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Expression of interferon gamma by a highly virulent strain of Newcastle disease virus decreases its pathogenicity in chickens

Leonardo Susta [a], Ingrid Cornax [a,1], Diego G. Diel [a,2], Stivalis Cardenas Garcia [a], Patti J. Miller [a], Xiufan Liu [c], Shunlin Hu [c], Corrie C. Brown [b], Claudio L. Afonso [a,*]

[a] Southeast Poultry Research Laboratory USDA ARS, 934 College Station Rd., Athens, GA 30605, USA
[b] Department of Veterinary Pathology, College of Veterinary Medicine, University of Georgia, Athens, GA 30605, USA
[c] Animal Infectious Disease Laboratory, School of Veterinary Medicine, Yangzhou University, Yangzhou, PR China

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ABSTRACT

The role of interferon gamma (IFN-γ) expression during Newcastle disease virus (NDV) infection in chickens is unknown. Infection of chickens with highly virulent NDV results in rapid death, which is preceded by increased expression of IFN-γ in target tissues. IFN-γ is a cytokine that has pleiotropic biological effects including intrinsic antiviral activity and immunomodulatory effects that may increase morbidity and mortality during infections. To better understand how IFN-γ contributes to NDV pathogenesis, the coding sequence of the chicken IFN-γ gene was inserted in the genome of the virulent NDV strain ZJ1 (rZJ1-IFNγ), and the effects of high levels of IFN-γ expression during infection were determined *in vivo* and *in vitro*. IFN-γ expression did not significantly affect NDV replication in fibroblast or in macrophage cell lines. However, it affected the pathogenesis of rZJ1-IFNγ *in vivo*. Relative to the virus expressing the green fluorescent protein (rZJ1-GFP) or lacking the IFN-γ insert (rZJ1-rev), expression of IFN-γ by rZJ1-IFNγ produced a marked decrease of pathogenicity in 4-week-old chickens, as evidenced by lack of mortality, decreased disease severity, virus shedding, and antigen distribution. These results suggest that early expression of IFN-γ had a significant protective role against the effects of highly virulent NDV infection in chickens, and further suggests that the level and timing of expression of this cytokine may be critical for the disease outcome. This is the first description of an *in vivo* attenuation of a highly virulent NDV by avian cytokines, and shows the feasibility to use NDV for cytokine delivery in chicken organs. This approach may facilitate the study of the role of other avian cytokines on the pathogenesis of NDV.

1. Introduction

Newcastle disease (ND), caused by virulent strains of Newcastle disease virus (NDV) is a widespread and economically significant disease of poultry [1,2]. The virus belongs to the order Mononegavirales, family *Paramyxoviridae*, subfamily *Paramyxovirinae*, genus *Avulavirus*. NDV, or avian paramyxovirus-1 (APMV-1), is a non-segmented, single-stranded, negative sense RNA virus. The virus genome is approximately 15,200 bases in length and contains six genes encoding for: nucleoprotein (NP), phosphoprotein (P), V protein (V), matrix (M), fusion (F), hemagglutinin-neuraminidase (HN), and large polymerase protein (L) [1].

Historically, NDV strains have been classified into velogenic (high virulence), mesogenic (moderate virulence), and lentogenic (low virulence) based on clinical signs induced in chickens [2]. Currently, the intracerebral pathogenicity index (ICPI) and the amino acid sequence of the fusion protein cleavage site are the standard tests to predict virulence of NDV isolates [3].

Although the pathogenesis of different NDV strains has been extensively studied [4–7], very little is known about the role of the host innate immune response during NDV infection. Previous

Abbreviations: NDV, Newcastle disease virus; ND, Newcastle disease; IFN-γ, interferon gamma; IFN-α, interferon alpha; IFN-β, interferon beta; IL-6, interleukin six; SAA1, serum amyloid protein A; NO, nitric oxide; iNOS, inducible nitric oxide synthetase; ICPI, intra cerebral pathogenicity index; MALT, mucosa associated lymphoid tissue; GFP, green fluorescent protein; ISRGs, interferon-stimulated response genes.

* Corresponding author. Tel.: +1 706 546 3642; fax: +1 706 546 3161.
E-mail address: Claudio.Afonso@ars.usda.gov (C.L. Afonso).
[1] Permanent address: Richard-Reimerschmid-Allee 24, 81241 Munich, Germany.
[2] Permanent address: Department of Pathobiology, College of Veterinary Medicine, University of Illinois, 2001 S Lincoln Ave, Urbana, 61802, USA.

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studies conducted in our laboratory have shown increased levels of IFN-γ, IFN-α/β and IL-6 mRNA in chicken splenocytes infected with the velogenic NDV strain California (CA), when compared to a lentogenic NDV strain [8]. In the same study, microarray gene expression profile on spleen samples of chickens infected with NDV strain CA, showed increased expression of the key cytokines IFN-γ, IL-6, and of numerous IFN-stimulated-response genes (ISRGs), at day 2 post infection (pi).

Additionally, we have observed increased levels of IFN-γ RNA in spleens of birds infected with velogenic NDV strains when compared to spleens of birds infected with mesogenic strains or mock-infected controls [9]. Increased expression of IFN-γ was consistent with elevation of nitric oxide (NO) levels in the sera of chickens infected with NDV strain CA at days 2 and 3 pi [8]. This increase was supported by a strong immunohistochemical staining for inducible nitric oxide synthetase (iNOS) in the spleen of infected birds [8]. Notably, IFN-γ is known to induce expression of iNOS in chicken macrophages [10]. Another study showed marked increase of IFN-γ mRNA in the peripheral blood of chickens infected with a velogenic NDV strain, at day 3 pi [11]. Furthermore, we have previously shown that a highly virulent strain of NDV (velogenic viscerotropic strain, ZJ1 [12]) induced high levels of IFN-γ in naïve chickens at day 3 post challenge (right before death), while vaccine-protected birds did not show increased expression of this gene [13].

Although the aforementioned studies have consistently shown increased expression of IFN-γ during NDV infection *in vivo*, it is unknown how the expression of this cytokine affects the pathological changes induced by NDV in tissues, and how it contributes to NDV pathogenesis. It is known that cytokines exacerbate numerous diseases by mediating tissue damage. Studies with highly pathogenic influenza viruses showed that an uncontrolled production of cytokines – defined as cytokine storm – plays a prominent role in the pathogenesis of the respiratory lesions caused by this virus in humans [14–16]. Particularly, in the acute respiratory disease caused by influenza virus, the cytokine storm is initiated by endothelial cells and amplified by interferon-producing cells [17]. Deleterious effect of the cytokine storm also contributes to morbidity of severe forms of poxvirus infection [18,19], and recently it has been shown that an IFN-γ dependent intestinal pathology contributes to lethality in toxic shock syndrome of mice [20]. Limited data are available on the role that cytokine storm or more specifically IFN-γ plays in the pathogenesis of avian infectious diseases. In chickens a disruption of the cytokine response to H5N1 highly pathogenic avian influenza has been described in the early stages of the disease; and an increase of IFN-γ transcription in the lung of chickens has been associated with decreased mean death time and wider tissue tropism of recombinant H5N1 infectious clones [21,22]. Additionally, IFN-γ has been associated with the pathogenesis and immunosuppression caused by highly virulent strains of Infectious Bursal Disease virus in chickens [23,24].

Chicken IFN-γ was first cloned in 1995 [25], and later it was mapped on chicken chromosome 1 [26]. Many similarities between mammalian and chicken IFN-γ have been reported, including the ability to activate macrophages, induce NO production, and to induce a Th-1 biased cytokine network response [10,27–30]. However, little is known about the function of IFN-γ in avian immunopathogenesis; and the knowledge about its role in various avian diseases, including Newcastle disease, remains still limited.

To assess the role of IFN-γ on NDV pathogenesis, a virulent NDV expressing high levels of chicken IFN-γ during replication was made and used to infect chickens.

2. Materials and methods

2.1. Animal research

This study was carried out in strictly accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of Southeast Poultry Research Laboratory (SEPRL) (FY2011, 2012, 2013).

2.2. Cells and viruses

DF-1 cells (Chicken embryo fibroblast cell line; ATCC CRL 12203) and HEp-2 cells (American Type Culture Collection, ATCC, Manassas, VA, CCL-23) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum (FBS). HD-11 cells (chicken bone marrow macrophages cell line, ATCC [31]), were maintained in Roswell Park Memorial Institute (RPMI) media, 5%–10% FBS and 1% streptomycin/penicillin. The recombinant modified *Vaccinia* virus Ankara expressing the T7 RNA polymerase (a generous gift of Bernard Moss, National Institute of Health) was grown in primary chicken embryo fibroblast cells. All recombinant NDV clones used in this study were rescued from plasmids, as described below.

2.3. IFN-γ cloning

Total RNA was extracted from chicken spleen using Trizol, as previously described [8]. Chicken IFN-γ cDNA was transcribed from total RNA using the SuperScript III One-Step RT-PCR System with Platinum Taq DNA polymerase kit (Invitrogen, Carlsbad, CA) with the primers IFN-γ1F and IFN-γ1R (Supplementary Table 1). Amplicons were cloned into the pCR2.1 vector (Invitrogen, Carlsbad, CA) and the correct sequence was confirmed by sequencing. The "gene start" (GS), "gene end" (GE) and the ApaI restriction sites sequences [12] were added to the IFN-γ gene by PCR amplification (High Fidelity PCR kit, Promega, Madison, WI) using primers IFN-γ2F and IFN-γ2R (Supplementary Table 1). The amplicon was cloned into the pCR2.1 vector. An additional stop codon was inserted at the end of the IFN-γ ORF to maintain the number of nucleotides between the two ApaI sites as a multiple of six. The resulting plasmid was named pCRIFNγ.

2.4. Construction of recombinant cDNA clones of rZJ1-IFNγ and rZJ 1-Rev

The whole genomic cDNA of the wild type NDV ZJ1 (Goose/China/ZJ1/2000) had been previously cloned into the expression vector TVT7 (TVT-FLNDV-ZJ1) and named pNDV/ZJ1 [12]. The chicken IFN-γ ORF containing the flanking regions (GS, GE and ApaI restriction sites) was inserted between the P and M genes of the ZJ1 genome, within the untranslated regions (UTRs) of the P gene. The strategy adopted was similar to what was previously described for insertion of the green fluorescent protein (GFP) coding sequence into TVT-FLNDV-ZJ1 (named pNDV/ZJ1GFP) [12]. Primers used are listed in the Supplementary Table 1.

To insert the IFN-γ gene into the ZJ1 backbone, the 2857–5637 region of the ZJ1 genome was amplified using primers Z6F and Z9R (Supplementary Table 1), and cloned into the pCR2.1 vector (Invitrogen, Carlsbad, CA). This region was subcloned into the pUC19 vector using HindIII and XbaI restriction enzymes, resulting in the plasmid pUCZJ1. The IFN-γ gene was then transferred from the pCRIFNγ plasmid into the pUCZJ1 plasmid through the ApaI restriction site, and the resulting intermediate plasmid was named