

«Research Note»

A Preventive Cytokine Treatment of the Viral Infectious Bursal Disease (IBD) of Chickens

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Infectious bursal disease (IBD) is a viral disease of young chickens that produce severe lesions in the bursa of Fabricius and other organs inducing immunosuppression and mortality in birds. This study indicates that oral administration of IFN- α and IL-2 during 16 days produced a significant reduction in animals' morbidity and mortality to IBD virus (IBDV) infection accompanied with a decrease in symptoms and bursal tissue damage. The treatment also increased body weight, not only in birds challenged with IBDV, but also in uninfected controls. Infected birds treated with cytokines presented the same bursal index and organs' weight that controls; since untreated animals showed a significant decrease in these parameters. Finally, cytokine administration represents a new alternative to IBDV vaccination.

Key words: cytokines, infectious bursal disease, poultry

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Introduction

Infectious bursal disease (IBD) is a viral immunosuppressive disease of young chickens attacking mainly the bursa of Fabricius, an important lymphoid organ in newly born birds. Emergence of new variant strains of the causative agent, the infectious bursal disease virus (IBDV), has made it more urgent to develop new treatment strategies against IBD. Use of recombinant vaccines is one of these strategies, but alternative preventive approaches are also a priority.

IBD is an acute, highly contagious and immunosuppressive disease of young chickens (Sharma *et al.*, 2000). The causative agent, IBDV, belongs to family Birnaviridae. The virus infects and destroys actively dividing IgM-bearing B cells in the bursa of Fabricius (Hirai *et al.*, 1981; Rodenberg *et al.*, 1994). Replication of IBDV in the bursa is accompanied by an influx of T cells (Tanimura and Sharma, 1997; Kim *et al.*, 1999, 2000; Sharma *et al.*, 2000). Although the bursal T cells are activated and proliferate *in vitro*, when stimulated by purified IBDV, there is strong evidence that T cells do not serve as targets for infection and replication of

IBDV (Kim *et al.*, 2000). However, there are reports that macrophages and monocytes may be susceptible to infection with the virus (Käufer and Weiss, 1976, 1980; Burkhardt and Müller, 1987; Komine *et al.*, 1989; Inoue *et al.*, 1992; Lam, 1998; Khatri *et al.*, 2005; Palmquist *et al.*, 2006).

Most commercially available conventional live IBDV vaccines are based on classical virulent strains. Those classified as "mild" vaccines exhibit only poor efficacy in the presence of certain levels of maternally derived antibodies and against very virulent IBDV. "Intermediate" and "intermediate plus" or "hot" vaccines have a much better efficacy and may break through higher levels of maternally derived antibodies, but they can induce moderate to severe bursal lesions, and thus, cause corresponding levels of immunosuppression (Mazariegos *et al.*, 1990; Tsukamoto *et al.*, 1995; Kumar *et al.*, 2000). These vaccines may not fully protect chickens against infection by the very virulent IBDV strains (Rautenschlein *et al.*, 2005) or by antigenic variants. Safety and efficacy of such vaccines still remain a major concern. In addition, the practical on farm administration of the conventional live vaccines to a large number of animals is also a technically demanding process, with difficulties inherent to farm-to-farm variability (variable chicks, variable farming conditions, variable skills in vaccination crews, etc.) that should not be underestimated, when assessing the results

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of vaccination programs. Non-replicating antigens, such as inactivated whole viruses, viral subunits or recombinant viral antigens, are not immunogenic enough unless they are combined with supporting adjuvants and administered in repeated injections, or follow suitable priming with a replicating antigen. Need for possible repeat injection obviously contributes to the implementation costs of these vaccines, and their use is usually restricted to highly valuable birds, such as future breeder birds, vaccination before lay provides passive immunity to the offspring by means of maternally derived antibodies. However, such vaccines have also been used occasionally in birds as young as 10 days old, particularly in areas heavily contaminated with virulent viruses (Wyeth and Chettle, 1990). Inactivated IBDV vaccines are mostly formulated as water-in-oil emulsions, usually combining several antigens. It has been observed that inactivated IBDV vaccines were also able to induce IBDV-specific T-cell and inflammatory responses in chickens (Rautenschlein *et al.*, 2002). It has been reported that inactivated IBDV vaccines must have either a high or an optimized antigenic content to induce an immunity in breeders that helps protect the progeny from infection by variant IBDV strains (Rosenberger and Cloud, 1989; Müller *et al.*, 1992). Inactivated vaccines are most efficiently used in a prime-boost regimen, using attenuated live IBDV as priming vaccine.

Cytokines are key communication molecules between host cells in the defense against pathogens. Bacterial and viral infections induce expression of multiple chemokines and proinflammatory cytokines. In this work, use of cytokines was evaluated as IBDV preventive treatment.

Materials and Methods

Reagents— Recombinant avian interferon α (IFN- α) and interleukin 2 (IL-2) were purchased from Alquimia Laboratory (Buenos Aires, Argentina).

Animals— White Cornish chicks were obtained from “Avicola Areco” poultry farm in Buenos Aires, Argentina. Eggs without vaccination from Gumboro-free egg-laying hens were randomly selected from the incubation plant. Two hundred 5-days old chicks were individualized by numbering, randomly separated in four groups and grown in distinct battery cages, fed with non-sterile pellets (coccidia-free) and water *ad libitum*. All animals were tested for maternally derived antibodies against IBDV.

Animal Treatments and Data Collection— White Cornish chicks were randomly divided into four identical groups (N = 50): control group 1 without treatment, group 2 treated with cytokines, group 3 challenged with IBDV, and group 4 treated with cytokines and challenged with IBDV. Cytokines viz., recombinant avian IFN- α and IL-2, were administered to groups 2 and 4 *ad libitum* mixed in the drinking water for 16 days. Three days after the first dose groups 3 and 4 were infected using an intraocular administration of the IBDV. Fig. 1A shows a timeline scheme of the mentioned experimental protocol. Animals were challenged using a standard IBDV, sample standard virulent type virus from the

serotype 1 (Remorini *et al.*, 2006). IBDV was maintained in specific pathogens free embryonated eggs. At day 8, chicks were infected with IBDV (10^4 infective doses per 50 ml) by a non-invasive intraocular inoculation technique. Cytokines were administered, from day 5, diluted in water at a final concentration of 1,000 U/ml of recombinant avian IFN- α and IL-2. The cytokines solution was administered *ad libitum* in poultry drinkers as the sole source of liquid available. The consumption of this solution was not significantly different from regular water consumption on the poultry farm. Chick body weight was determined every 5 days by two independent operators.

Histopathological Analysis— The chicks were euthanized by cervical dislocation. Internal organ weight, tissue sample preparation and microscopic analysis were performed. Bursa were collected, fixed in 10% formalin, dehydrated in a series of alcohol concentrations, embedded in paraffin wax, sectioned at 5 μ m thickness and stained with haematoxylin and eosin.

Scoring of Clinical Manifestations— The infected birds were euthanized and analyzed at day 25th. Bursae’ histopathological grades and numerical scores (I-III) were adapted from previous works (Johnson and Reid, 1970; Mattiello *et al.*, 2000) and defined according to the folds or plicae (F), pseudostratified columnar epithelium (PCE), lymphoid follicles (LF), and interfollicular areas (IA), as described below:

Grade I (normal): F, large, spike shape, plenty; PCE, straight, without goblet cell or cyst; LF, large, uniform, tightly packed; IA, scanty.

Grade II (moderate): F, short, non-uniform, separate; PCE, wavy invaginated, few goblet cell, cyst formations; LF, small, irregular, pallor reticular center; IA, wide with oedema.

Grade III (severe): F, small, deformed, scanty; PCE, irregular invaginated, plenty goblet cells, cyst formation; LF, small or absent, very irregular, cyst formations; IA, stromal fibroplasia.

Dead animals between the day 5 and 25 were classified as grade IV.

Statistics— Proportions of diseased animals were compared between groups using the Chi-square test and post-hoc comparisons. Distribution of scoring data was analyzed using Kolmogorov-Smirnov test followed by a Kruskal-Wallis non-parametric comparison. Analyses were performed using the SPSS Statistics software version 11.5 (IBM).

Results and Discussion

Effect of Cytokines on the Poultry Weight. Initial pool of chicks (5-days old) showed no significant differences in the body weight according to Kolmogorov-Smirnov test ($p=0.001$). After 25 days, the population did not present a normal distribution, so; all the statistical analyses were performed using non-parametric tests. On day 25, the population presented a significant weight differences according to a Kruskal-Wallis test ($p=0.009$). Post hoc analyses (Mann-

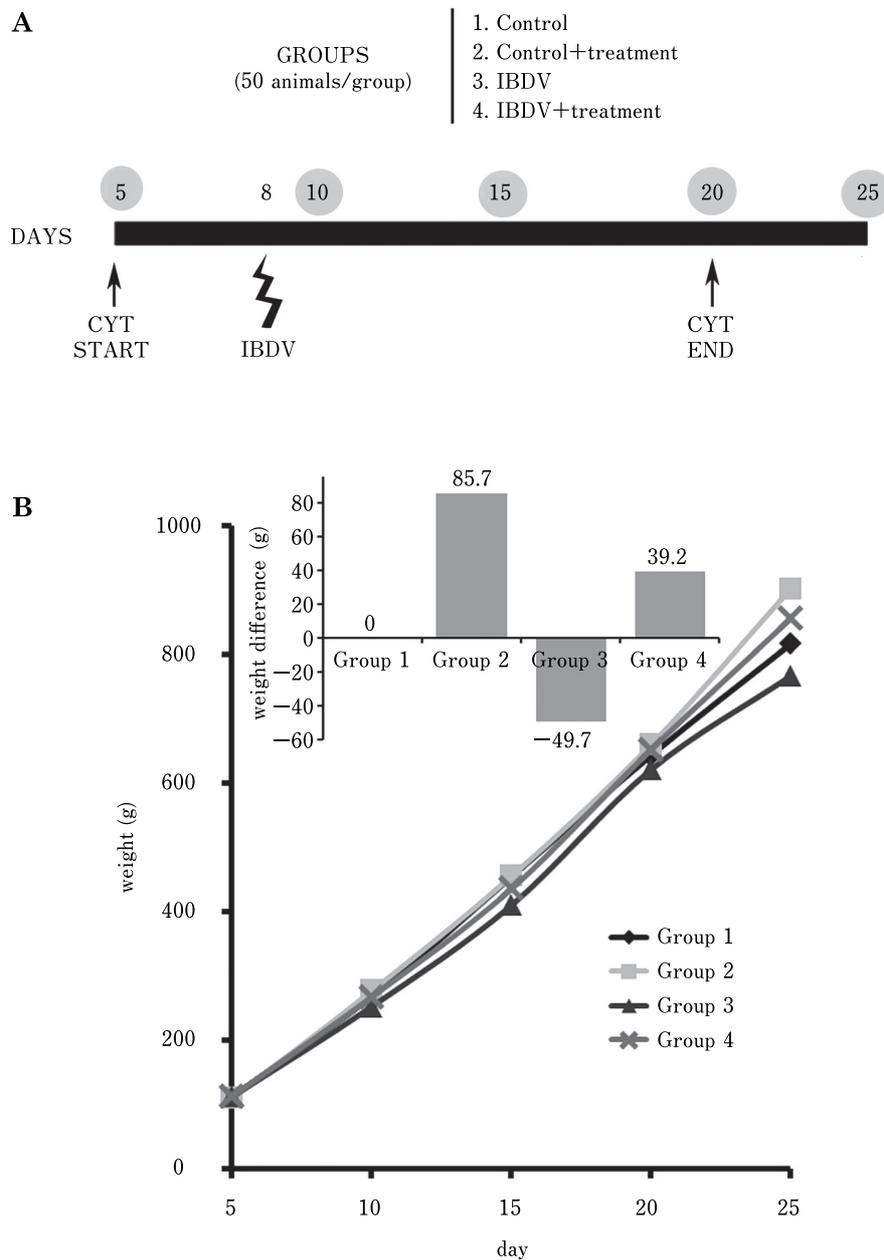


Fig. 1. *A) Experimental protocol scheme.* 200 White Cornish 5-days old chicks were divided into 4 groups. Cytokines were administrated from day 5 to day 20 to chicks from groups 2 and 4. At day 8, chicks from groups 3 and 4 were infected with IBDV. Chick body weight was determined each 5 days. *B) Weight measures.* Body weights from all the chicks were determined from day 5 to day 25 (large set). Weight differences over the control group 1 were calculated for day 25 (inset).

Whitney) showed significant differences between IBDV infected treated and untreated chicks ($p=0.03$) and also the controls treated and untreated ($p=0.037$). These results demonstrated that administration of cytokines produced an increase in the body weight of about 11–12% not only on

infected chicks, but also on normal animals (Fig. 1B). Increase in body weight of treated uninfected birds could be due to a protective effect of cytokines on other pathogens and protection of the intestinal flora thus improving the weight performance and feed conversion (Torok *et al.*, 2011).

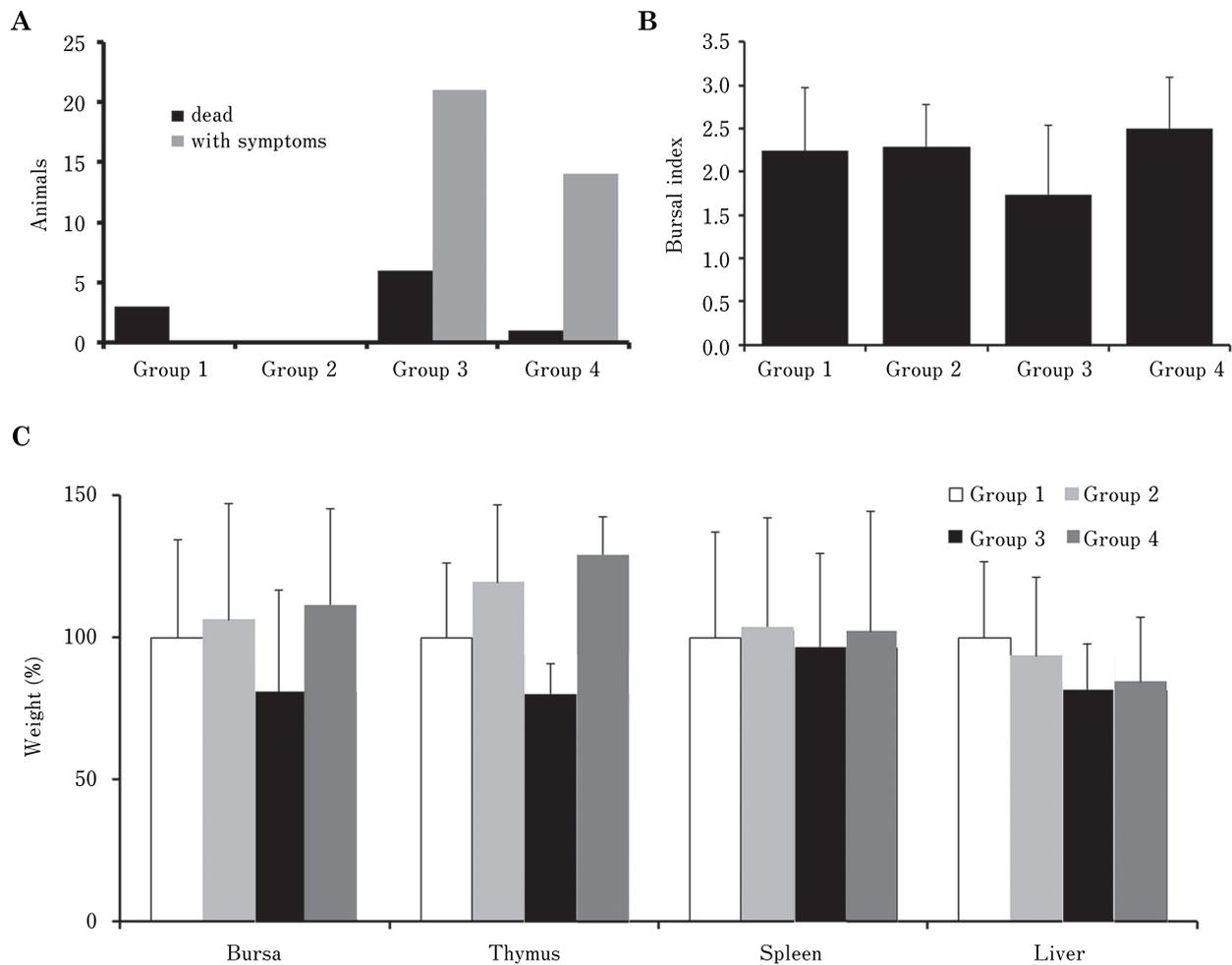


Fig. 2. *A) Animals morbidity and mortality.* The number of dead animals and symptomatic chicks per group were determined. *B) Bursal index.* The bursa weight/body weight ratio was calculated. *C) Organ differences.* Typical IBD affected organs were weighed post mortem. Error bars mean the standard deviation.

However, further investigation on this topic is required. Detailed weight differences over the control chicks (group 1) were 85.7 g, -49.7 g and 39.2 g of the groups 2, 3 and 4, respectively (inset Fig. 1B).

Animals' Morbidity and Mortality. On the other hand, death rates over 50 chicks per group were 3, 0, 6 and 1 (chi-square: $X(3)=8.84, p=0.031$) and the animals with IBDV symptoms were 0, 0, 21 and 14 (chi-square: $X(3)=45.818, p=0.0001$) for groups 1, 2, 3 and 4, respectively. The results demonstrated a significant difference in mortality and morbidity on animals treated with cytokines (Fig. 2A).

Finally, different organs were analyzed post-mortem. The bursa weight/body weight ratio (bursal index) showed that only group 3 is below the cut-off value of 1.8 (Fig. 2B). One-factor ANOVA and post hoc comparisons (DMS), showed statistically significant differences between IBDV infected treated and untreated chicks on the weight of the

bursa ($p=0.01$) and thymus ($p=0.01$), but not on spleen ($p=0.36$) and liver ($p=0.19$) (Fig. 2C). All bar graphics in Fig. 2 include the standard deviation.

Histopathological Analyses. Fig. 3 shows, the cellular structure of bursae from IBDV infected and control animals were completely different. Groups 1 (panel 1, control without treatment) and 2 (panel 2, treated control) were classified as grade I, presenting large and regular lymphoid follicles with an abundant population of lymphocytes. Bursae structures of group 1 presented follicle cortexes of moderate thickness with a normal follicular epithelium and a medullary zone with a regular quantity of lymphocytes. However, group 2 showed an increased follicle cortex width and a higher follicular epithelium which could be attributed to the cytokine treatment. Insets from panels 1 and 2 revealed large and abundant folds with a spike shape in both groups. Groups 3 and 4 were infected animals without (panel 3) or with (panel

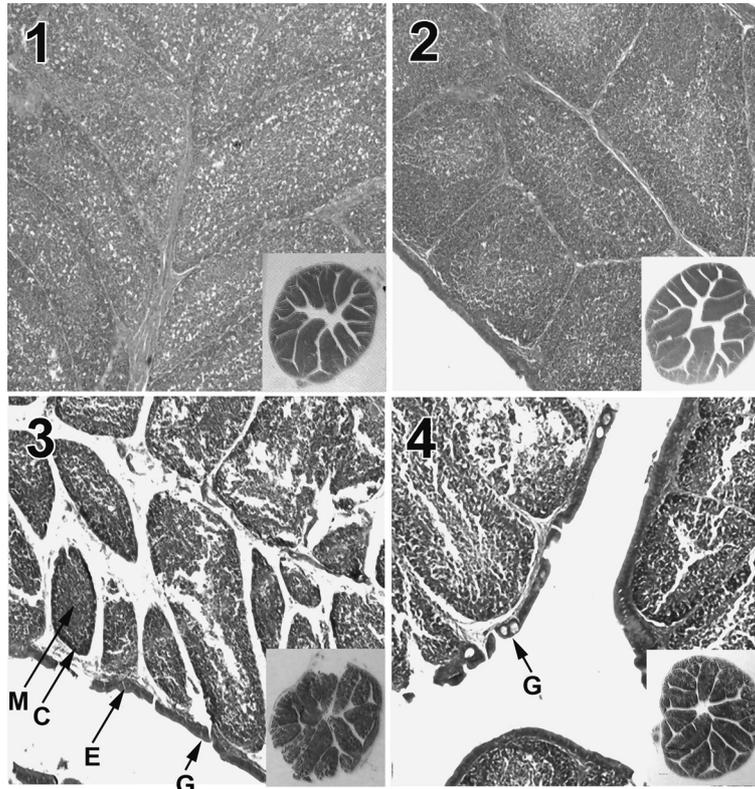


Fig. 3. **Histopathological analysis.** Cellular structure from bursa was analyzed by microscopic techniques. Each panel had the number from the corresponding group. A picture of the tissue sample is shown in the inset of each panel. Follicular epithelium (E), medullary zone (M), cortical zone (C) and goblet cells (G) were indicated with arrows.

4) cytokine treatment. As panel 3 showed, IBDV infection produced a significant decrease in the lymphoid follicles size, an irregular cell shape and a scarce lymphocyte population into the cortical and medullary zone. Bursae also presented a shorter follicular epithelium, interfollicular oedema, and hyperplasia of pseudostratified columnar epithelium. Bursae have scarce and small folds, and lymphoid follicles with a reduced number of lymphocytes (panel 3, inset). These samples were classified as grade III. Finally, comparing with group 3, the cytokine treatment produced an increase in the lymphocyte number into the lymphoid follicles (panel 4) and longer folds (panel 4, inset). These bursae were classified as grade II. In addition, goblet cells were observed in groups 3 and 4 (“G” arrows in panel 3 and 4).

Summarizing, the results demonstrated that oral administration of IFN- α and IL-2 to 5- days old chicks during 16 days reduced morbidity and mortality to IBDV infection, accompanied with decrease in symptoms and tissue damage in the bursa. We speculated that T-dependent lymphocyte system might be stimulated by IFN- α . Therefore, these activated T-lymphocytes present IL-2 receptors, and, external administration of recombinant IL-2 produces the stimulation of such selected clones. In addition, it could be hypothesized

that cytokine administration generates an augmented resistance to other viruses different to IBDV. For example, oral administration of chicken IFN- α inhibits avian influenza virus replication (Meng *et al.*, 2011). In other economically relevant animals, such as cattle, the same cytokines were used in combination with inactivated bacteria improving the bovine conjunctival immune response to the pathogen *Moraxella bovis*, the causative agent of infectious bovine keratoconjunctivitis (di Girolamo *et al.*, 2012).

An important feature of the treatment was the increase in body weight not only in IBDV infected animals, but also in controls. Considering the estimated production cost of cytokines for veterinary use, implementation of the treatment will be economically suitable for poultry producers, this industry worldwide, and especially in Argentina. In recent years, very virulent strains of IBDV, causing severe mortality in chickens, have emerged in Europe, Latin America, South-East Asia, Africa and the Middle East (Müller *et al.*, 2003); therefore, the cytokine treatment could be a new alternative to vaccination.

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