgacctcatgtgtcacgccactttcac 800
751gg.....t....c..t..... 800

801 catgctctcctgtctcccgtgagagttoccaattacaacatgattatca 850
801c.... 850

851 tggatgaagcacatttaccgatccagccagcatagcgcgagagggtag 900
851c.....gc..... 900

901 atctcaaccgagtgggcatgggtgaagcagctgcgatcttcatgacagc 950
901g..... 950

951 cactccccaggatcggaggcctttccacagagcaatgcagttatcc 1000
951 1000

1001 aagatgaggaaagagacattcctgagagatcatggaactcaggctatgag 1050
1001c 1050

1051 tggatcactgacttcccaggtaaaacagtctggtttgttccaagcatcaa 1100
1051t.....t.. 1100

1101 atcaggaaatgacattgccaactgcttaagaaagaatgggaaacgggtga 1150
1101t..... 1150

1151 ttcaattgagcaggaaaaacctttgatacagagtaccaaaaaacaaaaaac 1200
1151 .c.....a.....t.....g.....t 1200

1201 aacgactgggactatgtcgtcacaacagatatctccgaaatgggagcaaa 1250
1201 ..t.....c..t.....a..... 1250

1251 cttccgagccgacagggatagacccaagacgggtgtctgaaaccggtaa 1300
1251a.....t..... 1300

1301 tactaaaagatgggtccagagcgcgtcattctagccggaccgatgccagt 1350
1301t..... 1350

1351 actgtggccagtgctgccagaggagaggaagaattggaaggaaccaaaa 1400
1351c..... 1400

1401 caaagaaggtgatcagtacgtttacatgggacagcctttaataatgatg 1450
1401 t..g.....a.....c..c.... 1450

1451 aggatcacgctcattggacagaagcaaaaatgctccttgacaataataaac 1500
1451c..... 1500

1501 acaccagaagggatcatcccagccctctttgagccagagagagaaaagag 1550

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1501 .....t.....g.....g...a.. 1550
1551 tgcagcaatagacggggagtacagactgcggggagaagcaagaaaaacgt 1600
1551 .....a.....t.....g..... 1600
1601 ttgtggagctcatgagaagaggagatctacctgtctggctatcctacaaa 1650
1601 .c..... 1650
1651 gttgcctcagaaggcttccagtactctgacagaagatggtgctttgacgg 1700
1651 .....ag.....t.. 1700
1701 ggaaaggaacaaccaggtgttgaggagaacatggacgtggagatgtgga 1750
1701 .....c.....c.... 1750
1751 caaaagaaggagaacgaaagaaactacgacccccgctggctggatgccaga 1800
1751 .....a.....c..... 1800
1801 acatactcagaccctgcccctgcgcgagtttaagagtttgacagcagg 1850
1801 .....t.....a...t..... 1850
1851 aagaag 1856
1851 ..... 1856

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Figure 13: Alignment of the NS3 genes of Dengue 1 Singapore (top) and Dengue 1 Hawaii Nucleotide sequence (bottom)

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1 SGVLWDTSPPEVERAVLDDGIYRIMQRGLLGRSQVGVGFQDGVFHTMW 50
1 .....L.....E..... 50
51 HVTRGAVLMYQGKRLPSWASVKKDLISYGGGWRFQGSWNTGEEVQVIAV 100
51 .....E..... 100
101 EPGKNPKNVQTAPGTFKTPPEGEVGAIALDFKPGTSGSPIVNRGKLVGLY 150
101 ..... 150
151 GNGVVTTSPTYVSAIAQAKASQEGPLPEIEDEVFRKRNLTIMDLHPGSGK 200
151 ..... 200
201 TRRYLPAIVREAIRRNVRTLILAPTRVVASEMAEALKGMPVIRYQTTAVKS 250
201 .....K.KL..... 250
251 EHTGKEIVDLMCHATEMRLLSVPRVPNYNMIIMDEAHFTDPASIARRGY 300
251 .....R.....A... 300
301 ISTRVGMGEAAAIFMTATPPGSVEAFPQSNVAVIQDEERDIPERSWNSGYE 350
301 .....D 350
351 WITDFPGKTVWFVPSIKSGNDIANCLRKNGKRVIQLSRKTFDTEYQKTKN 400
351 ..... 400
401 NDWDYVVTTDISEMGANFRADRVDPVPRCLKPVILKDGPERVILAGPMPV 450
401 .....E..... 450
451 TVASAAQRRGRIGRNQNKEGDQYVYMGQPLNNDHHAHWTEAKMLLDNIN 500
451 .....I..... 500
501 TPEGIIPALFEPEREKSAIDGEYRLRGEARKTFVELMRRGDLPVWLSYK 550
501 .....G..... 550

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```

551 VASEGFQYSDRRWCFDGERNNQVLEENMDVEMWTKEGERKKLRPRWLDAR 600
551 .....K.....I..... 600

601 TYSDDLALREFKEFAAGR 618
601 .....Q..... 618

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Figure 14: Alignment of NS3 Amino Acid Sequences of Dengue 1 Singapore (top) and Hawaii (bottom). Yellow sections indicate areas of previously mapped epitopes. Pink indicates the amino acids in Dengue 1 that are different from the mapped epitopes of Dengue 2, 3, or 4.

Analysis of Recombinant Vaccinia Protein Expression by bulk CTL

In order to determine if the recombinant vaccinia viruses that were isolated after three rounds of screening were expressing the Dengue 1 Hawaii NS3 protein, a bulk culture assay using the PBMC of the donor FS was employed. The donor FS was a volunteer who was immunized with an experimental live-attenuated dengue virus type 1 vaccine (Green *et al.*, 1993). The CTL assay measures % specific lysis as a function of the release of ⁵¹Cr from target cells lysed by antigen-specific T-cells. Autologous B cells from the patient were transformed by infection with Epstein-Barr virus (BLCL). The targets were prepared by infecting BLCL with the recombinant vaccinia virus and the cells were then loaded with ⁵¹Cr. The positive control was cells infected with a vaccinia construct expressing the Dengue 3 NS3 protein and the negative control was cells infected with the wild type vaccinia virus. Effector T-cells were prepared by stimulating PBMC from the patient with Dengue 1 Hawaii virus for 7 days. The Effector to Target ratios (E/T) in the bulk culture CTL were 75:1 and 37.5:1. All data points were collected in triplicate, and minimum and maximum release data was also collected. As shown in Table 8, at an E/T ratio of 75:1, bulk culture T-cells recognized targets infected with VVII2A with 47.9% specificity compared to the targets expressing wild-type vaccinia proteins, at 18%. At an E/T ratio of 37.5, the VVIIA construct was not recognized at a level significantly higher than wild-type vaccinia. Therefore construct II2A is more likely to be expressing the Dengue 1 NS3 protein efficiently. This construct will be referred to as vD1VNS3.

Table 8: Recognition of Recombinant Vaccinia Virus Constructs by Bulk Culture CTL from Donor FS

	<u>ET 75:1</u>	<u>ET 37.5:1</u>
VI1A	29.9%	21.3%
VII2A	47.9%	31.1%
VD3VNS3	34.3%	26.1%
Vac D (wt)	18.1%	19.2%
Uninfected	5.2%	2.0%

Analysis of Recombinant Vaccinia Protein Expression by Protein Gel Electrophoresis

A pulse labeling experiment was performed to visualize the recombinant NS3 protein. The protein is 619 amino acids and is approximately 61 kDa. We should expect the protein to appear slightly larger because the cDNA of the gene did not originally contain a stop codon. Therefore the protein will most likely stop at the promoter of the flanking Lac Z gene which will contain an in-frame stop codon for protein expression. This may add a few thousand daltons to the molecular weight of the expressed protein. A short time course experiment was performed. Cell cultures were infected with vD1VNS3, vD3VNS3, Vac D, or Dengue Hawaii virus and were harvested and labeled with ³⁵S-Methionine at 24 or 48 hours post infection. Figure 15 shows the autoradiograph of the protein gel. Lane 2 shows the protein bands of cells lysed after 24 hours of vD1VNS3 infection. After comparison with Lane 4, the vaccinia background, it can be seen that there are two band differences between these lanes. The first is a band at about 100 kDa that is in the wild type vaccinia lane but not present in the recombinant construct cell lysates. This could be a part of the Thymidine Kinase protein that was lost upon homologous recombination. The second, more significant, difference is a band in the vD1VNS3 lane at about 70 kDa (see arrow). It can be hypothesized that this band is the dengue NS3 protein. This data, along with the bulk culture CTL results, indicate that the vD1VNS3 construct is expressing the dengue NS3 protein. A Western blot with dengue antibody would confirm this result. The protein gel also shows that by 48 hours of vaccinia infection, protein expression was much lower than at 24 hours.

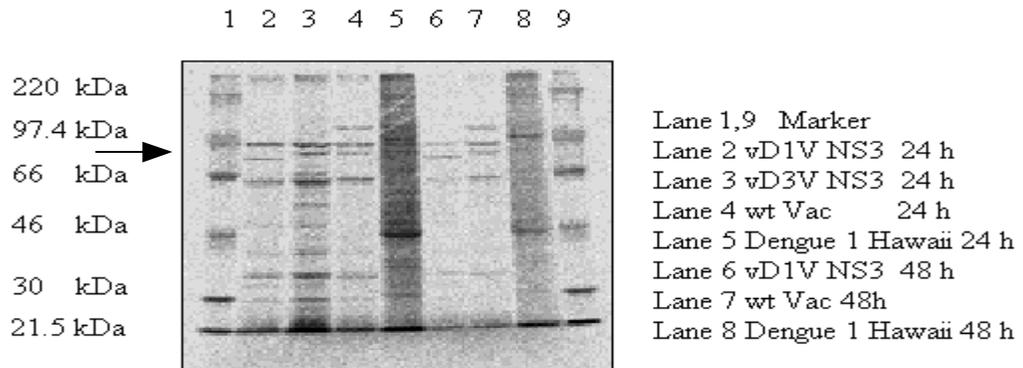


Figure 15: Polyacrylamide Gel Electrophoresis of ³⁵S-Met labeled Cell Lysates of recombinant vaccinia virus infected cells.

Use of previously isolated clones to test vD1VNS3 expression

Our lab has isolated many T-cell clones that demonstrate cross-reactivity to Dengue 2,3, and 4 NS3 proteins, but until this point none have been tested for Dengue 1 NS3 specificity using authentic NS3 protein. These T-cell lines were predominantly isolated from a Dengue 3 candidate vaccine recipient (JK) and their antigenic epitopes have been mapped. As shown in Figure 16, the epitope recognized by clone

JK34 shares 100% identity with the Dengue 1 Hawaii amino acid sequence at this epitope. The epitopes recognized by clones JK13 and JK 44 share 9/10 amino acids with the Hawaii sequence. The antigenic epitope of clone JK 43 is 78% homologous to the Dengue 1 Hawaii sequence. The epitope recognized by clone CB6.17 (isolated from a Dengue 4 infected individual, CB) shares 8/9 amino acids with the Hawaii sequence.

Clone	Epitope sequence from Serotype	Epitope Similarity
JK 34	Dengue 3	EIVDLMCHAT
Hawaii	Dengue 1
JK 44	Dengue 3	RKYLPAIVRE
Hawaii	Dengue 1	.R.....
JK 43	Dengue 3	VIGLYGNGV
Hawaii	Dengue 1	IV.....
JK 13	Dengue 3	WITDFVGKTVW
Hawaii	Dengue 1P.....
CB 6.17	Dengue 4	TPEGIIPTL
	Dengue 3A.
Hawaii	Dengue 1A.

Figure 16: Comparison of T-cell epitopes of D3 or D4 NS3 proteins with Dengue 1 Hawaii Sequence. Note: A single dot indicates identical amino acid in that position.

It would be expected that the more similar the Hawaii sequence is to the antigenic epitope of the T-cell clone, the more lysis will be observed. These clones were tested against autologous BLCL infected with recombinant vaccinia viruses expressing the D1V and D3V NS3 proteins, and the wild type vaccinia virus ("Vac D"). The % specific lysis is shown in Table 9.

Table 9: Recognition of recombinant vaccinia viruses by dengue virus-specific CTL clones. Vac D is the wild type vaccinia virus. ET ratios for all experiments were 10:1. ND indicates experiments not done. Background levels were particularly high for experiment 1.

Clone	V V	% Specific 51 Cr Release by:	
		Exp. 1	Exp. 2
JK 34	D1NS3	14	-6
	D3NS3	15	57
	Vac D	-31	4
JK 43	D1NS3	77	83
	D3NS3	63	98
	Vac D	-38	11
JK 44	D1NS3	66	76
	D3NS3	20	97
	Vac D	-33	4
JK 13	D1NS3	21	-4
	D3NS3	27	22
	Vac D	-28	5

CB 6.17	D1NS3	-1	0
	D4NS3	ND	71
	Vac D	24	1

JK 34 unexpectedly did not recognize the dengue 1 NS3 construct. The epitope of dengue 1 is exactly the same as the dengue 3 construct that was mapped for the clone. The clone did show recognition of the dengue 3 construct. Okamoto *et al.* (1998) reported that JK 34 is cross reactive for dengue 1 when stimulated with dengue virus antigen. The vaccinia virus backbone may be interfering with the eventual presentation of the epitope to this clone. It is unclear how this might occur.

JK 43 and 44 recognize the dengue 1 construct even though the mapped epitopes for these two clones are slightly different than the sequence of the dengue 1 Hawaii protein. The amino acid differences are not significant enough to alter presentation and recognition of the Hawaii epitopes, as is demonstrated in Table 9 in the second experiment. JK 43 had a specific lysis of dengue 1 NS3 targets of 83% and dengue 3 NS3 targets of 98%. JK 44 had similarly high recognition at 76% and 97% respectively. JK 44 dengue 1 protein has a lysine substituted for the arginine in the dengue 3 mapped epitope, as shown in Table 9. These two amino acids are basic. The epitope for JK 43 has a valine and an isoleucine at the first and second amino acid positions. These are isoleucine and valine in the first and second spots of the dengue 1 protein. These are both neutral non-polar amino acids and the swapping of their positions does not seem to influence recognition of the epitope to a great degree.

JK 13 was previously determined to be a dengue 3 specific clone (Zeng *et al.*, 1996). Although the background for targets expressing dengue 3 NS3 was high for both experiments, it can be seen from Experiment 2 in Table 9 that JK 13 recognizes dengue 3 NS3 to a higher degree than the dengue 1 protein.

CB6.17 was previously determined to be a dengue 4 specific clone (Livingston *et al.*, 1995). Experiment 2 shows that CB6.17 recognizes the dengue 4 NS3 protein with 71% specific lysis while it does not recognize the dengue 1 protein.

These experiments show that the recombinant vaccinia virus constructed in this study is expressing the dengue 1 NS3 protein. This protein can be recognized appropriately by clones that are reactive to the protein. Conversely, the presence of the protein does not significantly activate T-cell clones that are specific for the NS3 protein of other serotypes.

Attempted isolation of DIV NS3 specific T-cell Clones from FS PBMC

Limiting dilution of the FS T-cell population stimulated with Dengue 1 Hawaii virus was performed. These clones were diluted to 3, 1 or 0.3 cells per well and were allowed to grow for about 1 month. These clones were then screened by CTL against targets infected with vDIVNS3 or VacD. Four clones FS9, FS11, FS7.3 and FS8.3 were selected as possible candidates for DIV NS3 specificity. After

restimulation and a short growth period, these clones were retested against targets expressing D1VNS3, D3VNS3, and Vac D. Data (not shown) indicate that these clones are not Dengue 1 NS3 specific.

Precursor Frequency Assay

The precursor frequency assay is a quick technique to estimate the existence and abundance of a specific T-cell population in the original T-cell population. Donor FS PBMC were plated in 24 wells of a 96-well plate in decreasing cell dilutions and were stimulated with autologous gamma-irradiated PBMC and dengue 1 virus. They were allowed to incubate for 15 days until the cell buttons were sufficiently large for a CTL assay. A quarter of each well was removed to a clean 96 well plate and were tested against autologous BLCL infected with vaccinia recombinants expressing dengue 1 or dengue 3 NS3 proteins or wild type vaccinia.

Table 10: Mean and Standard Deviation Values for Dengue 1 NS3 Precursor Frequency Assay

Target		MEAN 24 wells	Standard Deviation	St. Dev * 3	Mean +3ST.D
vD1NS3	0 cells/well	315	15	45	360
vD3NS3	0 cells/well	272	25	76	348
VacD	0 cells/well	345	20	60	405

The mean of 24 replicate base-line wells (0cells/well) was determined, and the standard deviation was calculated. The percent lysis was determined with these values. A negative well was defined as any well having a percent lysis less than 10%. Negative wells were tallied for each of the 3 experimental targets. The proportion of negative wells was graphed versus the number of cells per well, as shown in Figure 17.

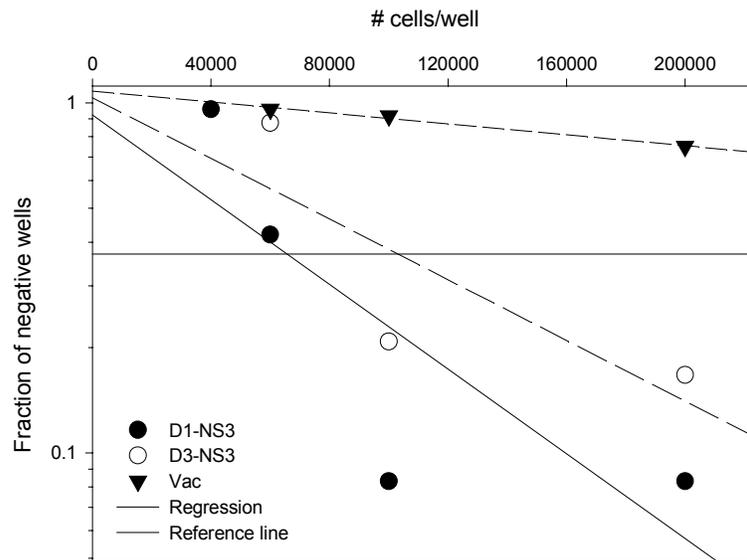


Figure 17: Precursor Frequency of dengue 1 NS3 specific T-cells. The equations for each line are: D1V [$\log 0.37 = -6.05 \times 10^{-6}(x) - 0.35$] D3V [$\log 0.37 = -4.333 \times 10^{-6}(x) + 0.0142$] Vac [$\log 0.37 = -7.8 \times 10^{-7}(x) + 0.0333$]

The precursor frequency is calculated from the equation of the best-fit logarithmic trend line for each data set. One can solve for the x value when the y value is set at 0.37. This value was determined by Sharrock, Kaminski and Man (1990). When 37% of the test cultures are negative, there is an average of one precursor per well. The T-cell frequency was highest for dengue 1 NS3, at 1 in 65,586 PBMC. The precursor frequency of dengue 3 NS3-specific T-cells is 1 in 103,002 PBMC and for vaccinia-specific T-cells, 1 in 596,280 PBMC.

Once the precursor frequency was calculated, each well was assessed for specific lysis of D1VNS3-expressing targets. This is calculated using the equation: $(\text{experimental cpm} - \text{minimum cpm}) / (\text{maximum cpm} - \text{minimum cpm}) * 100$. Minimum, or spontaneous, release is generated from 6 replicate wells with targets that are treated with medium only. Maximum release is generated from 6 replicate wells of targets treated with RENEX, which lyses all cells. Wells that had a significant amount of D1V NS3 specific lysis over D3VNS3 or VacD lysis were saved for further limiting dilution in order to isolate dengue 1 NS3 specific T-cell clones. The wells that showed significant dengue 1 NS3 lysis are shown in Table 11 comparing dengue 1 NS3, dengue 3 NS3, and wild-type vaccinia specific lysis.

Table 11: Comparison of Precursor Frequency well specific lysis. Only wells with significant recognition of Dengue 1 NS3 targets are shown here.

<u>Cell</u>	<u>Cells/well</u>	<u>D1NS3 % Lysis</u>	<u>D3NS3 %Lysis</u>	<u>vacD%lysis</u>
B1	2.00E+05	60	8	-2
B7	2.00E+05	73	14	4
C1	1.00E+05	108	16	0
C5	1.00E+05	22	-4	-6
C6	1.00E+05	74	29	9
D2	1.00E+05	25	4	-4
D10	1.00E+05	53	3	-9
E1	6.00E+04	18	-12	-13
E7	6.00E+04	74	-1	0
E8	6.00E+04	43	-12	-3
E9	6.00E+04	32	3	-6
F3	6.00E+04	21	-3	-8
F6	6.00E+04	20	-8	-13
F9	6.00E+04	28	5	-9
F10	6.00E+04	21	-3	-2
F11	6.00E+04	33	0	-3
G12	4.00E+04	26	-4	-6

NS3 specificity of previously established FS CD4+ CD8- clones

Donor FS was the recipient of an experimental live-attenuated dengue virus serotype 1 vaccine. The donor was a 38-year-old white male who had never traveled outside of the US and showed no flavivirus antibody response before vaccination. He received the vaccine strain DEN-1 45 AZ5 PDK passage 27. Four months post immunization, peripheral blood was obtained. This donor had a predominantly serotype specific T-cell response. A precursor frequency assay showed that 80% of the T-cell clones isolated from this donor were serotype specific while 20% were serotype 1 and 3 subcomplex specific, as determined by serotype antigen presentation (Green *et al.*, 1993).

Several CD4+ CD8- clones have been isolated from this donor. Clones FS 5, 7, 20, and 24 were determined to be dengue serotype 1 specific when stimulated with dengue virus antigen. Clones 10 and 15 are dengue serotype 1 and 3 subcomplex specific. Other CD4+ clones were isolated and were shown to recognize the NS1-NS2a proteins expressed by a recombinant vaccinia virus (Green *et al.*, 1997). Until this point no clones have been tested for NS3 specificity because of the lack of a recombinant vaccinia virus expressing this protein. This study tested clones FS 5, 7, 10, 15, 20 and 24 for dengue serotype 1 NS3 protein specificity. It should be noted that clones 5, 7, 10 and 20 were found to be non-reactive towards the NS1 or NS2a proteins. As is shown in Table 12, the spontaneous release (min) was high for this experiment, causing the % lysis values to be negative. None of the clones shown here are dengue 1 NS3 specific.

Table 12: Dengue 1 and 3 NS3 Specific Lysis by FS Clones 5, 7, 10, 15, 20 and 24.

	D1NS3 % Lysis	D3NS3 % Lysis	vacD % Lysis
FS 5	-3	-12	-13
FS 7	-3	-4	-7
FS 10	0	-4	-7
FS 15	-1	-6	-10
FS 20	-21	-5	-11
FS 24	-1	1	-7
Min	547	470	254.5
Max	1401.5	1493.5	708
Min/max	39	31	36

DISCUSSION-PART 2

The purpose of Part 2 of this study was to construct and use a recombinant vaccinia virus expressing the dengue type 1 NS3 protein in immunological studies. The vaccinia virus expression system has been well established for use in immunological studies. Antigenic specificity of cytotoxic T-cells can be studied using recombinant vaccinia virus infected cells. The benefits of using the vaccinia virus expression system include cytoplasmic transcription of the foreign gene and control of transcription by various vaccinia virus promoters. When a protein needs to be expressed in the context of the major histocompatibility complex class 1 molecule, a compound early/late promoter may be used.

The dengue 1 Hawaii NS3 gene was sequenced in this study. The Hawaii gene was shown to be 95% homologous to the dengue type 1 Singapore strain. The amino acid sequence of the Hawaii NS3 protein was 98% homologous to the Singapore strain. This sequence data confirms that the Hawaii strain is distinct from the other dengue type 1 strains. From this data, we can make educated hypotheses about what epitopes of the protein may or may not be recognized by T-cell clones.

The recombinant vaccinia virus constructed in this study was shown to be expressing the dengue type 1 NS3 protein. Electrophoresis of cellular proteins of cells infected with the recombinant vaccinia virus showed a band at the expected size of the dengue 1 NS3 protein, which is about 61 kDa. Additional evidence was provided by a bulk CTL assay with the PBMC of donor FS. The assay showed that the dengue 1 NS3 protein was being expressed and was recognized at a significant level by cytotoxic T-cells of the donor. In addition, this assay showed that dengue 1 NS3 specific T-cells are present in the PBMC of this donor. Further CTL assays using CTL clones from donors JK and CB showed that the dengue 1 NS3 protein does not activate clones that are not primed for the epitopes of the dengue 1 Hawaii NS3 protein. These experiments also showed that although an epitope may vary by one or two amino acids, it may still be recognized by a T-cell clone. We concluded that the recombinant vaccinia virus is properly expressing the dengue type 1 NS3 protein.

The donor FS was a volunteer experimental dengue 1 vaccine recipient. Several studies (Green *et al.*, 1993; Green *et al.*, 1997) have investigated this donor's cytotoxic and memory T-cell specificities. CD4+ T-cell clones were established and using recombinant vaccinia viruses, several clones were shown to be dengue 1 NS1 or NS2a specific. These studies established that 80% of the CD4+ clones isolated were dengue 1 specific and 20% were dengue 1 and dengue 3 cross reactive. No dengue 2 or 4 reactive clones were isolated. This can be explained by the high homology shared between dengue serotypes 1 and 3. However none of these clones were tested for dengue 1 NS3 specificity with authentic NS3 protein. It would be hypothesized that because this donor has a predominantly serotype specific T-cell response and because the NS3 protein is highly antigenic, there exist dengue 1 NS3 specific T-cells. The precursor frequency assay conducted in this study supports this hypothesis. Approximately 19 of the 96 wells of the

precursor frequency assay showed a significant percent specific lysis of dengue 1 NS3 targets over dengue 3 NS3 targets. These wells could, of course, contain a mix of dengue 1 specific and dengue 1 and 3 subcomplex specific T-cells. Isolation of dengue 1 NS3 specific T-cell clones is possible by limiting dilution of these positive wells.

In the present study, several of the previously established FS CD4⁺ clones were examined for dengue 1 NS3 protein recognition using the recombinant vaccinia virus constructed in the earlier part of this study (Green *et al.*, 1997). Six FS clones were tested and none were shown to be dengue 1 NS3 specific, although the small sample size is a factor to be taken into account. The precursor frequency for dengue 1 total antigen specific T-cells was established by Green *et al.* (1993) to be 1 in 1,686 PBMC, while the precursor frequency of dengue 1 NS3 specific T-cells was calculated to be 1 in 65,586 PBMC for this donor. This value is logical because the frequency of serotype specific protein specific T-cells is going to be much lower than just serotype specific T-cells. The values for the vaccinia specific T-cells was very low (1 in 596,280) because there should not be any Vaccinia specific T-cell stimulation when the T-cells are stimulated with Dengue 1 virus. Although it is currently unknown how the frequency of specific T-cell populations and illness recovery are correlated, precursor frequency is valuable for understanding immune response to dengue infection, whether induced naturally or by a vaccine.

Our laboratory has been studying dengue virus specific immune responses in order to further our knowledge of the role of T-lymphocytes in the pathogenesis of dengue diseases and disease recovery. Generally, PBMC from naturally infected or immunized individuals are examined for memory and cytotoxic T-cell abundance. T-cell clones are then established in order to study the T-cell repertoire that each dengue serotype induces, as well as map antigenic epitopes of the viral proteins. This gives us a better understanding of which proteins are the most antigenic, which then can serve as targets for vaccine development.

Until this point, studies have used vaccinia viruses expressing dengue 2, 3, and 4 NS3 proteins to investigate NS3 specific T-cell responses. NS3 has been shown to be one of the most antigenic viral proteins (Gagnon *et al.*, 1996; Mathew *et al.*, 1996). However, a vaccinia virus expressing the dengue serotype 1 NS3 protein has not been available. Without this construct, dengue NS3 studies have been incomplete. As shown in this study, a recombinant vaccinia expressing the dengue 1 NS3 protein is now available. Through CTL assays and protein gel electrophoresis, the current research demonstrates that the recombinant virus is expressing the NS3 protein in a form that can be recognized in the context of the HLA Class 1 molecule.

The development of such a vaccine has been a focus of infectious disease researchers for almost 20 years. Dengue diseases are a serious health problem in South America and Asian countries, where the mosquito species *Aedes aegypti* breeds. The dengue virus exists as four distinct serotypes 1, 2, 3 and 4. It has been shown that protective immunity to the serotype of the primary infection can provide life-long immunity but secondary infection with a heterologous serotype can cause severe illness in adults, including

Dengue Hemorrhagic Fever and Dengue Shock Syndrome. This phenomenon, called antibody dependent enhancement, complicates vaccine development. In addition, other flaviviruses such as Yellow Fever and Japanese Encephalitis cause some cross-reactive T-cell responses. It is unknown how the antibodies elicited by vaccines for these viruses will affect individuals that are immunized with a dengue virus vaccine.

The major reason that dengue virus research is ongoing is one of national security. Because the dengue virus is not indigenous to the United States, U.S. citizens are dengue naïve. A major concern when sending military troops overseas is the health threats they will encounter. Dengue illnesses are a major threat in many countries to which we have sent troops, such as Somalia, Haiti, and the Philippines. For example, during Operation Restore Hope in Somalia in 1992-1993, 43% of 96 soldiers hospitalized with a fever were diagnosed with Dengue Fever. (Sharp *et al*, 1995). Governmental agencies are interested in protecting our troops from being incapacitated by illness, including dengue diseases. A dengue vaccine would be very valuable to our government.

Another immediate concern is that the *Aedes aegypti* mosquito has been found in many of our southern states. As a result, the number of natively acquired dengue infections has been increasing. In 1997, seven cases of locally acquired dengue infection were reported in Texas (Rawlings *et al.*, 1998). As dengue epidemics occur closer to our southern borders, there is an increasing chance of an epidemic in states such as Texas, Louisiana, and Florida. The northward movement of the mosquito breeding grounds has been hypothesized to be correlated with the global warming effect (Reiter, 1996).

The development of an effective vaccine is not only enhanced by immunological research. Molecular biology can also provide useful research tools for the investigation of dengue pathogenesis. In the earlier part of this research, several genes were identified as being potentially upregulated due to dengue infection. These are genes that are not commonly thought to be associated with dengue infection, but the fact that they may be upregulated may lead to some interesting insight into the mechanism of some of the symptoms of dengue diseases and help identify targets for therapeutic intervention. Techniques such as PCR, gene sequencing and Differential Display can be used in a multi-focal analysis of dengue biology.

There are many directions in which dengue research is currently going. Experimental vaccine studies are on-going, as is clinical research of indigenous secondary infection in children in Thailand. Future research stemming from this study include isolation of dengue type 1 NS3 specific T-cells from donor FS and subsequent T-cell epitope mapping. This information would be added to the epitope data that already exist for the dengue virus. Of more immediate importance is the use of the recombinant vaccinia virus constructed in this study. It will be used to investigate CD4+ T-cell specificities in vaccine recipients. A correlation between the serotype causing infection and disease severity has yet to be shown but vaccinia constructs will be used to further this research and are valuable tools for labs researching dengue pathogenesis.

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