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Activation of the immune response against Infectious Bursal Disease Virus after intramuscular inoculation of an intermediate strain

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ABSTRACT

Infectious bursal disesase is a highly contagious, wide spread immunosuppressive chicken disease caused by the Infectious Bursal Disease Virus (IBDV). IBDV is a two segmented double-strand RNA virus, member of the *Birnaviridae* family. In order to study the interaction between IBDV and the immune system, chickens were exposed to an intermediate IBDV strain by intramuscular route, and using Real Time PCR the expression of a panel of avian cytokines and chemokines in duodenum, spleen and bursa of Fabricius was analyzed. Also, splenic nitrite (NO_2) production and the frequencies of different mononuclear cell populations were evaluated by Griess reaction and flow cytometry, respectively. Intramuscular (i.m.) IBDV inoculation promoted an over expression of proinflammatory cytokines IL-6, IL-15 and gIFN in spleen, which correlated with an increase of gIFN plasma concentration measured by ELISA, together with an increment of NO_2 concentration in splenocyte supernatants at 1 dpi. Results obtained in the present work showed that IBDV of intermediate virulence, given i.m., induced similar effects to those previously described for highly virulent IBDV in early innate immune responses. Considering that the i.m. route is the route of choice for the delivery of new generation vaccines, and that the use of recombinant antigens also requires the addition of adjuvants for proper immune stimulation, results presented here could contribute to identify suitable cytokines to be used or to be stimulated when utilizing subunit vaccines, for the improvement of prevention tools for avian health.

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Introduction

Infectious Bursal Disease Virus (IBDV) is a non-enveloped, icosahedral, two-segmented double-stranded RNA virus, member of the *Birnaviridae* Family (Dobos 1979; Sharma et al. 2000). The IBDV is endemic in most poultry producing areas worldwide, causing an acute, highly contagious, immunosuppressive disease in chickens (Eterradossi and Saif 2008). Two serotypes of IBDV (1 and 2) have already been described. Only serotype 1 viruses cause clinical signs and they are classified in increasing order of virulence as mild, intermediate, classical virulent and very virulent strains. Mild and intermediate viruses are used as live virus vaccines (Berg 2000). On the other hand, serotype 2 viruses may infect chickens and turkeys but they are non-pathogenic for both species (Jackwood et al. 1982; McFerran et al. 1980; McNulty and Saif 1988).

IBDV is a B-lymphotropic virus that infects and destroys dividing IgM bearing B-lymphocytes. Chickens infected with IBDV show both humoral and cellular immunosuppression. Humoral immunosuppression seems to be associated with the lysis of B-lymphocytes (Sharma et al. 1989). Cellular immunosuppression was evidenced by the ability of bursal T cells from IBDV infected chickens to inhibit ConA-mediated *in vitro* proliferation of normal splenocytes. Nevertheless, the mechanism of cellular immunosuppression induced by IBDV is still unclear (Kim and Sharma 2000).

The bursa of Fabricius is the principal target organ for IBDV infection and replication. Maturation and propagation of B-lymphocytes take place in this organ, which is present only in avian species.

Cytokines are essential effector molecules of innate and acquired immunity that initiate and coordinate cellular and humoral immune responses aimed at eradicating pathogens (Swaggerty et al. 2004). $CD4^+$ T helper (Th) cells play a major role in immune response. Th cells have been classified in Th1 and Th2 based on their cytokine balance (Janeway 1992). Like mammals, chickens show a Th1/Th2 immune response polarization as demonstrated by Degen et al. (2005) when infecting chickens with viral

Abbreviations: ConA, concanavalin A; dpi, days post-inoculation; EID, egg infectious dose; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IBDV, Infectious Bursal Disease Virus; IFN, interferon; IL, interleukin; i.m., intramuscular; mAbs, monoclonal antibodies; PBS, phosphate-buffered saline.

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Table 1
Oligonucleotides used to amplify cytokine and control coding regions.

Name	Sequence (5′–3′)	Access number (Gene bank)	Size of PCR product (bp)
LITAF Fw	CCATCTGCACCACCTTCA	NM_204267.1	184
LITAF Rv	TTGCTGCACATACACAGT		
IL-8 Fw	ATGAACGGCAAGCTTGGA	NM_205018.1	190
IL-8 Rv	GCAGTGGGGGCCGCTTGG		
IL-15 Fw	ACAGCCATTTCTTTTGCC	NM_204571.1	179
IL-15 Rv	CTCGTATGTGTTTGCAGT		
aIFN Fw	CTCACGCTCCTTCTGAAA	NM_205427.1	174
aIFN Rv	CAGGATGGTGTCGTTGAA		
gIFN Fw	CAAAGCCGCACATCAAACA	Y07922	259
gIFN Rv	TTTCACCTTCTTCACGCCATC		
IL-6 Fw	CAAGGTGACGGAGGAGGAC	AJ309540	254
IL-6 Rv	TGGCGAGGAGGGATTTCT		
GAPDH Fw	AGAACATCATCCCAGCGTCC	K01458	264
GAPDH Rv	CGGCAGGTCAGGTCAACA		

and helminth pathogens. Th1 cells have evolved to enhance clearance of intracellular pathogens through the production of a Th1 key role cytokine, gIFN and Th1 related cytokines such as aIFN, IL-15, IL-6, IL-12 and IL-17. Th2 cells are critical for the control of certain parasitic infections through the production of the clustered group of cytokines IL-4, IL-13 and IL-19. In addition, chemokines, another type of cytokines, such as IL-8, act as chemoattractant molecules (Kaiser et al. 1999).

Although oral route is the natural infection route for IBDV, systemic inoculation has demonstrated to be effective in producing high titers of neutralizing antibodies and protection in chickens, and this is the reason why several inactivated vaccines are delivered in this way. Also, different recombinant experimental vaccines have been tested using systemic inoculation. Francois et al. (2004) described the ability of a recombinant avian adenovirus expressing the VP2 protein of IBDV of inducing the effective production of neutralizing antibodies only when birds were immunized by systemic routes. Similar results were obtained by Sheppard et al. using another avian adenovirus (FAV 10) also expressing VP2. In this case, vaccination of chickens via the conjunctival sac (which finally results in a fluid delivered into the nasopharyngeal cavity) failed to produce any detectable antibodies against VP2. In contrast, systemic administration of the experimental vaccine yielded high titers of neutralizing antibodies and protected chickens against viral challenge (Sheppard et al. 1998).

Previous investigations have explored the pathogen–host relationship after IBDV exposure of virulent and very virulent strains (Eldaghayes et al. 2006; Khatri et al. 2005; Kim et al. 1998; Rautenschlein et al. 2007). The aim of the present work was to study the immune response elicited by the intramuscular administration of an intermediate strain of IBDV, commonly used as a live vaccine, in order to compare, in a near future, the results obtained, with those of vaccination experiments utilizing subunit antigens. The identification of suitable cytokines to be used or to be stimulated when utilizing subunit vaccines would contribute to the development of effective synthetic vaccines against IBDV.

Materials and methods

Animals and viruses

Specific-pathogen-free White Leghorn embryonated eggs were purchased from Rosenbusch S.A. (CABA, Argentina) and hatched in an automatic incubator (Yonar, CABA, Argentina). One day old chickens were kept in individual cages. Food and water were provided *ad libitum*.

The intermediate strain of IBDV was purchased from Laboratorios Inmuner (Entre Ríos, Argentina). The vaccine virus had a titer of 5×10^5 egg infectious dose (EID) per ml.

Experimental design

Twenty four three-week-old chickens were randomly designated into two groups. The experimental group was intramuscularly inoculated with 200 μl of the intermediate strain of IBDV. Negative control birds were mock-inoculated with an equal volume of phosphate-buffered saline (PBS).

At 1, 3 and 5 days post-inoculation (dpi), 3 chickens of each group were bled and euthanized. Pieces of 30 mg of spleen, duodenum and bursa of Fabricius, excised from exactly the same position of the organs, were kept immediately in RNAlater solution (QIAGEN, Valencia, CA) and stored at 4 °C. Spleens were harvested aseptically and they were used for NO_2 assay and flow cytometry. Rests of bursas were also kept for flow cytometry evaluation.

The 3 remaining birds of each group were bled weekly for 35 days to measure specific antibodies against IBDV.

RNA isolation and cDNA synthesis

RNA from each piece of tissue (spleen, duodenum, bursa of Fabricius) was obtained with the RNeasy kit (QIAGEN, Valencia, CA) according to the manufacturer's instructions and then it was stored at −80 °C until used. RNA was treated with DNAse I and reverse transcription was performed using SSIII Reverse transcription kit (Invitrogen, Carlsbad, CA) and random hexamers.

Quantitative RT PCR

Oligonucleotides used to amplify fragments of chicken cytokines and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) control genes were designed based upon sequences available at public databases (Table 1). Amplification and detection were carried out using equivalent amounts of RNA from each tissue.

Preparation of constructs and creation of standard curves for all cytokine genes used in this study, as well as for GAPDH gene, were performed as described before (Haghighi et al. 2008). Briefly, a fragment of aprox. 200 bp of the coding region of each gene was amplified by PCR using appropriate sets of primers (Table 1) and cloned in pGEM T vector (Promega Madison, USA). To calculate the number of copies of standard plasmids, the following formula was used:

$$\frac{\text{DNA concentration}(g/\mu l) \times 6 \times 10^{23}}{\text{Molecular weight of the recombinant plasmid}(g/mol)}$$

Serial 10-fold dilutions (containing from 10^9 to 10^1 DNA copies) were amplified using Real Time PCR with SYBR®Green Master Mix Kit in an ABI 7500 thermocycler (Applied Biosystems, Warrington, UK). Cycle threshold (CT) values were used to plot a standard curve.

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