CYTOKINE INDUCTION BY INFLUENZA VIRUS: A POSSIBLE CORRELATION TO PATHOGENICITY

By

SENAD DIVANOVIC

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Dean of the Graduate College

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NOMENCLATURE

BSA	bovine serum albumin
CD	cluster of differentiation
CMI	cell-mediated immunity
CTL	cytotoxic T-cell
EDTA	ethylene diamine tetra acetic acid
ELISA	enzyme-linked immunosorbent assay
EM	electron-micrograph
HA	hemagglutinin
IFN-γ	interferon-γ
IL	interleukin
MHC	major histocompatibility complex
NA	neuraminidase
$NaHCO_3$	sodium bicarbonate
NaOAc	sodium acetate
PBL	peripheral blood lymphocytes
PBS	phosphate-buffer saline
RT-PCR	reverse transcriptase polymerase chain reaction
SDS	sodium dodecyl sulfate
UV	ultra-violet

CHAPTER I

INTRODUCTION

1.1. Background

Reports coming from around the globe show an increase in the incidence of emerging diseases such as Ebola hemorrhagic fever, AIDS, Japanese encephalitis, West Nile virus encephalitis, tuberculosis, new variant Creutzfeld-Jakob disease, syphilis, viral hepatitis, diphtheria, measles. meningococcal disease, and others [46, 55, 89]. Despite armed with recent medical advances, these emerging diseases present a significant threat to humanity and posses a potential economic loss. Therefore, a better understanding of pathogens and the diseases they cause is pertinent in the development of preventive measures.

A pathogen is defined as any organism capable of invading another living organism, resulting in the development of disease in the host [4]. Pathogenicity is the ability of microorganisms to cause disease, while virulence is a measure of pathogens' ability to inflict severe disease [4]. Both pathogenicity and virulence are multi-factorial. Factors include the interactions between the host and the pathogen as well as other environmental factors [4]. Characteristics of influenza virus such as rapid mutation rate, antigenic shift and drift, airborne transmission, and the ability of avian viruses to directly infect humans define this virus as a significant emerging infectious agent.

Influenza is a highly contagious disease caused by influenza virus infection. Spread from person to person and within a community is very rapid, resulting in 20,000 deaths annually in the U.S. [11]. Fever, ranging from 38 to 40 °C, peaks within 24 hours of onset that lasts from one to five days is a common symptom in influenza. Other symptoms include chills, nausea, abdominal pain, diarrhea, a generalized feeling of weakness, muscle pain, and soreness of the respiratory tract [67]. Symptoms elicited by influenza virus infection are attributed to the release of cytokines by the immune response towards the virus [22, 33, 81].

Influenza viruses are divided into three types A, B, and C, based on their antigenicity. Influenza A virus, the most pathogenic of the three types, is further divided into 15 HA and 9 NA subtypes [19]. All of the influenza A virus subtypes are found in waterfowl [91, 93]. Two highly immunogenic viral surface proteins, viral hemagglutinin (HA) and neuraminidase (NA), have been associated with virus pathogenicity. The HA of highly pathogenic avian influenza virus (H7N7 and H5N2) has been implicated in the pathogenicity of these viruses [2, 41]. In addition, the internal NP gene, which plays a role in host range, has been implicated in contributing to disease development.

Surveillance of influenza virus (and influenza) by the World Health Organization (WHO), and by the Centers for Disease Control and Prevention (CDC), is a global effort to improve disease prevention and for vaccine formulation. A vaccine is available for influenza prevention. This vaccine, administered annually, contains two inactivated viruses of currently circulating influenza A viruses (subtypes H1N1 and H3N2) and one circulating influenza B virus. This vaccine provides only a short-term protection, and does not provide protection against future epidemic or pandemic influenza virus due to

antigenic drift and antigenic shift, respectively.

Despite the concern over a new pandemic, the molecular basis of influenza virus pathogenicity is not well understood. A better understanding of molecular factors involved in virus pathogenicity will assist in development of a better protection against influenza.

1.2. Research summary

1.2.1. Study problem

Why have only three (H1, H2, and H3) out of fifteen HA subtypes been circulating in humans? Why are some influenza viruses, such as H7N7, H5N2 and H5N1, but not other subtypes highly pathogenic in chickens? Is the hemagglutinin solely responsible for viral pathogenicity? If so, what is the mechanism?

1.2.2. Study hypothesis and speculations

The level of cytokine release, such as IL-6, has been shown to correlate with the severity of symptoms; i.e., a higher level of IL-6 results in a higher fever [22, 33, 81]. Furthermore, infection by influenza B and C viruses usually results in milder disease with less severe symptoms, than influenza A viruses. Therefore, there appears to be a correlation between pathogenicity and cytokine level. I hypothesize that, using an *in vitro* lymphocytic culture a more pathogenic influenza virus would induce a higher level of cytokines. I postulate that HA is an important factor in determining pathogenicity, and that the increased pathogenicity is a result of increased induction of cytokines. This can be assayed by the *in vitro* lymphocyte model. By using different influenza viruses, I will

be able to test this hypothesis.

1.2.3. Objectives

Since more pathogenic virus induces a higher level of cytokines in the host, the first objective will be to establish an *in vitro* model (using a human Jurkat T cell line and human umbilical cord blood lymphocytes) to mimic virus infection *in vivo*. The *in vitro* lymphocyte culture will be infected with influenza virus; the induction of cytokines will be measured both at the transcriptional and at the translational level. The second objective will be to determine if the levels of cytokine induced in this model using different influenza viruses correlate with their pathogenicity *in vivo*.

1.2.4. Limitations

In mammals including humans, the site of influenza virus infection is the upper respiratory tract. The cytokines are released from tissue dendritic cells. This results in development of immune response as well as common influenza symptoms. This *in vitro* lymphocyte model may not be a perfect model, as the ratio of dendritic cells are much lower.

1.2.5. Study approach

To eliminate a memory immune response due to previous influenza infections, I examined cytokine production using naïve equine (and human) peripheral blood lymphocytes, and a human Jurkat T cell line. All influenza viruses tested were UV-inactivated preventing viral replication, and therefore this model assays for the intrinsic

stimulation of the virus. Furthermore, to determine the importance of HA in cytokine induction, viruses of the same HA but different NA subtype (H3N2 and H3N8) were tested.

Briefly, naïve human (and equine) peripheral blood lymphocytes, and a human Jurkat T cell line cells were pulsed with UV-inactivated influenza A viruses and an influenza B virus. Following pulsing, stimulated lymphocytes were incubated for 3, 12 or 24 hrs prior to supernatant fluid collection and RNA extraction.

The amounts of RNA produced were assayed for using Reverse Transcriptase (RT) and Polymerase Chain Reaction (PCR). This assay is semi-quantitative. The amounts of cytokines released were determined using an Enzyme Linked Immunosorbent Assay (ELISA). Cytokines assayed include IFN-γ, IL-2, IL-4, IL-6, IL-10 and IL-12.

1.3. Future application/significance

The correlation between cytokine induction and severity of symptoms in influenza infection could be used as a potential marker for the pathogenicity and virulence of new influenza viruses. Additionally, an *in vivo* study of selected viruses regarding their pathogenicity would enable development of an improved and longer lasting vaccine strategy, for example, DNA vaccines [71, 92]. With education, and with massive immunization campaign, a repeat of a pandemic like that occurred in 1918 will be minimized if not prevented. Furthermore, this *in vitro* model could be used for determination of pathogenicity of other pathogens.

CHAPTER II

LITERATURE REVIEW

2.1. Introduction

2.1.1. Influenza

Influenza, sometimes called Flu or Grippe, is an epidemic, acute, viral infection of the upper respiratory tract [67]. Infection (in humans and other mammals) is initiated by the inhalation of aerosolized droplets containing the virus. Initial infection is limited to the epithelial lining of the respiratory tract. Onset of infection is sudden, and in the acute phase fever ranging from 38 to 40 °C is seen [11, 12]. These symptoms last from one to five days. Other symptoms include chills, nausea, abdominal pain, diarrhea, a generalized feeling of weakness, muscle pain, and soreness of the respiratory tract [12, 67]. Influenza virus infection can directly injure the nasal and tracheobronchial epithelium, possibly as a result of virus-induced cellular apoptosis [12, 67]. The loss of respiratory epithelial cells is a major reason for symptoms associated with influenza such as cough, depressed trachobronchial clearance, and altered pulmonary function [12, 67].

2.1.2. Influenza pandemics

Influenza A virus epidemics and pandemics have been recorded in European

literature since the 17th century. These records show large outbreaks in 1658, 1710, 1837, and 1889 [50]. In the twentieth century alone, three pandemics occurred in 1918, 1957, and 1968. The three different influenza virus subtypes responsible for the twentieth century pandemics were H1N1, H2N2, and H3N2, respectively. Because of the number of deaths, influenza virus can be considered one of the deadliest viruses of the twentieth century.

The first pandemic of the twentieth century, the "Spanish Flu," occurred in 1918 and caused, with conservative estimates, 20,000,000 deaths. It claimed the lives of as many as one in 100 of the world's population at the time [79, 91]. The virus spread very quickly through North America, Asia, and Europe. Even the Alaskan wilderness as well as the remote islands of the Pacific did not escape [91]. The disease was named the "Spanish flu" because it was first reported in a Spanish newspaper as a result of press censorship in all the other countries involved in World War I. Approximately 28% of the U.S. population was infected, and the mortality rate averaged 2.5%, with a 70% mortality rate seen in some isolated populations [91]. This was considerably higher than the mortality rates from previous epidemics, which were usually around 0.1% [91]. Besides the high mortality rate, the "Spanish flu" differed from other epidemics as its primary victims were young adults, a population thought usually to be unaffected by the disease.

The second pandemic, the "Asian Flu," occurred in 1957, and the third pandemic, the "Hong Kong Flu," occurred in 1968. These two pandemics were not as deadly as the pandemic of 1918. However, they caused much fear and suffering.

2.1.2.1. *Mortality and economical impact*

Mortality due to influenza can be quite high. The 1918 pandemic resulted in over 20,000,000 deaths. The 1957 pandemic caused an excess mortality of 70,000, while in the 1968 pandemic the excess mortality was 30,000 [8, 24]. Deaths due to influenza are not restricted to pandemics. An estimated 600,000 influenza-associated deaths have occurred during epidemics between 1958 and 1990, with annual 20,000 influenza-associated deaths in the United States alone between 1972 and 1991 [11, 14]. With the recent finding that some avian influenza viruses can directly cross the species barrier – from avian to mammal - and infect humans [47, 52, 68], makes the threat of the next pandemic ever more frightening.

Despite all the advances in medical technology and medical treatments projections of fatalities if a pandemic similar in nature to the 1918 pandemic would have occurred in 1998 are very high [50]. The projections, shown in Table 2-1, suggest that with shortened travel time, the virus could spread around the globe in just four days.

TABLE 2-1

PROJECTED FATALITIES FOR A 1918-LIKE INFLUENZA PANDEMIC IN 1998

Year	1918	1998
Population size	1.8 billion	5.9 billion
Principal type of transportation	Ships, Trains	Airplanes
Global spread of virus	120 days	4 days
Disease prevention	Gauze masks	Vaccines
Disease treatment	Bed rest, aspirin	Antiviral drugs
Estimated deaths	20 million	60 million

Adapted from [50].

In addition to the high mortality, the CDC has predicted a high economic impact, including medical costs, for the United States if such pandemic occurs [58]. The cost, forecasted to be around $ 70-166 billion, is summarized in Table 2-2. With the lack of a lasting and effective vaccine for influenza (in addition to only a small percent of the population being vaccinated) the economical impact is expected to be much higher [58]. Scientists at CDC argue that immunization of all "high-risk category" persons in the U.S. will be more cost-effective [58]. However, what virus is going to cause a new pandemic is unknown. Therefore, there is an urgent need to develop an *in vitro* model that allows the identification of a potentially pandemic influenza virus. The use of this model may minimize the medical and economical impact, if not prevent a new pandemic.

TABLE 2-2

AN ANNUAL ECONOMICAL IMPACT OF INFLUENZA VIRUS INFECTION IN THE U.S.

Causes	Cases
Death	89,000 - 207,000
Hospitalizations	314,000 - 734,000
Outpatient visits	18,000,000 - 42,000,000
Additional illnesses	20,000,000 - 47,000,000
Economic impact	$ 71.3 -166.5 billion

Modified from [58].

2.2. Influenza virus

2.2.1. Classification

Influenza virus is a member of the *Orthomyxoviridae* [19]. The term "myxo" refers to viruses that interact with the mucus of the body, while the term ortho is used to distinguish it from another group of negative-strand RNA viruses, the paramyxoviruses [19]. Influenza viruses are divided into three types: A, B, and C. Type A is the most pathogenic of the three, and is a principal etiological agent of influenza epidemics or pandemics [88]. Type B influenza virus on the other hand, causes a mild disease. However, it can cause localized winter outbreaks, mainly in children [88]. Type C influenza virus is of questionable pathogenicity for humans [88], therefore it is the least studied of all influenza viruses.

2.2.1.1. Virion and genome organization

The shape of virion of the influenza A virus is spherical to cylindrical, as shown in Fig. 2-1. The virion has an envelope made up of a lipid bilayer. Each virion contains eight linear negative-sense, single-stranded RNA molecules. The size ranges from 890-2341 nucleotides (nt). The virus nucleocapsid has a helical symmetry and is approximately 6-9 nm in diameter and 60 nm long. However, the virus itself has no defined shape, possibly as a result of budding during its release from the host cell [19]. The viral envelope contains matrix protein (M2), and embedded onto the lipid surface are the two surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA). The core contains eight RNA segments in association with a ribonucleoprotein (NP). Three different polymerases, PA, PB1, and PB2, are surrounded by matrix-1 (M1) protein

which forms the capsid. Two nonstructural proteins, NS1 and NS2, are important for proper virus replication. Each segment encodes a single polypeptide, however segments two and three both produce two templates by differential splicing. Therefore, a total of ten viral proteins are encoded in the eight-segment genome.

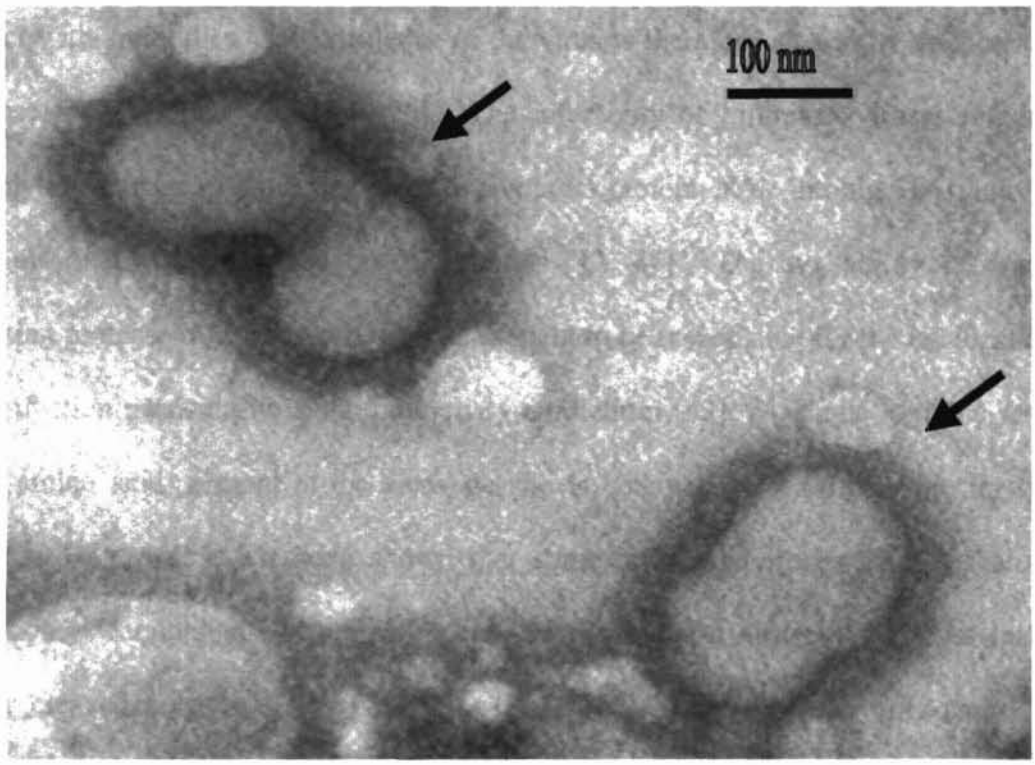

Fig. 2-1, Electron-micrograph of influenza A virus. Reproduced from [49].

2.2.1.1.1. Hemagglutinin (HA)

The hemagglutinin plays an essential role in viral entry for influenza viruses. It is a trimeric, type I, membrane glycoprotein, with an N-terminal ectodomain and a C-proximal anchor. The hemagglutinin binds to the sialic acid at the cell surface, hence mediates virus attachment to the cell receptor. Glycosylation of the HA is believed to

play an important role in viral pathogenicity [76]. HA glycosylation sites are determined by specific amino acid sequence.

The viral genome is replicated by an error-prone RNA polymerase, hence a high frequency of mutations. These mutations affect the number of glycosylation sites, and has been shown to affect the ability of the HA molecule to bind to cellular receptors or to interact with antibodies. The virulence of the virus is therefore altered by an increase or decrease in its binding capacity [76]. The infectivity of influenza viruses requires a proteolytic cleavage of the hemagglutinin precursor protein, HA_0, into the HA1 and HA2. HA1 and HA2 are linked by disulfide-bonds [42, 44]. The availability of this HA activating protease in various cell types is thought to be responsible for the localization of viral infection particularly in the pulmonary epithelium [43]. A single arginine residue (basic amino acid) present at the cleavage site is believed to be responsible for tissue tropism. However, it has been shown that some influenza viruses do not share this restriction. Mammalian and nonpathogenic avian influenza viruses have HAs that are usually cleaved only by a few cell types [36, 82]. Hence, they can only cause local infections [36, 82]. In contrast, highly pathogenic avian influenza viruses, such as H5 and H7, have HAs that can be cleaved by furin and other ubiquitous proteases present in multiple cell types, thus permitting these viruses to cause systemic infections [59, 82]. These highly-cleavable HAs have multiple basic amino acid residues forming a consensus sequence, Arg-X-Lys/Arg-Arg. However, the HA sequence of highly pathogenic 1918 (H1N1) virus differs from highly pathogenic avian influenza viruses [86]. Pathogenicity of 1918 (H1N1) virus has been attributed to viral adaptation within the human host, possibly several years before the 1918 pandemic [72].

2.2.1.1.2. Neuraminidase (NA)

Neuraminidase contains an N-proximal anchor and a C-terminal ectodomain. It cleaves the α-glycosidic linkage between sialic acid and an adjacent sugar residue, therefore preventing virus self-aggregation and allowing the release of the virus from an infected cell [27]. The sialic acid serves two functions: 1) it blocks viral assembly, and 2) it becomes incorporated into the mature virus particle. Several studies focus on the importance of NA in infection. For example, Goto *et al.* [27], proposed a possible mechanism for cleavage of the pandemic 1918 (H1N1) influenza virus involving NA. They suggested that the NA binds and sequesters tissue plasminogen, leading to a higher local concentration of this ubiquitous protease precursor [27]. This results in an increased cleavage of HA, hence leading to higher pathogenicity [27]. The structural basis for this unusual property appears to be by the presence of a lysine at the carboxyl-terminal end, and the absence of an oligosacharide side chain at position 146 [27].

2.2.1.1.3. Matrix proteins (M1 and M2)

Matrix-1 protein is the most abundant viral protein. It is located directly beneath the lipid envelope. The function of M1 is to form the capsid for the assembly of progeny virus. Matrix-2 protein is a product of an alternate spliced transcript of the RNA segment. It is an integral membrane protein, and believed to function as an ion channel for pH control. It is an essential factor for virus maturation and cell entry [39, 85].

2.2.1.1.4. Nucleoprotein (NP)

Nucleoprotein provides structural support to the RNA genome. It forms the backbone of the helical complex associated with each of the eight RNA segments, and

with three different virion polymerases.

2.2.1.1.5. Polymerases (PA, PB1, and PB2)

Polymerases are responsible for carrying viral transcriptase activities. Polymerase A (PA) is an acidic viral protein, and is believed to be responsible for virion RNA synthesis. Polymerase B1 (PB1) and polymerase B2 (PB2) are basic viral proteins, and they are implicated to complement RNA synthesis.

2.2.1.1.6. Nonstructural proteins (NS1 and NS2)

Non-structural protein-1 (NS1) and Non-structural protein-2 (NS2) are found in abundance in infected cells during virus replication. However, they are not incorporated into progeny virions. Both of these proteins are translated from alternate spliced mRNA transcripts.

2.2.1.2. Replication

Viral infection is initiated by the binding of the globular head of HA region to cell surface sialic acid residues. Following the binding, viruses are translocated into the cell by endocytosis. During endocytosis, the low pH in endocytotic vesicle leads to a conformational change in the HA molecule. This conformational change allows the amino terminus of HA2 to insert into the vesicular membrane and results in fusion of the viral envelope and the vesicle membrane. Release of virion content into cytoplasm soon follows. The NP migrates to the nucleus, and NP associated polymerase starts transcription of early viral proteins, NP and NS1. Since virion transcriptase complex is

unable to self-initiate viral mRNA synthesis, an RNA endonuclease cleaves the 5' terminal fragments from capped and methylated mRNA (10-14 nt long) from the host cell. Newly synthesized NP and NS1 proteins migrate to the nucleus, and initiate complementary RNA and negative viral RNA synthesis. RNA-dependent RNA polymerase is involved in the synthesis of positive-sense strands from the negative-sense template [19].

Newly synthesized viral RNA segments are encapsulated with NP, and used as secondary templates for the transcription of M1, HA, and NA proteins. Nucleocapsids are enclosed by M1, followed by transportation to the cytoplasm for assembly. Interaction between M1, HA, NA, and M2 appears to serve as the initiation signal for virus budding. Enzymatic activity of neuraminidase results in the virion release from the host cell, while extracellular cleavage of HA into HA1 and HA2 by host proteases is the final step of viral maturation [19]. A summary of influenza virus replication is shown in Fig. 2-2.

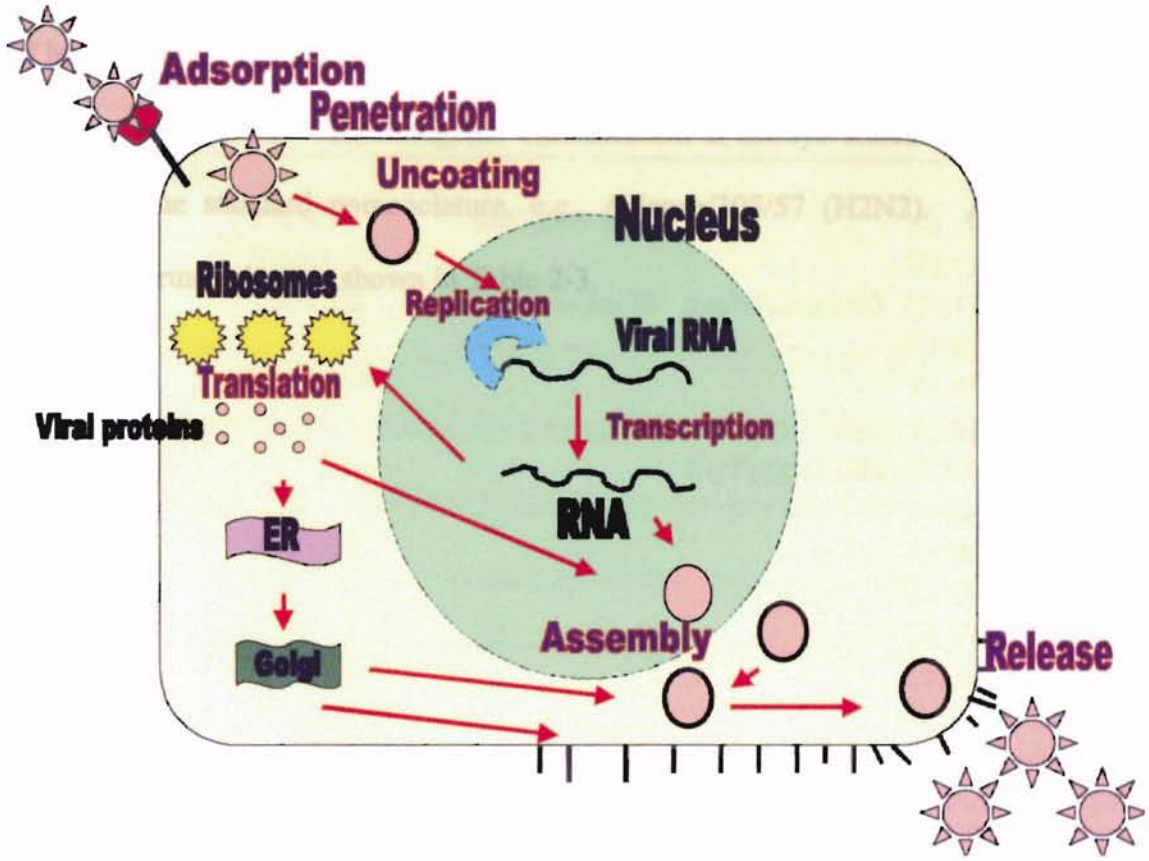

Fig. 2-2. Replication of influenza virus. Seven steps involved in virus replication are; 1) adsorption; 2) penetration; 3) uncoating; 4) viral genome replication and transcription of viral genes; 5) translation; 6) assembly; and 7) release.

2.2.1.3. Antigenic subtypes

Influenza viruses are divided into several subtypes, based on their antigenic differences for the HA and NA [19]. Antigenic differences and cross-reactivity are determined using post-infected ferret serum. So far, 15 different HA and 9 different NA subtypes, have been identified. All of these subtypes have been isolated from avian species, making avian species a natural host of influenza virus [3, 84]. Out of these subtypes, only five are pathogenic for humans and three of them have caused pandemics [3, 84].

The nomenclature for influenza virus is based on the virus type, the host species (if human virus, no host designation is necessary), the geographic origin, isolate number, and year of isolation. The antigenic classification is always stated in the parenthesis following the standard nomenclature, e.g., A/Japan/305/57 (H2N2). A summary of influenza virus isolates is shown in Table 2-3.

TABLE 2-3

ORIGIN OF SPECIES OF INFLUENZA A VIRUS SUBTYPES

	Humans	Swine	Horses	Birds
Hemagglutinin:				
H1	PR/8/34	Sw/Ia/15/30	-	Dk/Alb/35/76
H2	Sing/1/57	-	-	Dk/Ger/1215/73
H3	HK/1/68	Sw/Taiwan/70	Eq/Miami/1/63	Dk/Ukr/1/63
H4	-	-	-	Dk/Cz/56
H5	HK/156/97	-	-	Tern/S.A./61
H6	-	-	-	Ty/Mass/3750/65
H7	-	-	Eq/Prague/1/56	FPV/Dutch/27
H8	-	-	-	Ty/Ont/6118/68
H9	HK/1/99	-	-	Ty/Wis/1/66
H10	-	-	-	Ck/Ger/N/49
H11	-	-	-	Dk/Eng/56
H12	-	-	-	Dk/Alb/60/76
H13	-	-	-	Gull/MD/704/77
H14	-	-	-	Dk/Gurjev/263/82
H15	-	-	-	Dk/Austral/341/83
Neuraminidase:				
N1	PR/8/34	Sw/Ia/15/30	-	Ck/Scot/59
	HK/156/97	Sw/Taiwan/70	-	-
N2	Sing/1/57	-	-	Ty/Mass/3750/65
	HK/1/99	-	-	-
N3	-	-	-	Tern/S.A./61
N4	-	-	-	Ty/Ont/6118/68
N5	-	-	-	Sh/Austral/1/72
N6	-	-	-	Dk/Cz/56
N7	-	-	Eq/Prague/1/56	FPV/Dutch/27
N8	-	-	Eq/Miami/1/63	Dk/Ukr/1/63
N9	-	-	-	Dk/Mem/546/74

Primary isolated subtypes of influenza A virus. Of the equine influenza A viruses, equine-2 (H3N8) and equine-1 (H7N7), cause clinically more and less severe disease in horses respectively. For human influenza A viruses, H1N1, H2N2, and H3N2, have caused pandemics in 1918, 1957, and 1968 respectively. Isolation of H5N1 and H9N2 avian influenza viruses in humans indicates a possible direct species crossing between the two species. The H5N1 subtype appears to be highly pathogenic (33% case-fatality-rate), on the contrary, H9N2 causes only a mild disease. No virus isolate is presented by the dashed lines (-).

2.2.1.4. Antigenic Shift and Drift

The two known features of influenza virus that account for its epidemiological success are: 1) antigenic shift and 2) antigenic drift [19].

2.2.1.4.1. Antigenic shift

Antigenic shift occurs only in influenza A viruses, and is believed to be the mechanism responsible for the past pandemics [19]. It is defined as the sudden and drastic change in virus antigenicity, resulting in emergence of novel type A influenza viruses [19]. In antigenic shift, segments of the RNA genome from two genetically distinct strains of influenza virus that have infected the same cell become associated. The mixed and reassorted RNA results in generation of reassortant viruses [94]. This reassortment can occur in any species. However, swine seems to be involved because they are susceptible to both avian and human viruses [19]. The reassorted viruses do not have to emerge through the same lineage; therefore, several different viruses can reassort and emerge at once [97]. The HA and NA molecules of the newly, reassorted virus may differ, at the amino acid level, anywhere from 20-50% from the previously circulating strains [11]. Hence, the change in the surface antigens of the virus makes it unrecognizable to the antibody elicited by a previous infection or immunization process [3, 25]. This can lead to a higher attack rates for the new virus, and hence establish itself in humans.

2.2.1.4.2. Antigenic drift

Antigenic drift is limited to type A and type B influenza viruses [19]. It is defined

as a rapid and unpredictable antigenic change, a result of point mutations in viral surface proteins, mainly HA [19]. Antigenic drift is likely to occur as a result of the selection pressure exerted on the virus by host immunity. Viruses that evade host immunity by changing the amino acid composition at their epitope remain infectious, while unchanged viruses are neutralized by the host immunity. Antigenic drift variants are known to be responsible for annual epidemics that occur between pandemics [11, 90]. Influenza viruses isolated from aquatic birds show no evidence of net evolution over the past 60 years [17, 26]. However, their nucleotide changes (in their HA and NA) appear to occur at rates similar to human influenza viruses [17, 26], suggesting that immune selection is partially responsible for antigenic drift.

2.2.1.5. Geographic Determination and Reassortment Specificity

Since most of the pandemics start in Asia, it has been speculated that the next flu pandemic may also come from that region [25, 32]. One reason is that the lifestyle considered to be ideal for viral gene mixing (reassortment) is found in Asia, more specifically, in China [90]. Live bird markets, housing a variety of avian species and pigs in close proximity, are considered to be ideal place for such an interaction [90]. Swine species are considered "mixing vessels" for influenza viruses. Swine species exhibit a low species barrier to both avian and human influenza A viruses [3, 25, 53]. Pigs have both, NeuAc-2,3Gal (human) and NeuAc-2,6Gal (avian) receptors required for the attachment of influenza virus to epithelial cells. On the contrary, the avian influenza viruses are not readily introduced into humans, possibly because humans do not possess the α-2,3-sialyllactose (NeuAc-2,3Gal) receptors [5]. Continued replication of avian

influenza viruses in swine allows the avian viruses to mutate and to recognize human cell surface receptors [38], therefore increasing the chance of transmission to human species [37]. Following the infection of a new avian or mammalian hosts, influenza viruses evolve rapidly, usually causing a mild respiratory infection [53].

2.2.1.5.1. Direct crossing of the species barrier

Until 1997, it was believed that pigs were required as the "mixing vessels" for human infection. However, the recent introduction of avian influenza to humans in Hong Kong [47, 68, 84], suggest that this hypothesis has to be reconsidered. A new theory suggesting the possibility of direct barrier crossing between avian and human species may be happening more than previously thought.

During the 1997 "Chicken Flu" outbreak in Hong Kong, a previously unknown influenza virus, of subtype H5N1, was isolated from a three-year-old boy hospitalized due to influenza related symptoms. This novel human H5N1 influenza virus was shown to be identical to the avian H5N1 influenza virus [47, 80] causing high mortality in chickens at that time. This H5N1 virus is capable of causing systemic infection in chickens by replicating in neurons and vascular tissues [45]. Preventive measures were taken to reduce further spread of this highly pathogenic influenza virus. Since the initial outbreak of H5N1 in May 1997, Hong Kong, eighteen confirmed cases of human infection have been reported, of which six were fatal (case-fatality-rate of 33%) [80]. The high pathogenicity of H5N1 in humans and chickens, is probably attributed to a large proportion of amino acid substitutions in all gene products except in the hemagglutinin and neuraminidase genes [83].

A second introduction of avian influenza to humans occurred in April 1999, Hong Kong, when two girls were hospitalized with flu like symptoms [68]. The patients, a one-year-old-girl and a four-year-old girl, were admitted to the hospital with fever, malaise, anorexia, sore throat, headache, abdominal pain, vomiting, and inflamed oropharynx. Both patients recovered within three days and were discharged. A new strain of influenza virus (H9N2) was isolated, and identified to the previously-isolated avian virus [68]. The difference in clinical symptoms and mortality caused by H5N1 or H9N2 suggests a difference in their pathogenicity. Despite the lower pathogenicity of the H9N2 virus, a significant threat exists because H9N2 influenza A virus is now widespread in poultry in Asia. Avian H9N2 influenza virus has been shown to be a donor of the internal genes of H5N1 subtype [31] [52].

The last two episodes of H5N1 and H9N2 outbreaks indicate the capability of any avian influenza virus to infect humans. The high pathogenicity of H5N1 virus is a major concern, however the reasons for its low transmission are unclear. It is fortunate that H5N1 can rarely be transmitted from human to human, thereby preventing its spread.

Influenza pandemic occurs every 20-30 years. However, the ability of avian viruses to directly cross the species barrier, and the observable difference in viral pathogenicity, suggest that the next pandemic could occur at any time with devastating consequences [14]. The need for an *in vitro* model that can identify the potential pathogenicity of avian influenza viruses would be of great significance. Such a model will assist us to predict and prevent any future outbreaks, epidemics, or pandemics.

2.3. Immune response

2.3.1. Introduction

Infection by influenza elicits a cascade of immune responses, leading to a mucosal inflammation with the influx of plymorphonuclear cells, lymphocytes, and macrophages into the respiratory mucosa [67]. While this response leads to the resolution of the infection and protection against re-infection, it also contributes to the development of local systemic symptoms. The factors responsible for this are not completely understood [67]. A simple explanation implies that the respiratory symptoms of influenza result from direct cytopathic effects of the virus, and that the systemic symptoms are caused by production of cytokines [33].

During an infection, the innate, as well as both arms of adaptive immunity are activated. Innate immune responses are initiated early and do not depend on immunologic memory [73]. They are mediated by interferon type I and II cytokines [73]. The adaptive immune responses, on the other hand involve both, cell-mediated and humoral immune responses [66]. Adaptive immune responses require antigen recognition, processing, and presentation, and cellular activation and differentiation. Adaptive immune responses, therefore play a role later in infection than the innate responses.

2.3.2. The innate immune response

Little is known about the initial stages of the immune response in influenza virus infection prior to emergence of specific antiviral effector mechanisms. During the initial phase of infection, influenza virus interacts with the cells on the luminal side of the

airways to induce the release of immunoactive mediators. The chemokines attract infiltrating cells to the site of infection and process antiviral activities. This is an early defense against viral infection. Induction of pulmonary inflammation appears to be particularly important for antigen translocation to the lymphatic tissue from the lungs. This also leads to the recruitment, immigration. and activation of virus specific lymphocytes.

Innate immunity in influenza virus infection relies on macrophage responses. Macrophages-mediate lysis of infected cells and act as antigen presenting cells (APCs) [54], along with dendritic cells [96]. IFN-α, IFN-β, and IFN-γ release activates natural killer cells (NK) and prevents viral spreading by lysis of virus-infected cells. In addition. production of pro-inflammatory cytokines such as IL-1, IL-6, and TNF-α suppresses viral replication [6, 66, 70].

Another branch of the innate immune response is the complement system. It is activated by either the alternate pathway (in the absence of the antibody), or by the classical pathway (in the presence of antibody) and can lead to lysis of infected cells [73].

2.3.3. The adaptive immune response

There are two branches of the adaptive immune responses. They are cell-mediated immune response and the humoral immune response.

2.3.3.1. The cell-mediated immune response (CMI)

The cell-mediated immunity involves CD8+ lymphocytes, NK cells, ADCC, etc., antigen recognition, cytotoxic mediation such as TNF, and cytokine production by

activated cytotoxic T-cells [73]. This response system depends on antigen recognition and cytokine production by macrophages and T cells. CD8+ T-cell initiation of cell lysis requires the association of antigen with MHC [73]. This association leads to simultaneous stimulation of macrophages to produce IL-1 and activation of T-cells to produce IL-2. Interleukin-2 release leads to activation and proliferation of other T-cells resulting in additional cytokine release [21], [87] and eventually to destruction of an infected cell.

2.3.3.2. The humoral immune response

The humoral immune response is important for protection against cell-free virus, and prevention of re-infection by a homologous or antigenically-similar virus. The factors in the development of the humoral immune response are different from the cell-mediated immune response. The development of humoral immune responses is usually preceded by the cell-mediated responses [73]. However, antibody production provides a lasting and persistent protection [73]. Development of memory cells is essential in the activation of an immune response towards future infections. Th2 type cytokines promote proliferation and transformation of B-lymphocytes into antibody-secreting plasma cells. It has been shown that B-cell deficient mice have increased susceptibility to lethal infection by influenza viruses. [29]

Early in the humoral response, IgM antibody is produced [73], which is followed by the IgG synthesis and development of a mature immune response (secondary response). A summary of the cell-mediated and the humoral immune responses is shown in Fig. 2-3.

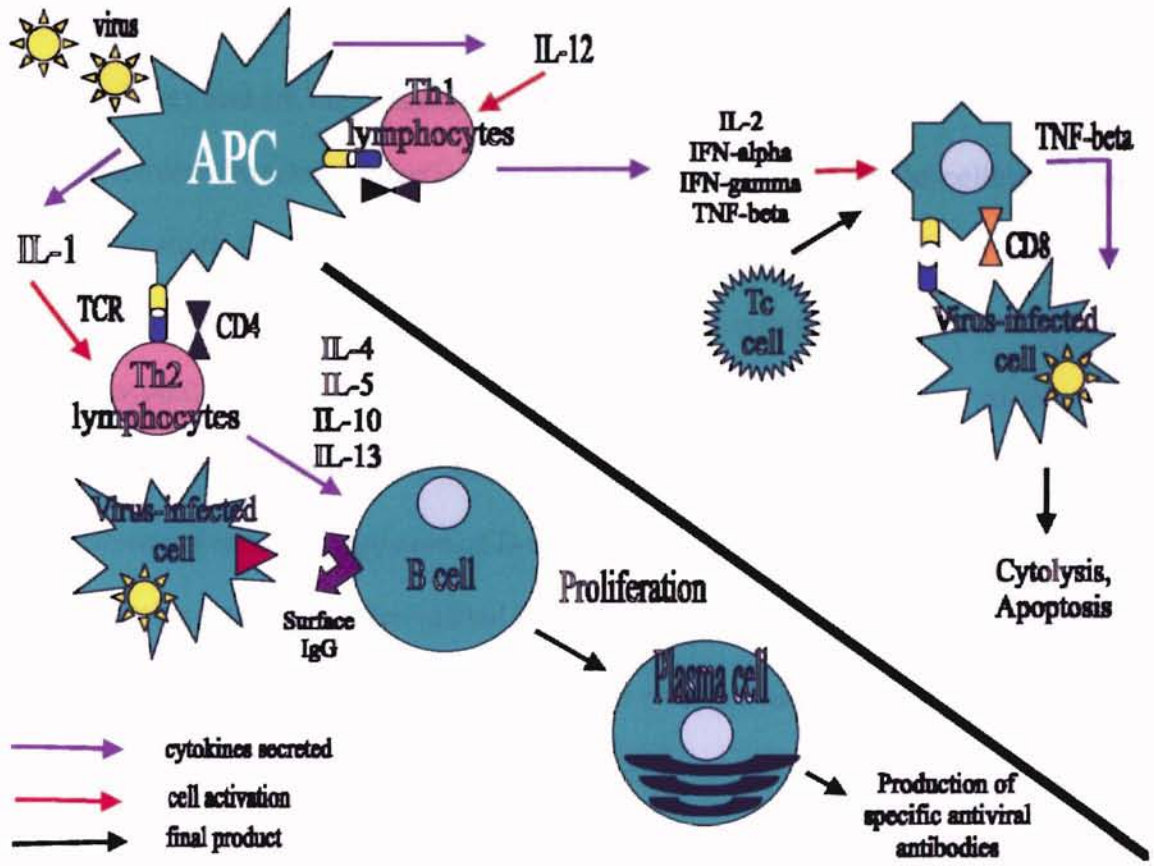

Fig. 2-3. Development and activation of the cell-mediated immune response and the humoral immune response. The upper right half shows development of CMI. The lower left half shows development of humoral immune responses. Both Th1 and Th2 cells receive their first activation signal from an interaction with an APC. The second activating signal for Th1 cells is the released IL-12, while Th2 cells are further activated by IL-1. Activation of helper T cells leads to development of either cell-mediated or humoral immune responses.

2.3.3.3. Th1 and Th2 dichotomy

The two types of adaptive immune response, the cell-mediated and humoral, complement each other in virus elimination, killing of virus-infected cells, and in recovery [73]. T-lymphocytes play a primary role in recovery from viral infections. Recently, T-helper cells have been classified into Th1 and Th2, based on the type of cytokines they secrete [65]. The functions of Th1 and Th2 cells are very different.

Although both Th1 and Th2 cells provide help for B-cell activation, Th2 cells play a more active role. On the other hand, the Th1 cells preferentially induce delayed-type hypersensitivity and are important for macrophage activation [29].

Recovery from viral infections is predominantly regulated by the cellular immune response, or more specifically, by CD8+ T cells. Cytotoxic T cells (CD8+) induce lysis of infected cells, hence preventing the production of progeny virus. CD4+ T cells respond to viral peptides presented on MHC II molecules by the release of cytokines. The cytokines have different functions; serving as growth factors for CD4+ and CD8+ T cells, maturation and differentiation of B-cells, and for inflammation initiation. CD4+ T-cells, after stimulation are differentiated into either Th1 or Th2. Th1 type produces large amounts of IFN-γ, which leads to the generation of cytotoxic T-cells (CTL), while Th2 cells produce other cytokines and control activation and suppression of CTL. B-cells, on the other hand, are activated by Th2 cytokines. However, in the presence of Th1 cytokines, B-cells are induced to produce complement activating isotypes (murine IgG2a). In contrast, non-complement activating mouse IgG1 is the predominant isotype in Th2 response. [61]

2.4. Cytokines

2.4.1. Roles in infection

Cytokines are small proteins secreted from a variety of cells in response to an infection or tissue damage [73]. Cytokines are mediators of a network of intercellular communication for immune cells. They are produced during the innate and the adaptive immune responses [73]. Cytokines induce localized effects, however excess production

results in systemic effects. Cytokines bind to specific receptor found on the surface of an effector cell. This binding leads to activation, proliferation, and cytokine release in the effector cell [16, 40, 66]. Cytokines have been shown to play an important role in defense against influenza virus infection and other pulmonary viruses [66, 69].

Influenza virus infects and replicates in macrophages, resulting in induction of cytokine. Cytokines may also contribute to the pathogenesis of the infection, by an increased production of inflammatory cytokines. In response to influenza virus infection, levels of IL-6, TNF-α, IFN-γ, IL-10, monocyte chemotactic protein-1 (MCP-1), macrophage inflammatory protein-1α (MIP-1α) and 1β (MIP-1β) are elevated in the nasal lavage fluid of human volunteers [20]. This elevation correlates with the magnitude and the duration of symptoms [20]. As shown by Hayden *et al.* [33], patients challenged with influenza virus have a peak level of IL-6 and IFN-α in nasal lavage fluid around day two. This correlates to maximum virus titers, elevated body temperature, increased mucus production, and higher symptom scores [20]. Skoner *et al.* [81], also report a correlation between the days of viral shedding and levels of IL-6 and other measures of symptoms. A summary of both Th1 and Th2 cytokines involved in pulmonary infections and of particular importance to our study is presented in Table 2-4.

TABLE 2-4

CYTOKINES ASSOCIATED WITH PULMONARY INFECTIONS

Cytokines	Production source	Mechanisms of action	Suppressed by	Activated by
Th1 type:				
IFN-γ	Macrophages and γδ T cells? NK cells and T lymphocytes	Antiviral activity through stimulation of MHC class I expression and immune cytotoxicity	IL-10	TNF, IL-2 IL-6
IL-2	T lymphocytes	Stimulation and differentiation of T and B lymphocytes, and activation of NK cells		IL-1
IL-12	Macrophages and B lymphocytes	Increase in activity of NK cells, CTL, and macrophages	IL-10	IFN-γ Th1cytokines
Th2 type:				
IL-4	Macrophages, mast cells, T and B lymphocytes, and basophils	Differentiation of B cells, B cells isotype switching to IgE and IgG4	IFN-γ	Th2 cytokines
IL-5	T lymphocytes, eosinophils, and mast cells	Stimulation and differentiation of B lymphocytes and eosinophils		Th2 cytokines
IL-6	Epithelial cells, endothelial cells mast cells, monocytes, macrophages, hepatocytes, fibroblast, and T lymphocytes	T cell activation and proliferation, CTL differentiation, B cell differentiation leading to mucosal IgA production, and pyrogenic effects	IL-4 IL-10	IL-1 IL-2 TNF-α
IL-10	Macrophages, monocytes, T and B lymphocytes	Stimulation of B lymphocytes, inhibition of macrophage activity leading to suppression of IFN-γ	IFN-γ Th1 cytokines	Th2 cytokines

Cytokines involved in pulmonary infections, assayed for in our study.

2.4.2. Th1 vs. Th2 summary

Th1 and Th2 cells indirectly regulate each other by cytokine production following the interaction with an APC [65]. Th2 cells inhibit the function of macrophages, which are the main source of Th1 response development [65]. On the contrary, Th1 cells inhibit the proliferation and function of B-cells necessary for the development of Th2 responses

[65]. As a result of such cross-regulatory phenomenon, cytokine mediated immune responses, can only be changed during the response development and not after the conversion has taken place [1, 10, 28, 51, 63, 65]. A summary of the cytokine interaction, cross-regulation, and function is shown in Fig. 2-4.

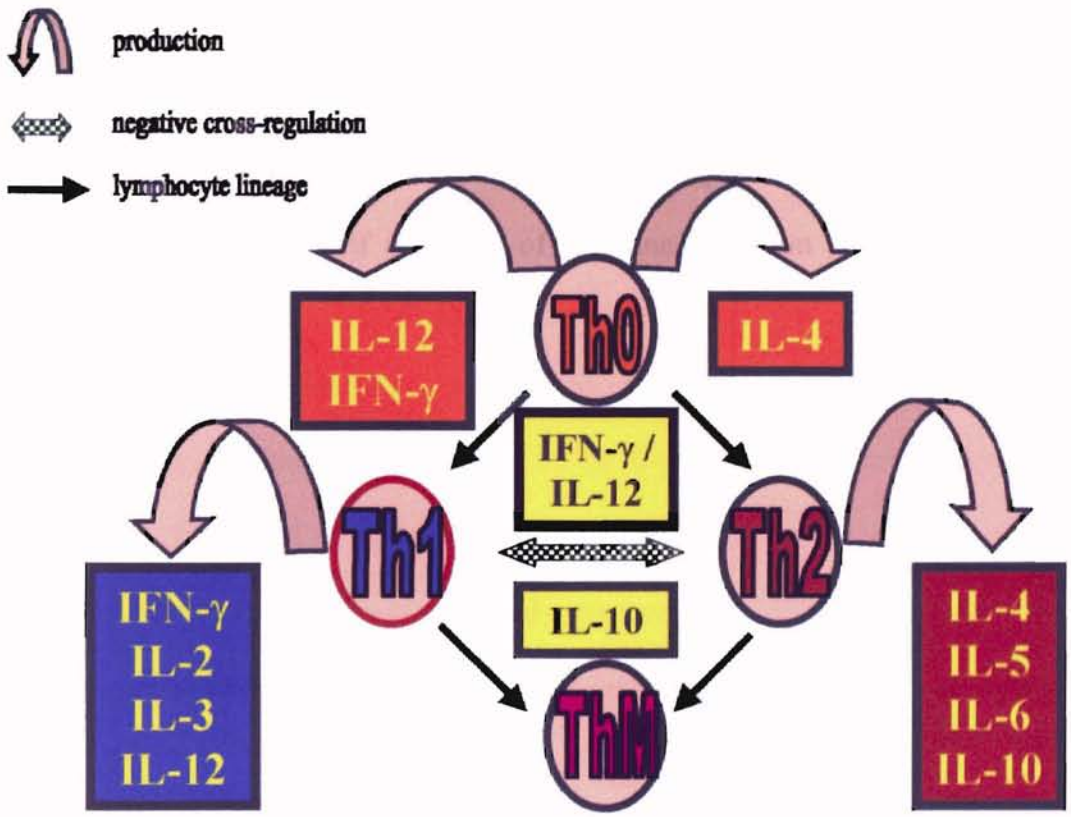

Fig. 2-4. Role of cytokines. The characteristics of each Th1 or Th2 cytokines is based on the type of the immune response they elicit. Th1 type cytokines (blue box), produced by Th1 lymphocytes elicit cell-mediated immune responses. Th2 type cytokines (red box), produced by Th2 lymphocytes, elicit humoral immune responses. Cytokines (yellow boxes) suppress production of the other type.

2.5. Concluding remarks

In this study, I attempt to establish an *in vitro* model for cytokine induction by influenza virus, using RT-PCR and ELISA. It is followed by determining if there is a

correlation to pathogenicity. Furthermore, I attempt to correlate this induction with virus pathogenicity. Production and secretion of cytokines during an immune response has been associated with influenza symptoms development [22, 33, 81]. Cytokines such as IL-1β and IL-6 are associated with chills and fever development [22], while IFN-γ is associated with weakness, muscle pain, and upset stomach. Although, cytokines such as IL-2, IL-4, IL-5, IL-10, and IL-12 are not directly implicated in the development of symptoms, they regulate the production of IL-1β, IL-6, and IFN-γ. Furthermore, by using a panel of influenza viruses with different degree of pathogenicity, this model provides a system to test if the level of cytokine induction is correlated to *in vivo* pathogenicity.

CHAPTER III

MATERIALS AND METHODS

3.1. Materials

3.1.1. Medium

3.1.1.1. RPMI:

RPMI 1640 (GibcoBRL, Grand Island, NY) was used for cell washing during lymphocyte purification. To the RPMI, 15 μg L-glutamine (Cellgro, VA) in 1 ml of medium and 1% NaHCO3 (Cellgro) were added. Antibiotics such as gentamycin (GibcoBRL), amphotericin, penicillin, and streptomycin (Cellgro) were added to a final concentration of 10 μg/ml, 0.5 μg/ml, 5 μg/ml, and 10 μg/ml of medium respectively.

3.1.1.2. Optimum RPMI:

Optimum RPMI medium was used for cell adaptation. It contained the following: RPMI 1640, L-glutamine, 1% $NaHCO_3$, gentamycin, amphotericin, penicillin, streptomycin, and 10% w/v FBS (Fisher, Pittsburgh, PA), in a final concentration of 15 μg/ml, 10 μg/ml, 0.5 μg/ml, 5 μg/ml, and 10 μg/ml of medium respectively.

3.1.1.3. Optimum Opti-MEM I:

Opti-MEM I media (GibcoBRL, Grand Island, NY), with L-glutamine, 1% $NaHCO_3$, gentamycin, amphotericin, penicillin, and streptomycin, in the final concentrations of 15 μg/ml, 10 μg/ml, 0.5 μg/ml, 5 μg/ml, and 10 μg/ml of medium respectively.

Both optimum RPMI and optimum Opti-MEM were used for cell adaptation and culturing at 37 °C and 5.0% CO_2 for 3, 12, and 24 hours.

3.1.2. Polymerase chain reaction (PCR)

3.1.2.1. Oligonucleotide primers for cytokine detection:

The cytokine oligonucleotide primers listed in Table 3-1 were used for amplification of cytokine cDNA using PCR .

TABLE 3-1

UNIVERSAL CYTOKINE OLIGONUCLEOTIDE PRIMERS

CYTOKINE	FORWARD	REVERSE	FRAGMENT
IL-2	GCACCTACTTCAAGCTCTAC	GATGCTTTGACAAAAGGTAATC	382 bp
IL-4	TATTAATGGGTCTCACCTACCA	TTGGCTTCATTCACAGAACAG	411 bp
IL-6	CTATGAACTCCCTCTCCACAA	TGCCCAGTGGACAGGTTTCT	711 bp
IL-10	TACTTGGGTTGCCAAGCCTT	TTCACAGAGAAGCTCAGTAAAT	495 bp
IL-12	AGATGCTGGCCAGTACACCT	TGATGATGTCCCTGATGAAGA	475 bp
IFN-γ	ATTTTGAAGAATTGGAAAGAGG	AAATTCAAATATTGCAGGCAGG	367 bp
G3PDH	CCTTCATTGACCTCAACTACAT	CCAAAGTTGTCATGGATGACC	256 bp

* Universal cytokine oligonucleotides are obtained from domestic mammal consensus sequences according to Rottman J. B. *et al.* [74].

3.1.3. Enzyme linked immunosorbent assay (ELISA)

3.1.3.1. Cytokine antibodies:

The antibodies for cytokine assay used in this study are listed in Table 3-2.

TABLE 3-2

PRIMARY AND SECONDARY ANTIBODIES FOR CYTOKINE ASSAY

Type	Cytokine	Produced in	Company
Primary			
	IL-2	Goat	R&D
	IL-4	Goat	Sigma
	IL-6	Goat	R&D
	IL-10	Rat	PharMingen
	IFN-γ	Rabbit	Chemicon
Secondary			
	IL-2	Rabbit anti-Goat IgG	Chemicon
	IL-4	Rabbit anti-Goat IgG	Chemicon
	IL-6	Rabbit anti-Goat IgG	Chemicon
	IL-10	Goat anti-Rat IgG	Sigma
	IFN-γ	Goat anti-Rabbit IgG	Chemicon
Standard			
	IL-2	Recombinant human	R&D
	IL-4	Recombinant human	Sigma
	IL-6	Recombinant human	R&D
	IL-10	Recombinant human	R&D
	IFN-γ	Recombinant human	Sigma

3.1.4. Cell types

3.1.4.1. Peripheral blood lymphocytes

3.1.4.1.1. Adult human peripheral blood lymphocytes

Blood was obtained from adult volunteers at the Student Health Center. Oklahoma State University, Stillwater, OK. Ten to fifty milliliters of blood was drawn (by the registered nursing staff) from the median cubital vein, followed by transfer into 10 ml "green top" test tubes containing sodium heparin anticoagulant (Becton Dickinson. Oxnard, CA).

3.1.4.1.2. Human umbilical cord blood lymphocytes

Human umbilical cord blood was obtained from the Maternity Ward at Stillwater Medical Center (SMC). Umbilical cord blood was collected by gravity, from discarded umbilical cords post-partum. Twenty to thirty milliliters of cord blood was collected into a 50 ml centrifuge tube containing 5 ml total volume of sodium citrate anticoagulant (32 mg/ml sodium citrate and 4.2 mg/ml of citric acid).

(A copy of OSU-IRB approval and a consent form can be found in the appendix section. Name, race, age, or sex of any party involved remains unknown).

3.1.4.1.3. Equine peripheral blood lymphocytes

Fifty to one-hundred milliliters of blood was obtained from non-vaccinated one to two year-old horses housed at Equine Research Park, Oklahoma State University, Stillwater, OK. The blood was drawn from the jugular vein then collected into 10 ml "green top" test tubes. The horses were purchased as foals. They have not been

vaccinated for influenza, or have any history of exposure to equine influenza virus. To exclude previous exposure to equine influenza virus, an HI test was performed on collected serum.

3.1.4.2. Chicken red blood cells

Chicken blood was obtained from the chicken facility at the Department of Poultry, College of Agriculture, Oklahoma State University, Stillwater, OK. The blood was drawn from the subclavian vein with a "butterfly needle" connected to a 5-ml syringe. Collected blood was transferred into a 5 ml "green top" test tube. To prepare a 2.5% chicken-red-blood-cell suspension, the erythrocytes were washed and diluted with PBS to 2.5 %. This chicken-red-blood-cell suspension is used for hemagglutination and hemagglutination inhibition tests.

3.1.4.3. Jurkat T cell line

The Jurkat cell line, a pseudoploid human T cell leukemia cell line (ATTC catalog number TIB-152), was kindly provided by Dr. Mats, College of Agriculture, Department of Biochemistry and Molecular Biology, Oklahoma State University, Stillwater, OK. Cells were maintained in optimum RPMI at 37 °C and 0.50% CO_2 in a water-jacketed incubator (Revco, Ultima). For virus infection, cells were synchronized at the log phase by passage 24 hours prior to infection.

3.1.5. Influenza viruses

Influenza viruses of different types, subtypes, and strains used in this study for infecting lymphocytic cells are listed in Table 3-3.

TABLE 3-3

INFLUENZA VIRUSES USED IN THIS STUDY

Type	Subtype	Host	Virus strains	Genbank accession no.
Influenza A				
	H1N1	humans	A/Puerto Rico/8/34/	V01088
	H1N1	humans	A/New Jersey/11/76	K00992
	H2N2	humans	A/Japan/305/57	L20407
	H3N2	humans	A/Aichi/2/68	J02090
	H3N2	humans	A/Panama/99*	ND
	H3N8	equine	A/Eq/Miami/1/63	M24719
	H3N8	equine	A/Eq/Saskatoon/1/90	AF197243
	H3N8	equine	A/Eq/Kentucky/1/97	AF197249
	H3N8	equine	A/Eq/Kentucky/1/98	AF197241
	H7N7	equine	A/Eq/Prague/56	X62552
Influenza B				
		humans	B/Lee/40	K00423

* A gift from Dr. Nancy Cox, CDC, Atlanta, GA. ND = not determined.

3.2. Methods

3.2.1. Lymphocyte purification

The protocol for lymphocyte purification adapted with modifications from Grimm *et.al.* [30], and Mawle *et.al.* [56]. Briefly, purification of lymphocytes from sialic acid containing erythrocytes was critical. Attachment of virus to the sialic acid on erythrocytes would interfere with the stimulation of lymphocytes, resulting in misrepresentation in quantity and type of cytokines induced following infection.

Lymphocytes were purified using Lymphoprep solution (NycoMed, Oslo, Norway). Thirty milliliters of lymphoprep medium were placed into a 50 ml tube, 15 ml of a blood sample was gently layered on the top of lymphoprep solution. Tubes were centrifuged at 500x g for 60 min. Using sterile pipettes, the lymphocyte-containing middle-white layer was collected and transferred to a new 50 ml centrifuge tube. Lymphocytes were washed twice with RPMI medium, gently shaken and centrifuged at 400x g for 10 min. Supernatant fluid was discarded, and the lymphocyte pellet was washed twice with 15 ml of RPMI medium and cultured in optimum RPMI medium at 37 °C and 5.0% CO_2 for cell synchronization.

3.2.2. Cell culture

3.2.2.1. Synchronization of lymphocytes

Lymphocytes, obtained from fresh blood, or from cell culture, were sub-cultured for 24 hrs in optimum RPMI. Supernatant fluid containing FBS could potentially be cross-reactive to cytokines, therefore it was removed by centrifugation at 400x g for 10 min, followed by an addition of RPMI medium.

3.2.2.2. Culturing of lymphocytes

Lymphocytes were resuspended in 1.0 ml of optimum Opti-MEM I medium, counted, and adjusted as necessary to a final concentration of $2.5x10^6$ cells/ml. Lymphocytes were pulsed with influenza viruses for 4 hrs, followed by 3, 12, or 24 hrs incubation at 37 °C and 5.0% CO_2 in 1.0 ml fresh, virus-free optimum Opti-MEM I medium. Following this incubation, cells were centrifuged for 5 min at 400x g, and

supernatant fluids were collected for quantification of cytokine production by ELISA. Cellular RNA was extracted, using a total RNA extraction method, and subjected to a semi-quantitative analysis by RT-PCR.

3.2.2.3. Cell count

The cell count protocol was adapted with modifications from George *et.al.*, [23]. Briefly, 20 µl of purified lymphocytes was mixed with 20 µl of Trypan Blue (Cellgro. VA) and incubated for 5 min at room temperature. Following the incubation, 10 µl of the cell-dye mixture was loaded onto a hemacytometer slide (Hausser Scientific, Horsham, PA) and observed at 400X under a compound light microscope (Fisher Scientific, Pittsburgh, PA). Cell number was determined and cell concentration was calculated.

3.2.3. Influenza viruses

3.2.3.1. Propagation of influenza viruses

The protocol for virus cultivation was adapted with modifications from Hierholzer *et.al.*, [34]. Briefly, influenza viruses were cultivated in the allantoic cavity of 9 to 11 day-old embryonated chicken eggs for 72 hrs at 37 °C. Following the incubation, allantoic fluid was harvested, and centrifuged at 400x g for 10 min to clarify the supernatant fluid. The clarified supernatant fluid was assayed by a hemagglutination test for virus concentration. Following the hemagglutination test, the allantoic fluid was diluted in PBS to 1:1 HA unit/ml before being UV-inactivated.

3.2.3.2. Hemagglutination test

Hemagglutination assay, protocol was adapted and modified from that described by Hierholzer *et.al.*, [34]. It was performed using 96-well microtiter Falcon plate (Becton Dickinson, Oxnard, CA). Fifty microliters of allantoic fluid were serially diluted with PBS, and 50 µl of 2.5% chicken erythrocytes were added to each well. The plate was incubated at room temperature for 30 min. Virus titer was determined by the presence of hemagglutination at the lowest dilution.

3.2.3.3. Hemagglutination inhibition

The hemagglutination inhibition test was performed according to the protocol from Chernesky [9], also using Falcon 96-well microtiter plates. Twenty-five microliters of equine antiserum was serially diluted with 25 µl of PBS. Following the dilution and 30 min room-temperature incubation, 50 µl of 2.5% chicken erythrocytes were added to each well. Thirty minutes after addition of erythrocytes, the titer was determined by the absence of hemagglutination inhibition at the lowest dilution.

3.2.3.4. Ultra-violet (UV) inactivation

One milliliter of purified allantoic fluid containing 1:1 HA unit/ml of influenza virus was aliquoted into a 35mm petri dish. The petri dish was placed on ice (with air-lid open), 15 cm from a 10 W ultra-violet (254 nm) light source, and irradiated for 10 min. Following the UV-inactivation, the HA titer was re-assayed.

3.2.3.5. Pulsing of cells with influenza viruses

Lymphocytes at a concentration $2.5x10^6$ cells/ml were pulsed for 4 hrs with either 1 or 10 units of UV-inactivated influenza viruses. Following a 4 hrs incubation, the cells were centrifuged at 400x g for 5 min, and the virus-containing supernatant fluids were removed. Cells were resuspended in fresh, virus-free, optimum Opti-MEM I medium and incubated at 37 °C and 5.0% CO_2 for 3, 12, or 24 hrs.

3.2.3.6. Controls

3.2.3.6.1. Negative control

Allantoic fluid obtained from virus-free 9 to 11 day-old embryonated chicken eggs incubated at 37 °C for 72 hrs was used as a negative control in human umbilical cord blood and Jurkat T cell line experiments. Tissue culture grade PBS (Boehringer Mannheim, Indianapolis, IN) was used as a negative control in human peripheral blood and equine peripheral blood experiments.

3.2.3.6.2. Positive control

Pokeweed and Concavalin A (ConA) mitogens (GibcoBRL, Grand Island, NY) were used as a positive control in umbilical cord blood and Jurkat T cell experiments at a final concentration of 50 μg/ml and 500 μg/ml. Lymphocytes were pulsed with equal amount (50 μl) of each mitogen, negative control, and viruses.

3.2.4. Assays

3.2.4.1. Total RNA extraction

The protocol was adapted with modifications from De Kossodo [15]. Briefly, to a 1.5 ml eppendorf tubes containing $2.5x10^6$ cells/ml, 100 µl of 10% SDS, 15 µl of 1 mg/ml ProteinaseK (GibcoBRL) and 0.5 µl of RNasin (GibcoBRL) were added. The tubes were gently vortexed, and incubated at room temperature for 15 min. Following the incubation, 10 µl of 3M NaOAc (pH 5.2), 500 µl of TRIZOL reagent (GibcoBRL, Grand Island, NY) and 100 µl of chlorophorm-isoamylalcohol mixture (24:1) were added. The tubes were vortexed and incubated on ice for 15 min, followed by centrifugation at 12000x g for 15 min. The upper aqueous layer was transferred to a new tube and mixed with 2X volume of 100% Isopropanol (Sigma, St. Louis, MO). To precipitate the RNA, tubes were stored at 4 °C for 15 min and centrifuged at 12000x g for 15 min. RNA pellets were washed with equal volume of 75% EtOH and centrifuged at 8000x g for 10 min. Following the centrifugation, supernatant fluids were removed, and the RNA pellets were air-dried. RNA pellets were resuspended in 50 µl sterile ddH_20 treated with of 0.2 units RNasin.

3.2.4.2. Genomic DNA removal

Six microliters of total RNA, 1.0 µl of 10X DNase I Reaction Buffer, 1 µl of 1 U/µl DNase I (GibcoBRL), and 2.5 µl of 0.2 units RNasin treated sterile ddH_20 were mixed and incubated for 15 min at room temperature. One microliter of 25mM EDTA pH 8.0 (GibcoBRL) was added to the mixture followed by 10 min incubation at 65 °C.

3.2.4.3. Reverse Transcription (RT)

Reverse transcription and PCR were performed according to the procedure described by Lai *et.al.*, [48]. Briefly, 10 µl of DNase-treated RNA, 1.0 µl of 2 mM Oligo (dT) primers, 8.0 µl of 0.2 units RNasin treated sterile ddH_20 were added and incubated at 70 °C for 10 min. Following incubation, samples were placed on ice for 3 min. Four microliters of 5X RT Buffer (GibcoBRL), 2.0 µl of 0.1 M DDT (GibcoBRL) and 4.0 µl of 2 mM dNTP were added and mixtures were incubated at 42 °C for 2 min. After incubation, 1.0 µl of 200 units/µl M-MLV Reverse Transcriptase (GibcoBRL) was added and the mixtures were incubated for 50 min at 42.0 °C, followed by incubation at 70.0 °C for 15 min. This cDNA was diluted in 80 µl of sterile ddH_20 treated with 0.2 units RNasin, and subjected to PCR.

3.2.4.4. Polymerase Chain Reaction (PCR)

The standard PCR conditions were: 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 0.2 mM dNTP, and 2.5 U *Taq* DNA polymerase, 5.0 µl cDNA, 1.0 µl of 0.5 pM cytokine specific forward oligonucleotide, 1.0 µl of 0.5 pM cytokine specific reverse oligonucleotide, 1.0 µl BSA, and 17 µl of water in 25 µl reaction mixture. The samples were exposed to 35 cycles, denatured at 94.0 °C for 1 min, and 95.0 °C for 45 sec, annealed at 55.0 °C for 45 sec, extended at 72.0 °C for 1 min, and 72.0 °C for 10 min in a thermocycler (Perkin-Elmer, GeneAmp PCR System 2400).

3.2.4.5. *Agarose gel electrophoresis*

Five microliters from RT-PCR sample tubes were mixed with 5 µl of tracking dye (0.25% (w/v) bromphenol blue, 0.25% (w/v) xylene cyanol, 20% glycerol, 0.1 M EDTA, pH 8.0) and loaded onto an 1.5% agarose gel. Five microliters of standard markers (1 Kb and 100 bp) were also mixed with the tracking dye and loaded onto gels. Electrophoresis was run on 100 V for approximately 35 min using 1.5% agarose gels and TBE buffer (89 mM boric acid, 89 mM Tris, and 2 mM EDTA, pH 8.0). Following electrophoresis, the agarose gels were stained in Ethidium Bromide (1.0 µg/ml) for 15 min. An Alpha Imager 2000 (Alpha Innotech Corporation) was used to photograph and document the agarose gel.

3.2.4.6. *Enzyme-linked immunosorbent assay (ELISA)*

ELISA was performed, by a modified protocol obtained from Meager [57]. Using a Microtiter 96-well Nunc-Immuno plates with MaxiSorp surface (Nunc Brand Products, Denmark), 15 µl of supernatant fluids were serially diluted with 135 µl of 50 mM $NaHCO_3$ (pH 9.6), and incubated overnight at room temperature. Each sample was assayed in triplicates.

Twenty-four hours after incubation, microtiter plates were washed with PBS (pH 7.4), and blocked with 2% BSA (Fisher Scientific, Pittsburgh, PA) in PBS and incubated for 1 hr at room temperature. Following incubation, plates were washed with PBS, 100 µl of 1:2000 dilution of the appropriate cytokine primary antibody (R&D Systems, Mineapolis, MN; Sigma, St. Louis, MO; PharMingen, San Diego, CA; and Chemicon, Tamecula, CA), in 2% BSA in PBS was added, and incubated at room temperature for 1

hr. Following incubation, plates were washed with PBS, 100 µl of 1:3000 dilution of appropriate cytokine secondary antibody (Sigma and Chemicon) in 2% BSA in PBS was added, and incubated at room temperature for 1 hr. Plates were then washed with PBS, followed by the addition of 100 µl p-NPP disodium hexahydrate (16 mg/ml; Bioworld, Dublin, OH) in Glycine Buffer (0.1M Glycine, 1 mM $ZnCl_2$, 1 mM $MgCl_2$, pH 10.4). ELISA microtiter plates were then incubated (in the dark) at room temperature for 24 hrs before absorbance at 405 nm was determined using an EL800 Universal Microplate Reader (Bio-Tek Instruments, Winooski, VT).

3.2.4.6.1. Data analysis

From the raw data collected on the Universal Microplate Reader, the mean and the standard error of the mean (SEM) were calculated. The mean and standard error of the mean were plotted against standard curves allowing the quantification of cytokine production. Graphics were produced using SigmaPlot software.

CHAPTER IV

RESULTS AND DISCUSSION

Experiments were designed to answer the following questions. First, why have only three (H1, H2, and H3) out of fifteen HA subtypes been circulating in humans? Second, is the HA responsible for viral pathogenicity? If so, what is the mechanism? I hypothesized that for a more pathogenic influenza virus, a higher level of cytokines will be induced in this *in vitro* lymphocyte model. I postulate that, since HA is an important factor in pathogenicity. By using different influenza viruses, the role of the HA is pathogenicity can be studied.

Most studies on cytokines are involved in testing their ability to elicit an immune response. However, few have investigated the intrinsic stimulation of cytokines by influenza viruses. Therefore, I need to determine the parameters for the RT-PCR and ELISA. To optimize the RT-PCR method, I used an adult human peripheral blood lymphocytes (PBL).

4.1. Pilot study using human adult peripheral blood lymphocytes (PBL)

Human adult PBL were used to set the parameters for the RT-PCR test and to test the mitogenic properties of influenza virus HA. RT-PCR was used to assay for the

transcriptional levels of IFN-γ, IL-2, IL-10, and IL-12. Results of these experiments are presented in Fig. 4-1A and Fig. 4-1B.

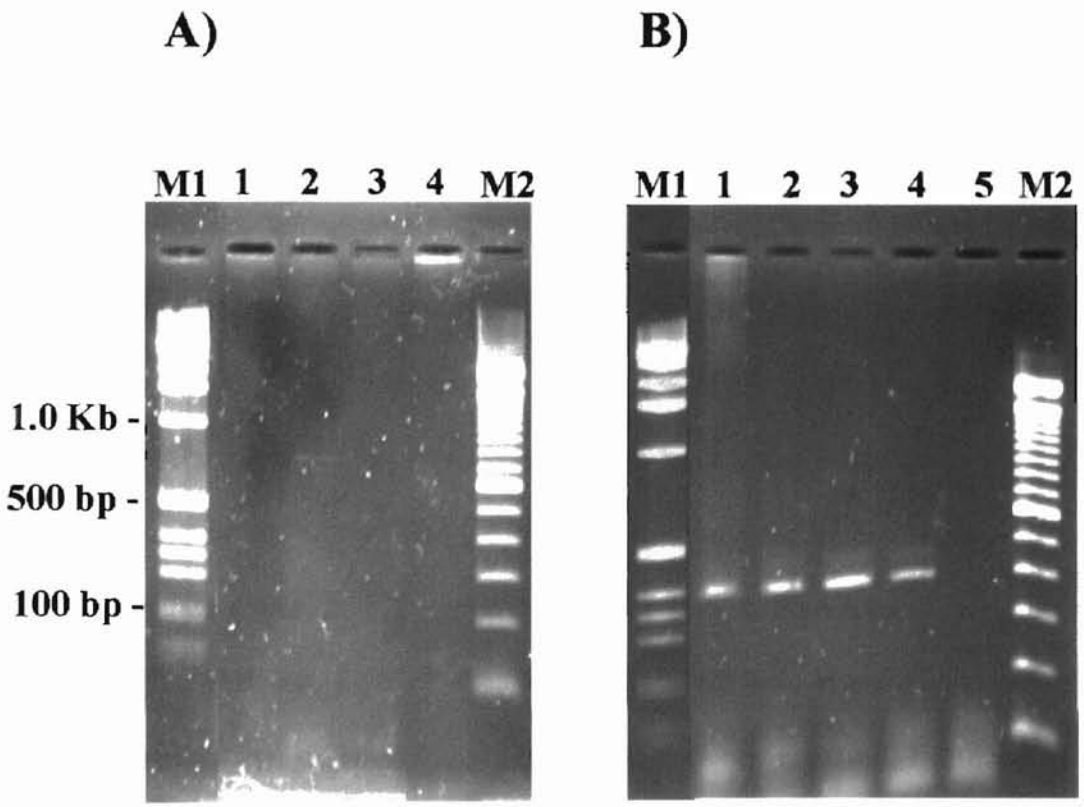

Fig. 4-1A. Production of cytokines using cytokine-specific primers by non- stimulated human adult PBL. RNA was isolated, subjected to RT-PCR, and electrophoresed in 1.5% agarose gel (100 V for 30 min). M1 = 1-Kb DNA ladder; M2 = 100 bp DNA ladder; lane 1 = IFN-γ; lane 2 = IL-12; lane 3 = IL-2; lane 4 = IL-10.

Fig. 4-1B. IL-2 production by human adult PBL 12 hrs post virus stimulation using IL-2 specific primers. RNA was isolated 12 hrs after infection, subjected to RT-PCR and electrophoresed in 1.5% agarose gel (100 V for 30 min). M1 = 1-Kb DNA ladder; M2 = 100 bp DNA ladder; lane 1 = A/Japan/305/57 (H2N2); lane 2 = A/Aichi/2/68 (H3N2); lane 3 = A/Eq/Kentucky/1/98 (H3N8); lane 4 = A/Eq/Prague/56 (H7N7); and lane 5 = PBS.

In Fig. 4-1A, production of either Th1 or Th2 type of cytokines by the non-stimulated human adult PBL was not detected. However, 12 hrs after stimulation, as shown in Fig. 4-1B, induction of IL–2 transcript (indicated by the 382 bp fragment) was

present when the human PBL were stimulated by A/Japan/305/57 (H2N2), A/Aichi/2/68 (H3N2), A/Eq/Kentucky/1/98 (H3N8), and A/Eq/Prague/56 (H7N7) influenza viruses. In contrast, cells pulsed with PBS alone did not produce IL-2, indicating that expression of IL-2 is a result of virus stimulation. Therefore, influenza virus HA has a mitogen-like property, and it can induce cytokine production in this PBL system.

However, I was unable to determine if the production of cytokines by this adult human PBL was a result of previous exposure to influenza viruses. Most adults have either been infected or have been vaccinated. This induction of IL-2 is possibly a recall response. However, it is known that less than 0.1% of all lymphocytic cells are memory cells [95]. Furthermore, most humans do not have memory towards equine H7N7 influenza virus or the human H2N2 subtype. Therefore, the level of cytokines detected was higher than expected if it resulted from memory cell re-activation. Therefore, I concluded that this induction of cytokines was an intrinsic response due to influenza virus. To ensure that there was no memory cell response involved, I utilized an equine system in which I was using naïve PBL.

4.2. Naïve equine peripheral blood lymphocytes (PBL)

Equine PBL, obtained from yearlings, were tested for serum crossreactivity towards equine influenza viruses. Absence of recall response allowed me to determine the parameters for cytokine gene transcription, and to determine the types of cytokines induced. Results of these experiments are presented in Fig.4-2A and Fig. 4-2B.

A)

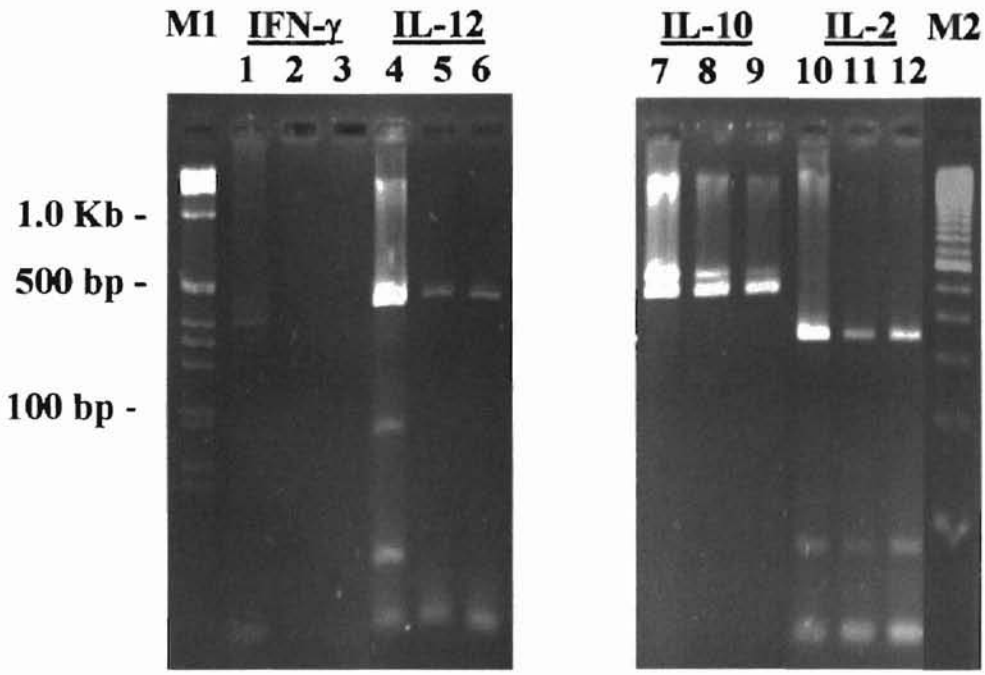

Fig. 4-2A. Induction of cytokines by equine-1 and equine-2 influenza viruses in equine naïve PBL. RNA was extracted 3 hrs after infection, subjected to RT-PCR and electrophoresed in 1.5% agarose gel (100 V for 30 min). M1 = 1-Kb DNA ladder; M2 = 100 bp DNA ladder. Lanes 1, 4, 7, 10 = A/Eq/Kentucky/1/98 influenza virus; lanes 2, 5, 8, 11 = A/Eq/Prague/56 influenza virus; lanes 3, 6, 9, 12 = PBS.

B)

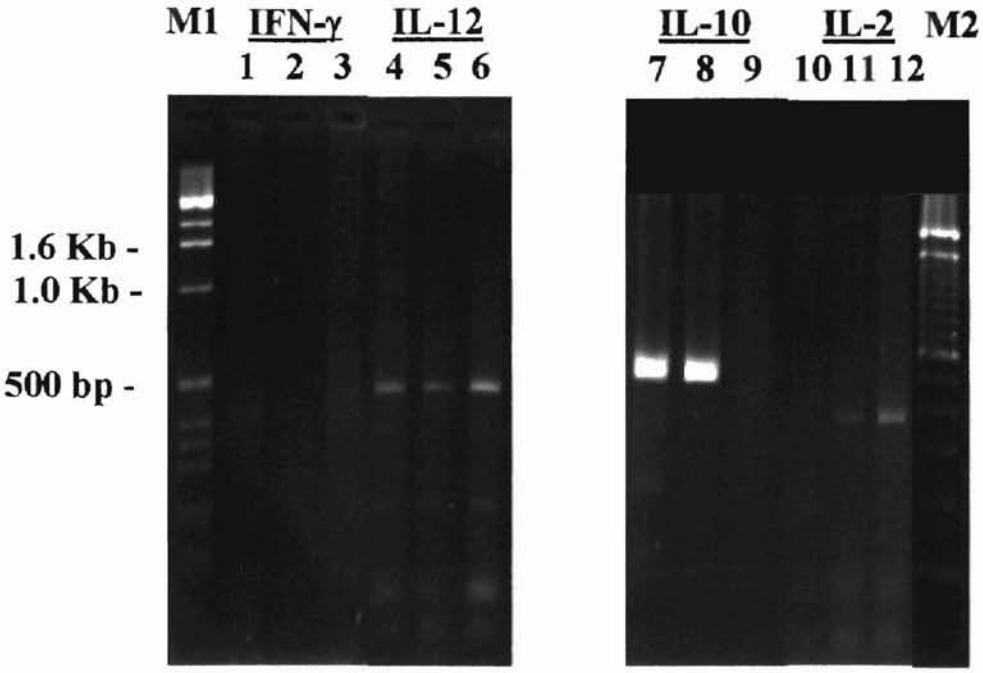

Fig. 4-2B. Induction of cytokines by equine-1 and equine-2 influenza viruses in equine naïve PBL. RNA was extracted 12 hrs after infection, subjected to RT-PCR and electrophoresed in 1.5% agarose gel (100 V for 30 min). M1 = 1-Kb DNA ladder; M2 = 100 bp DNA ladder. Lanes 1, 4, 7, 10 = A/Eq/Kentucky/1/98 influenza virus; lanes 2, 5, 8, 11 = A/Eq/Prague/56 influenza virus; lanes 3, 6, 9, 12 = PBS.

Three hours after infection naïve equine PBL produced both Th1 and Th2 type cytokines as shown in Fig. 4-2A. Production of IL-12 (475 bp fragment), IL-2 (382 bp fragment) and IL-10 (495 bp fragment) was observed. IFN-γ was induced in very low quantities, and only after infection with equine-2 (H3N8) influenza virus. Transcription of IL-12, IL-10, and IL-2 was highly increased following infection with the more pathogenic A/Eq/Kentucky/1/98 (H3N8) influenza virus. The less pathogenic A/Eq/Prague/56 (H7N7) influenza virus, however induced a lower level of cytokines.

Twelve hours after infection (Fig. 4-2B) equine-naïve PBL showed a decreased

Th1-type cytokine production, while Th2-type cytokine production remained constant. Production of IFN-γ and IL-2 was not detected by RT-PCR. Production of IL-12 is decreased when compared to 3 hrs after infection and attained the same level with either of the two equine influenza viruses. Induction of IL-10 (495 bp fragments) was increased when compared to 3 hrs samples. However, the difference between the two equine influenza viruses was not observed.

Production of both Th1 and Th2 cytokines was observed as early as 3 hrs following infection, and continued on at 12 hrs. This pattern of cytokine expression was attributed to the mitogen-like properties of influenza virus, or more likely due to the surface HA. Differences seen in cytokine induction between 3 and 12 hrs post infection suggested that kinetics of Th1 and Th2 type cytokine differ. It is probable that Th1 type cytokine production peaked before the 12 hrs. However, the decrease in Th1 cytokine production was possibly due to regulation by the induced IL-10.

The equine influenza viruses used, equine-2 A/Eq/Kentucky/1/98 (H3N8) and equine-1 A/Eq/Prague/56 (H7N7), have different pathogenicity. Clinical symptoms caused by equine-2 influenza virus are more severe than by the equine-1 influenza virus. Therefore, I attributed the difference in cytokine induction to difference in pathogenicity of these two viruses.

Detection of cytokine gene expression by RT-PCR was important, however a quantifiable assay, such as ELISA, is needed to quantify the true induction of cytokines. Lack of crossreactivity between human cytokine antibodies and equine-produced cytokines prevented me from performing such a quantifiable analysis on naïve equine PBL. Most equine cytokine-specific antibodies are not available , therefore I switched to

Jurkat human T cell line as human cytokine-specific antibodies are available commercially.

4.3. Jurkat human T cell line

Jurkat cells are a pseudodiploid human T cell line. They are not memory cells. Jurkat cells, if properly stimulated, produce a variety of cytokines [13, 62, 64, 77]. Therefore, Jurkat T cells represent a suitable model for studying *in vitro* cytokine induction.

Jurkat cells were cultured in RPMI with 10% (w/v) FBS and incubated at 37 °C and 5.0 % CO_2. Cells were diluted to 2.5 x 10^6 cells/ml and pulsed for 4 hrs with 50 µl (1 HA unit/2.5x10^6 cells) of control, B/Lee/40, A/Eq/Prague/56 (H7N7), A/Eq/Kentucky/1/98 (H3N8), A/Eq/Kentucky/1/97 (H3N8), A/Eq/Saskatoon/1/90 (H3N8), A/Eq/Miami/1/63 (H3N8), A/Panama/99 (H3N2), A/Aichi/2/68 (H3N2), A/Japan/305/57 (H2N2), A/swine/New Jersey/11/76 (H1N1), A/Puerto Rico/8/34 (H1N1) and mitogen. Four hours after infection, virus-containing supernatant fluids were removed, and 1.0 ml of fresh, virus-free, optimum OptiMEM I medium was added to the cells. They were incubated at 37 °C and 5.0 % CO_2 for 24 hrs before the supernatant fluids were collected and assayed by ELISA.

4.3.1. Th1 type cytokines

4.3.1.1. Interferon-γ (IFN-γ)

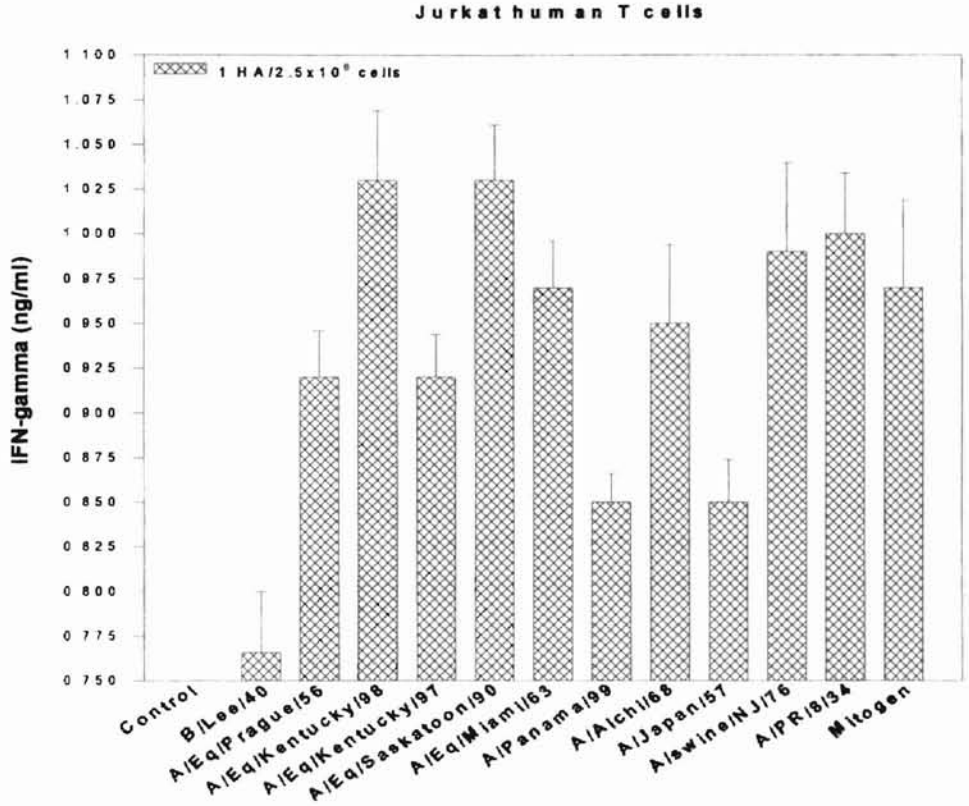

Fig. 4-3A. Production of IFN-γ by Jurkat T cells 24 hrs post-infection with UV-inactivated influenza viruses. Error bars represent the standard error of the mean for each virus tested.

As seen in Fig. 4-3A, influenza viruses and the positive control induced IFN-γ, while the negative control did not induce detectable amounts of this cytokine. Influenza viruses induced the following amounts of IFN-γ: B/Lee/40 (0.77 +/- 0.03 ng/ml), A/Eq/Prague/56 (H7N7) (0.92 +/- 0.03 ng/ml), A/Eq/Kentucky/1/98 (H3N8) (1.03 +/- 0.04 ng/ml), A/Eq/Kentucky/1/97 (H3N8) (0.92 +/- 0.02 ng/ml), A/Eq/Saskatoon/1/90 (H3N8) (1.03 +/- 0.03 ng/ml), A/Eq/Miami/1/63 (H3N8) (0.97 +/- 0.03 ng/ml), A/Panama/99 (H3N2) (0.85 +/- 0.02 ng/ml), A/Aichi/2/68 (H3N2) (0.95 +/- 0.04 ng/ml),

A/Japan/305/57 (H2N2) (0.85 +/- 0.02 ng/ml), A/swine/New Jersey/11/76 (H1N1) (0.99 +/- 0.05 ng/ml), and A/Puerto Rico/8/34 (H1N1) (1.0 +/- 0.03 ng/ml). Mitogen (positive control) induced 0.97 +/- 0.05 ng/ml of IFN-γ.

Influenza A viruses induced higher levels of IFN-γ than did influenza B virus. This may be associated with the difference in their surface HA and clinical pathogenicity. Influenza A virus subtypes, H1 and H3, with the exception of A/Panama/99 (H3N2) induced higher amounts of IFN-γ than the A/Japan/305/57 (H2N2) virus.

4.3.1.2. Interleukin-2 (IL-2)

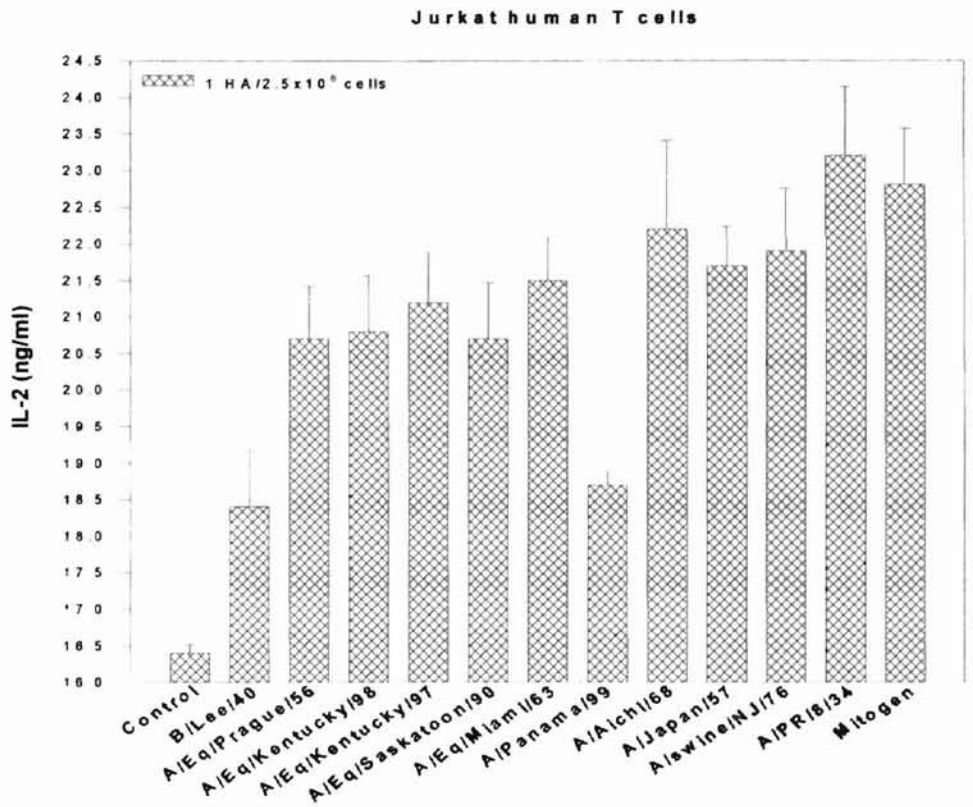

Fig. 4-3B. Production of IL-2 by Jurkat T cells 24 hrs post-infection with UV-inactivated influenza viruses. Error bars represent the standard error of the mean for each virus tested.

IL-2 was induced by all influenza viruses (Fig. 4-3B), however it was also produced by the mock-infected cells (16.4 +/- 0.12 ng/ml). Induction of IL-2 by mock-infected cells may have resulted from cell stress and cell death, and it was much lower than induced by influenza A viruses. Induction of IL-2 by influenza B virus (18.4 +/- 0.77 ng/ml) is different from mock-infected cells. However, whether it is biologically important remains to be determined.

Influenza A viruses induced the following quantities of IL-2: A/Eq/Prague/56 0(H7N7) (20.70 +/- 0.72 ng/ml), A/Eq/Kentucky/1/98 (H3N8) (20.80 +/- 0.77 ng/ml), A/Eq/Kentucky/1/97 (H3N8) (21.20 +/- 0.68 ng/ml), A/Eq/Saskatoon/1/90 (H3N8) (20.70 +/- 0.77 ng/ml), A/Eq/Miami/1/63 (H3N8) (21.50 +/- 0.58 ng/ml), A/Panama/99 (H3N2) (18.7 +/- 0.17 ng/ml), A/Aichi/2/68 (H3N2) (22.2 +/- 1.20 ng/ml), A/Japan/305/57 (H2N2) (21.7 +/- 0.54 ng/ml), A/swine/New Jersey/11/76 (H1N1) (21.9 +/- 0.85 ng/ml), and A/Puerto Rico/8/34 (H1N1) (23.2 +/- 0.95 ng/ml).

Influenza A viruses, with the exception of A/Panama/99, induced higher levels of IL-2 than did influenza B virus. IL-2 was induced in similar amounts by all influenza A viruses. This high level may be due to an accumulation of this cytokine, as it was induced early. In addition, IL-2, is a proliferative cytokine needed for cell activation, therefore it is not surprising that it is secreted in high amounts during an infection.

4.3.2. Th2 type cytokines

4.3.2.1. Interleukin-4 (IL-4)

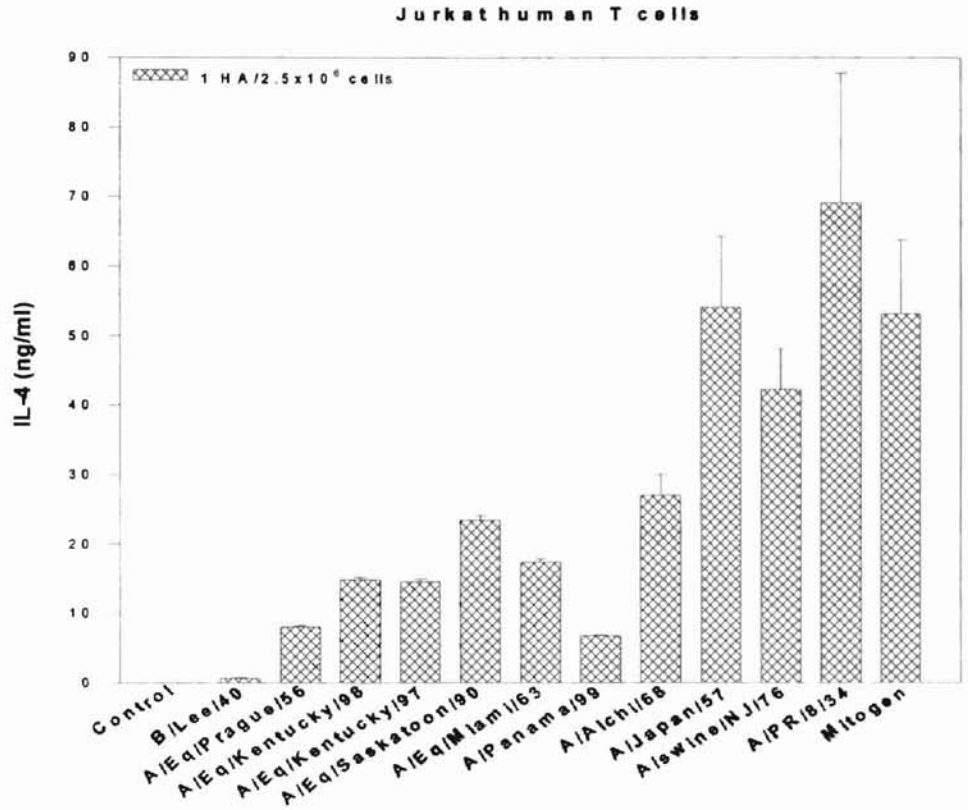

Fig. 4-3C. Production of IL-4 by Jurkat T cells 24 hrs post-infection with UV-inactivated influenza viruses. Error bars represent the standard error of the mean for each viruses tested.

As shown in Fig. 4-3C, influenza B virus (B/Lee/40) induced minimal quantities (0.66 +/- 0.02 ng/ml) of IL-4. Influenza A viruses induced high amounts of IL-4 with the following quantities: A/Eq/Prague/56 (H7N7) (8.10 +/- 0.13 ng/ml), A/Eq/Kentucky/1/98 (H3N8) (14.80 +/- 0.42 ng/ml), A/Eq/Kentucky/1/97 (H3N8) (14.60 +/- 0.31 ng/ml), A/Eq/Saskatoon/1/90 (H3N8) (23.40 +/- 0.63 ng/ml), A/Eq/Miami/1/63 (H3N8) (17.30 +/- 0.47 ng/ml), A/Panama/99 (H3N2) (6.75 +/- 0.06 ng/ml), A/Aichi/2/68 (H3N2) (27.0 +/- 2.97 ng/ml), A/Japan/305/57 (H2N2) (54.0 +/- 10.26 ng/ml), A/swine/New Jersey/11/76 (H1N1) (42.20 +/- 5.90 ng/ml), and A/Puerto Rico/8/34 (H1N1) (69.10 +/-

18.7 ng/ml). The high induction of IL-4 was also observed from mitogen stimulated cells (53.10 +/- 10.6 ng/ml).

IL-4 was induced in higher levels by influenza A viruses than by the influenza B virus. Among influenza A viruses H2 and H1 subtypes induced higher levels of IL-4 than did H3 and H7 subtypes. Difference in virus surface HA is potentially responsible for the observed variations. Among equine influenza A viruses, equine-2 (H3N8) influenza virus induced more IL-4 than did equine-1 (H7N7) influenza virus (Fig. 4-3C).

4.3.2.2. Interleukin-6 (IL-6)

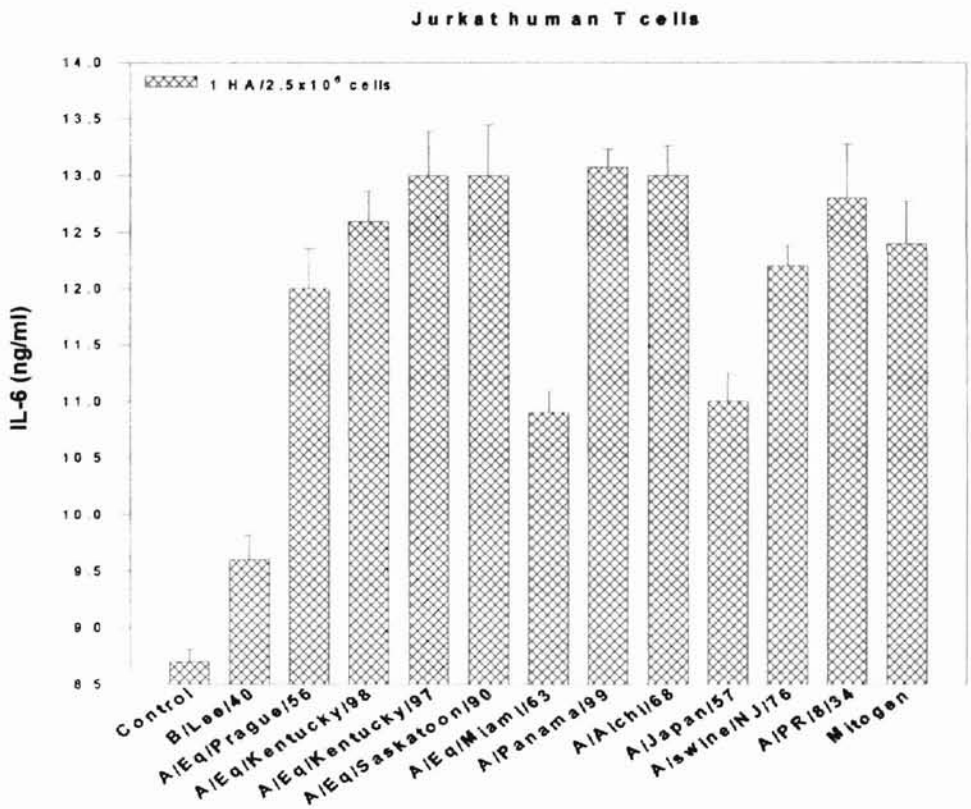

Fig. 4-3D. Production of IL-6 by Jurkat T cells 24 hrs post-infection with UV-inactivated influenza viruses. Error bars represent the standard error of the mean for each virus tested.

IL-6 was produced by both, mock-infected and influenza virus infected Jurkat T cells (Fig. 4-3D). Mock-infected cells, possibly due to cell stress and cell death, produced 8.7 +/- 0.09 ng/ml of IL-6. Influenza B virus (B/Lee/40) induced 9.6 +/- 0.22 ng/ml of IL-6, different from the mock-infected cells, however biological importance requires further testing.

Influenza A viruses induced the following quantities of IL-6: A/Eq/Prague/56 (H7N7) (9.60 +/- 0.22 ng/ml), A/Eq/Kentucky/1/98 (H3N8) (12.0 +/- 0.36 ng/ml), A/Eq/Kentucky/1/97 (H3N8) (12.60 +/- 0.26 ng/ml), A/Eq/Saskatoon/1/90 (H3N8) (13.0 +/- 0.39 ng/ml), A/Eq/Miami/1/63 (H3N8) (13.0 +/- 0.45 ng/ml), A/Panama/99 (H3N2) (10.90 +/- 0.19 ng/ml), A/Aichi/2/68 (H3N2) (13.0 +/- 0.26 ng/ml), A/Japan/305/57 (H2N2) (11.0 +/- 0.25 ng/ml), A/swine/New Jersey/11/76 (H1N1) (12.20 +/- 0.18 ng/ml), and A/Puerto Rico/8/34 (H1N1) (12.80 +/- 0.47 ng/ml) of IL-6. The importance of IL-6 induction is supported with similar induction by mitogen stimulated Jurkat T cells (12.40 +/- 0.37 ng/ml).

Influenza A viruses induced more IL-6 than influenza B viruses. Again, influenza virus subtypes, H1, H3, and H7, induced higher levels of IL-6 when compared to the H2 subtype.

4.3.2.3. Interleukin-10 (IL-10)

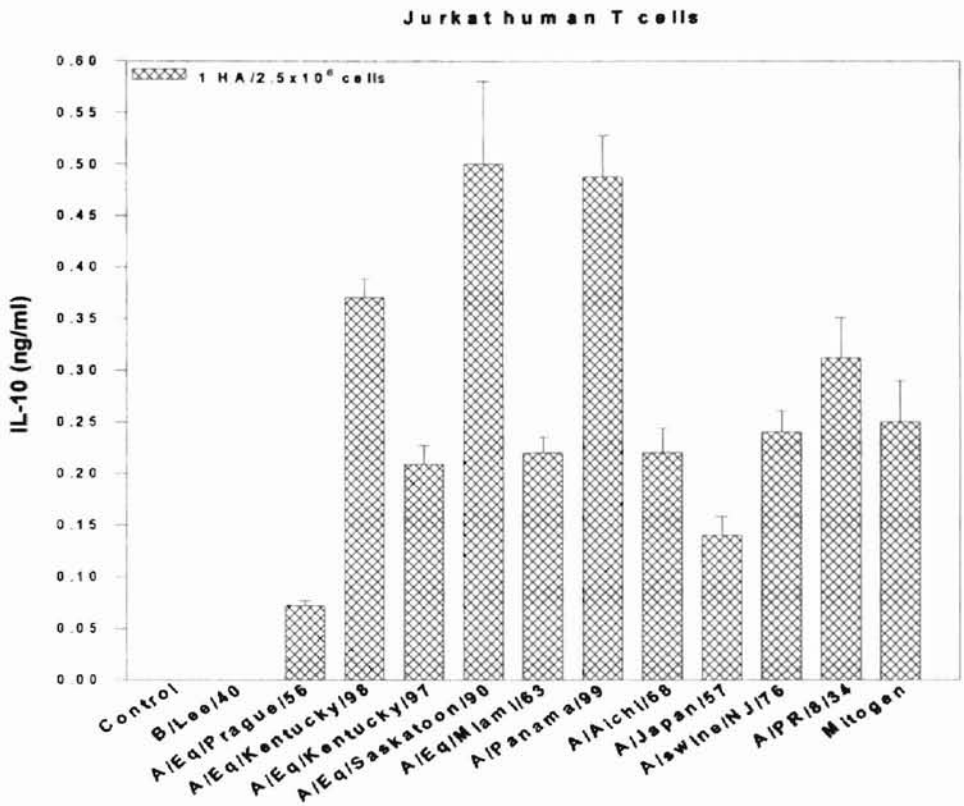

Fig. 4-3E. Production of IL-10 by Jurkat T cells 24 hrs post-infection with UV-inactivated influenza viruses. Error bars represent the standard error of the mean for each viruses tested.

Only influenza A viruses induced IL-10 (Fig. 4-3E): A/Eq/Prague/56 (H7N7) (0.07 +/- 0.01 ng/ml), A/Eq/Kentucky/1/98 (H3N8) (0.37 +/- 0.02 ng/ml), A/Eq/Kentucky/1/97 (H3N8) (0.21 +/- 0.02 ng/ml), A/Eq/Saskatoon/1/90 (H3N8) (0.50 +/- 0.08 ng/ml), A/Eq/Miami/1/63 (H3N8) (0.22 +/- 0.02 ng/ml), A/Panama/99 (H3N2) (0.49 +/- 0.04 ng/ml), A/Aichi/2/68 (H3N2) (0.22 +/- 0.02 ng/ml), A/Japan/305/57 (H2N2) (0.14 +/- 0.02 ng/ml), A/swine/New Jersey/11/76 (H1N1) (0.24 +/- 0.02 ng/ml), and A/Puerto Rico/8/34 (H1N1) (0.31 +/- 0.04 ng/ml). Mitogen (positive control) induced 0.25 +/- 0.04 ng/ml of IL-10.

Among type A influenza viruses, H3 and H1 subtypes induced more IL-10 than

did H2 subtype. This pattern was also seen with IFN-γ and IL-6. Comparing equine influenza A viruses, equine-2 (H3N8) induced higher level of IL-10 than did equine-1 (H7N7) subtype. H1 viruses induced similar amounts of IL-10, while H3 viruses were split into two groups. The first group, containing A/Eq/Kentucky/98, A/Eq/Saskatoon/90 and A/Panama/99, induced a high level of IL-10. These viruses are relatively new in circulation. The recent evolutionary changes, since 1990, in H3 subtypes may have resulted in higher induction of IL-10.

The advantage of using Jurkat T cell line makes it an efficient and economic choice for an *in vitro* model for cytokine induction. However, it is consisted of one cell type. Influenza virus infection *in vivo* is associated with several cell populations. Therefore, I decided to use the human umbilical cord blood lymphocytes. The presence of heterologous cell populations, absence of memory cells, make usage of umbilical cord blood lymphocytes a more proper model for the analysis.

4.4. Human umbilical cord blood

The absence of recall immunity, and being a mixed population of cells, composed of both B and T lymphocytes, phagocytic cells, and auxilary cells, allowed me to test the true intrinsic induction of cytokines as a result of similarity to an *in vivo* setting.

Lymphocytic cells obtained from human umbilical cord blood samples were diluted to 2.5 x 10^6 cells/ml, and pulsed for 4 hrs with 50 µl (1 HA unit/2.5x10^6 cells) and 500 µl (10 HA units/2.5x10^6 cells) of control, B/Lee/40, A/Eq/Kentucky/1/98 (H3N8), A/Panama/99 (H3N2), A/Aichi/2/68 (H3N2), A/Japan/305/57 (H2N2), A/swine/New Jersey/11/76 (H1N1), A/Puerto Rico/8/34 (H1N1) and mitogen. Four hours after pulsing,

virus-containing supernatant fluids were removed. One milliliter of fresh, virus-free, optimum OptiMEM I medium was added, and cells were incubated at 37 °C and 5.0% CO_2 for 24 hrs before the supernatant fluids were collected and assayed by ELISA.

4.4.1. Th1 type cytokines

4.4.1.1. Interferon-γ (IFN-γ)

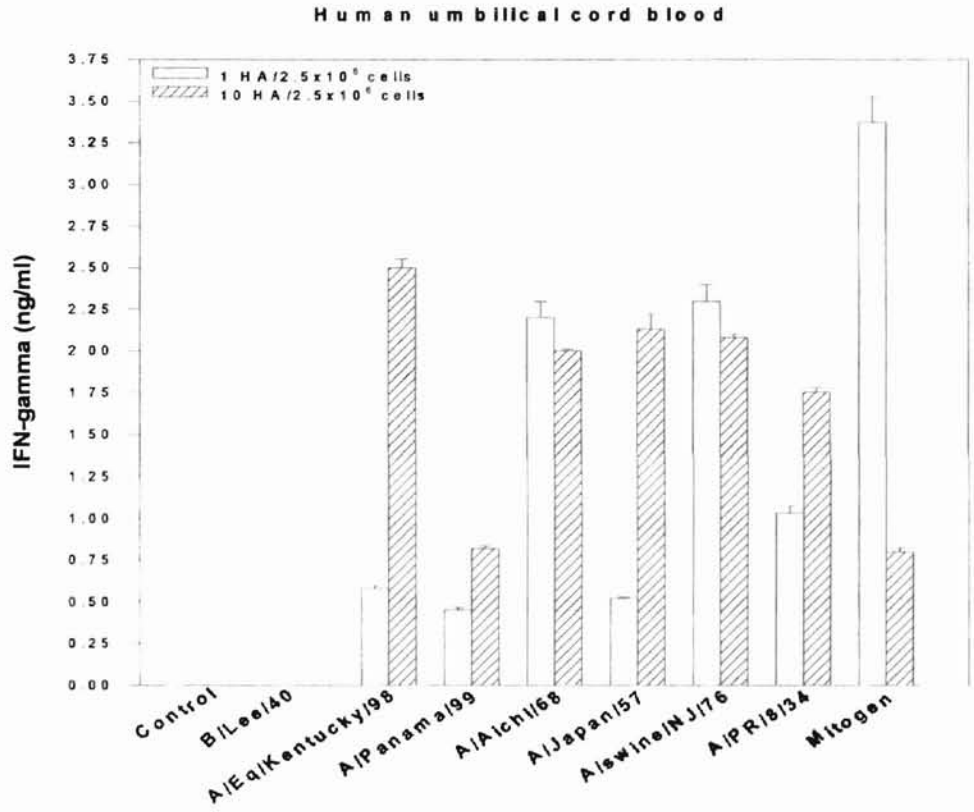

Fig. 4-4A. Production of IFN-γ by human umbilical cord lymphocytes 24 hrs post infection with UV-inactivated influenza viruses. Error bars represent the standard error of the mean for each virus tested.

Only influenza A viruses induced IFN-γ regardless of virus concentration (Fig. 4-4A). One HA unit of influenza A viruses induced the following quantities of IFN-γ: A/Eq/Kentucky/1/98 (H3N8) (0.58 +/- 0.01 ng/ml), A/Panama/99 (H3N2) (0.45 +/- 0.02

ng/ml), A/Aichi/2/68 (H3N2) (2.2 +/- 0.10 ng/ml), A/swine/New Jersey/11/76 (H1N1) (2.3 +/- 0.10 ng/ml), A/Puerto Rico/8/34 (H1N1) (1.03 +/- 0.05 ng/ml), and A/Japan/305/57 (H2N2) (0.52 +/- 0.01 ng/ml).

Ten HA units of influenza A viruses induced the following quantities of IFN-γ: A/Eq/Kentucky/1/98 (H3N8) (2.5 +/- 0.05 ng/ml), A/Panama/99 (H3N2) (0.82 +/- 0.01 ng/ml), A/Aichi/2/68 (H3N2) (2.0 +/- 0.01 ng/ml), A/swine/New Jersey/11/76 (H1N1) (2.1 +/- 0.02 ng/ml), A/Puerto Rico/8/34 (H1N1) (1.75 +/- 0.03 ng/ml), and A/Japan/305/57 (H2N2) (2.13 +/- 0.09 ng/ml) of IFN-γ.

Strong difference in IFN-γ induction between influenza A and influenza B viruses were observed. Among influenza A viruses, difference in cytokine induction is detected mainly in experiments using one HA unit of viruses. Subtypes, H3 (A/Aichi/68) and H1 induced four times as much IFN-γ than did the H2 subtype. The other two H3 subtypes did not induce as high levels of IFN-γ as A/Aichi/68. This may be associated with their attenuated pathogenicity resulting from 30 years of evolutionary changes.

Similar induction of IFN-γ by most influenza A viruses after stimulation with 10 units of influenza viruses, could be associated with high production of IFN-γ, or cell death due to high virus concentration. High induction of IFN-γ could have resulted in over-saturation of ELISA assay. Due to the high production of IFN-γ, the pH of the binding buffer is changed possibly preventing the protein binding to the microtiter plate. Followed by plate washing, the unbound proteins are washed off, resulting in a lower detection of true cytokine induction. This is best seen with A/Aichi/2/68 (H3N2) and A/swine/New Jersey/11/76 (H1N1).

4.4.1.2. Interleukin-2 (IL-2)

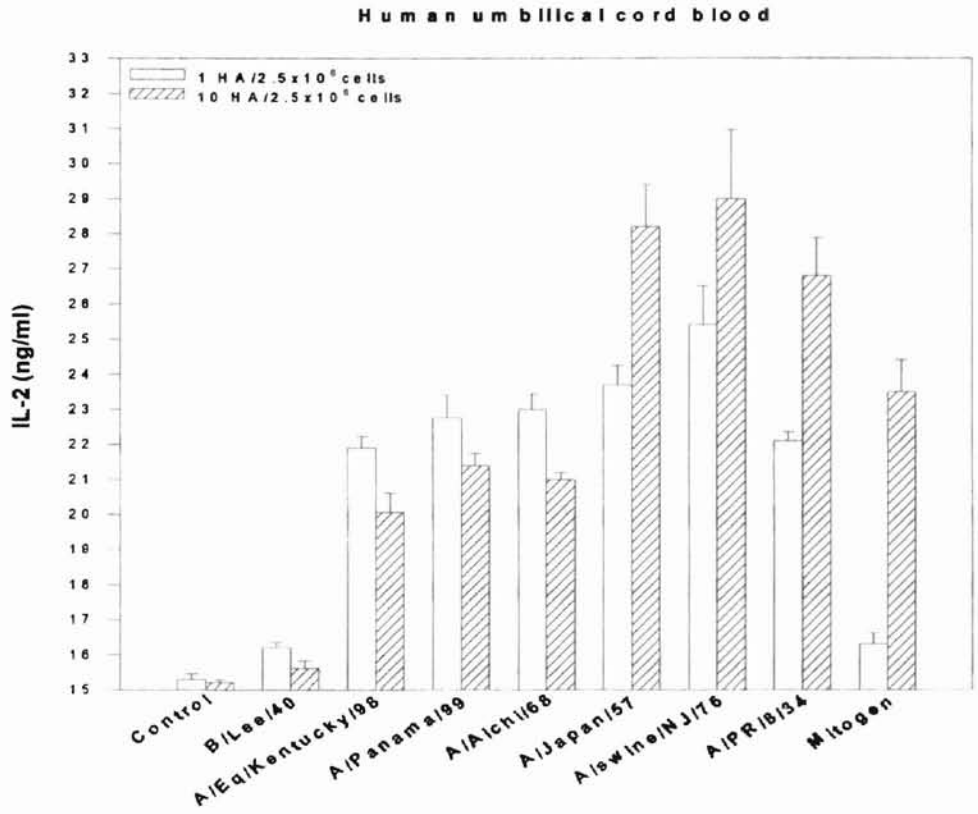

Fig. 4-4B. Production of IL-2 by human umbilical cord lymphocytes 24 hrs post infection with UV-inactivated influenza viruses. Error bars represent the standard error of the mean for each virus tested.

As Fig. 4-4B shows, high quantities of IL-2 were detected. IL-2 was produced by mock-infected cells (50 µl and 500 µl produced 15.3 +/- 0.153 ng/ml and 15.2 +/- 0.076 ng/ml respectively). Experiments using one HA unit of influenza A viruses indicated similarity in levels of IL-2 induction: A/Eq/Kentucky/1/98 (H3N8) (21.9 +/- 0.33 ng/ml), A/Panama/99 (H3N2) (22.75 +/- 0.66 ng/ml), A/Aichi/2/68 (H3N2) (23.0 +/- 0.44 ng/ml), A/swine/New Jersey/11/76 (H1N1) (25.4 +/- 1.11 ng/ml), A/Puerto Rico/8/34 (H1N1) (22.1 +/- 0.26 ng/ml), and A/Japan/305/57 (H2N2) (23.7 +/- 0.55 ng/ml). Influenza B virus (B/Lee/40), induced 16.2 +/- 0.15 ng/ml. This is more than induced by

mock-infected cells, however biological importance could not be determined.

Ten HA units of influenza A viruses induced the following quantities of IL-2: A/Eq/Kentucky/1/98 (H3N8) (20.1 +/- 0.56 ng/ml), A/Panama/99 (H3N2) (21.4 +/- 0.34 ng/ml), A/Aichi/2/68 (H3N2) (21.0 +/- 0.19 ng/ml) of IL-2, A/swine/New Jersey/11/76 (H1N1) (29.0 +/- 1.97 ng/ml), A/Puerto Rico/8/34 (H1N1) (26.8 +/- 1.10 ng/ml), and A/Japan/305/57 (H2N2) (28.2 +/- 1.20 ng/ml).

Higher levels of IL-2 were induced by the same viruses than the induced levels of IFN-γ. This may be attributed to IL-2's proliferative functions. Infection by influenza B virus, B/lee/40, in one HA unit or ten HA units showed the ability of this low pathogenic virus to induce IL-2 production. Despite the lower level of IL-2 induction by influenza B virus, induction of other cytokines, such as IL-6, can result in development of very mild influenza symptoms.

Influenza A viruses induced higher levels of IL-2 than did influenza B virus. Similar induction of IL-2 among influenza A viruses possibly resulted from early production of IL-2, resulting in its accumulation. Identical pattern was observed with influenza A virus stimulated Jurkat T cells. Experiments completed using ten HA units of viruses showed a similar result if interpreted with possibility that a lower IL-2 detection by H3 subtypes resulted from ELISA over-saturation.

4.4.2. Th2 type cytokines

4.4.2.1. Interleukin-4 (IL-4):

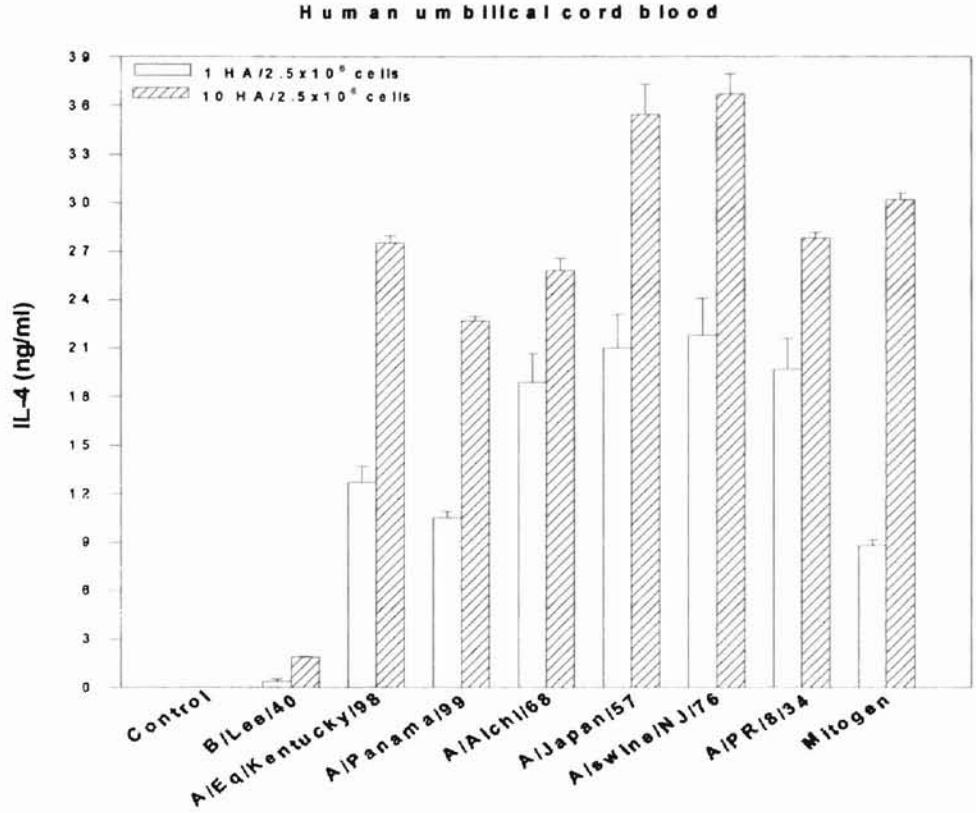

Fig. 4-4C. Production of IL-4 by human umbilical cord lymphocytes 24 hrs post infection with UV-inactivated influenza viruses. Error bars represent the standard error of the mean for each virus tested.

As shown in Fig. 4-4C, one HA unit of influenza viruses induced the following quantities of IL-4: B/Lee/40 (0.8 +/- 0.15 ng/ml), A/Eq/Kentukcy/1/98 (H3N8) (12.7 +/- 0.99 ng/ml), A/Panama/99 (H3N2) (10.5 +/- 0.39 ng/ml), A/Aichi/2/68 (H3N2) (18.9 +/- 1.75 ng/ml), A/Japan/305/57 (21.0 +/- 2.10 ng/ml), A/swine/New Jersey/11/76 (H1N1) (21.8 +/- 2.30 ng/ml), and A/Puerto Rico/8/34 (19.7 +/- 1.91 ng/ml).

Ten HA units of influenza viruses induced the following amounts of IL-4: B/Lee/40 (1.9 +/- 0.008 ng/ml), A/Eq/Kentucky/1/98 (H3N8) (27.5 +/- 0.44 ng/ml), A/Panama/99 (H3N2) (22.7 +/- 0.27 ng/ml), A/Aichi/68 (H3N2) (25.8 +/- 0.75 ng/ml),

A/Japan/305/57 (H2N2) (35.5 +/- 1.81 ng/ml), A/swine/New Jersey/11/76 (H1N1) and (36.7 +/- 1.25 ng/ml), and A/Puerto Rico/8/34 (27.8 +/- 0.361 ng/ml).

Differences observed between influenza A and influenza B viruses suggests biological importance. At ten HA units of all influenza viruses, including type B, a 2-fold increase of IL-4 is observed when compared to one HA unit of influenza viruses. Therefore, I conclude that cells stimulated with higher concentration of influenza viruses induce higher amounts of cytokines. IL-4 was induced in similar amounts among influenza A viruses.

4.4.2.2. Interleukin-6 (IL-6)

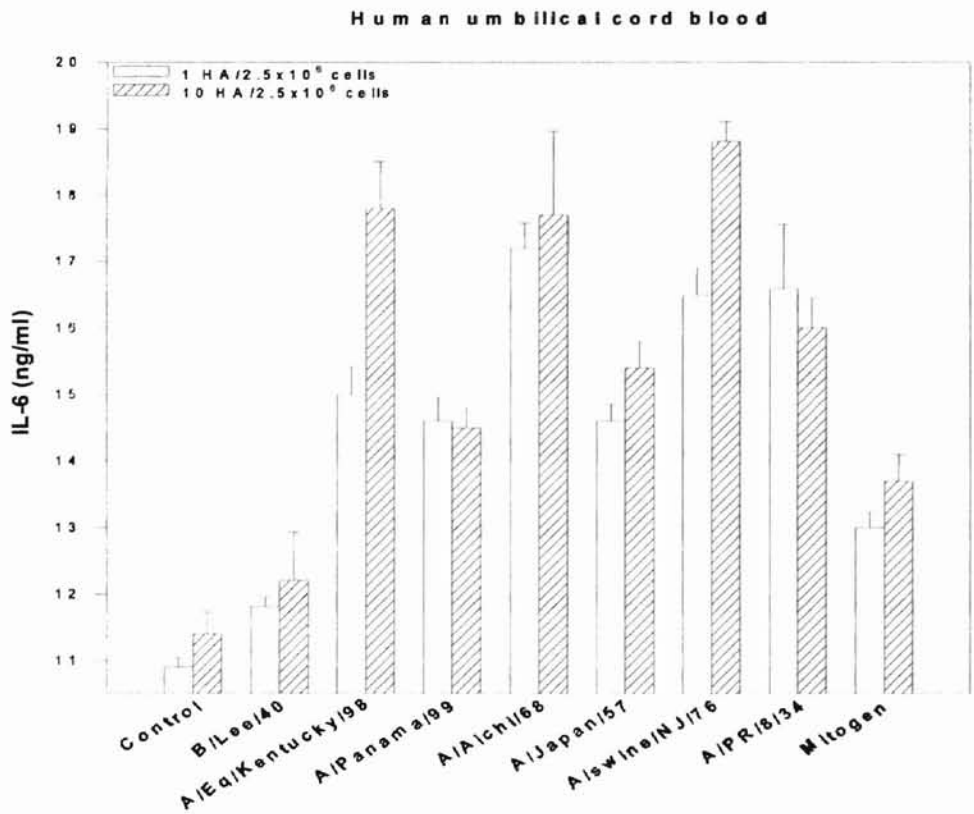

Fig. 4-4D. Production of IL-6 by human umbilical cord lymphocytes 24 hrs post infection with UV-inactivated influenza viruses. Error bars represent the standard error of the mean for each virus tested.

As shown in Fig. 4-4D, IL-6 was also produced by mock-infected cells. Fifty microliters of allantoic fluid induced 10.9 +/- 0.14 ng/ml, while 500 µl of allantoic fluid induced 11.4 +/- 0.33 ng/ml. Similar data was obtained with the Jurkat T cell line. One HA unit of influenza viruses induced the following quantities of IL-6: B/Lee/40 (11.8 +/- 0.15 ng/ml), A/Eq/Kentucky/1/98 (H3N8) (15.0 +/- 0.42 ng/ml), A/Panama/99 (H3N2) (14.6 +/- 0.35 ng/ml), and A/Aichi/2/68 (H3N2) (17.2 +/- 0.38 ng/ml), A/Japan/305/57 (14.6 +/- 0.26 ng/ml), A/swine/New Jersey/11/76 (H1N1) (16.5 +/- 0.40 ng/ml) and A/Puerto Rico/8/34 (H1N1) (16.6 +/- 0.96 ng/ml).

Ten HA units of influenza viruses showed similar induction: B/Lee/40 (12.2 +/- 0.73 ng/ml), A/Eq/Kentucky/1/98 (H3N8) (17.8 +/- 0.71 ng/ml), A/Panama/99 (H3N2) (14.5 +/- 0.29 ng/ml), A/Aichi/2/68 (H3N2) (17.7 +/- 1.26 ng/ml), A/Japan/305/57 (H2N2) (15.4 +/- 0.38 ng/ml), A/swine/New Jersey/11/76 (18.8 +/- 0.30 ng/ml), and A/Puerto Rico/8/34 (H1N1) (16.0 +/- 0.45 ng/ml).

Interleukin-6 was induced in high quantities following infection with influenza viruses. Influenza B virus induced lower levels of IL-6 than did influenza A viruses. Subtypes, H3 A/Aichi/68, and both H1 viruses induced more IL-6 than did the H2 subtype. Similar pattern was obtained with IFN-γ induction. Ten HA units of influenza viruses induced approximately two times as much IL-6 than did the mock-infected cells. Lower induction of IL-6 detected for A/Panama/99 (H3N2) and A/Puerto Rico/8/34 (H1N1) with ten HA units concentration could be attributed to ELISA over-saturation.

4.4.2.3. Interleukin-10 (IL-10)

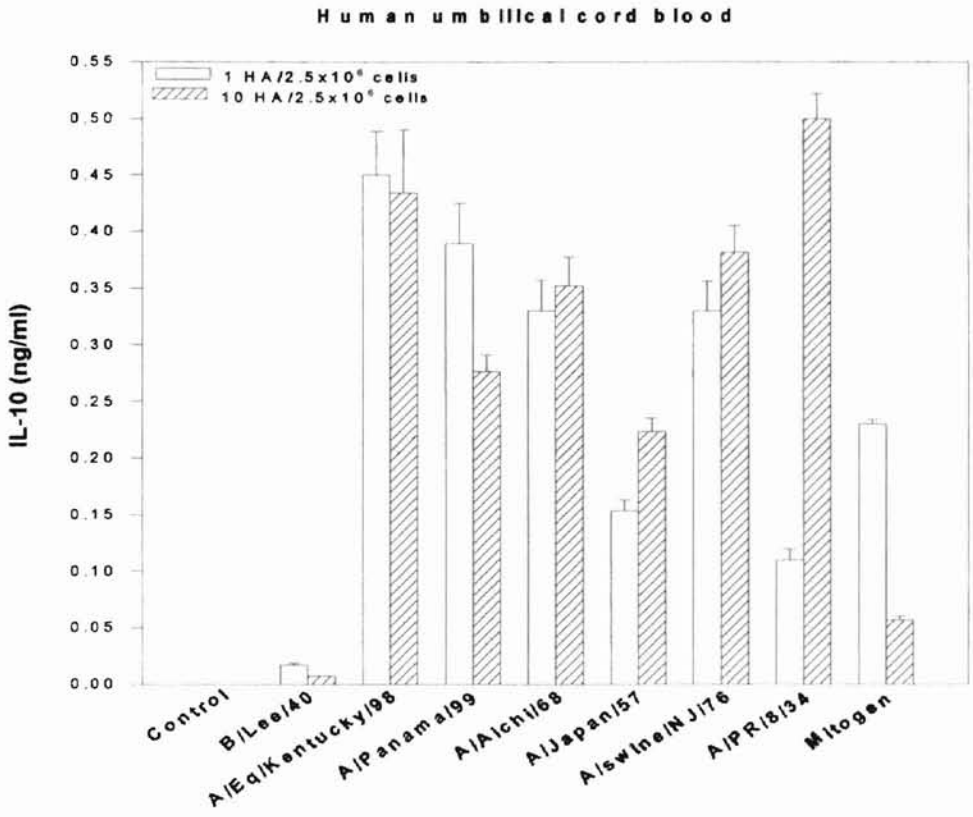

Fig. 4-4E. Production of IL-10 by human umbilical cord lymphocytes 24 hrs post infection with UV-inactivated influenza viruses. Error bars represent the standard error of the mean for each virus tested.

As shown in Fig. 4-4E, experiments conducted using one unit of influenza viruses showed induction of IL-10 in the following amounts: B/Lee/40 (0.017 +/- 0.001 ng/ml), A/Eq/Kentucky/1/98 (H3N8) (0.45 +/- 0.04 ng/ml), A/Panama/99 (H3N2) (0.39 +/- 0.03 ng/ml), A/Aichi/2/68 (H3N2) (0.33 +/- 0.03 ng/ml), A/Japan/305/57 (H2N2) (0.153 +/- 0.01 ng/ml), A/swine/New Jersey/11/76 (0.33 +/- 0.03 ng/ml) and A/Puerto Rico/8/34 (0.11 +/- 0.01 ng/ml).

Ten HA units of influenza viruses induced the following levels of IL-10: B/Lee/40 (0.007 +/- 0.0001 ng/ml), A/Eq/Kentucky/1/98 (H3N8) (0.434 +/- 0.06 ng/ml), A/Panama/99 (H3N2) (0.276 +/- 0.01 ng/ml), A/Aichi/2/68 (H3N2) (0.352 +/- 0.03

ng/ml), A/Japan/305/57 (H2N2) (0.223 +/- 0.01 ng/ml), A/swine/New Jersey/11/76 (H1N1) (0.382 +/- 0.02 ng/ml), and A/Puerto Rico/8/34 (H1N1) (0.50 +/- 0.02 ng/ml).

Interleukin-10 was induced in small but important quantities after influenza virus infection if compared to similar induction by mitogen stimulated cells. Influenza A viruses induced a higher level of IL-10 than did influenza B virus. Among influenza A viruses, subtypes H3 and H1 induced a two-fold more IL-10 than did the H2 subtype. Similar patterns were observed with IFN-γ and IL-6 induction. Therefore, the surface hemagglutinin of subtypes H3 and H1 may be higher inducers of both Th1 and Th2 type cytokines.

4.5. Summary

According to my data, a more pathogenic influenza A virus induced a higher level of cytokines than did a less pathogenic influenza B virus. Furthermore, it appears that the hemagglutinin plays an important role in cytokine induction, by comparing H3N2 and H3N8 viruses. Even-though, they are different in their NA subtypes, they elicit a similar level of cytokines. The more divergent the viruses are, the higher the difference in the levels of cytokine induction. Influenza A and influenza B viruses surface HA1 are 31.5% homologous on the amino acid level, while some of the influenza A viruses are as homologous as 98.4% on the amino acid level. Furthermore, A/Eq/Prague/56 (H7N7) shares the closest homology to the influenza B virus, and it induces the lowest levels of cytokines among any influenza A virus tested. A summary of influenza virus HA1 percent homology is shown in table 4-1.

TABLE 4-1

HA1 PERCENT HOMOLOGY

	1	2	3	4	5	6	7	8	9	10
1		78.9	65.7	58.5	62.5	65.0	64.9	64.8	57.1	56.4
2	*71.6*		64.5	56.5	60.3	62.2	61.8	61.8	56.4	53
3	*41.6*	*42.0*		58.4	61.4	62.0	61.9	61.9	56.1	55.1
4	*40.8*	*35.4*	*40.0*		77.3	76.5	76.4	76.1	56.9	55.0
5	*38.4*	*35.8*	*39.2*	*84.8*		92.3	91.4	91.4	60.7	56.9
6	*40.0*	*35.8*	*35.9*	*79.4*	*89.5*		97.7	97.7	61.8	59.4
7	*40.4*	*35.0*	*36.3*	*78.6*	*88.4*	*96.9*		98.1	61.9	59.5
8	*40.4*	*35.8*	*36.7*	*78.6*	*88.7*	*98.4*	*97.7*		61.8	59.5
9	*32.4*	*31.5*	*32.2*	*38.2*	*39.4*	*37.8*	*38.2*	*38.2*		50.1
10	*27.6*	*28.0*	*27.4*	*28.8*	*29.6*	*31.0*	*30.2*	*31.5*	*23.6*	

The upper half, white background, represents nucleotide homology in HA1 portion of virus surface HA. The lower half, gray background, represents amino acid homology in HA1 portion of virus surface HA. Influenza viruses used in this study are compared with the exception of A/Panama/99 (H3N2) virus, whose HA1 sequence is not available. The influenza viruses are ordered numerically; **1)** A/Puerto Rico/8/34 (H1N1), **2)** A/swine/New Jersey/11/76 (H1N1), **3)** A/Japan/305/57 (H2N2), **4)** A/Aichi/2/68 (H3N2), **5)** A/Eq/Miami/1/63 (H3N8), **6)** A/Eq/Saskatoon/1/90 (H3N8), **7)** A/Eq/Kentucky/1/97 (H3N8), **8)** A/Eq/Kentucky/1/98 (H3N8), **9)** A/Eq/Prague/56 (H7N7), and **10)** B/Lee/40.

One exception to this general observation, is that H3 and H1 subtypes are more divergent from each other than they are from the H2 subtype. Despite this difference, H3 and H1 induced more cytokines than did the H2 virus. Therefore, it is not the overall homology among HA proteins that determines their inductive capacities, but rather the specific amino acid sequences of the surface protein may determine the level of cytokine induction.

A high mutation rate of influenza viruses could be associated with difference or similarity in cytokine induction among different subtypes of influenza viruses. For

example, similar induction of IL-6 by A/Japan/57 (H2N2) and A/Eq/Miami/63 was determined. However, the other H3 subtypes induced higher amounts than of IL-6 than did H2 subtype. This may have resulted from the evolutionary changes in H3 influenza virus subtypes since 1963. Furthermore, among human influenza virus subtypes H3, A/Panama/99 induced less IFN-γ, IL-4, and IL-6, than did its ancestor A/Aichi/2/68. On the contrary, it induced more IL-10. Long duration and circulation of the human H3 subtypes, as well as thirty-one years of antigenic drift from A/Aichi/2/68 (H3N2) to A/Panama/99 (H3N2) may be responsible for the appearance of a less pathogenic influenza virus. Exact factors are not clear. A possible explanation is that influenza virus subtype H3, due to long evolution in humans resulted in a "co-adaptation" of this influenza virus subtype with the host. Therefore, the new human H3 influenza virus subtypes, such as A/Panama/99, are possibly less pathogenic, hence inducing a weaker immune response and a lower cytokine induction.

Reduced immune response would most likely result in the humoral immune response development. Therefore, the Th2 types of cytokines would be primarily induced. As seen with our data, two out of three Th2 type of cytokines are secreted in high amounts, especially the IL-4. As shown by Sharma *et. al.* [78], and Moran *et. al.* [60], high induction of IL-4 impedes virus clearance by activation of humoral immune response. Therefore, it is possible that co-evolution between influenza virus and its host may result in "co-adaptation" by a higher induction IL-4.

CHAPTER V

CONCLUSIONS

The success of influenza viruses is attributed to their capacity to mutate rapidly. Current circulating viruses, especially the H3 subtypes, have been shown to mutate at high rates [7]. Pathogenicity and virulence has been attributed to virus evolution. antigenic drift allows the virus to "re-infect" an immune individual. Using phylogenetic analysis Bush *et. al.* [7], and Hillis [35], have shown that, by using specific criteria, evolution of H3N2 virus may be "predicted". However, the criteria used in their studies is not suitable to test any potentially pathogenic virus for humans. This study is a first attempt to analyze whether there is a correlation between cytokine induction and pathogenicity of influenza viruses.

5.1. Conclusions

Adult human PBL must be stimulated to produce cytokines. Following infection, the mitogen-like properties of the influenza virus HA protein induced simultaneous release of Th1 and Th2 cytokines. Furthermore, influenza A viruses induced higher levels of IFN-γ, IL-2, IL-4, IL-6, and IL-10 than did influenza B virus. Among influenza A viruses: the equine-2 (H3N8) subtypes induced higher levels of IFN-γ, IL-4, IL-6, IL-10 and IL-12 than did equine-1 (H7N7) subtype. Furthermore, H3 and H1 influenza

virus subtypes were found to be higher inducers of IFN-γ, IL-6, and IL-10 than were the H2 or H7 influenza virus subtypes. Therefore, I concluded that more pathogenic influenza viruses induce higher levels of cytokines than the less pathogenic viruses. Furthermore, I concluded that the influenza virus hemagglutinin plays a major role in cytokine induction and in pathogenicity.

5.1.1. Concluding remarks

In vitro testing of human adult PBL, equine naïve PBL, the human Jurkat T cells and human naïve umbilical cord blood showed similar patterns in both the types and levels of cytokines induced following influenza virus infection. Both types (Th1 and Th2) of cytokines are simultaneously induced. This is in agreement with other studies, that reported simultaneous induction of Th1 and Th2 cytokines by influenza virus infection *in vivo* [18, 39, 75]. Therefore, I suggest that *in vitro* testing of human umbilical cord blood lymphocytes or Jurkat T cell-line for cytokine induction is a good model to correlate to *in vivo* infections.

5.2. Significance

The ability to test cytokine induction with a new influenza virus could lead to prediction of its pathogenicity and its pandemic potential. Screening of newly isolated influenza viruses can be done along with current antigenic analyses. This could help in the development of a better vaccine. A new immunization technique, such as DNA vaccines, capable of eliciting persistent and longer-lasting humoral and cell-mediated immune response to a variety of viral antigens [71, 92] can be developmed. Furthermore,

a similar approach could be used to determine pathogenicity of pathogens other than influenza, resulting in prevention of the corresponding diseases.

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APPENDIX

OSU-IRB APPROVAL and CONSENT FORM

OKLAHOMA STATE UNIVERSITY
INSTITUTIONAL REVIEW BOARD

Date: December 21, 1999 IRB# AS-99-071

Proposal Title: "CYTOKINES INDUCTION BY INFLUENZA A VIRUS HA"

Principal Investigator(s): Alexander Lai

Reviewed and Processed as: Full Board

Approval Status Recommended by Reviewer(s): Approved

Signature: Carol Olson

Carol Olson, Director of University Research Compliance

12/21/99
Date

Approvals are valid for one calendar year, after which tie a request for continuation must be submitted. Any modification to the research project approved by the IRB must be submitted for approval with the advisor's signature. The IRB office MUST be notified in writing when a project is complete. Approved projects are subject to monitoring by the IRB. Expedited and exempt projects may be reviewed by the full Institutional Review Board.

CONSENT FORM

I. _____ , hereby agree, after delivering of my baby, to donate the umbilical cord blood for medical research.

The material will be used by a research laboratory at Oklahoma State University. The purpose of the research is to identify new influenza viruses. A unique feature of influenza virus is it ability to change, and that's why we need a "flu shot" every winter. Sometimes influenza viruses from birds can cause illness in humans. When this happens, many people will be sick and some may die. Birds, particularly ducks and geese, carry many different influenza viruses. The scientists at OSU, by using the donated cord blood, are trying to identify which bird virus causes illness in humans. Once the virus is identified, vaccine will be made. This will save many people from getting sick.

Specifically, cord blood will be drawn by my physician (or his/her assistant), and the sample will be sent to the research laboratory, and:

- There is no pain associated with the procedure
- There is no extra procedure, nor requiring extra time from my doctor
- The procedure will not increase the normal risk associated with delivery
- Donation of the cord blood will not transmit any infectious agents to me or to my baby
- No medical history, nor any identification will be attached to the cord blood
- The cord blood will be used for medical research only

I understand that my agreement to allow the donated material be used for medical research is voluntary. It is for the benefit of scientific discoveries, as part of an investigation tilted " Cytokines induction by influenza A virus hemagglutinin". The research project is headed by Dr. Alexander CK Lai. The research is approved by the OSU Institutional Review Board.

If I have further questions, I may contact Dr. Alexander CK Lai at (405) 744-4449. I may also contact Ms. Sharon Bacher, IRB Executive Secretary, 203 Whitehurst, Oklahoma State University, Stillwater, OK 74078; or by phone at (405) 744-5700.

I have read and fully understand this consent form, and that my doctor, _____, has fully explain the purpose of the study and answer all my questions. I sign it freely and voluntarily. A copy has been given to me.

Signature Date

VITA

Senad Divanovic

Candidate for the Degree of

Master of Science

Thesis: CYTOKINE INDUCTION BY INFLUENZA VIRUS: A POSSIBLE CORRELATION TO PATHOGENICTIY

Major field: Microbiology, Cell and Molecular Biology

Biographical:

Personal Data: Born in Sarajevo, Bosnia and Herzegovina, August 25, 1976, son of Nahida and Dr. Kucuk-Alija Divanovic.

Education: Graduated from Memorial High School, Tulsa, Oklahoma, in May 1994; received Bachelor of Arts Degree in Biology and German from DePauw University, Greencastle, Indiana in May 1998; completed requirements for the Master of Science Degree at Oklahoma State University in December 2000.

Professional Experience: Teaching Assistant, Department of Microbiology and Molecular Genetics, Oklahoma State University, August 1998 to May 2000. Research Assistant, Department of Microbiology and Molecular Genetics, Oklahoma State University, June 2000 to August 2000.