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Interferon-gamma does not restrict influenza virus replication in RAW 264.7 and AMJ2-C11 macrophages through the mechanism involving activation of inducible nitric oxide synthase expression

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Interferon-gamma (IFN-γ) antiviral activity operates partly via inducible nitric oxide synthase (iNOS) induction. Whether IFN-γ affects influenza A virus (IAV) replication within macrophages through this pathway is unknown. For the investigation, RAW 264.7 and AMJ2-C11 murine macrophages were exposed to IFN-γ, live IAV (A/PR/8/34) or their combination. At 24 h post-exposure, cell viability was evaluated by trypan blue dye exclusion, nitrite levels in macrophage culture supernatants were measured using the Griess reagent, and cells were harvested for reverse transcriptase (RT)-PCR. The IFN-γ-activated macrophages incubated with live IAV demonstrated a considerably greater cytopathic effect as well as a significant viability reduction than those without IAV, or with IAV minus IFN-γ ($p < 0.05$). Simultaneously, iNOS gene expression and nitric oxide (NO) production were increased in both cell lines. These findings indicate that IFN-γ-induced iNOS expression and NO synthesis and IFN-γ do not inhibit but permit at least partial IAV replication within macrophages.

Key words: interferon-gamma, influenza virus, inducible nitric oxide synthase, nitric oxide, macrophages

INTRODUCTION

Viral infections induce a cascade of host immune responses, which includes production of two types of interferons (IFN-α/β and IFN-γ) functioning as antiviral and immunomodulatory cytokines. The ability of IFN-α/β to inhibit viral replication in host cells is mediated via the stimulation of cellular genes encoding such antiviral enzymes as double-stranded RNA-activated protein kinase (PKR), 2′,5′-oligoadenylate synthetases, and Mx proteins [1]. By the same mechanisms, IFN- α/β induces an antiviral state in lung epithelial cells and macrophages infected with influenza A virus (IAV), and thus can limit the virus spread within respiratory tract [2]. Noteworthy, IAV infection of macrophages *in vitro* results in unproductive (abortive) replication of the virus within this type of cell [3]. As distinct from IFN-α/β, the IFN-γ exerts antiviral activity through a different pathway. It has been demonstrated by experiments *in vitro* that IFN-γ restricts replication of DNA-containing viruses (ectromelia, vaccinia and *herpes simplex* type 1) in mouse macrophages through the activation of inducible nitric oxide synthase (iNOS) expression and increased nitric oxide (NO) generation [4]. However, it is unknown what effect IFN-γ exerts on macrophages infected with IAV. Therefore, we investigated the capacity of IFN-γ to suppress replication of IAV in RAW 264.7 monocytes / macrophages and AMJ2-C11 lung alveolar macrophages *in vitro* by upregulating iNOS expression.

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MATERIALS AND METHODS

Cell cultures

Murine RAW 264.7 and AMJ2-C11 macrophage cell lines were obtained from the American Type Culture Collection, Manassas,

VA, USA. Media and components (including antibiotics) for cultivation of the cell cultures were purchased from Invitrogen Corp., Grand Island, NY, USA. RAW 264.7 cells were cultured as reported previously [5]. AMJ2-C11 cells were maintained in Dulbecco's modified Eagle medium (DMEM) containing 5% heat-inactivated fetal bovine serum, penicillin (100 U/ml), streptomycin (100 μg/ml), amphotericin B (1.25 μg/ml), and 5 mM HEPES buffer solution. The cells were cultivated in a humidified incubator at 37 °C and 5% CO_2. In all experiments, cell viability was evaluated by trypan blue dye exclusion.

Virus infectivity titer

The mouse-adapted IAV strain A/PR/8/34 (H1N1), a kind gift of Dr. Bradley S. Bender (College of Medicine, University of Florida, Gainesville, FL, USA), was propagated in the allantoic cavities of 10-day-old embryonated chicken eggs as described by Lennette [6]. The infectivity titer was assessed in Madin–Darby canine kidney cells as reported [5], and it was determined as $10^{8.4}$ $TCID_{50}$/ml (50% tissue culture infectious dose per ml).

Experimental design

RAW 264.7 and AMJ2-C11 cells were seeded at 1×10^6 cells/well in 6-well tissue culture plates containing 5 ml of serum-free DMEM/well, and they were incubated for 18 h and 6 h, respectively. Then, both cell lines were exposed in triplicate fashion to mouse recombinant (r) IFN-γ (1 ng/ml) purchased from Sigma-Aldrich Co., St. Louis, MO, USA, live IAV (infectivity titer of 10^6 $TCID_{50}$/ml) or a combination of both (IAV plus rIFN-γ). Unexposed cells served as controls. The incubation was continued for an additional 24 h under standard conditions (37 °C and 5% CO_2). Afterwards, nitrite levels in RAW 264.7 and AMJ2-C11 macrophage culture supernatants were measured, and cells were harvested for RNA isolation and iNOS mRNA detection by reverse transcriptase (RT)-PCR.

Nitrite determination

Nitrite (NO_2^-) levels, an index of cellular NO production, were determined in macrophage culture supernatants using a modified Griess reagent (Sigma-Aldrich Co., St. Louis, MO, USA), as directed by the manufacturer. The optical density at 540 nm was measured using a microplate reader-spectrophotometer ($PowerWave_X$; Bio-Tek Instruments Inc., Winooski, VT, USA), and nitrite concentrations were calculated as described previously [5]. The nitrite detection limit was 0.24 μmol/ml.

iNOS mRNA detection by semiquantitative RT-PCR analysis

For total RNA isolation, cells in each treatment group were combined from three wells to increase the quantity of cellular RNA extracted. The total RNA was isolated using Trizol reagent according to the instructions provided by Invitrogen Corp., Grand Island, NY, USA. iNOS mRNA was detected using one-step reaction with the Access RT-PCR System kit (Promega Corp., Madison, WI, USA). The murine primers for iNOS and β-actin mRNAs detection were selected on the basis of the published nucleotide sequences [5]. β-actin mRNA expression was used as an internal control. RT-PCR was performed with 1 μg of total RNA from each sample in 25 μl reaction volumes according to the manufacturer's instructions, using a thermal cycler (GeneAmp PCR System 9600; Perkin-Elmer Applied Biosystems Corp., Norwalk, CT, USA) under the described conditions [5]. In the experiment with AMJ2-C11 cells, a total of 30% of the iNOS and β-actin reaction products were subjected to electrophoresis to improve detection of the bands resulting from the mRNA expression, following rIFN-γ and IAV exposures. The amplified PCR products were electrophoresed in 1.5% agarose gels, stained with ethidium bromide, and then gels were photographed under UV transillumination. 100 bp DNA ladder (Invitrogen Corp., Grand Island, NY, USA) was used as a marker for identification of sizes of iNOS and β-actin amplified products. Bands corresponding to iNOS and β-actin products were quantified by densitometry using Scion Image software, version Beta 4.03 (available from: http://www.scioncorp.com), and values were expressed as the iNOS / β-actin ratio.

Statistical analysis

Data are expressed as the means ± SEM of triplicates in a single experiment. Statistical evaluation was performed with SPSS program, version 12.0, using independent samples t test (two-tailed) for comparison of means. A p value less than 0.05 was considered statistically significant.

RESULTS

The viability of RAW 264.7 and AMJ2-C11 macrophages

Viability of unexposed and rIFN-γ-stimulated RAW 264.7 and AMJ2-C11 macrophages under experimental conditions was no less than 94%. However, challenge to IAV or a combination of IAV plus rIFN-γ greatly reduced the survival rate of the macrophages. At 24 h post-exposure, the viability of RAW 264.7 macrophages declined to 83 ± 1% and 70 ± 2% for the stimulations with IAV and combination of IAV plus rIFN-γ, respectively. The viability of AMJ2-C11 macrophages fell to 77 ± 2% with IAV-infected cells, and to 63 ± 2% with IAV plus rIFN-γ combined stimulation. In both cases, exposure to the combination of IAV plus rIFN-γ reduced survival rate of the cells significantly more than challenge with IAV alone ($p < 0.05$). This combination also revealed changes in the morphology of macrophages, which were characteristic of the viral cytopathic effect (CPE), including variation in size and shape, granulation (vacuolation), degeneration and lysis of cells (Fig. 1 A and B). The same changes were found in macrophage cultures treated with influenza virus, though to a lesser extent.

Effect of IAV on NO production and iNOS mRNA expression in RAW 264.7 and AMJ2-C11 macrophages

Unexposed RAW 264.7 and AMJ2-C11 macrophages did not generate a detectable amount of nitrite, and incubation with IAV for 24 h insignificantly increased formation of NO_2^- compared to the limit of detection ($p > 0.05$). The measured NO_2^- concentrations in IAV-infected macrophage cultures were 0.28 ± 0.03 μmol/ml (for RAW 264.7 cells) and 0.31 ± 0.09 μmol/ml (for AMJ2-C11 cells). In contrast, exposure of RAW 264.7 and AMJ2-C11 macrophages to rIFN-γ rose NO_2^- levels to 2.75 ± 0.34 μmol/ml and 0.65 ± 0.15 μmol/ml, respectively. RT-PCR analysis determined that IAV stimulated iNOS mRNA expres-

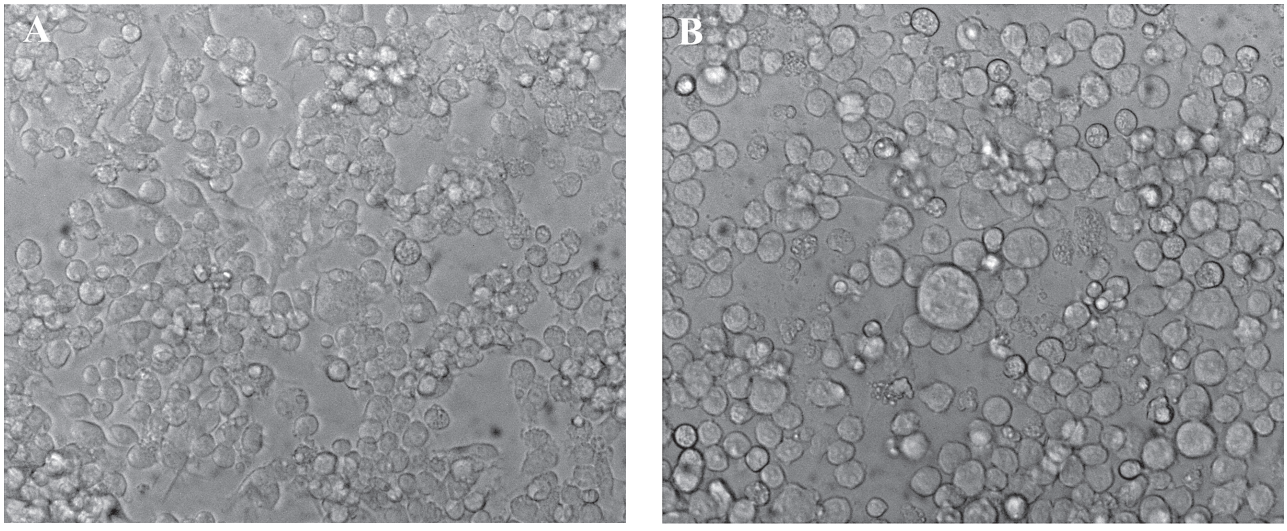

Fig. 1. Phase-contrast microscopy showing morphology of RAW 264.7 (A) and AMJ2-C11 (B) macrophages after 24 h of incubation with both IAV and rIFN-γ together. Magnification, × 40

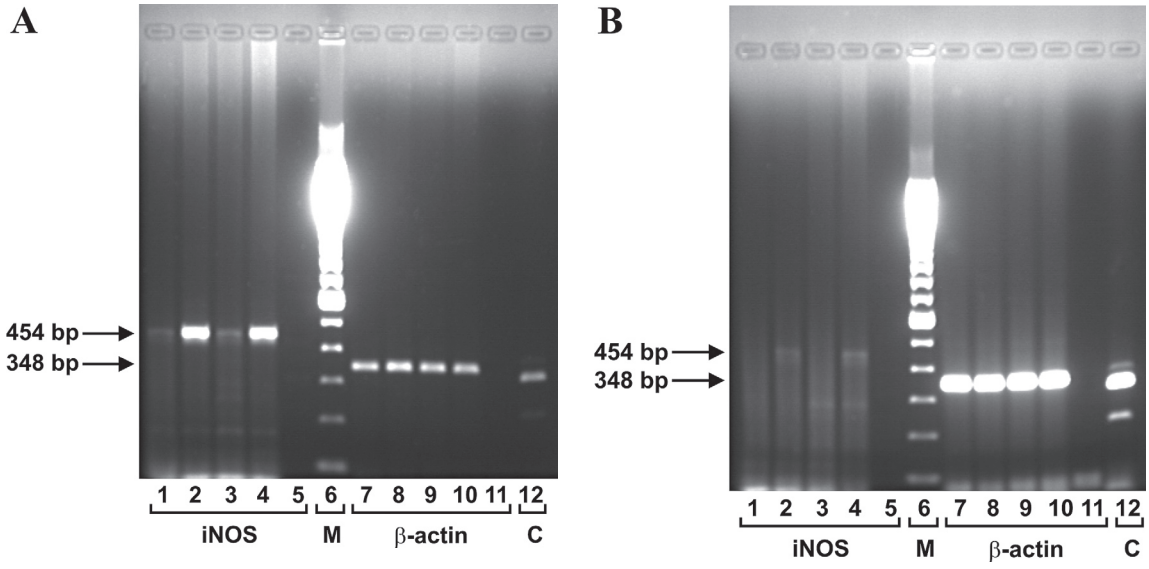

Fig. 2. Detection of iNOS mRNA expression by RT-PCR analysis in RAW 264.7 (A) and AMJ2-C11 (B) macrophages at 24 h after exposure to rIFN-γ, IAV or a combination of IAV plus rIFN-γ. The numbers represent lanes: 1 and 7 – for unexposed cells; 2 and 8 – for exposure to rIFN-γ; 3 and 9 – for exposure to IAV; 4 and 10 – for exposure to the combination of IAV plus rIFN-γ; 5 and 11 – negative control for RT-PCR performed with sterile nuclease-free water substituted for the RNA template; 12 (C) – positive control for RT-PCR performed with the RNA template from kit (323 bp); 6 (M) – molecular weight marker (100 bp DNA ladder). Arrows indicate the expected sizes of amplified products (454 bp for iNOS, and 348 bp for β-actin).

sion in RAW 264.7 macrophages (Fig. 2 A). According to densitometry, IAV-infected macrophages demonstrated the increased iNOS mRNA levels by 48% versus the mRNA levels expressed in unexposed cells (data not shown). However, iNOS mRNA expression in AMJ2-C11 macrophages after exposure to IAV was not detected (Fig. 2 B). Additionally, it should be noted that stimulation with rIFN-γ induced the iNOS gene in both cell lines independent of virus exposure (Fig. 2 A and B).

Effect of IAV plus rIFN-γ combined exposure on NO synthesis and iNOS mRNA expression in RAW 264.7 and AMJ2-C11 macrophages

Combined influenza virus plus rIFN-γ exposure of RAW 264.7 macrophages had a markedly greater NO production compared to IAV or rIFN-γ. In this regard, NO_2^- levels for stimulation with the combination overlapped 13.3-fold and 1.4-fold the NO_2^- levels for exposures to IAV and rIFN-γ, respectively. The increase of NO_2^- concentration in macrophage cultures, resulting from the combination exposure, was significant versus the NO_2^- concentration in IAV-infected cell cultures ($p < 0.05$). The combined IAV plus rIFN-γ challenge insignificantly increased NO_2^- formation in AMJ2-C11 macrophage cultures over exposure to either component alone ($p > 0.05$). Importantly, combination of IAV plus rIFN-γ activated expression of iNOS gene in both cell lines (Fig. 2 A and B). Densitometric analysis revealed that RAW 264.7 macrophages sustaining the combination exposure produced 87% and 22% greater iNOS mRNA levels than the macrophages challenged with IAV and rIFN-γ, respectively (data not shown). In AMJ2-C11 cells, stimulation with this combination resulted in an increase of iNOS mRNA expression by 44% compared to the rIFN-γ only exposure.

DISCUSSION

This study demonstrated IAV ability to cause infection of RAW 264.7 and AMJ2-C11 macrophage cell lines in the presence of rIFN-γ-induced increase of iNOS mRNA and nitrite formation. Furthermore, we have also found that IAV is independently capable to mediate iNOS gene induction in RAW 264.7 cells. The latter finding may be due to the replication of IAV within macrophages. A common feature of viral infections, including influenza, is that replication of virus involves generation of double-stranded RNA (DS-RNA) in the infected cell [7, 8]. Being the viral replicative intermediate, DS-RNA interacts with the PKR which, in turn, can activate such cellular transcription factors as nuclear factor-kappa B (NF-κB) and interferon regulatory factor-1 (IRF-1) [8, 9].

Since the promoter region of murine macrophage iNOS gene contains binding sites for both NF-κB and IRF-1 transcription factors [10], IAV likely triggered production of iNOS mRNA in RAW 264.7 macrophages in our experiments. In this context, it should be noted that IAV replication also upregulates expression of the iNOS gene in human airway epithelial cells [8].

The findings that IAV in combination with rIFN-γ can cause the CPE with a markedly reduced survival rate of both macrophage cell lines, despite the elevated iNOS mRNA and nitrite levels, suggest that IFN-γ does not inhibit replication of this virus within macrophages. Rather, IFN-γ may promote cell infection or be sufficiently permissive for viral toxicity such as by allowing virus entry and replication to the DS-RNA level. On the other hand, an excess generation of NO as in the macrophages stimulated with combination of IAV plus IFN-γ mediates various effects within the cellular system, including inhibition of the tumor suppressor protein p53 activity [11]. In this respect, an investigation of Turpin et al. [12] has shown that suppression of p53 activity results in enhanced IAV replication within human lung epithelial cells and mouse embryo fibroblasts, possibly because of the reduction in IFN signaling. Interestingly, the NO generated in mouse macrophages under encephalomyocarditis virus infection is able to downregulate the expression of IFN-α/β genes leading to the elimination of antiviral state in these cells [13]. As regards to antiviral activity of NO against IAV, it was reported by Yoshitake et al. [14] that neither endogenously synthesized nor exogenously derived NO has such effect on the RNA containing influenza and Sendai viruses. On the other hand, the attenuation of viability in macrophage cultures with IAV plus rIFN-γ combined exposure could be related to the cytotoxicity of NO, thereby masking and occurring in parallel with the virus-induced effects but not limiting its replication. Taken together with the data of discussed studies, our experiments indicate that IFN-γ can modulate conditions during IAV infection which may well allow the virus to partially or fully replicate within macrophages.

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GAMA-INTERFERONAS NESLOPINA GRIPO VIRUSO REPLIKACIJOS RAW 264.7 IR AMJ2-C11 MAKROFAGUOSE MECHANIZMU, AKTYVINANČIU INDUKUOJAMOS AZOTO MONOKSIDO SINTETAZĖS RAIŠKĄ

Santrauka

Gama-interferono (IFN-γ) priešvirusinis poveikis iš dalies yra susijęs su indukuojamos azoto monoksido sintetazės (iNOS) aktyvinimu. Tačiau nėra žinoma, ar IFN-γ gali paveikti A gripo viruso (GAV) replikaciją makrofaguose šiuo mechanizmu. Siekiant tai ištirti, RAW 264.7 ir AMJ2-C11 pelės makrofagai buvo paveikti IFN-γ, gyvybingu GAV (A/PR/8/34) arba jų abiejų kombinacija. Praėjus 24 val. ląstelių gyvybingumas buvo įvertintas trypan mėlynojo dažymo metodu, nitritų lygiai makrofagų kultūrose išmatuoti Griess reagentu ir surinktos ląstelės siekiant nustatyti iNOS iRNR atvirkštinės transkriptazės (AT)-PGR. Rezultatai atskleidė, kad IFN-γ aktyvintuose makrofaguose, inkubuotuose kartu su GAV, pasireiškė ryškus citopatogeninis efektas, taip pat ženkliai sumažėjo gyvybingumas palyginus su tais, kurie nebuvo paveikti GAV arba paveikti GAV be IFN-γ ($p < 0,05$). Tuo pat metu abiejose ląstelių linijose buvo padidėjusi iNOS geno raiška ir azoto monoksido (NO) gamyba. Šie duomenys rodo, kad IFN-γ bei dėl jo poveikio indukuota iNOS raiška ir NO sintezė neslopina, bet leidžia GAV bent iš dalies replikuotis makrofaguose.