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

Juan Manuel Carballeda[a,b], Silvina Chimeno Zoth[a,b], Evangelina Gómez[a], María José Gravisaco[a], Analía Berinstein[a,b,*]

[a] Instituto de Biotecnología, CICVyA, INTA, Castelar, Cc25 B1712WAA Buenos Aires, Argentina
[b] Consejo Nacional de Investigaciones Científicas y Tecnológicas (CONICET), Rivadavia 1917 C1033AAJ Ciudad de Buenos Aires, Argentina

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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.

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.

* Corresponding author at: Instituto de Biotecnología, CICVyA, INTA, Castelar, Cc25 B1712WAA Buenos Aires, Argentina. Tel.: +54 11 4621 1278.
E-mail address: aberinstein@cnia.inta.gov.ar (A. Berinstein).

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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.

Sample CT values were extrapolated in the standard curve in order to determine the initial amount of each particular transcript.

Lymphocyte isolation

Single cell suspensions were obtained from pools of spleens by mechanical disruption in RPMI 1640, and mononuclear cells were isolated by centrifugation over Histopaque density gradient (1.077 g/ml; Sigma, St. Louis, MO) at room temperature. Cells were isolated from the interface, washed, and live cells were counted using trypan blue exclusion.

Bursas were cut in very small pieces and mechanically disrupted by pressing with a syringe plunger, in RPMI 1640. Then, cellular suspensions were passed through a 40 μm mesh (Cell Strainer, BD) and mononuclear cells were isolated by centrifugation over Histopaque density gradient (1.077 g/ml; Sigma, St. Louis, MO) at room temperature. Cells were recovered from the interface, washed, and live cells were counted using trypan blue exclusion.

Flow cytometry analysis

Cells were diluted in staining buffer (PBS 1×, 10% FBS, 0.1% Sodium Azide) and 1×10^6 cells per well were seeded on 96 well-plates (V-shape), and washed twice with the same buffer. Staining was performed by resuspending the cellular pellet of each well with 100 μl of staining buffer including different combinations of antibodies, or as single-color stainings for compensation. Cells were incubated at 4 °C for 30 min and washed twice with staining buffer by centrifugation at 290 × *g* for 10 min.

Monoclonal antibodies (mAbs) (CD3-SPRD, CD4-PE, CD8α-FITC, CD8β-PE, KUL01-PE) were purchased from Southern Biotech. (Birmingham, AL). mAb 28.4 was kindly given by Dr Thomas Göbel (Ludwig-Maximilians-Universität (LMU) München) and a secondary antibody (goat anti-mouse-PE, Invitrogen) was also used. All antibodies were titrated in order to determine the optimal staining concentration of each one.

Positive cells were analyzed with a FACS Calibur flow cytometer (BD Biosciences, San Jose, CA) and CellQuest software. Both, lymphocyte and monocyte gates were defined by the forward/side scatter characteristics of the cells and 20,000 and 50,000 events were analyzed respectively for each sample.

Splenic nitrite production

Splenocytes were resuspended in RPMI medium supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 10 mM HEPES, 50 μM 2-mercaptoethanol and 10% FBS. 2×10^6 cells per well were seeded on 96 well-culture plates in the presence or absence of concanavalin A (ConA; 5 mg/ml) and incubated for 24 h at 41 °C in a 5% CO_2 atmosphere. Culture supernatants were collected and nitrite concentration was measured by the Griess reaction (Tsikas 2007). Briefly, 50 μl of culture media and serial fold dilutions of $NaNO_2$ [(from 125 to 1 μM) used as a standard curve] were displaced in 96 wells flat bottom microtiter plates (Nunc MaxiSorp®, eBioscience, CA, USA). Then, 50 μl of each Griess Reagent (Sulfanilamide and naphthylethylenediamine) were added and the 530 nm absorbance of each well was measured with a multiskan reader (Thermo Scientific Inc., MA, USA). NO_2 concentration was determined by extrapolation of the absorbance at 530 nm in the standard curve.

Plasmatic gIFN concentration

Plasma samples of treated chickens were analyzed by ELISA using the CytoSet Kit (Biosource, CA, USA) in 96 well plates, following manufacturer's instructions. Serial two-fold dilutions of recombinant gIFN were used to plot a standard curve, where OD values of each sample were extrapolated in order to calculate the plasmatic concentration of gIFN in each sample.

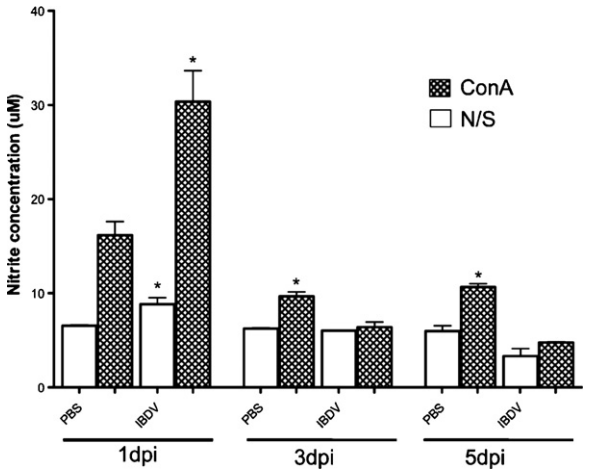

Fig. 1. Splenic NO_2 concentrations (mean ± SEM) in splenocyte cultures obtained from IBDV (or PBS) inoculated chickens. Splenocytes were isolated from disaggregated spleen tissues at 1, 3 and 5 dpi. After centrifugation over Histopaque density gradient (1.077 g/ml), cells were washed, counted and plated at 2×10^6 viable cells/well in triplicate in the presence (or absence) of ConA (5 mg/ml). Cells were incubated for 24 h at 41 °C with 5% CO_2. Supernatant fluid was collected and NO_2 concentrations were measured using Griess reagent. Each bar represents mean ± SEM of 3 replicates of each pool, constituted by samples obtained from 3 IBDV or PBS inoculated chickens at 1, 3 and 5 dpi. $^*p < 0.05$.

IBDV specific antibodies

Plasma of 3 IBDV or PBS inoculated chickens was obtained at 0, 7, 14, 21 and 35 dpi. Samples were analyzed using the FlowChek IBDV Kit (IDEXX, Maine, USA) following the manufacturer's instructions. Specific antibodies titers were calculated.

Statistical analysis

The Student's *t*-test was used to determine significant differences between mock-infected and IBDV-infected chickens. A value of $p < 0.05$ was considered to be statistically significant.

Results

Macrophage activation

Since the amount of NO_2 produced is related to macrophage activation (Jeurissen et al. 2000), the splenic nitrite production in ConA or mock stimulated splenocytes from IBDV or PBS inoculated chickens was measured. At 1 dpi a significant increase ($p < 0.05$) in NO_2 production in both ConA and mock stimulated splenocytes from IBDV inoculated chickens was observed (Fig. 1). At 3 and 5 dpi no differences in NO_2 production were registered in mock stimulated avian splenocytes. However, ConA stimulated splenocytes from IBDV treated chickens exhibited less NO_2 production than ConA stimulated splenocytes from control animals.

These results suggest an immunosupressive effect of IBDV at 3 and 5 dpi, in contrast to the activation observed at 1 dpi.

Cytokine response to IBDV inoculation

Spleen

Inoculation of chickens with an intermediate strain of IBDV resulted in the induction of proinflammatory cytokine genes

expression, such as IL-6 and IL-15, at 1 dpi in spleen (Fig. 2A). Also, animals inoculated with IBDV showed an increased amount of gIFN mRNA at 3 dpi. At this time, IL-6 still remained up regulated, whereas IL-15 was strongly down regulated. IL-6 was not up regulated at 5 dpi, although gIFN was still up regulated and IL-15 mRNA expression was strongly increased again (Fig 2A). Other mRNAs, such as aIFN, and the inflammatory innate immune related protein LITAF (Hong et al. 2006), were also up regulated at 5 dpi although in lower degree. IL-8 mRNA was measured as well and differences were only found at 1 dpi, when a down regulation was observed.

Duodenum

Since chickens have poorly developed lymph nodes and the major natural killer cell population is found in the intestine (Göbel et al. 2001) we analyzed cytokine gene mRNA expression in duodenum. As it is shown in Fig. 2B, an over expression of gIFN mRNA and of the antiviral cytokine aIFN at 1 dpi was observed. The IL-8 chemokine was also over expressed at this point, fact that was probably related to an antiviral response against IBDV. On the other hand, IL-15 was strongly down regulated at this time. IL-6, IL-8 and LITAF were strongly up regulated together with aIFN and gIFN which remained over expressed at 3 dpi (Fig. 2B). This result suggests a strong inflammatory immune response in duodenum.

At 5 dpi, IL-6, IL-15, aIFN, IL-8 and LITAF mRNA showed a notable down regulation. On the other hand, gIFN remained slightly up regulated (Fig. 2B).

Bursa of Fabricius

The bursa of Fabricius, where immature B cells undergo development, is a unique organ of avian species (Scott 2004). As it was stated before, IBDV replicates mainly in actively dividing B lymphocytes; thus, infection leads to the destruction of lymphoid cells in the bursa of Fabricius (Rautenschlein et al. 2002). Hence, we analyzed the cytokine mRNA expression pattern in this organ. At 1 dpi after IBDV exposure IL-6, gIFN and IL-8 were up regulated consistently with the results observed in duodenum. However, LITAF was down regulated (Fig. 2C). At 3 dpi IL-15 and LITAF mRNA expression was down regulated, while gIFN and IL-6 appeared to be up regulated. At 5 dpi, IL-8 was slightly down regulated and, in contrast to what occurred in duodenum, IL-6, gIFN and IL-15 were up regulated (Fig. 2C).

Plasmatic concentration of gIFN

To analyze the systemic immune response to IBDV inoculation at 1, 3 and 5 dpi, and to correlate the results with the tissue cytokine mRNA expression pattern and macrophage activation observed, the plasmatic concentration of gIFN was measured. An increase in plasmatic gIFN concentration at 1 dpi in the 3 IBDV inoculated animals was found, reaching values near to 1000 pM (Fig. 3). Plasmatic concentration levels of gIFN in PBS inoculated chickens at 1 dpi were undetectable, as they were in IBDV and PBS inoculated chickens at 3 and 5 dpi (data not shown).

Evaluation of cellular populations by flow cytometry

In order to study modifications in the frequencies of immune cells, mononuclear cells isolated from spleen and bursa were stained with different combinations of monoclonal antibodies and evaluated by flow cytometry. Inoculation of chickens with IBDV induced a notable increase of the percentage of all cellular populations studied, revealing a T-lymphocyte infiltration at 5 dpi in the bursa (Table 2). In spleen, an increase in the percentage of $CD4^+$ and $CD8\alpha^+$ lymphocytes was also observed in the gate; in contrast, NK

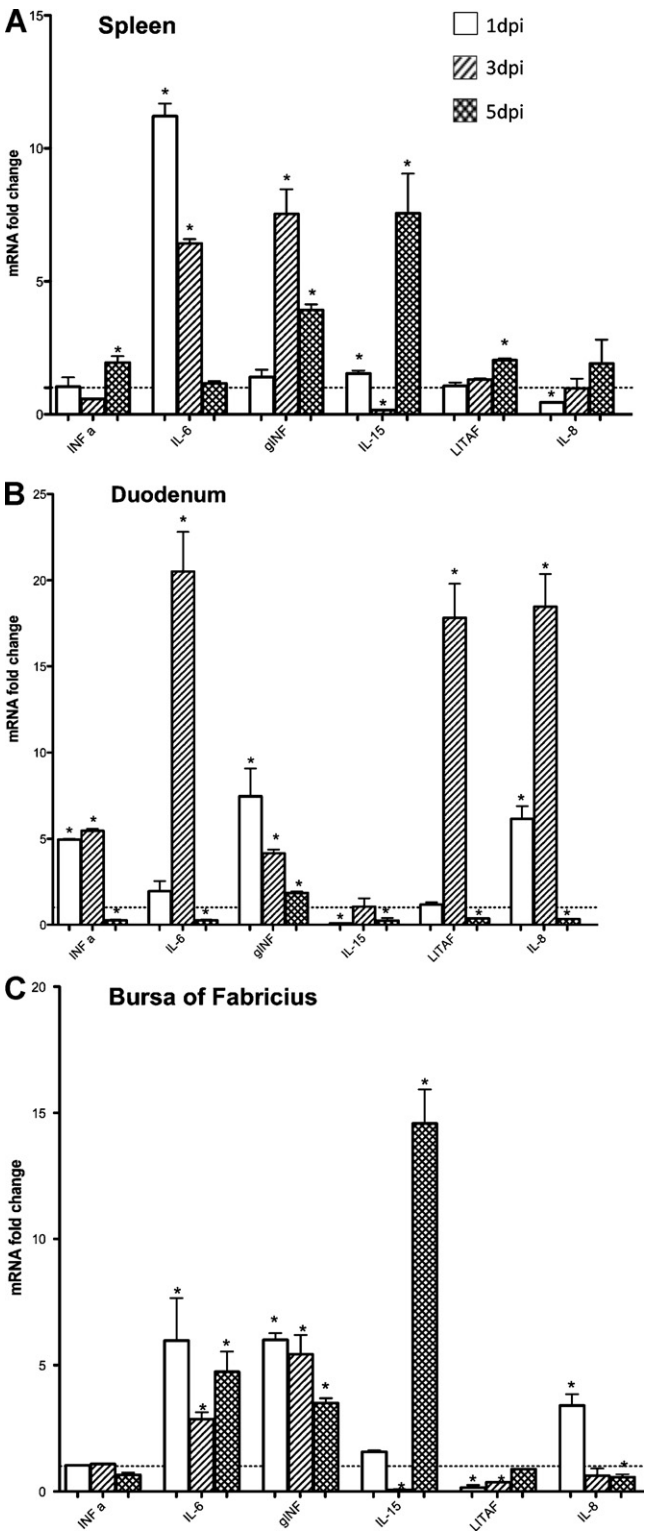

Fig. 2. Transcriptional pattern of cytokine genes. Total RNA was extracted from A: spleen, B: duodenum, and C: bursa of Fabricius of IBDV (or PBS) inoculated chickens at 1, 3 and 5 dpi and cDNA was synthesized. mRNA levels were determined by Quantitative Real Time PCR using specific primers and SYBR®Green method. The expression level of each mRNA was calculated in relation to the expression level of GAPDH mRNA and expressed as a fold increase between IBDV and PBS inoculated chickens. Each bar represents mean ± SEM of 2 replicates of each pool constituted by samples obtained from 3 IBDV or PBS inoculated chickens at 1, 3 and 5 dpi. $^*p < 0.05$.

Table 2
Evaluation of cell populations by flow cytometry.

Source of cells	% Positive cells in the gate				
	$CD3^+CD4^+$	$CD3^+CD8\alpha^+\beta^+$	$CD3^-CD8\alpha^+$	$28.4^+CD8\alpha^+$	$KULO1^+$
Spleen PBS	34.4	26.2	28.4	8.7	9.8
Spleen IBDV	41.6	28.2	22.6	5.5	1.9
Bursa PBS	0.7	0.2	0.3	0.4	19.7
Bursa IBDV	5.4	4.4	5.4	0.9	23.3

Chicken leukocytes were isolated from pools of spleens and bursas of IBDV- or PBS-inoculated animals at 5 dpi. Cells were stained with different combinations of antibodies and analyzed by flow cytometry. For $CD3^+$, $CD4^+$, $CD8\alpha^+$ and $CD8\beta^+$ the gating strategy was: location of the lymphocytes in a forward/side scatter-defined gate. For 28.4 mAb, following the identification of the lymphocytes, CD3-cells were gated and the presence of $28.4^+CD8\alpha^+$ cells was analyzed. $KULO1^+$ cells were studied in the monocyte gate defined in a forward/side scatter graph. Results were expressed as the percentage of stained cells in the gate.

cells (28.4^+ $CD8\alpha^+$) and monocytes/macrophages ($KUL01^+$) showed a drop in the percentage of the gate.

IBDV specific antibodies in plasma

In order to confirm the immune response triggered by intramuscular IBDV inoculation, we analyzed specific antibody production in sera of PBS and IBDV inoculated chickens at 0, 7, 14, 21 and 35 dpi. A specific anti-IBDV humoral immune response began to be detected at 7 dpi, and reached the maximum titer value at 21 dpi. The level of antibodies remained high until 35 dpi (Fig. 4).

Discussion

IBDV infects chickens by the oral route and initially may replicate in cells of the gut-associated lymphoid tissues (Vervelde and Davison 1997). For this reason, many live anti-IBDV vaccines are delivered orally, infecting birds trying to cause minimal adverse reactions. Nevertheless, intramuscular (i.m.) inoculation of animals also confers anti-IBDV immunity and the same live vaccines are used routinely by this inoculation route (OIE-*Manual of Diagnostic Tests and Vaccines for Terrestrial Animals, 2010,* http://www.oie.int/esp/normes/mmanual/a_index.htm).

On the other hand, almost all developed recombinant vaccines and subunit vaccines are delivered by intramuscular inoculation. In this way, the knowledge of the processes involved in the immune response obtained against IBDV when using the i.m. route results necessary and an important fact to take into consideration when designing novel recombinant and/or subunit vaccines.

In this study we analyzed several cytokine profiles produced in avian spleen, duodenum and bursa of Fabricius, key organs involved in anti-IBDV immune response. IBDV infection causes the production of proinflammatory cytokines by macrophages, which reaches their highest levels during the early phase of active virus replication (Khatri et al. 2005; Palmquist et al. 2006). We analyzed the mRNA expression of aIFN, gIFN, IL-6, IL-8, IL-15 and LITAF at 1, 3 and 5 days after IBDV i.m. inoculation. Considering that chicken NK cells develop in the bone marrow, mature in spleen and mainly reside in the intestine (Göbel et al. 2001), and taking into account that we observed an over expression of gIFN mRNA (probably secreted by activated NK cells and T lymphocytes) in duodenum and bursa of Fabricius (Fig. 2) and a high systemic gIFN concentration at 1 dpi (Fig. 3), we speculate that IBDV inoculation triggers NK cells activation in these organs. However, further investigations need to be performed to address this aspect.

Rautenschlein et al. (2007) reported that IBDV infection affects cytokine expression not only in the bursa of Fabricius and spleen, but also in caecal tonsils, supporting the speculation that IBDV infects through the gut-associated lymphoid tissue (GALT). According with this observation are our results which show that IBDV inoculation strongly affects the expression of cytokines, mainly IL-6, IL-8, LITAF at 3 dpi and gIFN and aIFN at 1 and 3 dpi, in duodenum, even though the virus was not administered by oral route.

Chicken spleen, as a secondary lymphoid organ, provides an essential microenvironment for interaction between lymphoid and non-lymphoid cells. The contribution of the avian spleen to the immune system as a whole may be more important than in mammals because of the poorly developed avian lymphatic vessels and nodes (Oláh and Vervelde 2008). The spleen, where a large macrophage population (main target of gIFN) exists, could be the target of the produced gIFN as we observed an increment of NO_2

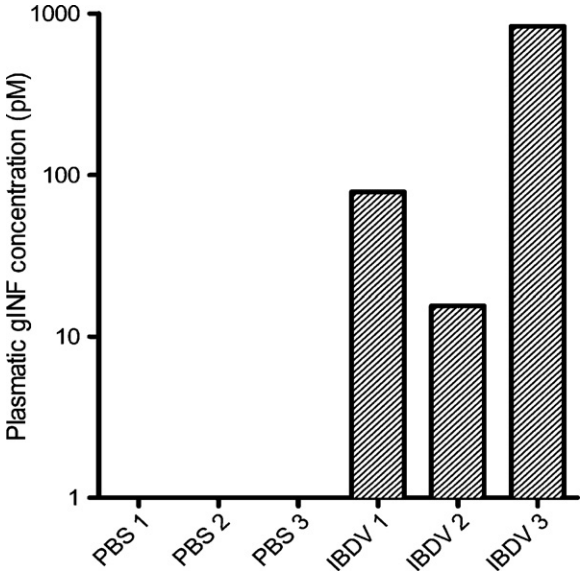

Fig. 3. Plasmatic gIFN concentration. Plasma samples of IBDV (or PBS) inoculated chickens were obtained at 1 dpi. Plasmatic concentrations of gIFN were individually determined by ELISA (CytoSet Kit, Biosource).

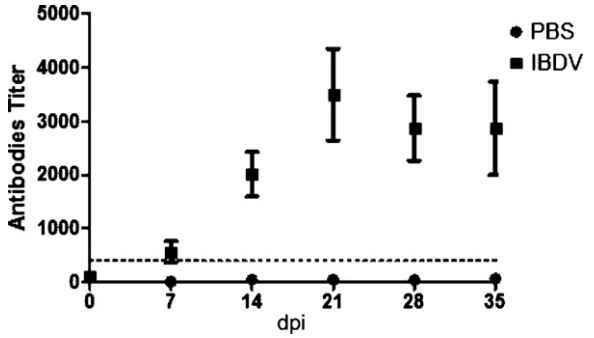

Fig. 4. Specific antibodies against IBDV. Serum samples were obtained from IBDV or PBS inoculated chickens at 0, 7, 14, 21 and 35 dpi and specific antibodies titers were individually determined by ELISA (FlowChek IBDV Kit, IDEXX). Each point represents the mean ± SEM of 3 animals at each time point. Line indicates the cut-off value of the assay.

production and an over expression of a variety of inflammatory cytokines mRNA, such as IL-6 at 1 dpi. This observation agreed with other studies (Kim et al. 1998), which demonstrated that virulent IBDV infection resulted in enhanced expression of IL-6 and elevated levels of NO_2 in splenic macrophages. Our results also showed that IL-6 expression level decreased at 3 dpi and remained low at 5 dpi. At this time, NO_2 production by splenocytes obtained from IBDV-treated chickens was lower than the one detected in the corresponding control sample (Fig. 1). In addition, a drop in macrophage population in the spleen was detected at 5 dpi by flow cytometry (Table 2). These results are in accordance with the results obtained by Palmquist et al. (2006) who demonstrated that virulent strains of IBDV induced a decrease in splenic macrophage population.

Flow cytometry assays performed on bursal cells, showed a notable infiltration of T cells ($CD4^+$ and $CD8^+$) in the bursa, induced by intermediate IBDV at 5 dpi (Table 2). $CD4^+$ percentage peaked from 0.7 to 5.4 and the percentage of $CD8^+$ varied from 0.2 to 4.4. Strong increases of T cells in the bursa had been previously described by Kim and coworkers in chickens infected with virulent IBDV (Kim et al., 2000).

Eldaghayes et al. (2006) reported the unexpected absence of increased levels of aIFN mRNA in the bursa of animals infected with virulent and very virulent strains of IBDV, while Gelb et al. (1979) previously demonstrated that attenuated and virulent strains of IBDV stimulated the production of an antiviral factor, assumed to be type I IFN. Results obtained in the present work also showed no significant increase in aIFN mRNA expression, according to Eldaghayes and coworkers observations.

As expected, intramuscular administration of a middle strain of IBDV induced high titers of specific antibodies against IBDV.

The results obtained in the present work demonstrated the ability of an intermediate strain of IBDV to produce an inflammatory effect in different organs of chickens at short times after i.m. inoculation, showing similarity with previous results described for virulent IBDV strains. Also, we noted the importance of studying different organs simultaneously to get a more comprehensive idea of the immune response induced by the virus in its natural host.

Being the intramuscular route the route of choice for the delivery of new generation vaccines, we believe that these results could be of great interest for the rational development of preventive tools in avian health.

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