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Interferon-gamma augments cytotoxicity in influenza virus-infected RAW 264.7 and AMJ2-C11 macrophages by upregulating the expression of inducible nitric oxide synthase

Tomas Kačergius[1],

Arvydas Ambrozaitis[1],

Yuping Deng[2],

Norine S. Kuhn[2],

Stefan Gravenstein[3]

[1] *Department of Infectious Diseases, Dermatovenerology and Microbiology, Faculty of Medicine, Vilnius University, Lithuania*

[2] *Glennan Center for Geriatrics and Gerontology, Department of Internal Medicine, Eastern Virginia Medical School, USA*

[3] *Division of Geriatrics, Department of Medicine, Warren Alpert Medical School of Brown University, USA*

Background. Interferon-gamma (IFN-γ) produced during influenza A virus (IAV) infection stimulates abundant generation of the cytotoxic free radical nitric oxide (NO) in macrophages that in turn damage pulmonary cells and tissues. However, it is unknown whether IFN-γ contributes to the cytotoxicity in the IAV-infected macrophages through the stimulation of inducible nitric oxide synthase (iNOS) expression.

Materials and methods. RAW 264.7 and AMJ2-C11 murine macrophages were exposed for 24 h to either IFN-γ or 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 Griess reagent, and cells were harvested for iNOS mRNA detection by reverse transcriptase (RT)-PCR.

Results. The added IFN-γ was associated with reduced viability of both cell lines only when infected with IAV, which was coincident with an elevated level of nitrite and iNOS mRNA. Furthermore, the increase of NO generation was significantly more when IFN-γ and IAV were not used together ($p < 0.05$).

Conclusions. Our results indicate that IFN-γ enhances cytotoxicity in the IAV-infected macrophages by increasing expression of iNOS gene and production of NO. These findings are important for understanding the mechanism by which IAV causes injury to cells and tissues in lungs.

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

INTRODUCTION

Influenza A virus (IAV) infection continues to be a serious worldwide health problem exhibiting great morbidity and mortality rates in spite of providing vaccination. The primary cause of deaths during the epidemics of influenza is related to the development of pneumonia (1). Unfortunately, the factors involved in the mechanism of pneumonia pathogenesis are incompletely characterized. An increasing number of experimental mouse model investigations, initiated in 1996 and proceeded by Akaike et al. (2, 3) as well as by other researchers, indicate that excessive generation of free radical nitric oxide (NO) by inducible nitric oxide synthase (iNOS) contributes to lung damage in the course of pneumotropic virus infections, including influenza (4, 5).

Correspondence to: Tomas Kačergius, Department of Infectious Diseases, Dermatovenerology and Microbiology, Faculty of Medicine, Vilnius University, Birutės 1, LT-08117 Vilnius, Lithuania.
E-mail address: tomas.kacergius@mf.vu.lt

The IAV infection induces a cascade of host immune responses that involve production of proinflammatory cytokines, such as interferon-gamma (IFN-γ), tumour necrosis factor-alpha, and recruitment of macrophages to the lungs (5, 6). These two and other proinflammatory cytokines stimulate expression of iNOS leading to high-output synthesis of NO, predominantly within macrophages (2, 7). In turn, the NO overproduction can elicit cytotoxic effect because of the induction of nitrative stress in cells (8). It is therefore noteworthy that IAV of mouse macrophages *in vitro* synergizes with IFN-γ in NO generation via iNOS induction (9). On the other hand, the IAV infection itself is cytopathic for epithelial cells and macrophages within lungs.

However, it is unknown whether the cytopathic and cytotoxic effects can be accelerated by IFN-γ via induction of iNOS gene expression. Therefore, we investigated the capacity of IFN-γ to augment the cytotoxicity of RAW 264.7 monocytes/macrophages and AMJ2-C11 lung alveolar macrophages infected with IAV *in vitro* through the increase of iNOS and NO production.

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 (9). AMJ2-C11 cells were maintained in Dulbecco's modified Eagle medium (DMEM) containing 5% heat-inactivated foetal 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 titre

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 DA (10). The infectivity titre was assessed in MDCK cells as reported (9), and it was determined as $10^{8.4}$ $TCID_{50}$/ml for the experiments with RAW 264.7 cells and $10^{7.5}$ $TCID_{50}$/ml for the experiment with AMJ2-C11 cells.

Experimental design

For four independent experiments, 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 and 1% heat-inactivated serum DMEM/well, respectively. Then, RAW 264.7 macrophages were incubated for 18 h, and AMJ2-C11 macrophages – for 6 h. Following the incubation, RAW 264.7 cells 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 titre of 10^6 $TCID_{50}$/ml) or a combination of both (rIFN-γ plus IAV). Stimulation of AMJ2-C11 cells was performed in the same manner except that the concentration of rIFN-γ was 10 ng/ml, and the infectivity titre of IAV – 2×10^6 $TCID_{50}$/ml. In all experiments, unexposed cells served as controls. The incubation was continued for an additional 24 h under standard conditions (37 °C, 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 (9). The nitrite detection limits were 0.24 μmol/ml for the experiments with RAW 264.7 cells and 0.98 μmol/ml for the experiment with AMJ2-C11 cells.

iNOS mRNA detection by semiquantitative RT-PCR analysis

For total RNA isolation, cells in each treatment group were combined from three wells in order 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, as previously described (9). 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 (9). 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 IAV infection. 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

The data are expressed as the means ± standard error of the mean (SEM). 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

Cell survival

Viability of unexposed and rIFN-γ-stimulated RAW 264.7 and AMJ2-C11 macrophages under experimental conditions was no less than 94% (Table). However, exposure to IAV or a combination of rIFN-γ plus IAV significantly reduced survival of RAW 264.7 macrophages ($p < 0.05$). Contrarily, IAV alone did not affect viability of AMJ2-C11 cells but the presence of rIFN-γ did markedly decrease it. In both cases, exposure to the combination of rIFN-γ plus IAV reduced survival rate of the cells considerably more than challenge with IAV alone. This combination also revealed changes in the morphology of macrophages, which were characteristic of the viral cytopathic effect.

Nitrite formation and iNOS mRNA expression in the cell cultures

During all experiments, unexposed RAW 264.7 and AMJ2-C11 macrophages did not generate a detectable amount of nitrite (Table). Incubation of RAW 264.7 cells with IAV for 24 h significantly increased nitrite levels *versus* the detection limit ($p < 0.05$). In contrast, nitrite formation within the IAV-exposed

AMJ2-C11 macrophage cultures was not detected. Furthermore, stimulation of both cell lines with rIFN-γ alone produced substantially greater nitrite levels (Table). RT-PCR analysis determined that IAV, as well as rIFN-γ, activated iNOS mRNA expression in RAW 264.7 and AMJ2-C11 macrophages (Figs. A and B). It should be noted that stimulation with rIFN-γ alone induced the iNOS gene considerably more than the virus challenge alone (Table).

Combined rIFN-γ plus IAV exposure of RAW 264.7 and AMJ2-C11 macrophages had significantly greater nitrite formation compared to either component alone ($p < 0.05$; Table). As seen in Figs. A and B, combination of rIFN-γ plus IAV activated expression of iNOS gene in both cell lines. Densitometric analysis revealed that RAW 264.7 macrophages sustaining the combination exposure produced 71% greater iNOS mRNA levels than the macrophages challenged only with IAV (Table). However, iNOS mRNA expression in response to the combination was slightly over the levels (by 12%) determined within rIFN-γ-activated RAW 264.7 cells during the experiments. In AMJ2-C11 macrophages, stimulation with this combination resulted in an increase of iNOS mRNA expression by 16% and 47% compared to the rIFN-γ and IAV exposures, respectively.

Table. **Cell viability (%), NO_2^- concentration (μmol/ml) and iNOS mRNA levels (iNOS/β-actin ratio) in RAW 264.7 and AMJ2-C11 macrophage cultures after 24 h of exposure to rIFN-γ, IAV or their combination**

Stimulus	Cell viability	NO_2^-	iNOS/β-actin ratio
RAW 264.7 macrophages			
Control	96.56 ± 0.38[a]	ND	0.21 ± 0.06[a]
rIFN-g	94.22 ± 0.49[a]	1.78 ± 0.31[a]	1.70 ± 0.45[a]
IAV	73.22 ± 2.58[a]	0.42 ± 0.04[a]	0.56 ± 0.14[a]
rIFN-γ + IAV	58.67 ± 3.16[a,*]	3.49 ± 0.17[a,*]	1.93 ± 0.52[a]
AMJ2-C11 macrophages			
Control	99.00 ± 0.00[b]	ND	ND
rIFN-γ	96.00 ± 0.00[b]	2.17 ± 0.10[a]	0.54 ± 0.00[b]
IAV	93.00 ± 0.00[b]	ND	0.34 ± 0.00[b]
rIFN-γ + IAV	69.00 ± 0.00[b]	3.60 ± 0.02[a,*]	0.64 ± 0.00[b]

[a] Values are the means ± SEM (n = 3–9).

[b] Values represent a single measurement.

ND – not detected.

* $p < 0.05$ compared to rIFN-γ and IAV.

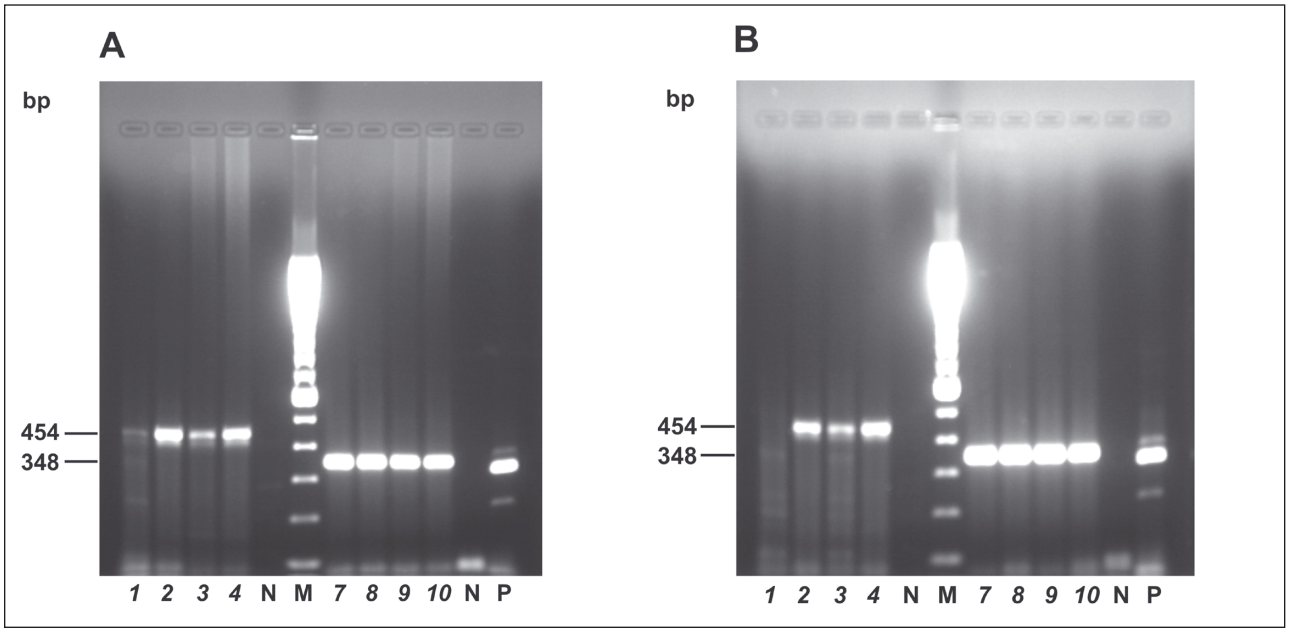

Figure. Detection of iNOS mRNA expression by RT-PCR analysis in RAW 264.7 (**A**) and AMJ2-C11 (**B**) macrophages at 24 h after exposure to rIFN-γ, IAV or their combination. Lanes *1–4* and *7–10* represents iNOS (454 bp) and β-actin (348 bp) amplified products, respectively. *1* and *7* – unexposed cells; *2* and *8* – exposure to rIFN-γ; *3* and *9* – exposure to IAV; *4* and *10* – exposure to the combination of rIFN-γ plus IAV; N – negative control for RT-PCR performed with sterile nuclease-free water substituted for the RNA template; P – positive control for RT-PCR performed with the RNA template from kit (323 bp); M – molecular size marker (100 bp DNA ladder). In photograph **A**, the gel is representative of three independent experiments

DISCUSSION

This study demonstrated IFN-γ ability to augment the cytotoxicity in IAV-infected RAW 264.7 and AMJ2-C11 macrophage cultures in a synergistic manner through mediation of the elevated iNOS mRNA expression and nitrite formation. Similar to the investigation of Imanishi et al. (11), we also found that live IAV is independently able to stimulate the iNOS gene in RAW 264.7 cells but further established that this occurs in AMJ2-C11 macrophages, too. The latter finding may be due to the replication of IAV within macrophages. Replicating virus generates a double-stranded RNA (DS-RNA) in the infected cell. Being the viral replicative intermediate, DS-RNA interacts with the protein kinase (PKR), which in turn can activate such cellular transcription factors as nuclear factor-kappa B and interferon regulatory factor-1. Eventually, these factors trigger the expression of iNOS gene in the cell (12).

The finding that IFN-γ in combination with IAV considerably reduced survival of both macrophage cell lines, indicates its potential to affect cytotoxicity via NO production. Our results showed that this was a necessary condition for cell damage *in vitro* because even a double concentration of the virus failed to affect viability of AMJ2-C11 macrophages. Together, IFN-γ and IAV exert a synergystic effect on cell survival, better reflecting the situation *in vivo* during IAV pulmonary infection, to which a lot of cytokines (including IFN-γ) are produced. The infection and consequent cytokine production then leads to the high-output generation of NO and superoxide (O_2^-) which react yielding a potent oxidizing and nitrating agent – peroxynitrite anion radical ($ONOO^-$). The latter induces cell apoptosis and necrosis through mitochondrial damage resulting in local (pulmonary) tissue injury (2, 8).

On the other hand, IFN-γ can modulate conditions during IAV infection which may well allow the virus to partially replicate within macrophages (13). Therefore, the observation that IAV alone did not decrease the viability of AMJ2-C11 cells as well as did not enhance detectable nitrite formation supports such speculation. In this respect, our research data differ from the study results of McKinney et al. (14), who found that IAV itself was sufficient to both cause the death of J774.1 macrophages by apoptosis and stimulate NO production. As regards to antiviral activity of NO against IAV, it was reported by Yoshitake et al. (15) that neither endogenously synthesized nor exogenously derived NO has such an effect on the RNA containing influenza and Sendai viruses.

CONCLUSIONS

In accordance with the data presented, we conclude that IFN-γ increases the cytotoxicity of RAW 264.7 and AMJ2-C11 macrophages in the presence of IAV infection by upregulating expression of iNOS gene and production of NO. Importantly, it provides new insight into the understanding of the factors which contribute to lung tissue damage in the course of IAV infection.

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INTERFERONAS-GAMA PADIDINA CITOTOKSIŠKUMĄ GRIPO VIRUSU UŽKRĖSTUOSE RAW 264.7 IR AMJ2-C11 MAKROFAGUOSE, SKATINDAMAS INDUKUOJAMOS AZOTO MONOKSIDO SINTETAZĖS RAIŠKĄ

S a n t r a u k a

Įvadas. Interferonas-gama (IFN-γ), pasigaminęs esant gripo A viruso infekcijai (GAV), stimuliuoja gausų citotoksiško laisvojo radikalo – azoto monoksido (NO) – gaminimąsi makrofaguose, kuris savo ruožtu pažeidžia plaučių ląsteles ir audinius. Tačiau nėra žinoma, ar IFN-γ turi įtakos citotoksiškumui GAV infekuotuose makrofaguose, stimuliuodamas indukuojamos azoto monoksido sintetazės (iNOS) raišką.

Medžiagos ir metodai. RAW 264.7 ir AMJ2-C11 pelės makrofagai buvo paveikti IFN-γ, gyvybingu GAV (A/PR/8/34) arba jų abiejų kombinacija. Po 24 valandų trypan mėlynojo dažymo metodu buvo įvertintas ląstelių gyvybingumas, panaudojant Griess reagentą išmatuoti nitritų lygiai makrofagų kultūrose, ir surinktos ląstelės, kad, pritaikius atvirkštinės transkriptazės (AT)-PGR, būtų nustatyti iNOS iRNR.

Rezultatai. Poveikis IFN-γ buvo susijęs su sumažėjusiu abiejų ląstelių linijų gyvybingumu tiktai tuomet, kada jos buvo užkrėstos GAV, ir tai sutapo su padidėjusiais nitritų ir iNOS iRNR kiekiais. Be to, šis NO kaupimasis buvo gerokai didesnis negu tada, kai IFN-γ ir GAV nebuvo naudojami kartu ($p < 0,05$).

Išvada. Mūsų rezultatai rodo, kad IFN-γ padidina citotoksiškumą GAV infekuotose makrofaguose, skatindamas iNOS geno raišką ir NO kaupimąsi. Šie duomenys yra svarbūs siekiant suprasti, kaip GAV sukelia ląstelių ir audinių pažeidimą plaučiuose.

Raktažodžiai: interferonas-gama, gripo virusas, citotoksiškumas, indukuojama azoto monoksido sintetazė, azoto monoksidas, makrofagai