Infiltration by CD4+ and CD8+ lymphocytes in bursa of chickens infected with Infectious Bursal Disease Virus (IBDV): strain-specific differences

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In order to investigate if there is any definite correlation between the degree of T-cell response in the bursa of Fabricius (BF) and the virulence of Infectious Bursal Disease (IBD) virus strains, chickens were infected with strains of different virulence i.e. mild (Lukert strain), intermediate (Georgia strain) or invasive intermediate (IV-95 strain). At various times post-inoculation, bursal samples were collected to study virus specific histopathological lesions, the distribution of viral antigen and the extent of T-cell infiltration in the bursa. Most severe bursal lesions were induced by IV-95 strain (the invasive intermediate strain), whereas Lukert, the mild strain caused the least severe lesions. The number of virus positive cells in the bursa was highest in chickens infected with IV-95 strain. Significant infiltration of CD4+ and CD8+ T-cells in the bursal follicles of virus-infected groups was observed from 4 d.p.i. onwards. The magnitude of T-cell response was more in the birds infected with intermediate (Georgia) or invasive intermediate strains of virus than chickens inoculated with mild (Lukert) strain, even when 10-fold higher doses of the inoculums were used. PHA responses to peripheral lymphocytes were found suppressed in all the groups of chickens only transiently. The results indicate that the magnitude of T-cell responses in BF during IBDV infection is influenced more by the virulence of virus strain rather than the quantum of viral load in BF. Over all these studies may have implications in understanding the role of T-cells in pathogenesis and immunity in IBD.

Keywords: Bursa of Fabricius, Bursal disease, Infectious, Immunity, Pathogenesis T-cell response.

Infectious Bursal Disease Virus (IBDV) is an important pathogen of chickens, which is responsible for a highly contagious disease characterized by immunosuppression. Chickens infected with IBDV develop reduced humoral and cellular immune responses and respond poorly to vaccines. The virus has a cytocidal effect on B cells leading to the reduced antibody production. The effect of virus infection on cell-mediated immunity is not fully characterized. Recently, CD4+ and CD8+ T-cells infiltration has been demonstrated in the bursa of Fabricius (BF) of IBDV infected chickens. These studies showed that T-cells begin to accumulate at the site of virus replication in the bursa as early as 24 hr post-infection (pi) and persisted for at least 12 weeks after infection. Kim et al. showed that both bursal CD4+ and CD8+ T-cells mediated suppression of mitogenic responses of normal splenocytes. Cyclosporin-A treatment in the chickens infected with IBDV resulted in increased virus replication in the BF, suggesting a role for T-cells in anti-IBDV immunity.

The aim of this study is to investigate the T-cell responses in the bursa of chickens infected with three strains of IBDV having different virulence.

Materials and Methods

Chickens—Day-old broiler chicks were obtained from the poultry farm of CCS Haryana Agricultural University, Hisar. The farm had no history of IBDV outbreak. The chicks were examined for anti-IBDV neutralizing antibodies before inclusion in the studies.

The chicks were raised together in departmental animal house till three weeks of age after which they were divided into different experimental groups to be kept in separate isolation units. All animal management protocols were undertaken in accordance with the requirements of ‘Blue Cross Society, COVS, Hisar’ (recognised by the Animal Welfare Board of India; Prevention of Cruelty to Animals Act 1960).

Virus—Three strains of infectious bursal disease virus (IBDV) were used. M/s Indovax Pvt. Ltd., Hisar, gifted the mild (Lukert), intermediate (Georgia) and invasive intermediate (IV-95), all vaccine strains
of IBDV. The isolates Lukert and Georgia were grown in chicken embryo fibroblast (CEF) cell culture and titrated before using for experimental inoculation. The IV-95 strain grown in chick embryo was used as indicated by the manufacturers.

Monoclonal antibodies—The FITC-conjugated anti-CD4+ and anti-CD8+ (both of IgG1K isotype) monoclonal antibodies (mAbs) were kindly gifted by Dr. A. Kaushik, University of Guelph, Canada. The anti-IBDV monoclonal antibody against VP2 (IgG2a isotype) was kindly gifted by Dr. J. Ignjatovic of CSIRO, Australia. Optimal dilutions of the above monoclonals were determined using checker-board titration.

Experimental design—At the age of three weeks chickens (96) were divided into four groups of 24 each. The chickens in different groups were inoculated with different strains of IBDV by oral and ocular routes as follows: Group I chickens were inoculated with \(10^5.5\) TCID_{50}/ml of Lukert strain, group II chickens with \(10^5.3\) TCID_{50}/ml of Georgia strain and group III chickens with \(10^5.3\) EID_{50}/ml of IV-95 strain (as this strain was adapted in chick embryo). Chickens in group IV were inoculated with phosphate buffered saline (PBS) and served as control. The birds were examined daily for clinical manifestations of IBD.

Sample collection—Blood was collected in heparin (used @20 IU/ml) and BF were collected in 10% neutral buffered formalin on days 0, 2, 4, 7, 10, 14, 21 and 28 pi.

Bursa was used to prepare 4 sets of paraffin-embedded tissue sections. One set was stained with Hematoxylin and Eosin to study histopathological changes caused by strains of IBDV differing in their virulence. Second set was used for demonstration of viral antigen in affected tissue and the third and fourth sets were used to demonstrate the distribution of CD4+ and CD8+ T-lymphocyte influx in bursal tissue sections, respectively.

Studies conducted

Detection of IBDV antigen in the bursal sections—IBDV antigen was detected, using indirect immunoperoxidase test, by the method of Cruz-coy et al., which was used with some modifications.

Briefly, the paraffin-embedded sections were deparaffinized in three changes of xylene followed by rehydration in graded (95, 90, 70 and 45%) ethanol. Sections were treated with 0.1% trypsin in PBS containing 0.1% calcium chloride (pH 7.8) for 15 min at 37°C followed by thorough rinsing under running water. Endogenous peroxidase activity was inhibited by placing slides in 0.75% hydrogen peroxide in methanol for 30 min followed by rinsing in PBS. Samples were blocked with 6% bovine serum albumin solution containing equal amounts of 1:20 dilution of normal goat serum. Sections were then treated with dilution of anti-IBDV mAb (1:40) for 60 min at 37°C followed by rinsing in PBS. Goat anti-mouse peroxidase conjugate was used in 1:40 dilution for 60 min at 37°C. After thorough washing, slides were incubated with peroxidase substrate dianinobenzidine tetrahydrochloride (Sigma, USA) for 3-5 min. Later slides were washed in tap water, and counter stained with Haematoxylin. Finally the sections were microscopically evaluated for number of positive cells, using a score from 1 to 3, where score 1 indicated that only a few lightly stained positive cells present; score 2 indicated widely scattered positive cells and score 3 indicated numerous dark staining cells. A total of 20 fields were counted per section and results were averaged. The slides were examined at random to exclude any personal bias.

Detection of CD4+ and CD8+ T-cells in the bursa—The presence of CD4+ and CD8+ T-lymphocytes in formalin-fixed, paraffin-embedded BF tissue sections was demonstrated by direct fluorescent antibody test. The slides were deparaffinized in xylene and rehydrated in graded alcohol followed by distilled water and PBS. Sections were treated with 0.1% trypsin solution containing 0.1% calcium chloride for 15 min. Tissue sections were then incubated with predetermined dilution (1:20) of mouse anti-chicken CD4+ or CD8+ mAbs for 10 min at 37°C. Slides were thoroughly washed in three changes of PBS, sections covered with 1:10 solution of glycerol in PBS, cover-slipped and observed under fluorescent microscope. The T-cell response in the sections of bursa was estimated on the basis of CD4+ or CD8+ T-cell accumulation by counting the percentage of affected follicles in 20 randomly selected follicles from each bursa as per the method followed by Tanmura and Sharma. The response was expressed as 0+ or more than 1/3rd of lymphoid follicles were affected. It was expressed as 1+ when 1/3rd to 2/3rd follicles were affected. The response was expressed as 2+ when more than 2/3rd of follicles had cell infiltration.
Lymphoproliferative responses (lp)—The proliferative responses of peripheral blood mononuclear cells (PBMC) from infected chickens were measured by MTT (3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyl tetrazolium bromide) assay. The method of Mosmann\textsuperscript{13} was used with modifications. Blood was collected in heparinized tubes with tetrazolium bromide) assay. The method of Mosmann\textsuperscript{13} was used with modifications. Blood was collected in heparinized tubes with 20 units of heparin per ml of blood and diluted in equal part of serum free RPMI medium. PBMC were separated from diluted blood by density gradient centrifugation over Histopaque-1077 and were suspended at a concentration of 5X10\textsuperscript{6} cells/ml in RPMI complete medium (containing 20nm HEPES buffer, 2mM L-glutamine, 0.5mM 2-mercaptoethanol, 3.5% sodium bicarbonate and 1% stock solution of antibiotics to ensure complete dissolution of formazan crystals, plates were normally read within 15 min of adding stop solution. Blastogenic responses for the assay were expressed as mean stimulation index calculated by dividing the mean O.D. of stimulated cultures with the mean O.D. of unstimulated cultures\textsuperscript{14}.

Statistical analysis—Duncan's multiple range tests of variables was used to assess the significance of difference between treatment groups.

Results and Discussion

Clinical signs—Following experimental inoculation with IBDV, birds showed dullness and there was decreased feed intake during the initial phase of infection, which lasted up to first-week post infection. Birds in the group inoculated with IV-95 strain were relatively more depressed. Birds from control group were healthy.

Pathology

Gross pathology—Haemorrhages on thigh and pectoral muscles appeared by 2 d.p.i. in chickens infected with Lukert, Georgia and IV-95 strains of IBDV. Bursal haemorrhages were not conspicuous in any group. The most marked change observed was bursal hypoplasia which was observed in all virus-inoculated groups from 7 d.p.i. onwards. Infection with Lukert strain caused decrease in size of bursae between 4 d.p.i. and 14 d.p.i. Lesions were milder as compared to other strains. Bursal hypoplasia was most severe in IV-95 inoculated group between 10 and 28 d.p.i. Bursal size was maximal on day 2 p.i. in Lukert infected group and on day 4 p.i. in Georgia or IV-95 infected groups, however, enlargement of bursa was not observed.

Histopathology—Changes in the bursa included mild depletion of lymphocytes and vesicle formation in the follicles along with the presence of heterophils in bursal follicles during early stages of infection. From 7 to 21 d.p.i., follicular lesion was characterized by decrease in size of bursal follicles and an increase in interfollicular connective tissue in Georgia virus infected group. However, few vesicles were detected in follicles. Most severe lesions were observed in birds infected with IV-95 virus. In this group, necrosis of lymphocytes along with presence of heterophils was observed as early as 2 d.p.i. From 4 to 7 d.p.i., a decrease in follicular size along with an increase in interfollicular connective tissue was observed. Later, severe decrease in interfollicular connective tissue along with decrease in size of follicles and cyst like structures were seen in the follicles.

Lesions in chickens inoculated with Lukert strain were mild when compared to other two groups. Bursae from control group of chickens did not show any lesions.

IBDV antigen positive cells in the bursa—The antigen positive cells were marked by intense brown colour in immunoperoxidase reaction. Viral antigen was detected as early as 2 d.p.i. in the bursa of infected chickens. The lesion scores based on intensity of colour and area involved are shown in Table 1. The frequency of positive cells as well as intensity of colour was maximum in IV-95 group. By 4 d.p.i., IBDV antigen could be detected in the cortex and medulla of most of the bursal follicles. Maximum
reaction was observed at day 4 p.i. in all virus inoculated groups. After day 10 p.i., the frequency of antigen positive cells diminished and at day 21 p.i. only a few positive cells could be detected in IV-95 infected group. In Georgia virus infected group no positive cell could be detected after 14 d.p.i. No IBDV antigen was observed in bursa of control chickens.

**Detection of T-cells in the bursa**—In uninoculated control chickens, a few T-cells were always present in the bursal follicles. In IBDV inoculated chickens, an increase in both CD4+ and CD8+ T-cells was observed as early as 1 d.p.i. The CD4+ cells were located at the boundary area between cortex and medulla, whereas, CD8+ cells were scattered throughout the lymphoid follicles. By 4 d.p.i., more than two-thirds of the lymphoid follicles were affected and a large proportion of lymphoid cells were T-cells. Although both CD4+ and CD8+ T-cells increased in numbers, there was a greater proportion of CD8+ T-cells on several occasions (Table 2). Infection with Lukert strain resulted in milder T-cell reaction compared to infection with the other two strains between days 2 and 10 p.i. (Fig. 1 a-d). However, the degree of T-cell response was not distinguishable between Georgia and IV-95 strains, though IV-95 produced more severe lesions and higher frequency of antigen positive cells in immunoperoxidase assay.

**Lymphoproliferative responses (lp)**—A transient depression in the proliferative responses was observed upon infection of chickens with IBDV within the first two weeks (Table 3). There was considerable variation in the blastogenic responses among individual chickens within groups. Infection with Lukert strain resulted in SI values of 2.56±0.24 and 2.24±0.76 against control values of 7.6 and 9.98±0.28 employing MTT assay on days 2 and 7 respectively. Similarly, infection with Georgia strain resulted in an early suppression of lymphoproliferative responses with SI value of 2.34±0.16 on 2 d.p.i. and 2.55±0.25 on 7 d.p.i. However, infection with IV-95 strain caused a late suppression of lp responses starting at 7 d.p.i. Maximum suppression was observed in this group at 14 d.p.i. with an SI value of 2.26±0.21 against a control value of 6.8±1.6. Control birds had high proliferative responses on all intervals.

### Table 1—Immu-no-histochemical evaluation of antigen positive cells in bursae of IBDV infected chickens

<table>
<thead>
<tr>
<th>DPI</th>
<th>Lukert</th>
<th>Georgia</th>
<th>IV-95</th>
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<tbody>
<tr>
<td>2</td>
<td>1.0</td>
<td>1.0</td>
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<td>4</td>
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<td>3.0</td>
<td>3.0</td>
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<td>2.0</td>
<td>3.0</td>
</tr>
<tr>
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<td>0.6</td>
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<td>1.6</td>
</tr>
<tr>
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<td>0.6</td>
<td>0.6</td>
<td>1.0</td>
</tr>
<tr>
<td>21</td>
<td>0.3</td>
<td>0.0</td>
<td>0.6</td>
</tr>
<tr>
<td>28</td>
<td>0.0</td>
<td>0.0</td>
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</tr>
</tbody>
</table>

### Table 2—CD4+ and CD8+ T-cells in the bursa of Fabricius of chickens infected with IBDV strains of different virulence

<table>
<thead>
<tr>
<th>DPI</th>
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<th>IV-95</th>
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<tbody>
<tr>
<td></td>
<td>CD4+</td>
<td>CD8+</td>
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<tr>
<td>21</td>
<td>+</td>
<td>++</td>
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</tbody>
</table>

### Table 3—Mitogen (PHA) specific lymphoproliferative responses of chickens inoculated with different strains of IBDV as measured by MTT assay

<table>
<thead>
<tr>
<th>DPI</th>
<th>Lukert</th>
<th>Georgia</th>
<th>IV-95</th>
<th>PBS</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>2.56±0.24&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.34±0.16&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.15±0.35&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.6&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>7</td>
<td>2.24±0.76&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2.55±0.23&lt;sup&gt;c&lt;/sup&gt;</td>
<td>6.15±1.05&lt;sup&gt;c&lt;/sup&gt;</td>
<td>9.98±0.28&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>14</td>
<td>10.25±1.65&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.6±0.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.26±0.21&lt;sup&gt;c&lt;/sup&gt;</td>
<td>6.8±1.6&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>21</td>
<td>5.49±0.69&lt;sup&gt;b&lt;/sup&gt;</td>
<td>9.15±1.25&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>7.75±0.45&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>6.43±1.47&lt;sup&gt;ab&lt;/sup&gt;</td>
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<tr>
<td>28</td>
<td>7.15±0.85&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.75&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8.7±0.45&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.5&lt;sup&gt;a&lt;/sup&gt;</td>
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Values with different superscripts (A, B, C, D) on a day are significantly different. Superscript 'a' indicates a single observation.
Infiltration of CD4+ and CD8+ T-cells in the BF has been reported in response to IBDV infection. In the present study, IBDV pathogenicity and T-cell response in the bursa of Fabricius was compared in chickens infected with three IBDV strains of different virulence with a view to correlate severity of lesions in the bursa with the degree of T-cell infiltration. A comparison of pathogenicity showed that Lukert strain, which is a mild vaccine strain, caused least severe pathological changes in the infected chickens in spite of the fact that a 10-fold higher amount of the inoculum was used to infect this group of chickens. The dose of the inoculum was adjusted so as to induce comparable virus load in the bursa based upon pilot experiments (results not shown). Histopathological changes in the bursa of chickens inoculated with Georgia were more severe when compared to Lukert infected birds. Most severe pathological changes were observed after inoculation of chickens with IV-95, the invasive intermediate vaccine strain.

The results of MTT assay show a significant depression of proliferative responses of PBMC from infected chickens between 2 and 14 d.p.i. The suppression was, however, transient and proliferative responses returned to normal after a period in all infected groups. A transient suppression of lymphoproliferative responses of lymphocytes from IBDV infected birds has been reported in the first two weeks of infection using IBDV strains of different virulence. In the present as well as other studies it
was not possible to show any correlation between the virulence of the virus and the degree or duration of suppression of cell mediated immunity employing T-cell proliferation assays.

The results of indirect immunoperoxidase test using an anti-IBDV mAb directed against VP2 protein of the virus were similar with all four strains of IBDV with maximum number of antigen positive cells seen in bursal tissues on 4 d.p.i. However, stronger stain intensity suggestive of higher degree of virus replication was observed with IV-95 strain, which also caused most severe histopathological lesions in the bursa of Fabricius. At 4 and 7 d.p.i., virus was mostly detected at the border of cortex and medulla in the bursal follicles. At around the same time, microscopic lesions consisted of depletion of lymphocytes in the cortical area. Thus, immunoperoxidase positive cells, which represent areas of viral replication, were associated with microscopic lesions. Similar results have been reported earlier where it was shown that severity of lesions in bursal follicles correlated with strong immunoperoxidase reaction.

An increase in T-cell accumulation in the bursal follicles of IBDV infected chickens as early as 1 d.p.i. Both CD4+ and CD8+ T-cells increased in numbers. Maximum T-cell influx was observed between days 4 and 7 p.i. for all the three virus inoculated groups. It has been demonstrated that IBDV replication in the bursal and caecal tonsils is accompanied by a rapid and profound appearance of CD3+ T-cells and was suggested that IBDV infection in the bursa recruited T-cells circulating in the blood or lymph vessels. Similarly, rapid increase in both CD4+ and CD8+ T-cells from 2 to 5 d.p.i. has been observed by others. Kim et al. noted intra-follicular T-cells within the first few days of IBDV infection, which persisted for at least 12 weeks p.i. In the present experiments it was observed that CD4+ T-cells were mostly present at the boundary area of cortex and medulla, however, CD8+ T-cells were distributed throughout the follicles. The number of CD8+ T-cells was higher than CD4+ T-cells as observed earlier. The number of T-cells remained higher in infected chickens compared to age matched controls till 21 d.p.i. Maximum antigen detection as well as maximum T-cell reaction was observed around the same time i.e., around 4 d.p.i. after infection of chickens, indicating that these T-cells accumulate in response to infection. There was no marked difference in the extent of T-cell reaction in response to Georgia or IV-95 strains. It was interesting to observe that in spite of almost similar number of IBDV antigen positive cells in the bursae of chickens inoculated with strains of intermediate or mild virulence, lower number of T-cell accumulation occurred in the bursa of chickens inoculated with the mild strain. Between 4 and 10 d.p.i., only one third of lymphoid follicles in Lukert infected chickens showed an increased accumulation of T-cells compared to two-thirds affected follicles during the same time in chickens inoculated with other two strains of IBDV. Taken together with the results of Poonia and Charan where it was shown by cyclosporin-A treatment of IBDV infected chickens that T-cells play a role in limiting the severity of disease and replication of IBDV in the bursa, the present findings suggest a definite correlation between the virulence of IBDV strain and degree of T-cell reaction in the bursa. However, phenotype of the infiltrating cells did not show any correlation with the virulence of IBDV strain used for infection. The extent of T-cell accumulation seems to be influenced by their requirement at the site of pathology, however, all T-cells that infiltrate the bursa in response to IBDV infection may or may not be crucial. In experimental encephalomyelitis (EAE) in mice, it has been shown that in addition to the few T-cell clones which are responsible for inciting the disease, a whole lot of inflammatory T-cells infiltrate brain and spinal cord on the onset of paralysis. When treated with an altered peptide ligand, the heterogeneous T-cell infiltrate disappears from the brain, with only the T-cells that incited the disease remaining in the original lesion. Similarly, whether all T-cells that accumulate in the BF during IBDV infection contribute towards limiting the severity of disease or virus replication and what proportion of these cells are antigen-specific would be interesting to investigate further.

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References


