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In vitro Characterization of Avian Influenza Virus isolates with a truncated NS1 gene segment

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Abstract

Avian Influenza Virus represents a significant threat to the world poultry population, and is a potential threat to humans due to the possibility of cross-species AIV infection. Our approach is to characterize a number of avian virus populations with respect to their content of biologically active particles that include hemagglutinating particles (HAP), plaque forming particles (PFP), interferon inducing particles (IFP), interferon induction-suppressing particles (ISP), defective-interfering particles (DIP), cell-killing particles (CKP) and non-infectious cell killing particles (niCKP) using unique in vitro assays developed for avian influenza virus in the Marcus-Sekellick Laboratory. Specifically, we will use a strain of Avian influenza virus, identified as A/TK/OR/71delNS1, which contains a truncated NS1 gene that is being evaluated as a potential Live Attenuated Influenza Vaccine (LAIV). Our long term goal is to determine whether the amount or ratio of any of the virus subpopulations correlates with an improved or more efficacious influenza virus vaccine.

Introduction

Avian derived influenza viruses pose a significant threat to the world poultry population, and an increasingly real threat to humans. The first recorded human case came on May 14th, 1997. A three-year old boy named Lam Hoi-ka was admitted to Queen Elizabeth Hospital in Hong Kong with a high fever. Seven days later, Hoi-ka was dead and the world was faced for the first time with a threat of global pandemic from an influenza virus derived from birds (1). The threat wasn't recognized immediately, but when virologist Albert Osterhaus attempted to ascertain the type of the virus that had killed Lam Hoi-ka, he discovered the hemagglutinin molecule protruding from the viral membrane was of the type H5, previously thought only to cause disease in avian species (2). The cross-species leap marked a significant increase in the dangerousness of the disease.

When a virus replicates in a host species it adapts over time to the host. This evolutionary change is usually correlated with decreased virulence, as it is evolutionarily adaptive for the virus to keep its host alive, so that the host may continue to produce more virus (3). When the virus finds a new host, however, it is poorly adapted and may cause very severe disease in the new host . H5N1 avian influenza virus caused acute respiratory, kidney, and liver failure in Hoi-ka, as well as something called “disseminated intravascular coagulopathy”, a curdling of the blood. Worse still, humans have limited immunity to avian influenza viruses (2).

Avian influenza virus can carry several mutations that make it exceptionally dangerous, and thus an important target for research. The favorability of virus activation is drastically enhanced by an increase in the number of basic amino acids close to the cleavage point of the Hemagglutinin's fusion protein (4). Mutations to this area also results in the ability of Avian Influenza virus to be activated by multiple different kinds of cellular proteases (4). Additionally, substitutions in the PB2 protein (Glu627Lys) causes enhanced replication, and a substitution in the NS1 gene (Asp92Glu) causes an increased resistance to the action of cellular cytokines such as interferon and tumor necrosis factor α

(4). Pharyngeal swabs revealed H5N1 virus titers greater than ten times that of H3N2 or H1N1 viruses at 4 through 8 days post infection (4). All things considered, this has resulted in an overall 60-70% fatality rate for Human infections with Avian Influenza (1,3).

The potential for a pandemic avian influenza has grown exponentially since 1997. The truly alarming issue is that, much like the infamous Spanish Influenza of 1918, many deaths have occurred in otherwise healthy individuals, as contrasted with many cases of conventional influenza virus, which is associated with either the very young or the very old (3). In this time of high speed intercontinental travel, this virus can be spread rapidly from continent to continent across the world. While human to human contact is a minor case of the transmission of the virus, animal to human and environment to human can all contribute to the spread of this virus.

While the literature discussed to this point has focused on the H5N1 virus seen first in Hong Kong in 1997, other serotypes which can cause human disease have been identified including viruses containing the H7 and H9 avian derived hemagglutinin (1). The need for an effective vaccine for these high pathogenicity avian influenza viruses is of extremely high priority, especially considering recent outbreaks of H7N9 avian influenza viruses in China, which as of April 2013 have caused 104 infections and 21 deaths (5). However, the process of vaccine design itself is costly and time consuming. The use of *in vivo* study systems (for example, chickens) may cost large amounts of money, and non-human primates may cost almost $5000 per animal, not including the cost of feeding and maintaining the animals as well as the cost associated with the use of laboratory space in a designated primate center. An important step forward in the vaccine process would be the development of a set of criteria to screen effective from ineffective live attenuated vaccine strains *in vitro* (6). The major value in this screening process would be the efficient selection of viable live attenuated vaccine candidates. This work has been done in mixed plaquing phenotypes of an H7N3 Avian influenza virus (A/TK/OR/71delNS1) attenuated by reductions in the length of the carboxy-terminus of the NS1

protein by Lee *et. al.* at Ohio State University (7) . This strain of virus was then separated into four genetically stable isolates (5,6).

These isolates were designated pc1, pc2, pc3, and pc4, and utilized in a vaccine trial in SPF chickens (7). Two of these isolates, pc3 and pc4, produced high antibody titers and were protective against heterologous virus challenge. These were deemed to be vac^+, while the remaining isolates were vac^- (6). These four isolates were analyzed in order to determine the relative amounts of virus particles they contained. The total number of physical particles was determined by hemaglutanin assay. A plaque assay was performed to give the number of plaque forming particles. The number of interferon inducing particles (IFP) was determined by performing an interferon assay, from which the titer of IFPs could be calculated using the poisson distribution. A dose response for interferon production was was determined by plaque reduction assay using a defective interferon particle (DIP)-free helper virus. Finally, the number of non-infectious cell killing particles (niCKPs) was determined by use of the clonogenic assay (6).

Upon plaquing on monolayers of primary CEK cells, it was observed that these viruses produced mixtures of large and small plaque phenotypes (6). Plaques were harvested using the pêcher method (6). A 1 milliliter tuberculin syringe was used to isolate plaques of a single phenotype (6). Isolates were prepared from two large plaques, L1-12 and LB-1 (pc2 and pc3 respectively), and two small plaques, S3-11 and SG-3 (pc2 and pc3 respectively). The pc2 isolates were prepared by Elise English, and the pc3 by Jason Peterson, previous honors undergraduate students in the Marcus/Sekellick Laboratory.

Our experiment aims to produce sub-particle titers for these isolates. Through an analysis of the relative ratios of HA, PFP, IFP, and ISP in selected isolates, we will aim to correlate plaque size with amount of interferon produced, decide if plaque size can be correlated to vaccine efficiency, both in mammalian and avian cell types.

Materials and Methods

Cell Culture and Media

Primary chicken embryo cells (CEK) were obtained from Charles River SPAFAS, Inc. (Storrs, CT) were incubated in DMEM +10% Fetal Bovine Serum as well as fungizone (Amphotericin B) at a concentration of 1µg per ml for 24 hours at 38.5°C to achieve confluency. MARC cells (a mammalian cell line) were grown in DMEM+5%FBS containing fungizone (Amphotericin B) at a concentration of 1µg per ml. Chick embryo fibroblasts (CEC) were obtained from Charles River SPAFAS, Inc. (Storrs, CT) and incubated in Attachment Solution (AS), which contained NCI +6% Calf Serum as well as fungizone (Amphotericin B) at a concentration of 1µg per ml.

LAIV

In a previous study, four isolates were derived from serial passage of a stock of A/TK/OR/71delNS1 (H7N3) virus in embryonated eggs. Plaque purification confirmed the stability of these viruses, and plaques of a single phenotype (small or large) were identified and grown in embryonated eggs. Four distinct strains were identified, designated LB-1 and SG-3, the pc3 mutants, and L1-12 and SG-13, the pc2 mutants.

This study first confirmed the plaque phenotype (large or small) of each virus stock. Then, the stocks were passaged in embryonated eggs. All stocks were stored at -80°C, thawed rapidly and held on ice for use.

Plaque Assay

Primary CEK cells were obtained and allowed to grow to monolayer confluency at 38.5°C in DMEM+10%FBS+Fungizone in 60 millimeter dishes at a concentration of 2 million cells per dish. After 24 hours, the medium was aspirated from the dishes and replaced with 100 microliter doses of varying concentrations of diluted virus stock (in NCI) as well as 200 microliters of AS (Attachment

Solution: NCI+6% Calf Serum). The virus was allowed to attach at 37°C for one hour. After attachment, the virus was aspirated and an overlay containing 0.6% Agarose in NCI medium and fungizone (Amphotericin B) at 1 µg per ml. The plates were allowed to cool for 15 minutes and placed in the 37°C incubator for 3-5 days until plaques developed. If plaques were not easily visible, the monolayers were stained with a vital stain (neutral red). Plaques counts were obtained and then multiplied by the inverse of the virus dilution to determine the plaque titer of the virus stock, expressed in PFP/mL.

Aging Experiment and Interferon Induction

MARC cells were plated at an approximate density of 2.0×10^6 cells per 60 mm dish. Cells were aged for ten days at 37°C in either DMEM+5%FBS or MEM+5%FBS. Three plates were removed from the incubator on days 2, 4, 6, 8, and 10. A hemacytometer was used to obtain a cell count after trypsinization of one plate. The medium was removed by aspiration in the two additional plates, and a dose of VSV/IN/T1026R1 was applied corresponding to an m=5. AS was added to a final volume of 300 microliters. The virus was allowed to attach for one hour at 37°C. After attachment, the virus was removed by aspiration and replaced with 3 milliliters of either DMEM+5%FBS or MEM+5%FBS. 24 hours after virus induction, the medium was removed and frozen at -18°C until the experiment was completed. Interferon samples were processed by acidification with 1.5 M perchloric acid. 36 hours after the addition of perchloric acid, the samples were centrifuged for 10 minutes at 2000 rpm to remove any precipitate. The samples were then "neutralized" by dropwise addition 4 M potassium hydroxide followed by 1.5 M perchloric acid.

Interferon Assay

96 well microtiter trays were prepared containing MARC cells at a concentration of 4×10^4 cells per well. The trays were to incubate at 37°C until the cells reached confluency. 24 hours was sufficient in all experiments. Interferon samples were applied to the trays, allowing a cell and virus

control column. The remaining columns were treated with interferon samples, and a twofold serial dilution was carried out from an initial dilution of 1:50 in column three. Plates were incubated for approximately 24 hours at 37°C. After two days, the plates were challenged with a 1:500 dilution of VSV/IN/HR-W+ (4/24/2000). After 48 hours, the plates were stained with a vital stain, neutral red and allowed to dry for several days. The neutral red was solubilized by the addition of 100 microliters of 3 M Guanidine to each well. A BIOTEK plate reader (BIOTEK Instruments, Inc. Winooski, VT) was used to obtain absorbance data from the resulting suspended neutral red. The 50% endpoint of the assay was calculated using the BIOTEK Plate reader software (BIOTEK Instruments, Inc., Winooski, VT) based on the dilution of interferon which was sufficient to reduce the cytopathicity of the VSV wildtype by half as compared to the cell and virus controls. This dilution is by definition equal to one unit of interferon.

Egg Incubation

9 day old **e**mbryonated chicken eggs were obtained from Charles River SPAFAS, Inc (Storrs, CT). After inspecting the eggs, the outer surface of the eggs were cleaned with 95% ethanol and the eggs were injected with doses corresponding to a viral multiplicity of 10 and 100 PFP per egg respectively. This was done for each of the four pc2 and pc3 isolates. The virus was injected directly into the chorioallantoic fluid. After 68 hours of incubation at 34°C, the eggs were removed from the incubator, refrigerated overnight, and the resulting fluid was harvested.

Hemaglutination Assay

Chick red blood cells (ckRBC) were obtained from Charles River SPAFAS, Inc (Storrs, CT). The cells were counted using a hemacytometer, diluted in PBS-7 and inoculated at a concentration equaling 2×10^6 cells per well into specialized round bottom microtiter 96 well plates. Virus stocks were then added on to the cells and diluted serially across the plate. The clumping of the cells was then

observed. The reciprocal of the dilution at which the cells ceased to form a clumped, rounded mass at the bottom of the well is read as the HA titer.

Interferon Induction and Generation of Dose Response Curves

MARC cells were plated in 60 millimeter dishes (at a concentration of 2.0×10^6 cells per dish) and aged without medium change for 7 days in DMEM+5%FBS at 37°C, as established in previous experiments. In separate experiments, CEC cells were plated at a concentration of 2.0×10^6 cells per 60 millimeter dish and aged without medium change for 9 days at 38.5°C. Medium was aspirated, and representative egg-derived LAIVs were added to separate plates in dosages of 5, 10, 25, 50, and 100 microliters. The virus was allowed to attach in AS for one hour in a total volume of 300 microliters (at 37°C for MARC and 40.5°C for CEC). The unattached virus was removed by aspiration from each plate. The plates were "washed" twice using a small amount of NCI containing no serum. 3 milliliters of DMEM+5%FBS were provided to each plate, for the MARC cells, and 3 milliliters of NCI for the CEC cells. After 24 hours, the plates were removed from the incubator and the medium was collected in polypropylene tubes and processed as described previously. Interferon assays were performed as previously described.

Results

We first hypothesized that these isolates would produce plaques that were uniform in their appearance. To examine this, we performed a plaque assay on four pc2 and pc3 plaque isolates. One large and one small plaque isolate was used from each strain. For the pc2, L1-12 and S3-11 were used. For the pc3, LB-1 and SG-3 were used. These four virus isolates were plaqued on twelve 60 millimeter dishes containing monolayers of primary CEK cells. Each virus stock was diluted serially to give a final dilution of 10^{-5}, 10^{-6}, and 10^{-7} on the plates. Due to the limited availability of CEK cells, we were

only able to use a single plate for each virus dilution. We found that the plaques produced were consistent with the expected phenotype: each large plaquing isolate produced large plaques, and each small plaquing isolate produced small plaques. The experiment indicated a relatively high titer for each stock since each plate contained too many plaques to count. The experiment was repeated using virus dilutions 10^{-6}, 10^{-7}, and 10^{-8} on fresh CEK cells prepared as described previously. Each dilution was performed on two duplicate plates. The plaque phenotypes remained consistent with the expected—large isolates produced large plaques and small isolates produced small plaques—and the plaque titers were determined mathematically (Table 1).

Next, we wanted to investigate the growth of the pc2 and pc3 isolates in MARC cells. These cells are of mammalian origin (monkey kidney), and are good producers of interferon. We hypothesized that the addition of the protease trypsin would aid in the cleavage of the fusion protein of the hemaglutanin, allowing for the entry of influenza virus into these cells. For this plaque assay, we used the 10^{-6}, 10^{-7}, and 10^{-8} dilutions, which produced a countable amount of plaques in the previous assay (on CEK cells). We used a large plaquing isolate, LB-1 (pc3) and a small plaquing isolate SG-3 (pc3). Each dilution was prepared in duplicate. While no cell lysis was observed, some areas of the cell monolayer had the appearance of fusion.

Next, we attempted to learn more about the MARC cell line by identifying time of incubation without media change that would produce the best yield of interferon. This process has been investigated and established in CEC cells by Sekellick (9), Marcus (10), and others. In order to accomplish this, 20 plates initially containing 2.0×10^{6} MARC cells each were prepared. The plates were incubated continuously without media change for 10 days. At two day intervals, three plates were removed from the incubator. One plate was used for a cell count performed with the aid of a hemacytometer. (Figure 1) The remaining two plates were infected with VSV-T026R1, a mutated strain of vesicular stomatitis virus that is known to be a good inducer of interferon at a multiplicity of 5

infectious particles per cell. The virus was allowed to attach for one hour in 300 microliters of Attachment Solution (AS). After an hour any unattached virus was removed by aspiration, and the plates were washed using NCI. The plates were given 3 mL of fresh media, which was collected after 24 hours and then used in an interferon assay. The ability of the interferon samples in the supernatant, which were purified by acidification of the media, to protect MARC cells from challenge with a wildtype VSV was determined after a heterologous virus challenge. Neutral red was used to visualize cells that had been protected from the challenge virus by the interferon sample. Using the BioTek Plate Reader and associated software, it was possible to calculate the interferon yield of each sample, where one unit of interferon was defined as the amount sufficient to protect 50% of the cells from the challenge virus. The interferon yield was then used to calculate the amount of interferon produced per 2.0×10^6 cells as a standardized measure (Figure 2). The next step was to use the data obtained from this experiment, age MARC cells for six days, and compare the amount of interferon produced by induction with different virus strains (Figure 4).

Next, the interferon induction of four avian influenza viruses (AIV/B/MD/06, AIV/B/Yamanashi/05, AIV/A/Udorn/W+/06, and AIV/A/TK/OR/W+/06) was analyzed. MARC cells were aged 6 days, as per the results of the previous study. Two dose levels were used: a 10 microliter dose was designated “low dose”. A 100 microliter dose was designated “high dose”. Due to a procedural error, four plates were prepared that had been infected with one dose each of two separate viruses. These four plates were replaced (so that there were a total of 22 plates in this assay). In order to test the action of trypsin, two plates were prepared for each dosage, one containing trypsin and one without. The final concentration of trypsin was 1.5 micrograms/milliliter. The virus was attached for one hour at 37.5 degrees Celsius. The remaining unattached virus was removed by aspiration, and the plates were washed with NCI. The plates were given 3 milliters of fresh media (DMEM+5%FBS) and allowed to incubate for 24 hours. The resulting supernatant was collected and acidified in order to

purify the interferon samples as described previously. The interferon samples were assayed as in the previous experiment. The interferon production for each sample was calculated and reported in Table 2.

The next step in characterization of these viruses was to prepare sufficient high titer stocks of the virus isolates to perform assays to assess the sub particle populations. This was accomplished by growing the virus in 9 day old embryonated chicken eggs. Thirty eggs were prepared for this purpose. Each was inoculated with a diluted sample of the current virus stocks so that one set of eggs received a multiplicity of 10 infectious particles, and the other received 100 infectious particles. The virus was inoculated directly onto the chorioallantoic fluid of the egg. After incubation for 68 hours at 34 degrees Celsius, the chorioallantoic fluid was harvested at the end of incubation, and a hemagglutination assay was performed on each sample. This allowed us to determine the number of physical particles in each virus stock by assessing the number of hemagglutinating particles (HAP).

The next step, once HA titers were obtained for each egg-derived stock, was to perform plaque assays to determine the titer of each collected virus stock. Initially, there was some degree of difficulty with the procedure for plaque assay of AIV. It was found that insufficient removal of serum proteins from the plaque monolayers was responsible for this setback. Several high quality stocks were identified, based on a high plaque titer, a good HA titer, and a large volume of sample collected.

In order to confirm the aging necessary to perform a proper interferon induction in the MARC cells, we repeated the aging experiment from last semester, while adding a second variable. In order to decide which media was best for the aging of MARC cells, we provided one set of plates with DMEM+5%FBS and the other with MEM+5%FBS. We found that the number of cells in each sample was consistently lower in the MEM cells. The amount of interferon induced by each sample (in two day intervals) was determined by interferon assay. It was determined that the MEM cells produced a lower amount of interferon than the DMEM cells. The peak yield was unfortunately not determined, and that plate will be repeated in the future to provide a more complete data set.

Plaque titers were used to generate a dose-response curve for each of the identified high quality stocks as compared to several control viruses. Once the calculations had been performed, it was determined that it would be more practical to use a constant dose for each virus stock. We used doses of 50, 25, 10, and 5 µl of each stock. This allowed us to look at a standardized view of each isolate's capacity to induce an interferon response in MARC cells (a mammalian cell line). Five isolates were used in this study, which were identified as candidates based on a high HA titer, a relatively high plaque titer, and a large amount of available stock.

MARC cells were aged in 60 millimeter dishes for 7 days as established in previous experiments. On day 7, the cells were exposed to virus at varying dosages supplemented with Attachment Solution in a final volume of 300 microliters. The virus was allowed to attach at 37.5°C for one hour. After this period, the AS was aspirated from the cells and the cells were washed with NCI. 3 milliliters of DMEM+5%FBS was provided. The resulting samples were collected 24 hours post inoculum and processed as described previously. An interferon assay was performed using these samples, and the results are displayed in Figure X.

A further survey of the samples was undertaken in order to screen more of the isolates for interferon production. 13 isolates were selected (displayed in table 4). From the pc2 mutant, 4 large plaquing and 3 small plaquing isolates were selected. From the pc3 isolate, 3 large plaquing and 3 small plaquing isolates were selected. For reference, VSV/IN/T1026R1 was used to compare interferon yields. A 10 microliter and 100 microliter dose was used for each isolate. Using 60 millimeter dishes aged 7 days, an interferon induction was performed as described previously. Once collected the interferon samples were tested as described previously. The results are displayed in table Y. The data demonstrate that the isolates from pc3— interestingly enough, both the large and small plaquing isolates—produced significantly higher yields of interferon for both the 10 microliter and 100 microliter doses. This is consistent in part with results of the Marcus/Sekellick laboratory and others

(6)(7). In 2008, it was determined that pc3 isolates of AIV/TK/OR/71delNS1 were good vaccine candidates, and further determined that a high interferon induction capability (in the form of a favorable proportion of Interferon Forming Particles to Interferon Suppressing Particles) is a property conducive to excellent live attenuated avian influenza virus vaccine potential. However, the results of this study may be incongruous with John Ngunjiri's (11) findings that INF induction is reversed in chicken and mammalian cells—The 2010 Marcus study utilized CEC cells to induce interferon (6). However, since time constraints precluded the performance of the screening process in CEC cells as well, it is possible that interferon induction in CEC by the screened isolates might produce a higher amount of interferon.

Discussion

After three semesters of work, we are able to say that we have successfully characterized several properties of the MARC cell line. These include the necessity of using trypsin to ensure proper infection with AIV, and the optimum aging time to generate the maximum yield of interferon. We have also been successful in creating several high titer stocks of derived from the passage of isolates L1-12, LB-1,S3-11, and SG-3 in embryonated chicken eggs, which we have begun to assay in order to determine the relative amounts of various virus sub particles.

One interesting observation during an attempted plaque assay of MARC cells was evidence of fusion seen in the presence of trypsin. Further research revealed that according to Huang *et. al*. (8), influenza viruses can cause this effect under slightly acidic media conditions, which were present in the AS used for attachment. The other requirement for this state is the addition of a proteolytic enzyme. In this case, trypsin was used. However, the property of a virus that allows cell-cell fusion to outcompete infectivity varies by virus strain. This fusion was seen to vary with the concentration of the virus, (only

seen at the 10^{-6} dilution) but was seen clearly in both pc3 isolates used, and less clearly in the wildtype A/TK/OR virus control. It would be interesting to follow up this assay with a similar investigation under the same conditions (MARC + trypsin) in the two pc2 strains.

In terms of areas that might merit further study, the phenomenon of cell-cell fusion in the MARC (mammalian) cell line may shed some light on the trans-species transmission of avian influenza viruses. While there are many factors that influence whether or not an influenza virus may infect a certain cell line, Wang *et. al.* (7) have found that the interferon production in MARC cells is sufficient to drastically reduce the ability of avian influenza viruses containing a mutated or non-functional NS1 gene to replicate. I would suggest attempting to infect VERO cells, which are of similar origin to MARC cells, but do not produce interferon, with our pc2 and pc3 mutants, in order to see if they are able to enter the cells (most likely in the presence of trypsin). Since this ability was seen only in our pc3 mutants, and not in our wildtype virus, I would be interested to see if it was similarly conserved in the pc2 mutants in the MARC system.

We have identified several high titer egg derived LAIV and confirmed through repeated plaque assays (both this semester and last) that a single plaque phenotype remains in each strain. Additionally, we have confirmed that 7 days is the optimum time of aging without media change for the MARC cell line, and that DMEM+5%FBS provides the best environment for MARC cells to age. We were also able to confirm that in the MARC system, a better ability to induce interferon was correlated with the effective vaccine strains (pc3) (6) (Table 4). Interestingly, it appeared that in the MARC system, plaque size was correlated with interferon induction. Those isolates that produced a large plaque on average induced a larger amount of interferon (Table 4). On average large plaquing isolates produced 78.50% more interferon than small isolates (pc3 produced 132.58% more and pc2 produced 24.43% more). While it seems that large plaque size is correlated with greater interferon production, further experiments should aim to confirm that the plaque phenotype remains constant in the MARC cells.

Ngunjiri *et. al.* (11) concluded that the ability to induce interferon varied with cell type. While we attempted as a part of this study to investigate this property, but our results were largely inconclusive. Performing the same "screen" of isolates—that we had done in MARC cells—using CEC cells might produce more information about this property. This merits further investigation because one possible application of this research is the design of LAIV vaccines for both humans and animals. This inverse relationship may help researchers more successfully identify potential mammalian vaccine candidates that may have produced small amounts of interferon in avian systems. Our results showed a larger degree of interferon production in the pc3 isolates. According to Ngunjiri, we should have seen a more interferon induced by the pc2 isolate in MARC cells.

If more time had been available for this project it would have been helpful to more thoroughly examine the titers of Interferon suppressing particles, defective interfering particles, and non-infectious but cell killing particles. Increased characterization of these sub particle populations should lead to an increased understanding of the properties of AIV/TK/OR/71delNS1. It might have been more efficient to have started the analysis of interferon induction earlier in the project. This would have allowed a greater amount of flexibility in terms of error as well as providing a better opportunity to study the ISP, DIP, and niCKP titers. This additional time could also have been used to analyze additional data, which would have produced a conclusion of a larger scope.

Figures and Tables

Table 1

Isolate Designation	Plaque Titer (CEK) (PFP/mL)	Plaque Assay in MARC cells
(Pc2) L1-12	6×10^{8}	N/A
(Pc2) S3-11	5×10^{9}	N/A
(Pc3) LB-1	9×10^{8}	No CPE, Fusion in 10^{-6}
(Pc3) SG-3	3.7×10^{8}	No CPE, Fusion in 10^{-6}

Table 1- Plaque titers of the four isolates were determined after growth on primary chicken embryo cells. The plaque titer is determined by taking the average number of plaques observed in a dish, and multiplying by the reciprocal of the virus dilution. The designation PFP/mL refers to the number of plaque forming particles per milliliter of virus stock.

Table 2

Sample	Dose (Microliters)	Trypsin (+/-)	Interferon Yield (Units/mL)
MD-7	10	-	<50
MD-7	100	-	31
MD-7	10	+	14
MD-7	100	+	59
Y4-3	10	-	<50
Y4-3	100	-	1
Y4-3	10	+	<50
Y4-3	100	+	50
TK/OR/W+	10	-	<50
TK/OR/W+	100	-	38
TK/OR/W+	10	+	<50
TK/OR/W+	100	+	<50
Udorn W+	10	-	<50
Udorn W+	100	-	<50
Udorn W+	10	+	<50
Udorn W+	100	+	8
VSV/T1026R1	100	-	232
VSV/T1026R1	100	+	351
Cell Control	0	-	<50
Cell Control	0	+	<50
Y4-3+TK/OR/W+	10+10	-	<50
Y4-3+TK/OR/W+	10+100	+	7
Y4-3+TK/OR/W+	100+100	+	47
Y4-3+TK/OR/W+	100+100	+	58

Table 2- The interferon yields for the four isolates is shown above. None of these viruses was particularly effective at inducing interferon. The VSV control however, showed a respectable amount of interferon induced. Three of the four viruses had comparable HA titers (given as 1:2560) (MD-7,Y4-3, and TK/OR/W+). The Udorn W+ had a HA titer of 1:640, or roughly one quarter. While the numbers are all extremely low, none of the Udorn W+ samples produced a measurable yield of interferon (a yield less than the starting dilution of 1:50).

Table 3

Isolate	Stock Designation	Volume Harvested from 1 Egg (mL)	HA Titer	Plaque Titer PFP/mL
LB-1	A1-1	5.5	1:1280	$7.9x10^8$
	A1-2	12.5	1:2560	$2.7x10^8$
	A2-1	3	1:1280	$1.51x10^9$
	A2-2	11	1:2560	$9.6x10^8$
	A2-3	11	1:2560	$4.5x10^8$
	A2-4	6	1:2560	$1.26x10^9$
SG-3	B1-1	3.5	1:5120	$2.42x10^9$
	B1-2	5	1:1280	$2.6x10^8$
	B1-3	11	1:1280	$4.2x10^8$
	B1-4	11.5	1:2560	$2.15x10^8$
	B2-1	2	1:5120	$7.9x10^8$
	B2-2	5.5	1:2560	$4.8x10^8$
	B2-3	4.5	1:2560	$8.9x10^8$
	B2-4	11	1:2560	$1.37x10^9$
L1-12	C1-1	11	1:2560	$2.9x10^8$
	C1-2	12.5	1:2560	
	C1-3	12.5	1:320	
	C1-4	12.5	1:320	
	C2-1	11	1:320	
	C2-2	12	1:320	
	C2-3	9.5	1:640	$5.2x10^8$
	C2-4	11	1:320	$6.05x10^8$
S3-11	D1-1	11.5	1:640	$1.14x10^8$
	D1-2	10	1:640	$4.78x10^8$
	D1-3	11	1:640	$4.6x10^8$
	D1-4	13	1:1280	$2.81x10^8$
	D2-1	10	1:160	
	D2-2	11.5	1:160	
	D2-3	10	1:320	
	D2-4	10	1:640	

Table 3- HA titers and Plaque titers were determined for Egg-derived LAIV. So called "high quality stocks" are highlighted. These stocks will be tested further for interferon induction and other properties. Stocks C1-2 and after have not yet had plaque titers determined.

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Table 4

Mutant	Isolate	HA Titer	PFP/mL	Plaque Phenotype	INF Yield (10µl)	INF Yield (100µl)
LB-1 (pc2)	A1-2	1:2560	2.7 x 10^8	Large	97	153
	A2-2	1:2560	9.6 x 10^8		142	642
	A2-3	1:2560	4.5 x 10^8		336	ND
	A2-4	1:2560	1.26 x 10^9		167	334
Average		*1:2560*	*7.35 x 10^8*		*185.5*	*376.3*
SG-3 (pc2)	B1-1	1:5120	2.42 x 10^9	Small	70	99
	B2-2	1:2560	4.8 x 10^8		153, 185	ND
	B2-3	1:2560	8.9 x 10^8		178	511
		1:3413.3	*12.6 x 10^8*		*146.5*	*305*
L1-12 (pc3)	C1-2	1:2560	7.3 x 10^8	Large	198	534
	C2-3	1:640	5.3 x 10^8		731	1305
	C2-4	1:320	6.05 x 10^8		465	8447
		1:1173.3	*6.21 x 10^8*		*464.66*	*3428.6*
S3-11 (pc3)	D1-1	1:640	1.14 x 10^8	Small	392	1105
	D1-2	1:640	4.78 x 10^8		333	2956
	D1-3	1:640	4.6 x 10^8		80	156
		1:640	*3.5 x 10^8*		*268.3*	*1405.6*
n/a	VSV/T1026R1	*n/a*	*1 x 10^9*	*n/a*	*1240*	*1460*

Table 4- 13 isolates of AIV/TK/OR/71delNS1 were used to induce interferon in MARC cells. The results of HA assay and Plaque assay are also shown for clarity.

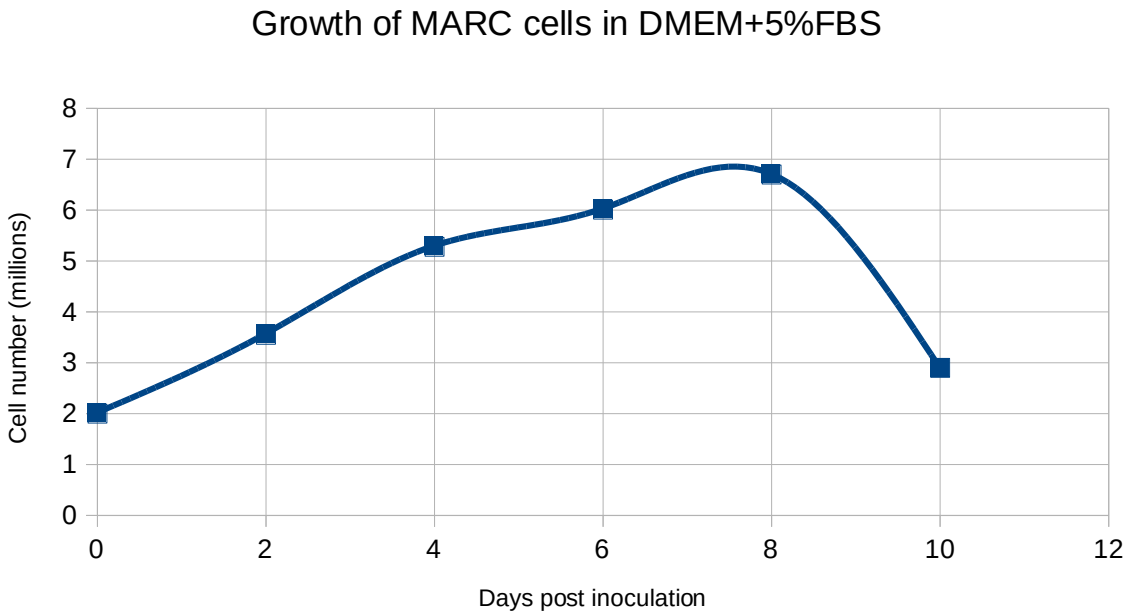

Figure 1- The cell count was performed every two days using a hemacytometer. Note the peak cell number at approximately day 8 of growth with no media change. Subsequent days yielded samples containing significant numbers of cells that had the appearance of being dead or dying.

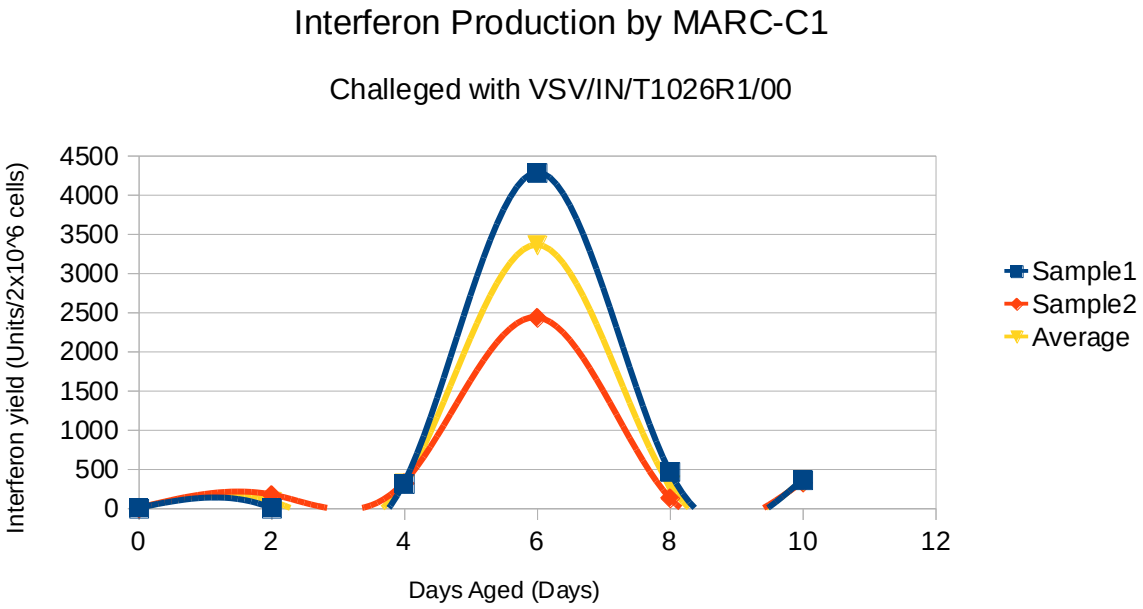

Figure 2- MARC cells aged as described in *Figure 1* were challenged with VSV/IN/T1026R1/00. The Interferon Yield was calculated from the 50% cell killing endpoint as read using the BioTek Plate reader software. This curve was generated by determining the total yield of interferon from each sample, dividing it by the number of cells in that culture dish, and then multiplying the resulting number by 2 x 10^6. The peak of interferon production is at day six p.i.

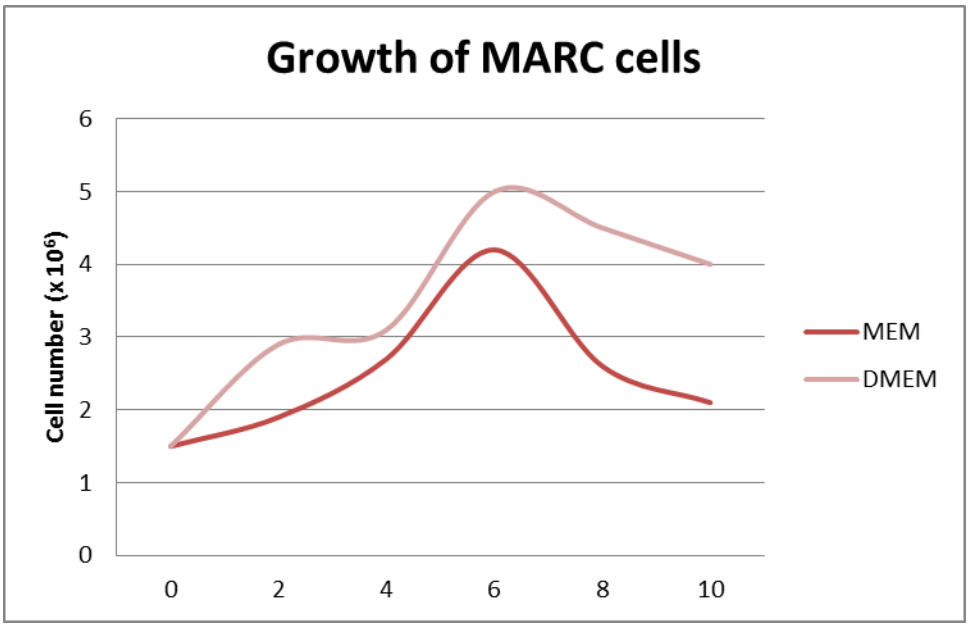

Figure 3-MARC cells aged in either MEM+5%FBS or DMEM+5%FBS. The cell count was performed every two days using a hemacytometer. Note the peak cell number at approximately day 8 of growth with no media change. Subsequent days yielded samples containing significant numbers of cells that had the appearance of being dead or dying.

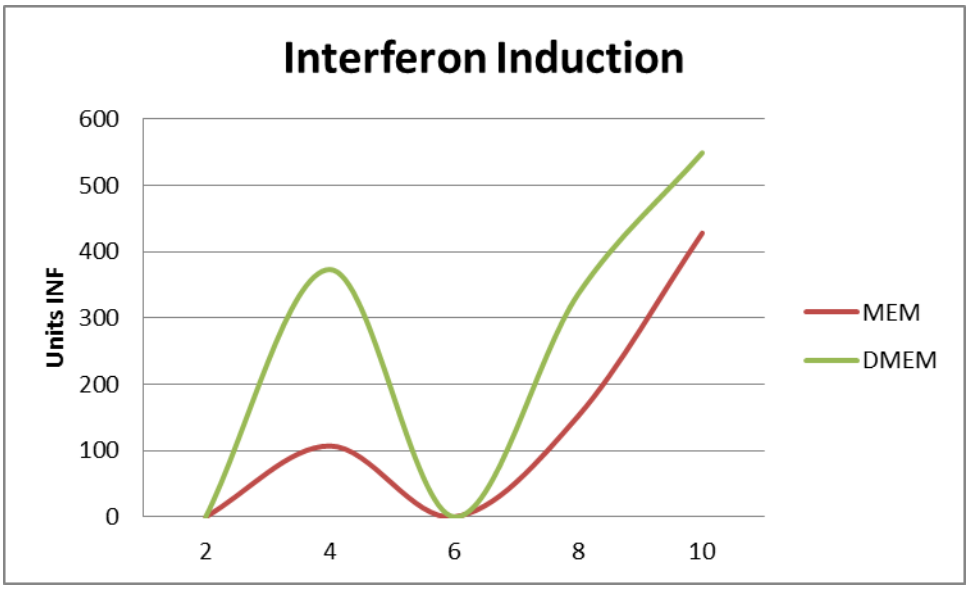

Figure 4-The induction of interferon in aged MARC cells. This diagram is incomplete because it lacks values for day 6, the predicted peak yield. Interesting is the high values for induction seen in the day 10 cells, not seen in the original aging experiment.

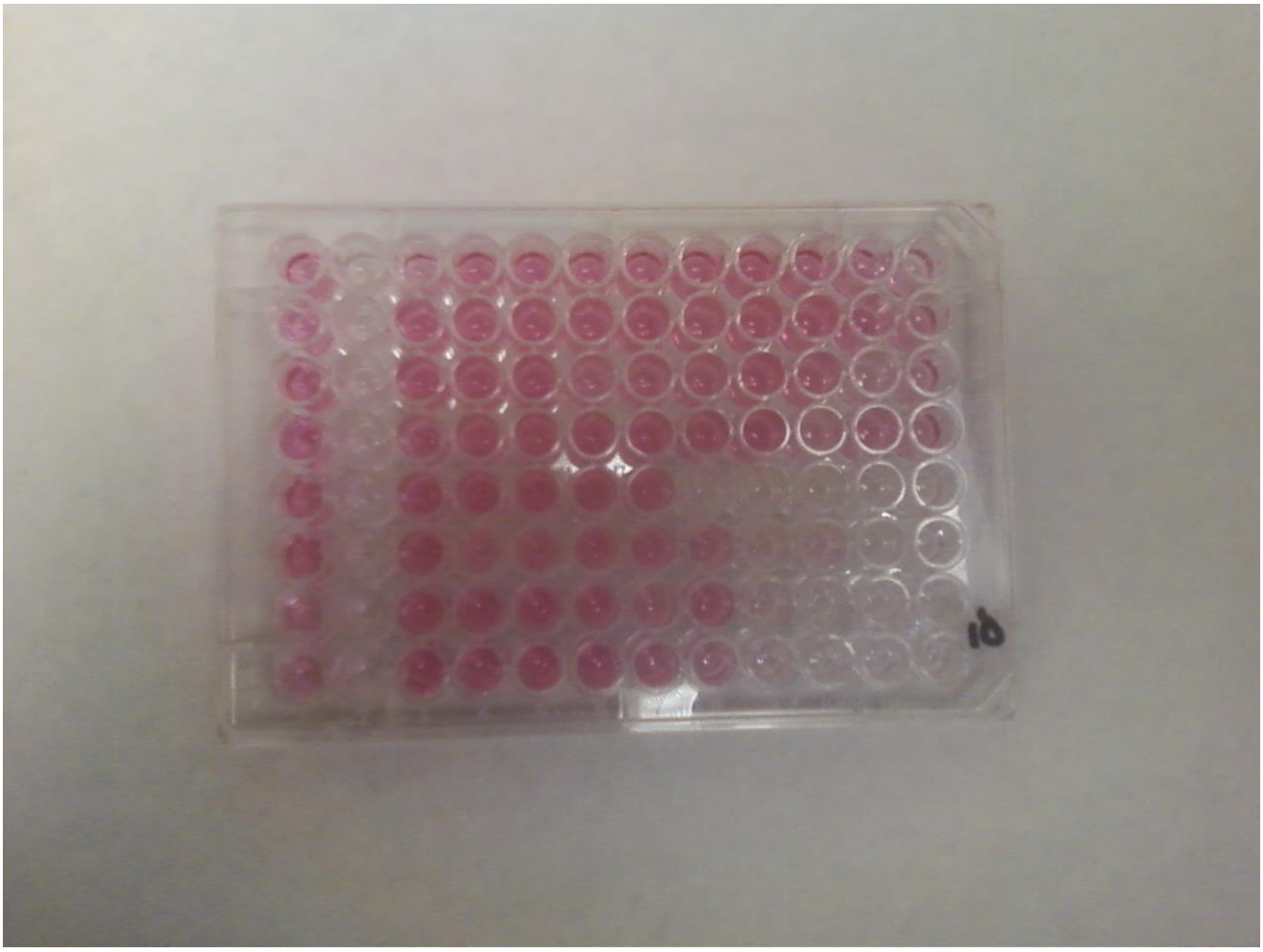

Figure 5- MARC cells at the final stage of an interferon assay. These cells have already been exposed to both interferon, a challenge virus, been stained with neutral red, and solubilized with 3M Guanidine. Red coloration indicates living cells. Column 1 on the left represents a cell control. Column 2 represents a virus control. Columns three through twelve represent two-fold dilutions of an interferon sample. In this case, a 1:25 dilution was used in column 3. The top four rows indicate a sample produced by the 100 microliter dose of AIV, the bottom four indicate a 10 microliter dose.

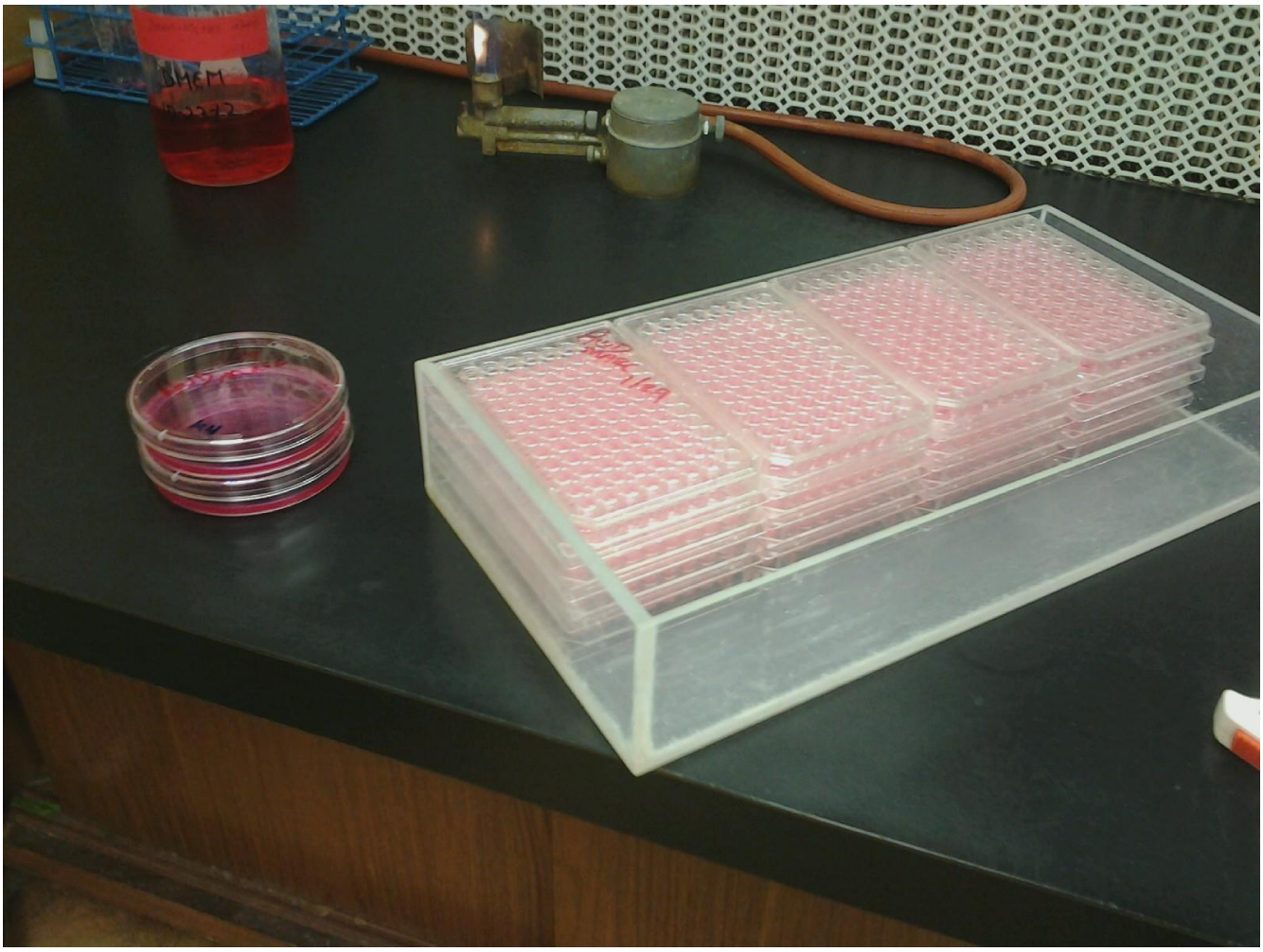

Figure 6- MARC cells plated in 100 millimeter dishes (left) and 96-well microtiter trays (right)

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