IBDV Challenge with an Intermediate Strain for Evaluation of Immunogens in Chickens: Seeking for Better Animal Welfare Conditions

1,2Silvina Chimeno Zoth, 1,2,3Juan M Carballeda, 1María J Gravisaco, 1,2Evangelina Gómez, 1María S Lucero, 1,2Matías Richetta and 1,2Analía Berinstein

1Instituto de Biotecnología, CICVyA, INTA, Castelar, Cc 25, B1712WAA, Buenos Aires, Argentina
2Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Rivadavia 10 1917, C1033AJA, CABA, Argentina
3Present address: Fundación Instituto Leloir- Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Buenos Aires, Argentina

Abstract

Gumboro is a highly contagious, worldwide spread immunosuppressive chicken disease caused by the Infectious Bursal Disease Virus (IBDV). The severity of the disease depends on characteristics of the infected bird and the virulence of the infecting virus. The disease causes very severe signs and long-lasting immunosuppression, letting the animals more susceptible to other diseases, including those that do not normally affect healthy chickens. To avoid the use of extremely virulent IBDV, and the consequences suffered by the birds, we present here an alternative method to perform a first evaluation of immunogens against IBDV. We inoculated White Leghorn chickens with 2500, 10000 and 25000 EID₅₀ of an intermediate virus and studied cellular and histopathological lesions in bursa. We analyzed different cytokine profiles by qRT-PCR, bursal lesions by classic histopathology methods, and T cells infiltration by flow cytometry at 3 and 6 days post inoculation. Taken all together, the results obtained allowed us to determine that 25000 EID₅₀ of the IBDV strain used is the adequate dose to use in order to reproduce typical aspects of infection in Bursa of Fabricius, without very virulent virus manipulation, avoiding the exposure of birds to very virulent IBDV.
weeks of age. The disease has a sudden onset, and the mortality rate in the flock increases rapidly. Clinical signs of disease include dehydration, trembling, ruffled feathers, vent pecking, and depression. Also, the disease can cause severe, long-lasting suppression of the immune system. Chickens that are immune suppressed by early IBD infections do not respond well to vaccination and are more susceptible to other diseases, including those that do not normally affect healthy chickens. On necropsy, the main lesions are found in the Bursa of Fabricius. This organ is exclusive of birds and it is a specialized and essential organ for the amplification and differentiation of B cells. Despite different assays like ELISA are believed to correlate with the level of protection elicited by vaccination [8], sometimes it is desirable to test the effectiveness of a given vaccine by performing a challenge experiment. However, during challenge, animals are exposed to tissue damage and pain produced by infection and, depending on the disease working with, special challenge conditions are requested, like the use of isolated cages or to work in BSL 3 laboratories, conditions that are not always available. We present here an alternative method to first evaluate immunogens against IBDV.

Materials and Methods

Animals and virus

Specific-pathogen-free (SPF) White Leghorn chickens (eggs from Rosenbusch S.A., CABA, Argentina) were used all through the research work. All procedures involving the use of animals were performed in agreement with institutional guidelines and approved by the Institutional Committee for the care and use of experimental animals (CICUA-E – CICVYA – INTA. Authorization reference number: 4/2011). Animals were maintained in individual cages, with water and food provided ad libitum. IBDV intermediate virus LZD was obtained from Laboratorios Inmuner S.A., Entre Ríos, Argentina.

Experimental design

Twenty four SPF chickens of 21 days of age were randomly divided into four groups. Three groups received 50, 200 and 500 µl of the mentioned virus (5 x 10⁴ EID₅₀/ml) and the control group received the highest volume of vaccine diluent. Three animals of each group were sacrificed at 3 and 5 days post inoculation. (d.p.i.) and the bursas were removed. A fragment of 30 mg of bursa was submerged in RNA stabilizing solution (RNAlater, Qiagen). The rest of the organ was divided into two similar fragments. One of them was submerged in RPMI media for subsequent lymphocytes isolation, and the other was submerged in fixing solution for histological processing.

RNA Isolation and cDNA synthesis

RNA was obtained from each sample utilizing RNAeasy kit (Qiagen) following manufacturer’s instructions. The purity and concentration of each RNA obtained was determined using the absorbance 260/280nm relation. After treatment with DNAseI, reverse transcription was done using SSIII kit (Invitrogen) and random hexamers.

Quantitative RT PCR

Primers used for amplification of interferon gamma (IFNg), alpha (IFNa), interleukin 6 (IL-6) and GAPDH (glyceraldehyde 3-phosphate dehydrogenase, internal control) genes were designed based on available reported sequences (Table 1, 9). To quantify mRNA of the mentioned genes, recombinant plasmids containing a ~200bp fragment of each codifying region were constructed to create standard curves [9]. Quantification was carried out with the SYBR®Green Master Mix Kit (Applied Biosystems, Warrington, UK). Cycle Threshold (CT) values were used to plot a standard curve in which the CT values decreases in linear proportion with the log of the template concentration. Sample CT values were extrapolated in the standard curve in order to determine the initial amount of each particular transcript.

Histopathological observation of bursa

A fragment of each bursa was processed for histopathological observation following standard procedures.

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5' - 3')</th>
<th>Access number (Gene bank)</th>
<th>Size of PCR product (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFNa Fw</td>
<td>CTCACGCTCCTTCTGAAAA</td>
<td>NM_205427.1</td>
<td>174</td>
</tr>
<tr>
<td>IFNa Rv</td>
<td>CAGGATGGTGTCGTTGAAA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IFNg Fw</td>
<td>CAAAAGCGGCACTCAAAACA</td>
<td>Y07922</td>
<td>259</td>
</tr>
<tr>
<td>IFNg Rv</td>
<td>TTTACCTCTCTCGACCACTC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-6 Fw</td>
<td>CAAGGTGACGGAGGAGGAC</td>
<td>AJ309540</td>
<td>254</td>
</tr>
<tr>
<td>IL-6 Rv</td>
<td>TGGCGAGGAGGGATTTCT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GAPDH Fw</td>
<td>AGAACATCATCCCAGGCTCC</td>
<td>K01458</td>
<td>264</td>
</tr>
<tr>
<td>GAPDH Rv</td>
<td>CGGCAGGTCAAGGTCAAACA</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 1 Oligonucleotides used to amplify cytokine and control coding regions.
Briefly, samples were submerged in fixing solution, transferred to successive solutions with increasing concentrations of ethanol, incubated with xylene and embedded in paraffin. Histopathological analysis was performed by staining bursa sections with the hematoxylin/eosin method.

**Lymphocyte Isolation and flow cytometry analysis**

Another fraction of the organ was processed for mononuclear cells isolation by ficoll gradient centrifugation and subsequent staining with specific monoclonal antibodies for lymphocyte markers conjugated to different fluorochromes and flow cytometry analysis as described before [9,11]. Briefly, bursas were cut in very small pieces and mechanically disrupted by pressing with a syringe plunger. Cellular suspensions were passed through a mesh (Cell Strainer, BD) and mononuclear cells were isolated by centrifugation over Histopaque density gradient (1.077 g/ml; Sigma, St. Louis, MO). Cells obtained were resuspended in Staining Buffer (PBS 1x, 10 % FBS, 0.1 % Sodium Azide) and 1x10⁶ cells per well were seeded on 96 well-plates. Staining was performed by resuspending the cellular pellet of each well in 100 µl of staining buffer including different combinations of antibodies, or as single-color staining for compensation. Monoclonal antibodies (CD3-SPRD, CD4-PE, CD8α-FITC, CD8β-PE, KUL01-PE) were obtained from Southern Biotech (Birmingham, AL). 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 30,000 and 50,000 events were analyzed respectively for each sample.

**Results and Discussion**

To determine the optimal IBDV dose capable of producing alterations at cellular and histopathological levels in the bursa, we inoculated different amounts of an intermediate virus and studied different parameters in the chicken. First, we evaluated the expression of IFNg, IFNa, IL-6 and GAPDH by real time-PCR. A very strong over expression of inflammatory cytokines (IFNg, IL-6) and IFNa was observed (Figure 1). This effect was found to be dose dependent, being stronger when animals were inoculated with 500 µl (2.5 × 10⁴ EID₅₀) of IBDV on day 3 (Figure 1). Previous studies using virulent and very virulent strains showed the up regulation of IL-6 and IFNg in bursa of IBDV treated chickens. However, the authors observed that type 1 IFNs were not up regulated or were even down regulated when infecting chickens with very virulent IBDV strains [10]. In this way, the absence or presence of IFNa in infected chicken samples could give a clue on the degree of virulence of the infecting virus. The microscopic observation of the bursas showed a gradual increase of histological damage in relation with the dose of IBDV received, being the effect observed for the lowest dose used (50 µl, 2.5 x 10⁵ EID₅₀) undistinguishable from the negative control (Figure 2). Higher doses produced, in increasing degree, alterations compatible with IBDV infection. Animals which received 200 µl (1 x 10⁶ EID₅₀) of IBDV showed diffused apoptosis spots. These observations were more pronounced in animals that received 500 µl of IBDV at 5 d.p.i., when more histological damage was observed, i.e. lymphoid depletion, presence of dense apoptotic bodies regions, modification of the cortico-medullar epithelia and follicular stromal thickening, infiltration of inflammatory cells, etc. (Figure 2).
Figure 2: Bursal tissue sections stained with hematoxylin and eosin. A: normal bursa; B, C and D: Bursa of animals infected with 50, 200 and 500 µl of an intermediate strain of IBDV strain, respectively.

Table 2 Evaluation of cellular populations by flow cytometry

<table>
<thead>
<tr>
<th>Cell subpopulation</th>
<th>CD3+</th>
<th>CD3+CD8α+</th>
<th>CD3+CD4+</th>
<th>CD8α+CD8β+</th>
<th>KUL01+</th>
</tr>
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<tbody>
<tr>
<td>IBDV dose</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-</td>
<td>3.25</td>
<td>0.66</td>
<td>0.72</td>
<td>0.51</td>
<td>24.1</td>
</tr>
<tr>
<td>50µl</td>
<td>2.49</td>
<td>0.44</td>
<td>0.5</td>
<td>0.26</td>
<td>25.47</td>
</tr>
<tr>
<td>200µl</td>
<td>3.11</td>
<td>0.87</td>
<td>0.42</td>
<td>0.67</td>
<td>20.92</td>
</tr>
<tr>
<td>500µl</td>
<td>6.58</td>
<td>3.37</td>
<td>1.85</td>
<td>3.15</td>
<td>25.59</td>
</tr>
</tbody>
</table>

Lymphocytes isolated at 5 dpi from pools of bursae of IBDV-infected and PBS-treated chickens were stained with different combinations of antibodies and analyzed by flow cytometry. For CD3+, CD4+, CD8α+ and CD8β+ the gating strategy was location of the lymphocytes in a forward/side scatter-defined gate. KULO1+ cells were studied in the monocytes/macrophages gate defined in a forward/side scatter graph. Results are expressed as the percentage of stained cells in the gate.

cells and edema, among others (Figure 2). Finally, results of the cytometry analysis of bursal lymphocytes are shown in table 2. The presence of T cells was only detected in bursae from chickens inoculated with 500 µl of IBDV (Table 2). It is already known that in virulent and very virulent IBDV infected chickens, there is an increase in the number of intrabursal T cells, compared to the bursas of uninfected chickens [12-14]. In the same way, we found intrabursal T cells augmented when chickens were infected with the highest dose of classical IBDV. Practically, none T cells were found in the bursae of the rest of the animals. In nature, affected birds shed large amounts of virus for about 2 weeks after infection. IBDV is shed in faeces and spreads between birds or by contact with a contaminated environment. IBDV is very stable and difficult to eradicate. Mealworms and litter mites may harbor the virus for 8 weeks. In this context, avoidance of the use of virulent virus for experimental challenge is interesting, as it reduces the risk of virulent virus dissemination. In a recent study on vaccination approaches against IBDV, Arnold and coworkers used the classical IBDV strain. Edgar for challenge experiments and subsequent bursal lesion score analysis [15]. In their case they had to use 100 EID<sub>50</sub> to produce a significant disease. Each challenge virus may have to be evaluated to determine the optimal dose to be used, but the approach is being incorporated in different laboratories.
Taken all together, our results allowed us to determine that $2.5 \times 10^4$ EID$_{50}$ of an intermediate strain of IBDV is the adequate dose to use to reproduce typical aspects of infection in Bursa of Fabricius, without very virulent virus manipulation, avoiding the exposure of birds to very virulent IBDV. We could reproduce infection without disease manifestation using a high dose of the virus. This method brings a safer and less traumatic option for first evaluating IBDV vaccines.

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**References**


