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Review Article

Proteins of Influenza Virus: A Review

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ABSTRACT

Influenza virus infections are important both epidemically as well as pandemically and have great threat to avian species and humans especially, from last few decades. Certain pandemics of influenza virus have been reported causing severe damages. Once the host infected with influenza virus, it becomes immune-compromised and susceptible to secondary bacterial infections. Genome of influenza virus consists of eight segments of RNA, producing eleven types of proteins. Virulence and pathogenecity greatly depend upon its surface glycoproteins such as neuraminidase (NA) and hemagglutinin (HA). Other proteins of influenza virus are nucleoprotein (NP), 2 types of matrix proteins (M1 and M2), 2 non-structural proteins (NS1 and NS2), and polymerase subunit (PA) i.e. PB1, PB1-F2 and PB2. NP and M proteins are responsible for further classification of Influenza virus into influenza virus A, influenza virus B and influenza virus C. The aim of the review article is to discuss the structural importance of proteins of influenza A viruses.

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INTRODUCTION

Influenza virus is involved in epidemic and pandemic diseases particularly respiratory system of both humans and animals. In 1918, influenza pandemically disturbed 50 million people and is still considered a great threat (Boivin *et al*., 2010). It belongs to family *orthomyxoviridae* and genus *influenzavirus A*. Influenza virus is an envelope, negative sense, single stranded virus with segmented RNA that produces 11 proteins (Hale *et al*., 2008). Waterfowls are considered to be the natural host for influenza A viruses (Jourdain *et al*., 2010). Virulence and pathogenecity of influenza virus greatly varies with its surface glycoproteins such as neuraminidase (NA) and hemagglutinin (HA) (Tumpey *et al*., 2005). NAs are nine in number while haemagglutinin proteins (HA) are sixteen in number (Fouchier *et al* 2005: Glaser *et al*., 2005: Yen *et al*., 2008). Other proteins of influenza A viruses include, nucleoprotein (NP), two types of matrix proteins i.e. M1 and M2, two non-structural proteins, i.e. NS1 and NS2 also called as nuclear export proteins (NEP), polymerase subunit (PA), PB1, PB1-F2 and PB2 (Ghedin *et al*., 2005). Based on the antigenic differences in NP and M, influenza viruses can be classified into three genera: influenza virus A, influenza virus B and influenza virus C. Two others, still not well accepted genera include isavirus and thogotavirus (Potter., 1998). Influenza A is mainly concerned with pandemic infections. It is reported that influenza viruses are pleomorphic in nature with mean diameter of 100–150 nm and the shape varies from filamentous to spherical (Roberts and Compans., 1998).

Certain pandemics have been reported most of which are; H1N1 Spanish flu in 1918, H2N2 Asian flu reported in Singapore in 1957, H2N2 epidemic in Japan in 1962 and Taiwan in 1964, H3N2 in Hong Kong flu in 1968 (Matrosovich *et al*., 2000; Shimada et al., 2009), H1N1 swine flu in Jersey in 1976, H1N1 Russian flu in USSR in 1977, H1N1 Swine flu in California in 2009 (Garten., *et al*., 2009: Smith *et al*.,2009). Influenza virus involved in immunosuppression was first time reported in 1908 (Kantzler *et al*., 1974). After infection with influenza virus, host becomes immune-compromised and susceptible to not only secondary bacterial infections but also other concurrent infections. Influenza virus damages the respiratory epithelial cells that ultimately result in invasion of secondary bacterial infections. Moreover, it was reported that Haemagllutinin (HA) was involved in inhibition of LPS-induced interleukin 12 (IL12) and p70 at transcriptional level making environment suitable for secondary bacterial infections (Noone *et al*., 2005). It has also been reported that influenza infections are fatal in patients having cardiac and pulmonary infection (Steininger *et al*., 2002). Influenza virus adapts different ways to circumvent host immune system, either by down regulation of host immune system or by enhancing its replication process to counteract with host immune system.

The basic purpose of this review article is to highlight the structural importance of proteins of influenza A viruses in relation to pathogenic standing, virulence and immunosuppression. Discussion will be made related to each protein of influenza A virus and their relationship with virulence; immune response of host, antigenic and pathogenic nature of virus.

LIFE CYCLE

The detailed life cycle of influenza virus has been elaborated in Figure 1. There are three surface proteins of the influenza virus i.e. M2 protein, hemagglutinin (HA), and enzyme sialidase (NA). Influenza virus is an enveloped virus, it binds to α2-6 sialic acids (human influenza viruses) and to α 2-3 sialic acids (avian influenza viruses) via HA surface glycoproteins (Thompson *et al*., 2004). The enzymatic action of breaking α-ketosidic linkages by Neuraminidase (NA) (Hughes *et al*., 2000) leads the virus to enter into cell by endocytosis. This endocytosis occurs through receptor-mediated mechanism. After entry, virus binds with endosomal membrane. Endosome changes into lysosome due to decrease in pH up to 6 and HA

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molecule unfolds resulting into exposure of hidden hydrophobic chain of HA known as fusion peptide. Finally viral nucleocapsid releases into cytosole (Wharton *et al*., 1995) of the host cell. After release of nucleocapsid, protein synthesis occurs in cytoplasm. Finally virus releases via budding. sialidase elutes virus from cell surface and virus can now infect other cells (Itzstein, 2007).

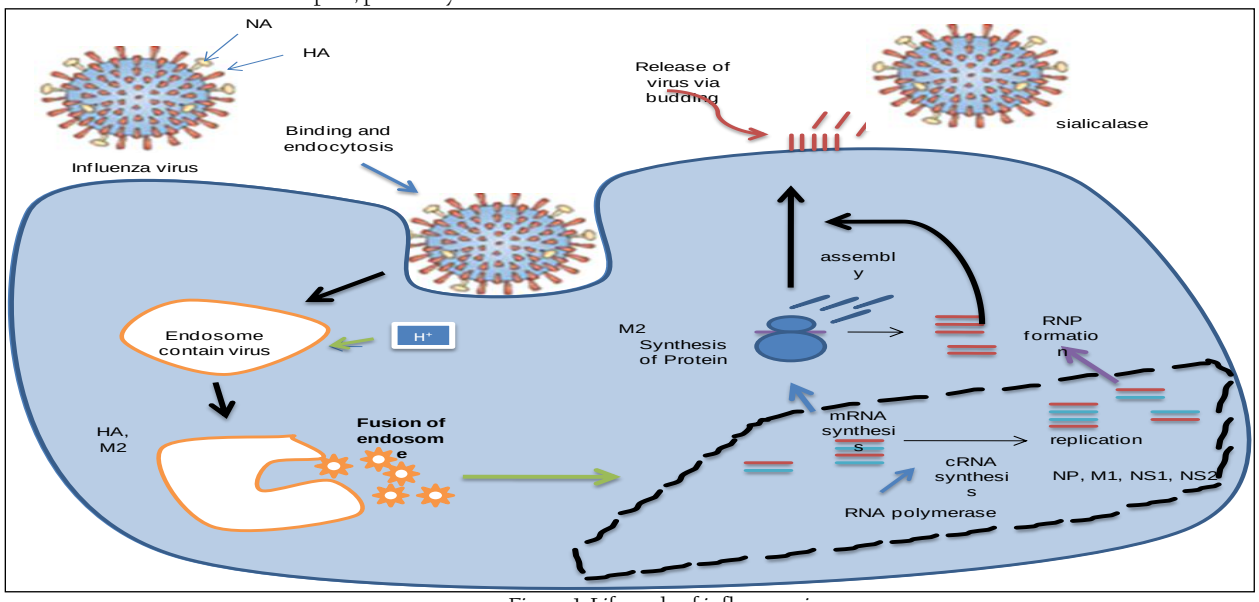

Figure 1: Life cycle of influenza virus

HEMAGGLUTININ (HA)

Influenza hemagglutinin (HA) is a glycoprotein, presented on the surface of virus. The molecular weight of HA is 165447.31 Dalton. Three chains H, J, L for polymer 1 with 328 amino acids and three chains I, K, M for polymer 2 with 160 amino acids have been reported. Extinction coefficient and theoretical pI have been calculated as 270060 and 6.15, respectively (Gamblin *et al*., 2004). HA protein has trimer structure, 135 Å at its tip. There are three subunits with two chains, so from final six chains, three are HA1s and three are HA2s. Monomer of HA1 consists of 328 residues, with elongated structure up to N-terminus of viral membrane; it ends after turning down at C-terminus. Distal portion of HA1 is jellyroll motif with eight anti-parallel beta sheets; α helix via forming loop separates jellyroll at position 3 and 4 forming pocket of sialic acid at top. Part of the distal end of HA1 forms an 8-stranded anti-parallel beta-sheet motif termed a "jelly roll." A short α-helix forms in the loop that separates 3rd and 4th strands of the jellyroll. HA2 consists of 221 residues with two anti-parallels α-helix having size of 75Å each. Single disulphide bond forms connection between HA1 and HA2. Whole molecule is stabilizing through van der Waals force (Wilson and Wiley., 1981).

There are four antigenic sites (Figure, 2) on hemagglutinin monomer; site A with 8 Å down from surface of molecule, site B binds externally to the residues of α-helix forming pocket or sialic acid binding, site C with 60 Å from lower tip of molecule, and site D with two β-sheets in jelly roll. Finally it can be concluded that any mutation in these antigenic sites can be changed in to virulent and antigenic properties of influenza virus (Schweiger *et al*., 2002). Influenza virus causes agglutination of red blood cells due to its hemagglutination ability. Based on which HA inhibition test is used for determination of virus titter (Madeley *et al*., 1971). The HA is also the receptor-binding site of the virus to initiate the infection as discussed before. Presently, 16 subtypes of HA are known ranging from H1 to H16. Recently, H16 was discovered from influenza A virus in black headed gulls in Sweden and Norway (Fouchier *et al*., 2005). The H1, H2, H3 are more prevalent in humans. H5N1 is more pathogenic in humans. It has also been studied that mutation in single amino acid of H5 of avian origin can change receptor acceptance to H5N1 in humans (Suzuki, 2005; Gambaryan *et al*., 2006).

NEURAMINIDASE (NA)

Three dimensional structure of NA at 2.2 Å indicates that it has single polypeptide chain A, four domains, with molecular weight of 46502.45 Dalton, Theoretical pI: 6.48 and Ext. coefficient of 85005 (Varghese and Colman, 1991). Structurally NA is a spikes square boxlike head and a stalk on surface of virus envelope. NA can also identify sialic acid (Itzstein *et al*., 1993) and has a role in catalyzation and cleavage of α-ketosidic linkage in terminal sialic acid residue and neighbouring residue of carbohydrate chain (Lentz and Air, 1986), so helping for the release of virus which results into spread of infection (Schulman and Palese., 1977). Active site of NA is present on its head (Colman *et al*., 1983). As a result of repeated infection, mutation occurs (Shu *et al*., 1993) due to which nine subtypes of NA has been developed (Kendal, 1987). There are two groups of NA: group 1 consists of, N1, N4, N5 and N8 while group 2 consists of N2, N3, N6, N7, and N9 (Russell *et al*., 2006).

The NA is glycoprotein and it is involved in antigenic variations such as antigenic drift or shift (Webster and Laver., 1975; Webster *et al*, 1982). The mutations have important role in circumventing immune responses. Such a shift was reported in NA of influenza A in 1957, which resulted into Asian influenza (H2N2) (Deshairs *et al*., 1986). Mutations in NA results into drug resistance so there is always need to improve drugs and vaccine with the passage of time (Thompson *et al*., 2004). Currently, there is an upsurge in the research to explore the possible mechanisms behind this resistance development especially for the drugs targeting the NA like Oseltamivir (TamiFlu).

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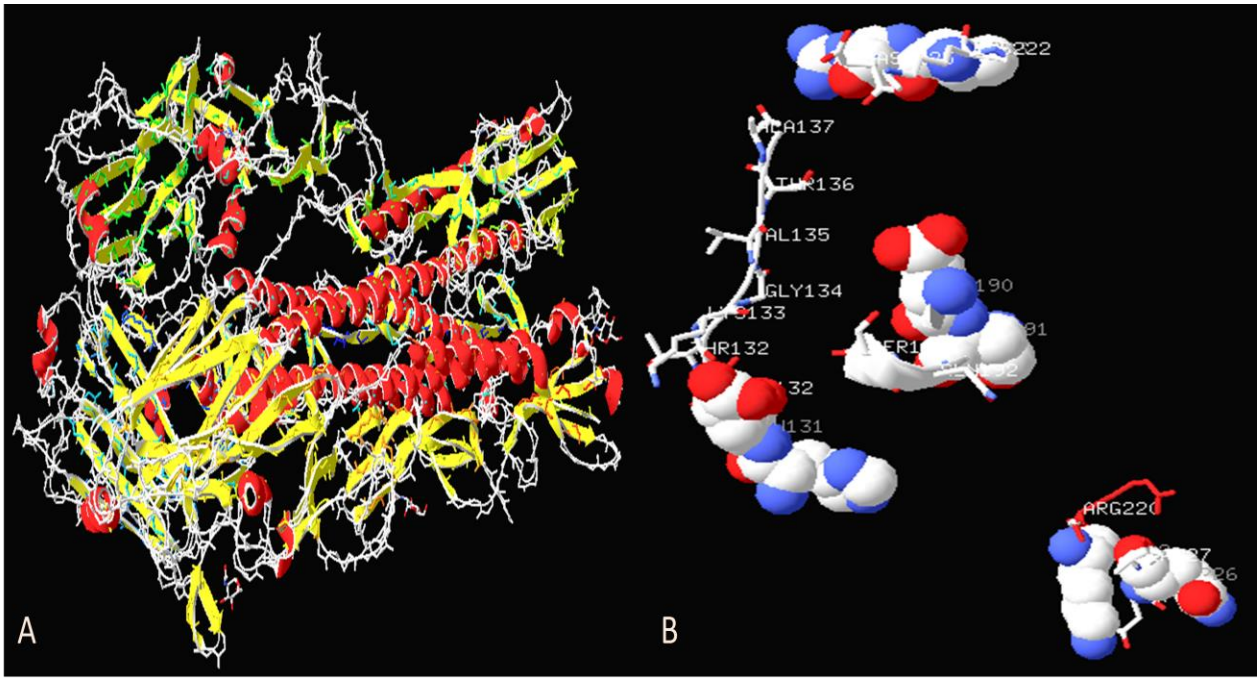

Figure: 2 indicate protein structure of HA. (A) Shows ribbons in red colour and helix in yellow colour while loops and side chains are indicated with white colour. (B) Shows four antigenic sites of HA.

NUCLEOPROTEINS (NP)

The NP is classified as viral nuclear proteins with molecular weight 170419.81 Dalton. It is a polypeptide nucleocapsid protein having one polymer, three chains i.e. A, B and C, with total length of 499 amino acids, theoretical pI: 9.24 and ext. coefficient of 167175. It is reported that at 3.2 Å, crystalline structure indicates that shape of protein is crescent like with a head, body and a flexible tail loop for attachment (Tarus et al., 2012). The RNA binding grove is present in the middle of head and body, outside the NP oligomer. There is a binding site for viral polymerase at body domain of NP. Furthermore tail loop can be a potent point for antiviral advancement (Ye *et al*., 2006). Eight RNA segments are rolled on NP subunits resulting formation of viral ribonucleoprotein (vRNP).

NP is positively charged, basic protein, present in 5th segment of mRNA (Hutchinson *et al*., 2009) and required for replication of virus (Huang *et al*., 1990). It is reported that using 32p orthophosphate labelling NP shows phosphorelation and has a role in viral replication and monitoring of viral yield (Kistner *et al*., 1989). Gene replacement shows that NP proteins are involved in host range determination (Altmuller *et al*., 1989) and site-specific phosphorylation (Kistner *et al*., 1985). It was studied on nucleoprotein primed mice that priming of NP could not stop infection but it can help in recovery via cross- reactive cytotoxic T cells (Wraith *et al*., 1987), CD_8 T-cell gives cross-defence to influenza A (Zhong *et al*., 2010). Terminal part of 5th segment is involved in packaging of reporter genes. This transports between cytoplasm and nucleus via signals called nuclear localization signals (NLSs) (Neumann *et al*., 1997). Two NLSs have been reported for NP. NLS_1 is conscientious for NP attachment to karyopherins α_1 and α_2 (Palese and Neill, 1997), found in residues 3 and 13. NLS_1 is not only required for nuclear localization of NP but also involved in transport of vRNA to nucleus. NLS_2 can be found at residues 198 to 216 in heart of NP and it functions as reporter protein and vRNA transcription (Weber *et al*., 1998). Moreover, NLS_2 is essential for viral replication despite of NLS_1 (Ozawa *et al*., 2007).

MATRIX (M) PROTEINS

M gene is responsible for matrix proteins including M1 and M2 proteins and membrane proteins. Segment 7 of influenza virus encodes for these M proteins. The size of M gene is 1027 bps. Furthermore M1 is found at 26 to 784 and M2 is located on nucleotide position 26 to 51 and 740 to 1007 (Lamb *et al*., 1981). These two proteins are discussed below.

M1 PROTEIN

The X-rays crystalline structure at pH 4.0, and 2.08-Å resolution of influenza A M1 protein indicates that N-terminal ranging amino acid from 2 to 158 is dimeric in shape with positive charge on its hydrophobic surface (Harris *et al*., 2001). The M1 protein is located at the inner side of virus in the shape of dimmer, creating a link among membrane proteins and inner core. The M1 has L domain motif that has important role in the assembly of viral part and budding of virus (Nayak *et al*., 2004). Gomez-Puertas *et al*., (2000) demonstrates that M1 proteins are involved in formation of virus-like particles i.e. VLPs instead of ribonucleoproteins. Function of M1 protein along with vRNA is not only the assembly of RNP in nucleus but it also transports it to cytoplasm (Huang *et al*., 2001). Latex agglutination test has been developed using chicken serum against M1 protein for identification of antibodies against influenza strains such as H3, H5, H7, and H9 (Wang *et al*., 2010).

M2 PROTEIN (The ion channel protein)

M2 protein has total 97 amino acids (Zebedee and Lamb, 1989) out of which there are five main amino acids at position 26, 27, 30, 31, and 34 that are important for drug resistance especially against adamantine (Liu *et al*., 2010). M2 is expressed on cell surface and integrated in virus. M2 is important for replication of virus at lower levels.

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In nineties, it was believed that M2 protein is concerned in assembly of viral particles (Zebedee and Lamb, 1989), but latter it was also reported that M2 proteins was involved in regulation of ion channels particularly of monovalent ions. Ion channel function is concerned with acidification, which is vital phenomenon for virus entry. Hydrogen ions break linkage of vRNP and M1 resulting into RNP entry into cytoplasm after endocytosis of virus (Chen *et al*., 2008). Mutation in M2 protein can be resulted in drug resistance because of disturbance in ion channels regulation. Studies have shown that M2 protein forms trans-membrane domain. M2 ion channels are also affected by pH (Pinto *et al*., 1992). In Asia and USA anti-viral drug resistance against AIV has been reported due to single amino acid replacement i.e. serine to Asparagine (S31N) mutation in M2 protein (Nelson *et al*., 2009).

PB1, PB2 and PA PROTEINS

PB1 is located on segment 2 of the mRNA of influenza virus with two overlapping open reading frames (ORF) including PB1 and PB1-F2. These are located in mitochondria of eukaryotic cells and are responsible for intracellular localization of PB. It is also reported that another polypeptide is synthesized from second segment of mRNA named as PB1 N40 (Wise *et al*., 2009). PB1-F2 is made up of 90 amino acids, and is located in cytosol, nucleus and mitochondria especially in infected cells. It has been reported that PB1-F2 is involved in forming protein channels in mitochondrial membrane and for this function; c-terminal has great importance (Henkel *et al*., 2010). Khiabania *et al*., (2009) found that PB1 had a role in re-assortment events equally via inter and intra host. They also found that re-assortment patterns were same in pandemic influenza strains of 1957 and 1968 in humans. Human H2N2 and H3N2 re-assortment was only due to PB1 segment. Interaction of PB1 by its both N- and C-terminal with PA and PB2 resulting into heteromeric polymerase complex has been reported. Along with these traits, PB1 also has conserved RNA-dependent RNA polymerase motifs, viral RNA, complementary RNA, and nucleotide binding domain. Therefore, re-assortment and adaptation may be due to viral polymerase genes, which were later proved by Li *et al*., (2009). It was studied that PB1 may be absent in many influenza viruses leaving question mark on its importance in viral survival. So evolution of PB1 studies on truncated PB1 from different isolates of virus shows that it has effect on replication, and virulence but studies are needed to understand the effect of PB1-F2 on virus fitness (Trifonov *et al*., 2009). Structural weight of PB1-PA complex is 63530.73 Dalton. PB1-PA has two polymer chains, polymerase acidic protein polypeptide L chain A with 478 amino acids and RNA-directed RNA polymerase catalytic subunit polypeptide chain B with length of 81 amino acids (Obayashi *et al*., 2008).

The vRNA is catalyzed via complex processes with the help of viral RNA-dependent RNA polymerase that is made up of trimeric complex having three subunits, PB1, PB2 and PA in nucleus (Deng *et al*., 2005). PB2 has role in viral transcription via N terminal and is a cap binding protein (Gastaminza *et al*., 2003). Main protein is PB1 on which N-terminal is attached with PA and C-terminal is attached with PB2. One group of thought believed that PB1 and PA interacts in cytoplasm and then moves to nucleus with the help of RanBP5 while PB2 enters in nucleus, as monomer with help of Hsp90 (Momose *et al*., 2002) and then interacts with PB1 and PA complex, resulting in functional unit of PA-PB1-PB2 (Naito *et al*., 2007). Second opinion is that the PB1 and PB2 first intermingles in cytoplasm and enters in nucleus via Hsp90 protein and then PA protein attaches in the nucleus resulting in formation of functional complex but this concept is controversial. Hemerka *et al*., (2009) has developed a method called Bimolecular Fluorescence Complementation Assay (BiFC) which can analyze both PA-PB1 and PB1-PB2 interaction and have demonstrated that both are formed in cytoplasm and then travels to nucleus. They have also demonstrated that 100 amino acids on N-terminal region of PA protein are responsible for attachment to PB2 (Hemerka *et al*., 2009). PA, PB1, and PB2 are polymer in nature and along with NP; these are involved in forming ribonucleoprotines (RNP). RNPi further helps in RNA transcription, and replication (Ka-Leung *et al*., 2008)

Recent research shows that PB2 has role in inhibiting expression of interferon-β but its exact mechanism is unknown in addition of major virulence of influenza (Graef *et al*., 2010). PB2 is required for replication of virus and integral part of viral polymerase complex. PB2 has a role in determining host range and pathogenicity, proved by using squirrel monkeys, mice, mammalian cells, and pig (Manzoor *et al*., 2009). PA is phosphoprotein and functions as protease due to third amino terminal present closely to its nuclear localization signals. Mutations in PA result in down regulation of cRNA production from vRNA as polymerase action decreases.

NON-STRUCTURAL PROTEINS (STRUCTURE AND FUNCTIONS)

There are two non-structural proteins of influenza virus, i.e. NS1 and NS2 produced from 8th segment of RNA. These proteins are known as non-structural proteins as these proteins are produced in infected cell without integration into virion. These proteins are discussed in detailed as below.

NS1

NS1 protein has many functions with respect to virulence, importance in the disease pathogenesis, and studying host pathogen relationship. There are two domains of NS1 protein, i.e. N-terminal structural domain also known as RNA-binding domain (RBD) and effector domain (ED). The RBD has function in virus protection from host immune response via restricting cellular anti-viral proteins of special interest is 2'-5'-oligo (A) synthetase/RNase L pathway so inhibiting INFα/β production (Krug *et al*., 2003). Effector domain prevents development of host antiviral activity at cellular mRNAs level via preventing attachment and removal of mRNAs and polyadenylation specificity factor (CPSF) resulting in to inhibition of poly(A)-binding protein (PAB II) activity (Wang *et al*., 2002). It has also been reported that two domains of NS1 has a role in interaction with RNA and cellular proteins.

One most studied and important role of NS1 is to circumvent the host immune response via antagonizing IFN during viral replication. NS1 inhibits the production of IFN-inducible antiviral proteins: 2-5-oligoadenylate synthetase and dsRNA-dependent protein kinase R (PKR). dsRNA is required for the activation of 2´-5´OAS and PKR. The physical binding of NS1 to dsRNA results in inhibition of cellular 2-5 OAS and also in the inhibition of activation of PKR (Reviewed in Hale *et al*., 2006).

NUCLEAR AND CELLULAR LOCALIZATION OF NS1 PROTEIN

NS1 molecules travel between cytoplasm and nucleus of eukaryotic cells via nuclear pores. Nuclear localization depends on many factors including cell type, cell signals or nuclear localization signals (NLS), nuclear export signals (NES), time of exposure, and expression of NS_1 protein, virus strain, and polarity of cells (Wen *et al*., 1995). Two nuclear localization signals have been reported for NS1 protein named as NLS1 and NLS2. NLS1 contains amino acids Asp-Arg-Leu-Arg-Arg codons

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from 34 to 38 and NLS2 is prevalent in region 203 and 237 amino acids (Greenspan *et al*., 1998).

NS1 has effect on nuclear post-transcriptional events either by inhibiting export of poly A mRNAs or via inhibiting splicing of pre mRNA. On the other hand, NS1 also effects viral mRNA translation in cytoplasm due to the binding of viral RNA with NS_1 protein. This will affect phosphorylation of eukaryotic translation initiation factor 2α. NS1 protein contains NES which has similar hydrophobic spacing particularly leucine. Furthermore, replacement of alanine with leucine abrogates activity of nuclear export (Yongzhong *et al*., 1998).

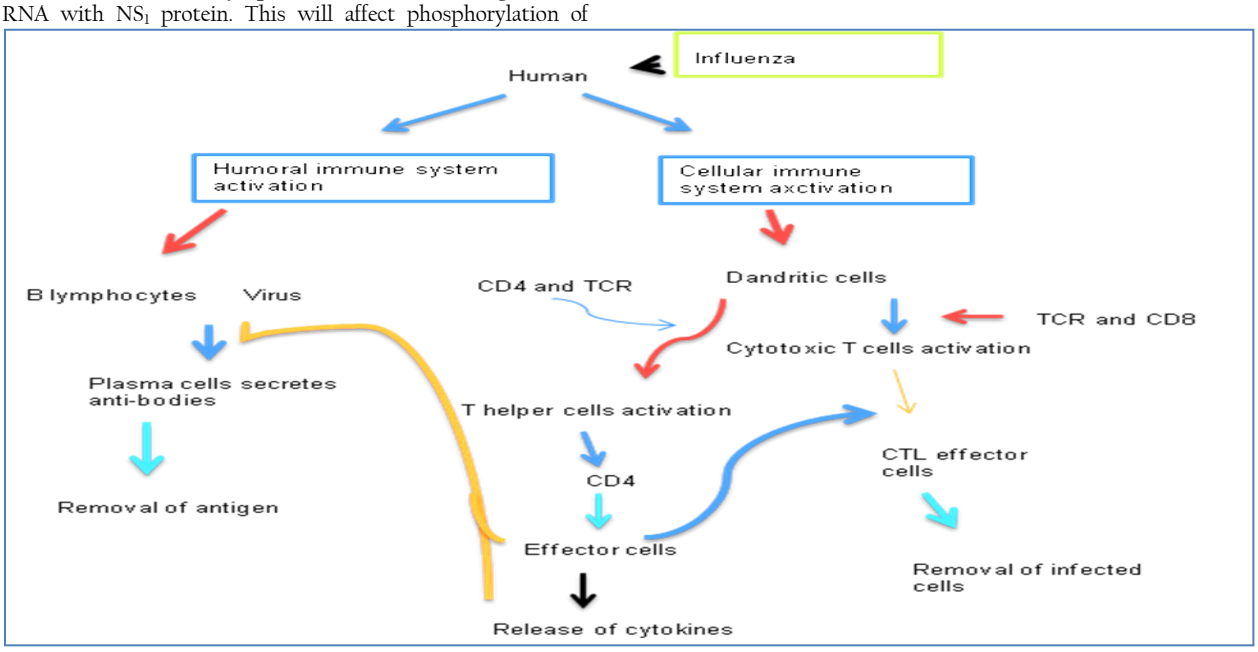

Figure 3: Presentation of both humoral and cellular immune response against influenza virus. Humoral system consists of B-lymphocytes, producing antibodies via plasma cells in response to influenza infection (left side of figure). On the other hand, cellular immune response initiates via antigen presentation by MHC I and II that further take action by dendritic cells by activation, production and separation of specific T cells i.e. CD^{4+} or CD^{8+}. These cells then release cytokines via effectors cells (rite side of figure), which finally kills antigen (Modified from Flint *et al*., 2004).

NS2

NS2 is composed of 121 amino acids and translates from NS gene through mRNA splicing. It is reported that NS2 can be found in purified virus, and interacts with M1. NS2 along with M1 participates in viral RNPs nuclear export (Lommer and Luo, 2002; Iwatsuki-Horimoto *et al*., 2004), so NS2 is also known as nuclear export signal. Crm1 participates in this mechanism. Hence NS2 mediates interaction of M1 via its C-terminal and Crm1 by its N-terminal. In addition to these functions, it is also reported that NS2 alters RNA level resulting in decreased accumulation of transcription products and elevates the levels of viral replication hence it regulates viral transcription and translation (Robb *et al*., 2009).

EXPRESSION OF MX PROTEIN AND INTERFERON

Interferon alpha/beta (IFN-α/β) has relation with host defence system. There are three proteins involved in this system including protein kinase R, oligoadenylate synthetase, and Mx proteins (Stark *et al*., 1998). Mx protein is widely expressed as a response of viral infection in manyof the vertebrates. There are two types of Mx, including MxA and MxB found in nucleus and cytoplasm. It is reported that Mx protein and IFN-α/β shows its expression against influenza infection (Jung and Chae, 2006).

MECHANISM OF IMMUNITY IN INFLUENZA AND RECOVERY VIA CYTOKINES

In influenza virus infection, body response to combat the viral infection comes in the form of cell-mediated immunity. HA are main proteins involved in immunogenicity via humoral immunity. During influenza infection, antibodies against HA not only increase but also transfer to next generation passively, which ultimately enhance recovery. It is also reported that antibodies against NA have also a role in decreasing virus load and cell-to-cell proliferation. Experiments showed that antibodies produced against M1 and NP are not so important in recovery (Jakeman *et al*, 1989). Both HA and NA are responsible for neutralizing antibodies due to wide variations in their antigenic nature (Fu-Shi Quan *et al*., 2008).

It was believed that T cells are mainly involved in handling infections of influenza but experiments on mice proved that B cells were also involved in addition to T cells. So it is better to produce those vaccines that are capable to produce memory T cells and antibodies (Rangel-Moreno *et al*., 2010). Details of immune system activation and removal of influenza virus and infected cells have been described by flint *et al*., (2004) and has been modified in figure 3.

CONCLUSIONS

Avian influenza occurrence is a reminder of our helplessness for emerging pandemic diseases. Vast verity of genetic shift and drift makes influenza virus dynamic, not only in causing infections but also producing mutations. Influenza virus contains eight segments of RNA, producing different eleven types of proteins. Every protein has unique characteristic and

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function in relation to virulence, pathogenic activity, and immunosuppression. Functional relationship of these proteins and immune system enables us to produce drugs and vaccines against mutated viruses like influenza virus. This review highlights the importance of influenza proteins in survival and development of virus with changing environment and its contribution in development of infection.

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