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Interferons and Alphavirus Pathogenesis:

Implications for Developing Medical Countermeasures

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Defence R&D Canada – Suffield

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Abstract

Our immune system can launch a quick defence against a large number of unforeseen viruses. This front line of defence restricts virus spread and buys time for our body to develop specific and long-lasting immunity. One of the major weapons in this defence system is interferons (IFNs) that are proteins with broad-spectrum antiviral activity. Therefore, understanding the interplay between IFNs and viral agents may provide an insight into the development of a single medical countermeasure effective agasint multiple viral agents. As an initial step to tap the IFNs for developing therapeutics against multiple strains of alphaviruses, this memo presents recent advances in studying the role of IFNs in alphavirus pathogenesis. I first review IFNs, then focus on IFN responses against three BW agents in the alphavirus family: Venezuelan equine encephalitis virus (VEEV), eastern equine encephalitis virus (EEEV), and western equine encephalitis virus (WEEV). I conclude with discussion of how to apply this knowledge for therapeutic and vaccine development.

Résumé

Notre système immunitaire est capable de déployer rapidement des défenses contre un grand nombre de virus imprévus. Cette défense de première ligne consiste à limiter la dissémination du virus et donne le temps à notre corps de développer une immunité spécifique et durable. Une des armes les plus importantes de ce système de défense sont les interférons (INF) qui sont des protéines ayant une activité anti-virale à large spectre. Une meilleure compréhension de l'action réciproque entre les INF et les agents viraux pourrait ainsi favoriser la mise au point d'une contre-mesure médicale unique efficace contre des agents viraux multiples. L'étape initiale de l'étude des IFN pour la mise au point d'une thérapeutique contre les souches multiples d'arbovirus du groupe A consiste à présenter dans ce mémo les progrès récents accomplis par l'étude du rôle des INF dans la pathogenèse des arbovirus du groupe A. Je vais premièrement examiner les INF puis me concentrer sur les réponses des INF contre trois agents de guerre biologiques appartenant à la famille des arbovirus A : le virus encéphalitique du cheval de Venezuela (VECV), le virus de l'encéphalite équine de l'Est (VEEE) et le virus de l'encéphalite équine de l'Ouest (VEEO). Je conclue avec une discussion au sujet de l'application de ces connaissances à la mise au point d'une thérapeutique et d'un vaccin.

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Executive Summary

IFNs were discovered more than 45 years ago as proteins that interfere with virus growth. Since their discovery, a number of different kinds of IFNs have been found. IFNs are classified into three types according to different cellular receptors to which IFNs bind. IFN-alpha (IFN-α) and IFN-beta (IFN-β) are two major members in the type I IFN family. These two IFNs play a central role in front line of defence against virus invasion. Thus, this memo focuses on these two IFNs in the pathogenesis of VEEV, EEEV, and WEEV.

Overview of IFN-α and -β

IFN-α and -β are synthesized immediately after virus infection. To detect the virus infection, cells deploy different molecular sensors along the pathway of viral invasion. These sensors send off signals to warn the host of virus attack and allow cells to produce IFN-α and -β before viruses get a foothold in the host. IFN-α and -β are produced first as precursor proteins that contain secretory signal sequences. These sequences are cleaved off before mature IFN-α and -β are secreted from the cells. In humans only one species of IFN-β has been discovered, however, for IFN-α, there are a total of 13 species. In general, little differences in biological functions are found among different species of IFN-α. It remains unclear why we need so many IFN-α subtypes. A possible explanation is that multiple IFN-α species might allow cells to better control and regulate IFN-α production.

IFN-α and -β themselves do not have a direct effect on virus replication instead they stimulate cells to express varieties of genes that suppress viral replication. Not surprisingly, viruses have evolved various strategies to counteract IFN-α and -β. These strategies, which help viruses establish infection in host and significantly enhance their virulence, include blocking IFN synthesis, binding and inactivating IFNs, inhibiting IFN-induced gene expression, and neutralizing IFN-induced anti-viral proteins.

IFN-α and -β and alphavirus infection

Alphaviruses are a large family of RNA viruses that share a common structure: a lipid bilayer envelope bearing viral glycoproteins, underneath this, a protein capsid, and inside the capsid, a viral RNA genome. VEEV, EEEV, and WEEV, members of the alphavirus family, are considered as potential BW agents due to their high infectivity via aerosol. Current medical countermeasures against VEEV, EEEV, and WEEV are inadequate. No commercial vaccines and anti-viral drugs are available and treatment only focuses on easing clinical symptoms.

The importance of IFN-α and -β in early protection against VEEV and EEEV infections is demonstrated in mice that lack IFN-α and -β activities. These mice are highly susceptible to VEEV and EEEV infections and have high mortality rate after infection. Virulence of different strains of VEEV and EEEV is also correlated to their sensitivity to IFN-α and -β. For example, in VEEV, enzootic strains produce little or no viremia and little disease, while epizootic strains achieve high-titer viremia and cause encephalitis and are responsible for major epidemics in humans and horses. It was reported that resistance to IFN-α and -β

may be one of the hallmarks of virulence of epizootic strains of VEEV. Mutations in the 5' noncoding region of the viral genome and in viral E2 glycoprotein have been identified as molecular determinants of VEEV virulence. So far little is known about the possible involvement of IFN-α and -β in virulence of different strains of WEEV.

IFN-α has long been used as a drug for treating patients with hepatitis C and AIDS. The therapeutic potential of IFN-α for VEEV was demonstrated in mice. IFN-α conjugated with polyethylene glycol (PEG) protects mice from either a subcutaneous or an aerosol infection of virulent VEEV.

Conclusion

Understanding the role of IFN-α and -β in the pathogenesis of VEEV, EEEV, and WEEV will shed light on the development of therapeutics and vaccines. IFN-α and -β possess an immediate, broad-spectrum antiviral activity. Thus, they have a great potential in prevention and rapid control of acute infections caused by multiple strains of alphaviruses. Combining IFN-α and -β therapy with vaccination may provide a quick and long-lasting protection. Because certain viral genes relating to counteraction of IFN activity can be the major determinants of virulence, identification of these genes and subsequent disruption of them through recombinant DNA technology should allow us to design better live attenuated vaccines for VEEV, EEEV, and WEEV. Indeed, this approach has been successfully applied to design vaccines against influenza and respiratory syncytial viruses.

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Sommaire

Les INF ont été découverts, il y a plus de 45 ans, comme étant des protéines qui interfèrent avec la croissance d'un virus. Depuis cette découverte, on a trouvé plusieurs sortes d'INF que l'on a classifié en trois types selon les différents récepteurs de cellules auxquels s'attachent les INF. Les INF-alpha (IFN-α) et INF-bêta (INF-β) sont les deux membres les plus importants de la famille INF du type I. Ces deux INF jouent un rôle central sur la première ligne de défense contre l'invasion des virus. Ce mémo se concentre donc sur ces deux INF dans la pathogenèse des VECE, VEEE et VEEO.

Un aperçu des INF-α et -β

Les INF-α et -β sont synthétisés dès l'infection par le virus. Pour détecter l'infection par le virus, les cellules déploient des capteurs moléculaires différents le long de la voie de l'invasion virale. Ces capteurs envoient des signaux d'arrêt pour avertir l'hôte que le virus attaque et ils permettent aux cellules de produire des INF -α et -β avant que les virus ne soient ancrés dans l'hôte. Les INF -α et -β sont d'abord produits comme protéines précurseurs contenant des séquences secrétoires de signaux. Ces séquences sont coupées avant que des INF -α et -β mûrs soient secrétés à partir des cellules. Chez les humains, une seule espèce de INF -β a été découverte mais en ce qui concerne les INF -α il existe un total de 13 espèces. Normalement on ne trouve que peu de différences entre les fonctions biologiques des différentes espèces de INF -α. Nous ne comprenons toujours pas très bien pourquoi nous avons besoin de si nombreux sous-types de INF -α. Une des explications possibles consiste en ce que les espèces multiples de INF -α permettent aux cellules de mieux contrôler et réguler la production de INF -α.

Les INF -α et -β n'affectent pas directement eux-mêmes la réplication du virus mais ils stimulent les cellules pour exprimer des variétés de gènes qui suppriment la réplication virale. Il n'est pas surprenant que les virus aient développé des stratégies variées visant à contre attaquer les INF -α et -β. Ces stratégies qui aident les virus à établir l'infection chez un hôte et améliorent de manière signifiante leur virulence consistent à bloquer la synthèse des INF, à lier et inactiver les INF, inhiber l'expression de gènes induits par un INF et neutraliser les protéines antivirales induites par les INF.

Les INF-α et -β et l'infection par les arbovirus du groupe A

Les arbovirus du groupe A font partie d'une grande famille de virus à ARN qui ont une structure en commun : une enveloppe bicouche lipidique portant des glycoprotéines virales, une capside protéique au-dessous de ceci et à l'intérieur de la capside, un génome ARN viral. Les VECV, VEEE et VEEO, étant membres de la famille d'arbovirus du groupe A sont considérés comme des agents de guerre biologiques potentiels du fait de leur grande infectiosité par aérosol. Les contre-mesures médicales actuelles contre les VECV, VEEE et VEEO ne sont pas adéquates. Aucun vaccin ni médicament antiviraux commerciaux ne sont disponibles et le traitement consiste seulement à adoucir les symptômes cliniques.

L'importance des INF -α et -β comme protection précoce contre les infections par VECV et VEEE est démontrée chez les souris qui manquent d'activité INF -α et -β. Ces souris sont très susceptibles aux infections VECV et VEEE et possèdent un haut taux de mortalité après l'infection. La virulence des différentes souches de VECV et VEEE est aussi en corrélation avec leur sensibilité aux INF -α et -β dans le cas du VECV par exemple, les souches d'enzootie produisent peu ou aucune virémie et peu de maladie alors que les souches épizootiques produisent des virémies aiguës, causent l'encéphalite et sont responsables des épidémies importantes chez les humains et les chevaux. On a dit que la résistance aux INF -α et -β. pourrait être la marque de la virulence des souches épizootiques de VECV. Des mutations dans la région non codante 5 du génome viral ainsi que dans la glycoprotéine E2 virale du VECV ont été identifiées comme étant des déterminants moléculaires de la virulence du VECV. Jusqu'à présent, nous en savons peu au sujet du rôle potentiel que jouerait les INF -α et -β au niveau de la virulence des différentes souches du VEEO.

Les INF -α sont depuis longtemps utilisés comme médicaments pour traiter les patients atteints d'hépatite C et du SIDA. Le potentiel thérapeutique des INF -α pour le VECV a été démontré chez les souris. Les INF -α en conjugaison avec le polyéthylène glycol (P.E.G.) protègent les souris des infections soit sous-cutanées soit en aérosol par des VECV virulents.

Conclusion

Une meilleure compréhension du rôle des INF -α et -β dans la pathogénèse des VECV, VEEE et VEEO facilitera la mise au point des thérapeutiques et des vaccins. Les INF -α et -β possèdent une activité antivirale à large spectre. Ils ont ainsi un potentiel important en prévention et en contrôle rapide des infections graves causées par les souches multiples des arbovirus A. Combiner la thérapie des INF -α et -β avec la vaccination peut offrir une protection rapide et durable. Certains gènes viraux liés à la contre réaction de l'activité de l'IFN pouvant être les déterminants les plus importants de la virulence, l'identification de ces gènes et leur disruption ultérieure par la technologie de l'ADN recombinant pourraient nous permettre de concevoir de meilleurs vaccins atténués vivants pour les VECV, VEEE et VEEO. Cette méthode a certainement été prouvée dans la conception des vaccins contre la grippe et les virus respiratoires syncytiaux.

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Introduction

In 1957, Alick Isaacs and Jean Lindenmann discovered that chicken embryo cells exposed to heat-inactivated influenza virus produced a substance that blocked the growth of influenza virus [1, 2]. They coined the term interferon due to its ability to interfere with virus replication. Further studies found that IFNs are highly species specific, i.e. mouse IFNs hardly have any antiviral activity on human cells and vice versa [3] and inhibit a wide spectrum of viruses [4]. In addition to anti-viral activity, IFNs inhibit parasites and intracellular bacteria [5, 6], regulate immune responses and cell growth [7].

IFNs contain multiple members that can be classified into type I, type II, and type III IFNs according to their use of different cellular receptors (Table 1). Type I IFNs include multiple IFN-α species, single IFN-β, and many other members [8]. In contrast, type II IFN only has one member, IFN-gamma (IFN-γ). Type III IFNs, which include IFN-lambda 1,2 and 3 (IFN-λ1, -λ2, -λ3), are the newly defined member of IFN family [9, 10]. IFN-λ1, -λ2, -λ3 have similar intrinsic antiviral activity to the type I IFNs but are otherwise structurally and genetically distinct [11, 12].

IFN-α and -β are major components in the first line of defence against virus invasion. Because of their pivotal role in launching initial immune protection, viruses have evolved various strategies to suppress IFN-α and -β, which include blocking their synthesis, binding and inactivating secreted IFN-α and -β, inhibiting IFN-induced signaling and IFN-induced anti-viral proteins [13-15]. These strategies help viruses establish infection in host cells and sometimes significantly enhance their virulence. For example, SARS-CoV, the pathogen of severe acute respiratory syndrome (SARS), avoids attacks by the IFN system by blocking the transportation of the transcription factor essential for IFN promoter activity from the cytoplasm to the nucleus, which shuts down the production of IFNs [16]. Avian influenza virus H5N1, a potential global pandemic strain, is highly virulent when compared to other human, avian and swine influenza viruses. One of the reasons for its high virulence is its resistant to the antiviral effects of IFNs and the viral gene encoding NS1 protein is a primary culprit for IFN resistance [17, 18]. Similar viral IFN-invasion strategies are discovered in dengue virus [19, 20], rabies virus [21], hepatitis C virus [22], and West Nile virus [23]. Based on these studies, Palese and co-workers proposed a new concept to generate live viral vaccines by altering viral IFN-resistance genes to attenuate virulence while retaining immunogenicity [24]. They demonstrated that influenza A and B viruses containing altered viral IFN antagonist genes are highly attenuated in mice, yet provide protection from challenge with wild-type viruses [24]. A similar approach has been used to make vaccines against respiratory syncytial viruses, a major cause of respiratory disease in young children [25].

Because of the significance of IFN-α and -β in viral pathogenesis and vaccine development, the study of IFN-virus interaction has become one of the most active research areas. Many review articles have been published in this field [13, 14, 26-28]. This memo focuses on three BW agents in the alphavirus family VEEV, EEEV, and WEEV. I describe the current state of knowledge of IFN-α and -β in the pathogenesis of VEEV, EEEV and WEEV and discuss potential application of this knowledge in development of therapeutics and novel vaccines against these viruses.

Table 1. *Interferon family*

Types	Number of Genes
Type I IFNs	
IFN-alpha (α)	Multiple
IFN-beta (β)	One (except cow)
IFN-kappa (κ) [29]	One
IFN-epsilon (ε) [30, 31]	One
IFN-omega (ω) [32]	One (primates) Multiple (cat, pig, cow)
IFN-delta (δ) [33]	Multiple
IFN-tau (τ) [34]	Multiple
IFN-zeta (ζ) [35]	Multiple
Type II IFN	
IFN-gamma (γ)	One
Type III IFNs	
IFN-lambda (λ)	multiple

Overview of IFN-α and -β

Genes and proteins

IFN-α is encoded by multiple genes. These genes together with the single IFN-β gene form an IFN-α/β gene superfamily. Genes encoding INF-α and -β are located on chromosome 9 in the human and chromosome 4 in the mouse [36, 37]. In humans, there are 13 IFN-α genes plus one pseudogene [8]. The pseudogene has a similar DNA sequence to IFN-α genes but does not encode any IFN-α protein. In mice, there are 14 IFN-α genes and three pseudogenes [8, 38]. DNA sequences of all the IFN-α genes share a high degree of homology, strongly indicating they evolved from a single ancestral gene. The ancestral gene for IFN is considered to have formed some 500 million years ago; Around 400 million years ago, the single IFN gene split into INF-α and IFN-β [39]. Since then, the IFN-α gene duplicated many times to generate the multiple IFN-α genes found in present-day mammals [40-42]. In general, there are little differences in biological functions among different IFN-α proteins. So why do we need so many IFN-α genes? A possible explanation is that multiple IFN-α genes allow better control of IFN expression. This is demonstrated that different IFN genes were sequentially activated and regulated following virus infection [43].

The structures of IFN-α and -β genes are similar. Each gene can be divided into the 5' flanking region containing a promoter and regulatory sites for binding transcription factors, an open reading frame encoding IFN protein, and the 3' flanking region containing polyadenylation site [7]. An outstanding feature of IFN-α and -β genes is lack of introns in the open reading frame [44]. Therefore, mRNA can be generated from the coding region without RNA splicing. Another feature is that a large number of motifs ATTA and TTATTAT exist in the 3' flanking region. It has been suggested that these motifs may contribute to the relative instability and short half-life of IFN-α and -β mRNAs [7].

IFN-α and -β are produced as precursor proteins that contain N-terminal secretory signal sequences. These sequences are then cleaved off before mature IFN-α and -β are secreted from the cells. Mature IFN-α and -β contain 165-172 amino acids with molecular mass between 17 and 26 kDa. The amino acid sequences of different IFN-α proteins are closely related. For instance, 50% of amino acids are identical among different human IFN-α [7]. Interestingly, both human and mouse IFN-α contain highly conserved cysteine residues which form disulfide bonds. All the IFN-β are found to be glycosylated. With respect to IFN-α, the majority are glycosylated in mice whereas only two are glycosylated in human [38, 45, 46]. Glycosylation is not required for the intrinsic bioactivity of IFN-α and -β. Unlike other proteins, IFN-α and -β are stable in acid (pH2) solution [38]. It is not clear whether this remarkable feature is associated with disulfide bonds and glycosylation of the proteins.

Synthesis of IFN-α and -β in response to virus infection

Virus infection stimulates cells in higher vertebrates to synthesize IFN-α and -β. To detect the virus infection, host cells deploy molecular sensors along the pathway of viral invasion. These sensors send off warning signals to the host, leading to rapid production of IFNs before the virus get a foothold in the host. Currently, two types of sensor have been discovered in cells. The first is the membrane-bound sensors. These sensors are strategically located in the membranes of cells or endosomes, which are the gateways of viral invasion. An excellent example for this type of sensor is CC-chemokine receptor 3 or CXC-chemokine receptor 4. These cell surface receptors interact with glycoproteins of herpes simplex virus to signal cells to produce IFN-α [47]. Toll-like receptors are set up in the endosomal membrane for detecting viral RNAs that enter cells through endocytosis [48-51]. Another type of sensor, located in cytoplasm, detects viral double-stranded RNA (dsRNA) produced during viral replication. For instance, cytoplasmic RNA helicases such as retinoic acid-inducible gene I (RIG-I) and melanoma differentiation associated protein-5 (MDA-5) act as sensors for detecting viral dsRNA in cytoplasm [52, 53].

IFN-α and -β are synthesized by two steps in most cells [43, 54]. (Figure 1) First, IFN-β and one species of IFN-α, IFN-α4, are produced immediately after viral infection. Their production depends on the IFN regulatory factor 3(IRF-3), a member of a growing family of transcription factors engaged in regulating immune-response genes [55]. IRF-3 is constitutively expressed in the cell and normally present in the cytoplasm. Viral infection activates IRF-3 through phosphorylation cascades. Together with other transcription factors, such as nuclear factor-κB and AP-1, the activated IRF-3 moves into the nucleus and binds to the regulatory sites of IFN-α4 and -β promoters to activate transcription. After production of IFN-α4 and -β, these IFNs further stimulate the synthesis of another IRF termed IRF-7 that is normally expressed at very low level in most cells. Induction of IRF7 by IFN-α4 and -β subsequently produces other species of IFN-α, which include at least IFN-α2, 5, 6 and 8. Therefore, viral infection initially activates the release of IFN-α4 and -β, which then sensitize the cells and trigger the production of numerous IFN-α species leading to a large scale IFN response.

Not all the cells use this two-step mechanism to produce IFN-α and -β. For example, immediately following viral infection (4 hr after infection), plasmacytoid dendritic cells in lymphoid tissue rapidly produce large quantities of IFN-α independent of synthesis of IFN-α4 and -β due to high level of IRF7 pre-existed in these cells [56, 57]. This could provide a survival advantage for the host when facing systemic viral infection that requires a rapid and effective response. The sudden surge of IFN-α and -β, however, may cause systemic reactions such as fever, headache, muscle pain and malaise, typical of flu-like symptoms [58].

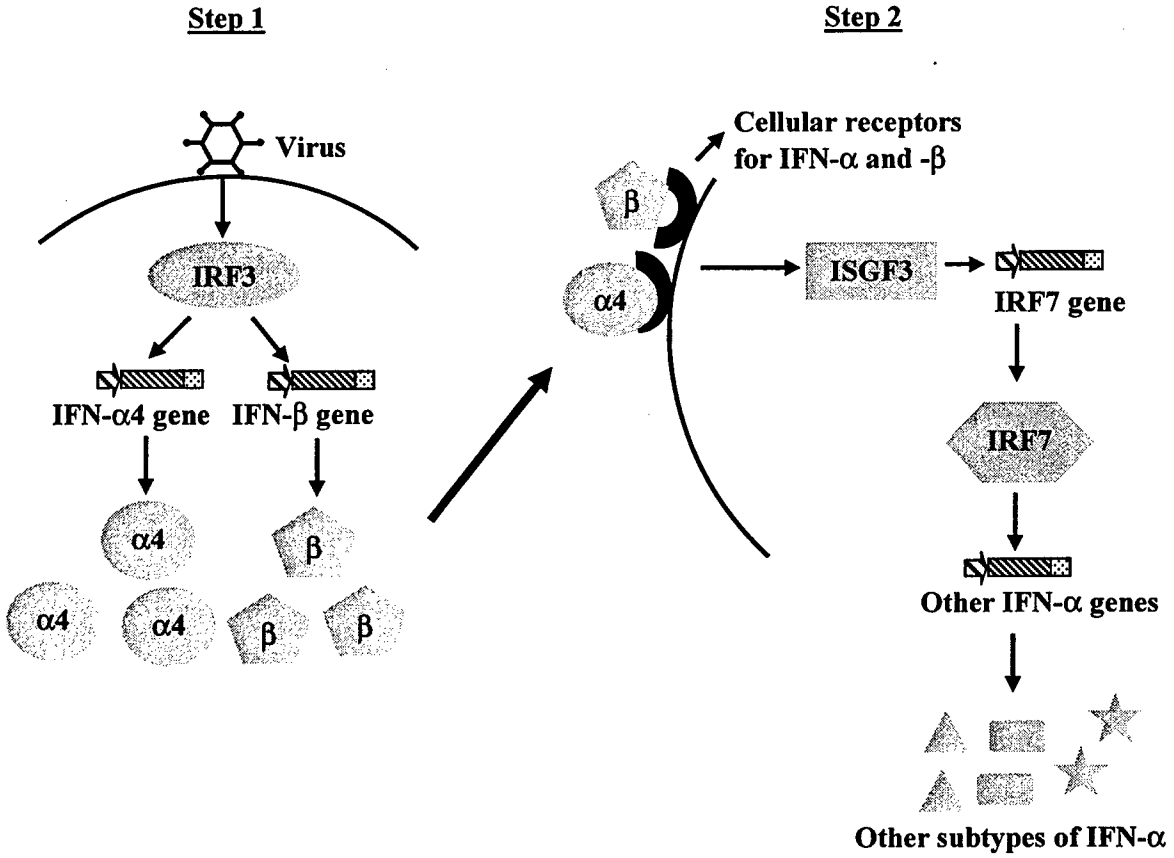

Figure 1. *Synthesis of IFN-α and -β by two-step mechanism. IRF3, IFN regulatory factor 3; ISGF3, IFN stimulated gene factor 3; IRF7, IFN regulatory factor 7.*

Antiviral proteins induced by IFN-α and -β

IFN-α and -β themselves do not have anti-viral function instead they induce expression of IFN-stimulated genes (ISGs) that encode anti-viral proteins [59, 60]. The molecular mechanism leading to expression of ISGs is well understood [61]. As illustrated in Figure 2, IFN-α or -β first binds to a specific cell-surface receptor to activate Janus kinases (JAKs) that subsequently phosphorylate the signal transducers and activators of transcription (STATs) in the cytoplasm. The phosphorylated STATs recruit IRF9 to form a complex known as IFN stimulated gene factor 3 (ISGF-3), which then is transported into the nucleus to activate ISGs. More than 300 ISGs are up- or down- regulated by IFN-α and -β, suggesting a highly complicated host defense system against virus infection [62, 63].

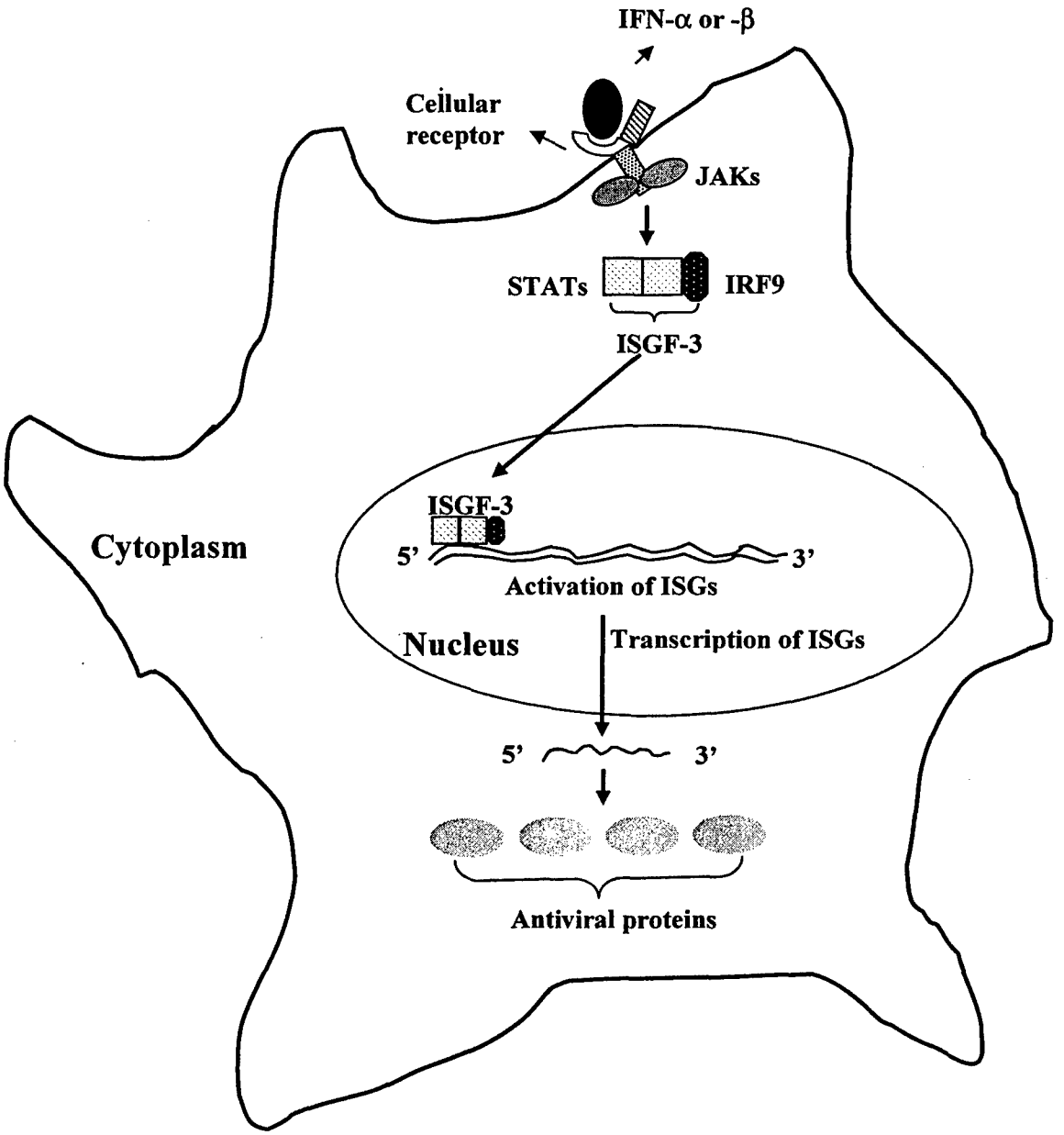

Figure 2. *Induction of antiviral proteins by IFN-α and -β. JAKs, Janus kinases; STATs, the signal transducers and activators of transcription; ISGF-3, IFN stimulated gene factor 3; ISGs, IFN stimulated genes.*

Three antiviral proteins encoded by ISGs have been well characterized. PKR (Protein kinase RNA regulated), previously known as the dsRNA-dependent protein kinase, is a serine-threonine kinase [64]. The protein contains two major motifs: the N-terminal regulatory motif that binds to dsRNA and the C-terminal catalytic motif. PKR is normally inactive, but following binding to dsRNA, it is activated by autophosphorylation. The activated PKR subsequently phosphorylates a wide range of substrates. One of them is eukaryotic initiation factor eIF2 that involves in the initial step of protein synthesis. Phosphorylation of eIF2 by PKR inhibits viral protein synthesis. Another important substrate of PKR is the inhibitor of NF-κB (I-κB), which binds to transcription factor NF-κB to block its function. PKR phosphorylates I-κB, resulting its release from NF-κB and allowing NF-κB to activate a number of immune responsive genes.

Another well characterized ISG-encoded protein is the 2'-5' oligoadenylate synthetase (2-5 OAS) [65]. Upon virus infection, this enzyme is activated by viral dsRNA, resulting in synthesis of oligomers (three to five units) of adenosine. These oligomers then stimulate endoribonuclease L (RNase L) that degrades viral RNAs and induces the transcription of genes that suppress virus replication [66].

The third extensively characterized antiviral protein is the myxovirus-resistance (Mx) protein. The Mx protein was first reported by Jean Lindenmann [67], who co-discovered IFNs with Alick Isaacs. Lindenmann and his colleagues demonstrated that mice carrying the Mx gene were resistant to the infections of several orthomyxoviruses, such as influenza A virus, and that the action of Mx was IFN-dependent [68]. Mx proteins are GTPases that belong to the superfamily of dynamin-like GTPases [69]. Although Mx proteins have been shown to inhibit a wide range of RNA viruses, the molecular mechanism has not yet been understood. Some researchers demonstrated that Mx proteins could trap viral nucleocapsid proteins of bunyaviruses in cytoplasmic inclusions, resulting in blockage of virus assembly [70]. Additionally, Mx proteins inhibit virus polymerase that is required for synthesis of viral DNA or RNA [71].

Besides these three well studied, IFN-induced antiviral proteins, several new anti-viral proteins have been identified recently [72, 73]. For example, ADARs (adenosine deaminases acting on RNA) recognize viral dsRNA and modify viral dsRNA by deaminating adenosine to produce inosine [74]. Because many RNA viruses replicate through a dsRNA-based intermediate, modification of viral dsRNA by ADARs alters the functional activity of the viral RNA genome. In addition, ADAR degrades the modified viral RNAs in the presence of an inosine-specific ribonuclease [75].

IFN-α and -β in regulating immune responses

In addition to inducing antiviral proteins, IFN-α and -β profoundly affect immune responses. Natural killer (NK) cells are populations of large granular lymphocytes that eliminate virus infection through killing virus-infected cells. IFN-α and -β up-regulate the NK perforins that are involved in cell killing mechanisms [76, 77]. Major histocompatibility complex (MHC) molecules of antigen-presenting cells (APCs) present viral peptide to cytotoxic and helper lymphocytes that are key components of host cellular immune responses. IFN-α or -β

increase expression of MHC molecules and act as a critical signal for activating APCs [78, 79]. Another role of IFN-α and -β in regulating immune responses is to influence antibody class switching. Class switching is an important step for antibody-producing B cells to produce different kinds of antibodies bearing distinctive functions. For example, IgA antibodies block virus entry via respiratory and intestine tracts. By promoting the production of certain classes of antibody, IFN-α and -β enhance the host defence against certain viruses [4, 59].

IFN-α and -β and alphavirus infection

General properties of alphaviruses

Alphaviruses consist of a large family of RNA viruses, which share common structural elements: an envelope composed of a glycoprotein-bearing lipid bilayer, underneath the envelope, a capsid, and inside the capsid, a single-stranded RNA genome [80]. Like cellular mRNA, the alphavirus RNA is capped with a 7-methyguanosines at its 5' terminus and is polyadenylated at its 3' terminus (Figure 3). The 5' two thirds of the viral RNA genome encodes the nonstructural proteins required for transcription and replication of the viruses. The 3' one third of the viral genome encodes the capsid proteins and envelope glycoproteins. In addition, both the 5' and 3' termini of the genome contain noncoding regions that are important for initiating viral replication [81].

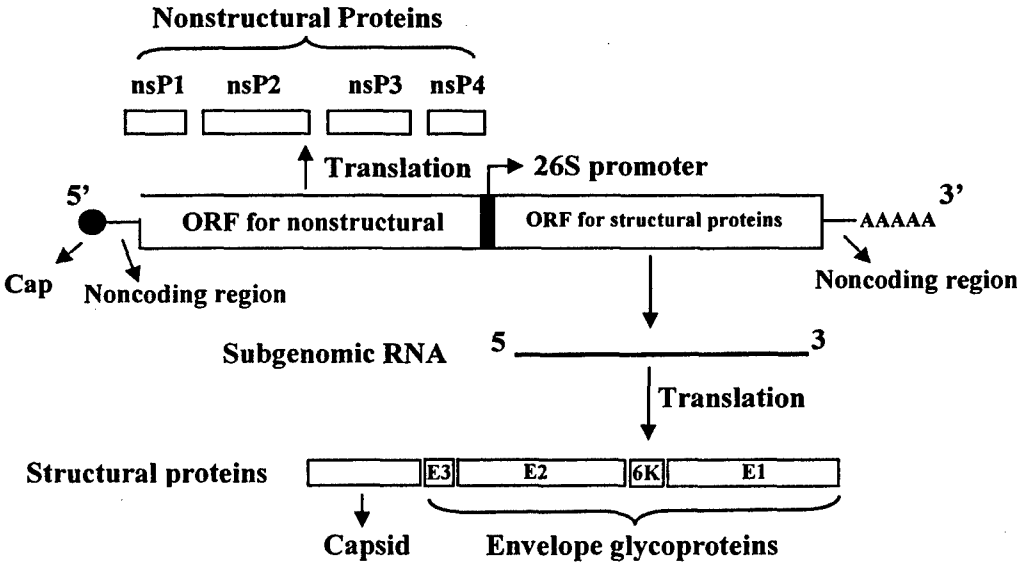

Figure 3. *Organization of the alphavirus genome and synthesis of viral proteins.*

Pathogenesis of alphavirus infection

The pathogenesis of VEEV infection has been extensively studied in different animal models. In horses, VEEV first replicates in lymphoid tissues with a high serum viremia followed by invasion of the central nervous system leading to an often lethal encephalitis [82]. Aerosol exposure of nonhuman-primate to VEEV causes fever, viremia, lymphopenia, and encephalitis in 70% of infected animals [83]. The clinical signs and pathology in these infected animals are similar to those in humans [84]. In mice, following a subcutaneous infection, VEEV initially infects skin dendritic cells known as Langerhans cells and these infected cells then migrate rapidly to the draining lymph nodes [85]. The virus primarily replicates in lymphoid tissue and then enters the blood system to spread out to nonlymphoid tissues. By 48 to 96 h after infection, the amount of virus begins to decline in infected tissues and by 72 to 96 h, virus is cleared from the peripheral tissues. However, at this point virus has already spread to the brain via the olfactory and trigeminal nerves and causes a fatal encephalitis 7 to 10 days after infection [86, 87]. In mice following an aerosol exposure, VEEV appears to enter the brain by directly infecting the olfactory nerves without causing viremia [87-90].

Similar to VEEV, after aerosol exposure of cynomolgus macaques to WEEV, the virus was only found in the brain, indicating that WEEV may also infect the brain by directly traveling up the olfactory nerves [91]. EEEV, injected by subcutaneous route, appears to enter the mouse brain by a vascular route, rather than via peripheral nerves [92]. In mice, EEEV first infects and replicates within fibroblasts and skeletal muscle myocytes at the inoculation site and within osteoblasts in bone resulting in a transient high-titer viremia [92]. The viral infection was observed as early as 12 h after infection and peaked at 1 day after infection. At this point, the virus was also first detected in the brain leading to death by day 4 after infection. The very rapid onset and apparently random and massive infection of the brain without affecting olfactory neuroepithelium suggest a vascular route for EEEV invasion of the brain [92, 93]. Further study is needed to show whether EEEV enters the brains via the olfactory nerves in aerosol challenged animals.

Alphaviruses as BW agents and current medical countermeasures

VEEV, EEEV, and WEEV are the potential BW agents in the alphavirus family because they are relatively stable in natural environments, they are highly infectious by aerosol, they are easy to produce in large quantities, they can be used as either incapacitating or lethal agents, they contain multiple strains that complicate vaccine development, and there are no commercial anti-viral drugs or vaccines available [94]. VEEV, EEEV, and WEEV cause encephalitis in humans with different mortality rate [94, 95]. EEEV is the most virulent with case fatality rates of 30% to 40%. In fatal cases, patients usually die 2 to 10 days after onset of encephalitis. Compared to EEEV, WEEV appears to be less virulent with case fatality rate of 10%. The fatal encephalitis often occurs in infants and in young children. VEEV usually causes an acute incapaciting illness with fever, chills, headache, muscle pain, diarrhea and vomiting, which appear 2 to 5 days after exposure to the virus. The incidence of encephalitis is generally less than 5% and case fatality rate less than 1%. The encephalitis and death often occur in children.

Current medical countermeasures against VEEV, EEEV, and WEEV are inadequate [94-96]. No anti-viral drugs are available and treatment only focuses on easing clinical symptoms. A live attenuated VEEV vaccine TC-83, developed by serial passage of the virulent Trinidad donkey strain in fetal guinea pig heart cells [97], protects laboratory workers from infection. The shortcoming of this vaccine is that 15% to 30% of vaccine recipients develop fever, malaise and headache and half of them are so severe that bed rest is required. Investigational vaccines derived from killed EEEV and WEEV are available only for laboratory workers at risk of exposure to these viruses. These vaccines require multiple injections and an annual boost. A new approach is badly needed to develop anti-viral drugs and vaccines.

Role of IFN-α and -β in host defence against alphavirus infection

The importance of IFN-α and -β in the induction of early protection against VEEV infection was first demonstrated by Grieder et al [98]. They showed that mice depleted of IFN-α and -β by injecting anti-IFN-α and -β antibody reduced average survival times by 3-fold after VEEV infection [98]. This finding indicates that blocking IFN-α and -β accelerates VEEV infection. The finding was confirmed in mice lacking cellular receptors for IFN-α and -β [99-101]. These mice are highly susceptible to subcutaneous infection with both virulent VEEV and attenuated VEEV TC-83 strain, resulting in death within 24 and 48 h, respectively [99].

For EEEV, higher titer viremia and shorter survival times were seen in mice deficient in receptors for IFN-α and -β than in wild-type mice, suggesting that these IFNs are also important in controlling EEEV replication [102]. In addition, mice given poly(I-C), a nonspecific IFN-α and -β inducer, exhibited dose-dependent protection against lethal EEEV and WEEV challenges [102, 103], further confirming that IFN-α and -β are important in controlling disease. Taken together, IFN-α and -β appear to play an important role in the protection of host against alphavirus infection.

IFN-α and -β and virulence of alphaviruses

VEEV can be divided into enzootic and epizootic strains based on virulence [104]. The enzootic strains, including VEEV subtypes II to VI and subtype I varieties ID to IF, produce little or no viremia and little disease. These viruses circulate continually in cycles among rodent reservoir hosts and mosquito vectors [105]. On the contrary, the epizootic strains, including subtype I varieties AB and C, achieve high-titer viremia and cause encephalitis that are responsible for major epidemics in humans and horses [106]. Genetic analysis supports the theory of enzootic strains periodically evolving to produce epizootic strains [107].

Correlation between IFN-α and -β and the virulence of VEEV was first revealed more than 30 years ago by Jordan [108] and Jahrling et al. [109]. In their studies, the epizootic strain Trinidad donkey and attenuated vaccine strain TC-83 were compared for their sensitivity in cell culture. TC-83 was found to be more sensitive than Trinidad donkey strain to IFN-α and -β [108, 109]. Spotts et al. extended these studies by comparing the IFN-α and -β sensitivities of the TC-83 vaccine strain with 24 enzootic and epizootic VEEV strains in cells [101]. They found that the IFN-resistant and -sensitive phenotypes correlated well with epizootic and

enzootic property, respectively, suggesting that resistance to IFN-α and -β may be one of the adaptations leading to the emergence of epizootic strains [101]. Another study, however, showed that the sensitivities to murine IFN-α and -β did not differ appreciably between an epizootic and an enzootic VEEV strain [110]. This discrepancy may be explained by differences in the methods used to test viral sensitivity to IFN-α and -β.

Molecular determinants of VEEV virulence differ among animals. For example, changes in nonstructural protein nsP3 and E2 glycoprotein are most frequently associated with equine virulence [111], while the 5' noncoding region in VEEV RNA genome and the viral E2 glycoprotein are important determinants of virulence in mice [101, 112-114]. The 5' noncoding region in VEEV consists of 45 nucleotides, which appears to form a stem-loop structure that is conserved across the alphaviruses [81]. Johnston's group constructed a recombinant VEEV, designated as V3043, which has a single nucleotide change in the 5' noncoding region compared to the virulent strain Trinidad donkey [112]. This change renders V3043 avirulent in normal adult mouse model. However, in mice lacking receptors for IFN-α and -β, infection of V3043 resulted in 100% mortality, suggesting that IFN-α and -β play a major role in the attenuation of V3043. Additionally, although there were no differences in the induction of IFN-α and -β between V3043 and Trinidad donkey strain, the recombinant virus was more sensitive to IFN-α and -β in *in vitro* assays. Why does the 5' noncoding region affect viral sensitivity to IFN-α and -β? The 5' noncoding region likely participates in initiation of translation and synthesis of viral dsRNA through interaction with host and/or viral proteins. As previously described, viral dsRNA is a strong inducer of IFN-α and -β. The increased sensitivity to IFN after mutations in the 5' noncoding region may be due to higher levels of viral dsRNA synthesis, leading to increased induction of IFN-α and -β and their ISGs [112]. Another possibility is that mutation in 5' noncoding region of VEEV genome causes slower viral inhibition of host protein synthesis, which allows continued production of key antiviral proteins in infected cells [112].

The possible involvement of IFN-α and -β in the virulence of EEEV was recently reported by Weaver's group [102]. EEEV strains from North America cause severe illness in both humans and horses. In contrast, EEEV strains from South American cause fatal encephalitis only in horses, not in humans [95, 106]. Weaver's group found that in cell culture EEEV of North American strains are more resistant to IFN-α and -β than South American strains, indicating that the high virulence of North American viruses may be associated with their resistance to IFN-α and -β [102]. However, this hypothesis was not consistent with *in vivo* data, which showed no difference in the induction of IFN-α and -β between these two strains in mice [102]. Further studies in a better animal model such as primate are needed to confirm the *in vitro* results. Similar to VEEV and EEEV, epizootic WEEV strains are generally more virulent for mice and guinea pigs than are enzootic strains and the epizootic strains may arise from nonvirulent enzootic strains [115-117]. However, so far very little is known about the possible role of IFN-α and -β in WEEV virulence [118, 119].

IFN-α as an immunotherapeutic for alphavirus infection

IFN-α has long been used as a drug for treating patients with hepatitis C, AIDS, papillomavirus warts and cancer [120-122]. Early studies showed that pre-treatment of mice with IFN-α had no effect on average survival times in mice challenged with virulent VEEV [99]. Later on, Lukaszewski and Brooks reported that conjugation of IFN-α to polyethylene glycol (PEG) protected mice from either a subcutaneous or an aerosol infection of virulent VEEV [123]. No virus was detected in PEG IFN-α-treated mice over the first 6 days post-infection, while large amount of viruses was detected in blood in untreated mice over the same period [123]. It appears that conjugation of IFN-α to PEG increases the half-life of IFN-α in the serum and delays clearance of IFN-α by kidney compared to native IFN-α [123]. Therefore, the potency of IFN-α is greatly enhanced. Single daily injection of native IFN-α results in a seesaw pattern of IFN-stimulated anti-viral proteins in the host. Thus VEEV could replicate significantly between doses of native IFN-α. PEG-conjugated IFN-α maintains serum levels of IFN-α for a longer period, which continuously stimulates the synthesis of anti-viral proteins [123]. By the same token, the half-life and potency of IFN-α can also be increased by continuously expressing IFN-α gene *in vivo* using viral delivery vectors [124-126]. It will be interesting to find if IFN-α delivered by viral vector can also protect mice from alphavirus infection.

Conclusion

The significance of studying the interaction between IFN-α and -β and alphavirus infection are two folds. First, the growing numbers of emerging viral pathogens and possible genetically engineered viruses with enhanced virulence represent big challenge for developing medical countermeasures against viral BW agents. To meet this challenge, we need a new strategy that emphasizes on developing single countermeasure against multiple known or unforeseen viral agents [127]. IFN-α and -β possess an immediate, broad-spectrum antiviral activity. Thus, they have a great potential to develop into therapeutics to prevent and rapidly control acute infections caused by multiple viral agents. In addition, combining IFN therapy with vaccination may provide a quick and long-lasting protection against acute infection of viral BW agents. The feasibility of this strategy has been recently demonstrated by rapidly controlling foot-and-mouth disease outbreaks [128-130]. Second, because viral genes related to counteraction against IFN system can be the major determinants of virulence of alphaviruses, identification of these genes and subsequent disruption of these genes through recombinant DNA technology could lead to design better live attenuated vaccines for VEEV, EEEV, and WEEV. This approach has been successfully applied to design vaccines against influenza and respiratory syncytial viruses [24, 25, 131].

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List of Symbols/Abbreviations/Acronyms/Initialisms

ADAR	Adenosine deaminases acting on RNA
APCs	Antigen-presenting cells
BW	Biological Warfare
dsRNA	Double-stranded RNA
EEEV	Eastern equine encephalitis virus
IFNs	Interferons
I-κB	Inhibitor of NF-κB
IRF	Interferon regulatory factor
ISGs	Interferon stimulated genes
JAKs	Janus kinases
MHC	Major histocompatibility complex
Mx protein	Myxovirus-resistance protein
NK cells	Natural killer cells
2-5 OAS	2'-5' oligoadenylate synthetase
PKR	Protein kinase RNA regulatec
RNase L	Endoribonuclease L
STATs	Signal transducers and activators of transcription
VEEV	Venezuelan equine encephalitis virus
WEEV	Western equine encephalitis virus

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Our immune system can launch a quick defence against a large number of unforeseen viruses. This front line of defence restricts virus spread and buys time for our body to develop specific and long-lasting immunity. One of the major weapons in this defence system is interferons (IFNs) that are proteins with broad-spectrum antiviral activity. Therefore, understanding the interplay between IFNs and viral agents may provide an insight into the development of a single medical countermeasure effective agasint multiple viral agents. As an initial step to tap the IFNs for developing therapeutics against multiple strains of alphaviruses, this memo presents recent advances in studying the role of IFNs in alphavirus pathogenesis. I first review IFNs, then focus on IFN responses against three BW agents in the alphavirus family: Venezuelan equine encephalitis virus (VEEV), eastern equine encephalitis virus (EEEV), and western equine encephalitis virus (WEEV). I conclude with discussion of how to apply this knowledge for therapeutic and vaccine development.

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