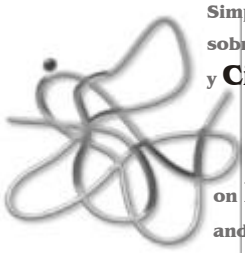

Simposio Internacional sobre **Interferón** y **Citoquinas**

International Symposium on **Interferon** and **Cytokines**

La Habana, Cuba.
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Havana, Cuba.
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Este simposio centra su atención en las bases moleculares de la cascada de señalización del IFN y en el papel que juegan algunos mediadores de la respuesta antiviral a nivel molecular. Además, se abordan elementos acerca de los mecanismos moleculares de la resistencia al IFN desarrollados por algunos virus como el de la Hepatitis C y el virus Papiloma Humano. Algunos tópicos son: la enzima dependiente de ARN de doble cadena (PKR), los receptores para citokinas y las quinasas Tyk así como los factores IRFs y NFkB entre otros. En este simposio han sido invitadas prestigiosas figuras inter- nacionales en el campo de las investigaciones sobre IFN y Citocinas.

This symposium focuses its attention on the molecular basis for the IFN signaling pathways and the role of some IFN-induced antiviral mediators at the molecular level. Attention is also focused to the molecular mechanisms for the IFN-resistance developed by viruses like Hepatitis C and Human Papillomavirus. Some topics include: the dsRNA-dependent kinase (PKR), Cytokine Receptors and Tyk kinases, the IRFs and NFkB between others. Very prestigious figures on IFN research have been invited to participate as speakers on this symposium.

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HCV NS5A Protein: A Viral Swiss-army Knife Against IFN Response?

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The development of effective antiviral therapies for the treatment of chronic hepatitis C virus (HCV) continues to be a pressing concern. Although the combined use of interferon (IFN)-α and ribavirin has improved therapeutic outcome for some patients, the majority of HCV-infected individuals remain unresponsive to therapy. However, advances are being made in our understanding of the mechanisms by which HCV evades the antiviral actions of IFN, with increasing interests on the viral nonstructural 5A (NS5A) protein. HCV NS5A was initially linked to IFN responsiveness in HCV-infected patients. Subsequent studies have shown that NS5A may block the IFN response by interacting with and inhibiting the IFN-induced PKR protein kinase, a primary mediator of the IFN-induced antiviral response.

Other modes of action have also been proposed, including the subversion of intracellular signaling pathways through NS5A interaction with cellular signaling molecules. Thus NS5A may employ multiple strategies to counteract IFN response and may be a potential target in antiviral drug discovery. Further defining the ability of NS5A to modulate intracellular signaling and the molecular mechanisms of IFN resistance may provide additional insights into the design of anti-HCV therapeutics.

Antiviral Mechanisms of Human MxA GTPase

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Interferon (IFN)-induced Mx proteins are members of the dynamin superfamily of large GTPases. They have antiviral activity against a number of RNA viruses including orthomyxo- and bunyaviruses, and block virus multiplication by recognizing the nucleoprotein component of the viral nucleocapsids.

In the case of Rift Valley fever virus (RVFV, a bunyavirus), MxA forms tightly packed fibrillary bundles with the nucleocapsid protein and sequesters the newly synthesized viral protein into perinuclear complexes that resemble aggresomes.

As a consequence, an essential viral component is trapped by MxA in the cytoplasm of infected cellsand is no longer available for the generation of new virus particles.

In the case of Thogoto virus (THOV, an influenza-like virus transmitted by ticks) MxA recognizes the incoming viral nucleoprotein and prevents the viral nucleocapsids from being transported into the nucleus, where THOV transcription and replication takes place. In both cases, GTP-binding and carboxy-terminal effector functions of MxA are required for viral target recognition. Essential components of both viruses are trapped by MxA in the cytoplasm of infected cells and become unavailable for the generation of new virus particles, suggesting that a common mechanistic principle of large GTPases is at work. To avoid induction of this efficient host defense system, some viruses have evolved escape strategies. For example, RVFV encodes a nonstructural protein (NSs) that antagonizes IFN production and thereby prevents induction of MxA.

The Growth Factor Midkine is a Cytokine that Inhibits HIV Infection by an Autocrine and Paracrine Action

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The growth factor midkine (MK) has been reported to bind heparan sulfate and nucleolin, two components of the cell surface implicated in the attachment of HIV particles.

Using recombinant and synthetic preparations of MK, we demonstrated that infection by various T lymphocyte- and macrophage-tropic HIV-1 isolates is inhibited in cell cultures in a dose dependent manner. This latter is due to inhibition of HIV particle anchorage to the surface of MK-pretreated cells. MK mRNA is systematically expressed in adult peripheral blood lymphocytes from healthy donors, while its expression becomes markedly but transiently increased upon *in vitro* treatment of lymphocytes with IL-2 or IFN-γand activation of T lymphocytes by PHA or through the engagement of the CD28 antigen. In MK producing lymphocytes, MK is detectable at the cell surface where it colocalizes with the surface-expressed nucleolin.

MK binds cells specifically since this binding is inhibited by excess MK but not by other related growth factors. On the other hand, MK-binding to cells is drastically inhibited by the nucleolin-binding HB-19 pseudopeptide. Accordingly, MK colocalized with the cell-surface expressed nucleolin as revealed by laser scanning confocal microscopy. Interestingly, MK binds specifically to Chinese hamster ovary cells deficient in heparan and chondroitin sulfate proteoglycans, and that such binding is inhibited by HB-19, thus pointing out that the main target of MK on the cell surface is nucleolin.

The potent and the distinct anti-HIV action of MK along with its enhanced expression in lymphocytes by various physiological stimuli, point out that MK is a cytokine that could be implicated in HIV pathogenesis. Consistent with this, we show that CD4+ CEM T-cell clones expressing recombinant MK resist HIV infection by the inhibition of virion attachment to cells. Moreover, CD4 positive HeLa cells resist HIV infection when cocultured with MK producing CD4 negative cell clones, since MK becomes transferred from CD4 -ve to CD4 +ve cells.

Activation of Antiviral and Apoptotic Cascades by the NF-kB and IRF Factors during Viral Infection

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Virus infection of host cells activates multiple signaling pathways that orchestrate the activation of cytokine genes involved in antiviral defense and immune response. Among the kinases induced by paramyxovirus infection are Akt, JNK1(SAPK), p38α, IKK, MEKK1 and the virus activated kinase (VAK) responsible for IRF-3 and IRF-7 C-terminal phosphorylation. IRF-3 and IRF-7 in turn regulate a sequential cascade of Type 1 IFN gene activation, as well as the RANTES chemokine gene. Using a series of point mutations, we have investigated the temporal order of IRF-3 phosphorylation in the C-terminal regulatory domain and demonstrate that Ser396 is a critical phosphoacceptor residue.

Chimeric proteins that combined different IRF-7 and IRF-3 domains were also tested and provided evidence of distinct DNA binding properties of IRF-3 and IRF-7. Interestingly, some of these fusion proteins led to supraphysiological levels of IFNA promoter activation. Strategies to use highly active forms of IRFs as immunomodulatory agents in conjunction with plasmid DNA vaccination strategies or as pro-apoptotic agents are also being investigated.

We further characterized the regulatory control of RANTES transcription during virus infection using *in vivo* genomic footprinting analyses. IRF-3, IRF-7 and NF-κB are identified as important *in vivo* binding factors required for the cooperative induction of RANTES transcription after virus infection. To further characterize the involvement of NF-κB in RANTES induction, retroviral-mediated transfer was used to introduce a dominant form of IKKγ/NEMO subunit (C-IKKγ) into U937 cells. C-IKKγ expression inhibits LPS-, TNF- and virus-induced IKK activity and NF-κB binding activity as well as decreased RANTES gene expression.

To examine the range of genes activated by IRF-3, we combined Tet-inducible expression of a constitutively active form of IRF-3 with a DNA microarray analysis of 8,500 unique genes. Interestingly, many of the genes activated by IRF-3(5D) included IFN stimulated genes involved in antiviral defense such as ISG-15, -54, -56, and -60. Subsequent analysis of ISG-56 expression demonstrated high level induction by IRF-3, mediated through the ISRE element of the ISG-56 promoter. Together these results imply that IRF-3 activates not only immediate early Type 1 IFN genes, but also a subset of IFN responsive genes that establish the antiviral state.

Nuclear Localization of the Tyrosine Kinase Tyk2

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The type I IFNs (IFN-α/β are produced by a variety of cell types upon infection with viral and non-viral pathogens. They are leading cytokines of the innate immune response that also shape the adaptive response. Activation of type I IFN receptors occurs through ligand-driven oligomerization of two transmembrane components (IFNAR1 and IFNAR2) pre-associated with the tyrosine kinases Tyk2 and Jak1. Reciprocal phosphorylation of these kinases leads to their activation and to phosphorylation of substrates, namely the receptor themselves and STAT family members.

As all JAK proteins, Tyk2 possess an amino-terminal region bearing the structural determinants for receptor binding, a regulatory kinase-like domain and a C-terminal tyrosine kinase domain. We have investigated the subcellular distribution of Tyk2 and Tyk2-GFP in human Tyk2-deficient cells. Immuno staining or direct fluorescence showed a diffuse localization of Tyk2, including in the nuclear compartments.

No detectable re-localisation of Tyk2 was observed upon cytokine treatment. Given the 134 kDa mass of Tyk2, its nuclear import is likely to require a discrete region(s) of the protein. We therefore monitored the localization of several truncated and mutated Tyk2 forms stably expressed in Tyk2-deficient cells.

Altogether, our results demonstrate that nuclear localization of Tyk2 relies on a 22aa Arg-rich motif located in the amino-terminal region and that this NLS-like motif is part of the receptor-binding domain of Tyk2.

The rescuing ability of a membrane-targeted form of Tyk2 demonstrate that the nuclear Tyk2 pool is dispensable for IFN-α/β induced transcriptional and anti-VSV activities. In parallel experiments we found that Jak1 is excluded from the nucleus. We are presently monitoring the subcellular distribution of chimeric Tyk2/Jak1 proteins in order to identify the minimal Tyk2 region sufficient to direct the chimera into the nucleus.

Essential Role of the dsRNA-dependent Protein Kinase, PKR, in Innate Immunity to Viral Infection

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The interferons (IFNs) comprise an essential element of innate immunity to viral infection by inducing a large amount of cellular genes that serve to directly inhibit viral replication and to initiate and direct subsequent adaptive immune responses.

Although many of the key IFN-inducible genes remain to be identified and/or characterized, one such gene product referred to as the dsRNA-dependent protein kinase, PKR has been proposed to play a critical role in IFN-mediated antiviral function. Interaction with dsRNA causes PKR to autophosphorylate and to catalyze the phosphorylation of substrate targets, the best characterized being the alpha subunit of eukaryotic initiation factor 2 (eIF2α), leading to the inhibition of protein synthesis in the cell. We have recently demonstrated the importance of PKR in antiviral host defense by showing that mice lacking PKR are extremely susceptible to intranasal (in.) infection with vesicular stomatitis virus, VSV. Interestingly, IFN treatment protected fibroblasts lacking PKR to VSV infection, indicating the induction of alternate antiviral genes, but failed to protect intranasally infected PKR knockout mice, suggesting a suppressed IFN system in the respiratory tract.

Aside from being sensitive to VSV infection, PKR-deficient animals were found to be ten times more sensitive to lethal respiratory infection with influenza virus (WSN). High level viral replication and significant viral-induced apoptosis in tissues was detected in the pulmonary tract, as well as in other organs. Collectively, our data indicate that the IFN-inducible PKR is an essential and non-redundant component of innate immunity that acts early in host defense prior to inception of the acquired immune response.

Design and Application of a Cytokine Receptor-based Interaction Trap

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Protein interactions underlie the structural and functional integrity of many subcellula Processes. The notion that most of the proteins within a cell are part of higher order complexes regulating signal transduction, gene expression, apoptosis and other crucial events, becomes generally accepted.

Existing methodologies for large-scale protein interaction mapping include yeast two-hybrid, phage display and mass spectrometric analysis. Although these approaches have found wide application, each of them suffers from intrinsic limitations, not at least from the fact that interactions are analyzed in a non-physiological context.

We have developed a cytokine receptor-based two-hybrid method in mammalian cells. Incorporation of an interaction trap in a signalling-deficient receptor allows identification of protein-protein interactions using a STAT-dependent complementation assay. Interaction between 'bait' and 'prey' leads to ligand-dependent STAT activation, which is detected by the use of STAT-dependent reporter/selector genes. Using this mammalian protein-protein interaction trap (MAPPIT) we were able to demonstrate both modification independent and tyrosine-phosphorylation dependent interactions.

Data will be presented on the application of this concept as a screening tool.This MAPPIT procedure places protein-protein interactions in their normal physiological context and may be particularly instrumental for the *in situ* analysis of signal transduction pathways.

La inhibición de la proliferación de las células 32D tratadas con interleucina-1 beta (IL-1b) es parcialmente mediada por el factor de necrosis tumoral alfa (TNF-a)

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Existe una gran cantidad de artículos que muestra que la IL-1β modula positivamente la hematopoyesis, sin embargo, en los últimos años, el grupo de Makio Ogawa, aportó evidencias de que también suprime la proliferación de células troncales hematopoyéticas totipotenciales de médula ósea de ratón. Empleando a las células 32D, una línea hematopoyética multipotencial de ratón, dependiente de IL-3 y ampliamente usada en estudios de la hematopoyesis normal, nuestro grupo encontró que la interleucina-1 beta también bloquea su proliferación a pesar de la presencia de IL-3.

Hasta el momento no se conoce si la inhibición de la proliferación de las células 32D es un efecto directo de la IL-1β o es vía la inducción a la producción de clásicos inhibidores de la hematopoyesis.

Este estudio tiene la finalidad de mostrar si las células 32D tratadas con IL-1β producen TNF–α y si este molécula contribuye en el bloqueo de la proliferación de las células 32D.

Para realizar el estudio, las células 32D fueron cultivadas durante 48 h con IL-3 en presencia o ausencia de IL-1β, el sobrenadante (MC) fue colectado y posteriormente adicionado o no a las células L-929, una línea celular empleada para detectar TNF. La proliferación de las células 32D expuestas a IL-3 con o sin IL-1β, en presencia o ausencia de anticuerpos anti-TNF-α, fue evaluada para analizar la contribución del TNF en el bloqueo de la proliferación de las células 32D tratadas con IL-1β.

Solo el sobrenadante de las células 32D tratadas con IL-3 e IL-1β indujeron muerte de las células L-929, al mismo nivel que el TNF-α recombinante. La adición del anticuerpo anti-TNF-α al cultivo de las células 32D con IL-3 e IL-1β revirtió el bloqueo de la proliferación al 50%, mientras que la misma dosis de anticuerpo revirtió completamente el efecto inhibitorio de 10 ng/ml de TNF recombinante de ratón.

La IL-1β promueve la producción de TNF-α en las células 32D. La inhibición de la proliferación de las células 32D tratadas con IL-1b, es en parte mediada por el TNF.

Proyecto apoyado por DGAPA IN215199, [1]becarios de PROBETEL UNAM, [3]becada por el proyecto IN215199, [2]becario de CONACYT.

El caseinato de sodio induce la expresión del gen del factor estimulador de colonias de macrófagos (M-CSF) y de su receptor (M-CSFR) en las células 32D

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El caseinato de sodio (CasNa) es una sal de la caseina, principal proteína de la leche. El CasNa es usado frecuentemente para inducir la migración de leucocitos a la cavidad peritoneal en varios animales. Recientemente nuestro grupo mostró, a nivel celular, que acelera la transición de neutrófilos en banda a segmentados.

Por otro lado, inhibe la proliferación e induce la diferenciación hacia el linaje monocito-macrófago de las células 32D, una línea hematopoyética multipotencial de ratón, dependiente de IL-3 y ampliamente usada en estudios de la hematopoyesis normal. Hasta el momento no se conoce si en la diferenciación hacia el linaje monocito-macrófagos de las células 32D tratadas con CasNa, existe inducción a la expresión del gen para M-CSF y su receptor (M-CSFR), dos moléculas que indican comprometimiento de las células hacia el linaje monocítico.

Este estudio tiene la finalidad de mostrar si las células 32D expresan el gen para el M-CSF y su receptor cuando son tratadas con CasNa. Para realizar el estudio, las células 32D fueron cultivadas durante 24 h con IL-3 en presencia o ausencia de CasNa, el RNA obtenido fue aislado y usado para la reacción de transcriptasa reversa (RT). El cDNA así obtenido fue amplificado por reacción en cadena de la polimerasa (PCR) con oligos específicos para revelar la presencia del gen del M-CSF y del M-CSFR, empleando al gen de b-actina como control interno de la expresión.

Después del corrimiento electroforético, se encontró que solo las células 32D tratadas con CasNa expresan el RNAm para el M-CSF. Las células 32D sin tratamiento muestran una ligera expresión de RNAm para el M-CSFR pero se incrementa fuertemente en presencia del CasNa.

El CasNa induce la expresión del gen del M-CSF, mientras que incrementa la expresión del M-CSFR en las células 32D.

Proyecto apoyado por DGAPA IN215199, [2]becarios de PROBETEL UNAM, [1]becada por el proyecto IN215199, [3]becario de CONACYT.