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Response of Gut Associated Lymphoid Tissues of Chickens to Very Virulent Infectious Bursal Disease Virus of Malaysian Isolate

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Abstract: An experiment was conducted to determine the response of the gut associated lymphoid tissues (GALT) to very virulent IBD virus (vvIBDV) of Malaysian isolate. Twenty eight days old specific pathogen free (SPF) chickens were divided into two groups namely: the control and IBD groups. The chickens each in the IBD group were inoculated orally with vvIBDV of Malaysian isolate at a dose of $10^{4.5}EID_{50}$/ml/chicken. Clinical abnormalities were recorded and three chickens in the IBD group were sacrificed at 0, 1, 2, 3, 4, 5, 6 and 12hrsand days 1, 2, 3, 4and 6 post infections (pi). Three chickens each in the control group were scarified at days 0, 1, 2, 3, 4 and 6 of the trial. On necropsy, the body weight, bursa weight and gross lesions were recorded. Samples at the junction of oesophagus and proventriculus, junction of proventriculus and gizzard, Meckel's diverticulum, middle part of ileum, caecal tonsil and bursa of Fabricius were collected and processed for histopathology. Blood was collected for detection of IBD antibody using enzyme linked immunosorbent assay (ELISA). Typical clinical signs of IBDV infections were observed in chickens from the IBD group at days 2 and 3 pi. Histopathology examination showed highly increased of lymphoid cells at the junction of oesophagus and proventriculus at 4, 5, 6 and 12 hrsand days 1 and 2 pi, with moderate increased thereafter when compared to the control group. Marked lymphoid cells increment was also observed at the junction of proventriculus and gizzard at 6 and 12 hrsand days 1 and 2 piand mild to moderate increased observed thereafter. No significant changes observed in the Meckel's diverticulum throughout the trial. Tissues from the middle part of ileum was absent of any lymphoid aggregation in both the control and IBD groups. Moderate increased of lymphoid cells with some cells showed degeneration and necrosis occurred in the caecal tonsils at 5, 6 and 12 hrsand days 1 and 2 pi. At days 3 and 4 pi, moderate to severe follicular degeneration and necrosis with mild congestion or haemorrhages were recorded. The bursa of Fabricius showed moderate to severe lesions at days 2, 3, 4 and 6 pi. Immunoperoxidase staining of the bursa showed intense positive reaction in the reticular epithelial lining and medulla of the bursa follicles at 4, 5, 6 and 12 hrs pi. At days 1, 2 and 3 pi, the cortex of the bursa follicle stained more intensely then the medulla. The control group was without any clinical signs, gross or microscopic lesions throughout the trial. IBD antibody titre was first detected in the IBD group at day 4 pi (552 ± 489) and further increased at day 6 pi (1496 ± 137). It was concluded that vvIBDV of Malaysian isolate can cause lesions in the GALT. The virus is first replicate in the GALT including in the lymphoid cells aggregation at the upper part of the gastrointestinal tract, at the junction of oesophagus and proventriculusand junction of proventriculus and gizzardand at the caecal tonsil leading to primary viraemia and replication of the virus in the bursa of Fabricius following oral route of vvIBDV infections. The response of GALT at the middle part of ileum could not determine due to absent of the lymphoid cells aggregation in the samples examined, whilst no significant response of the Meckel's deverticulum to vvIBDV was recorded.

Key words: Gut associated lymphoid tissues (GALT), infectious bursal disease (IBD), IBD virus, immunoperoxidase staining, ELISA

Introduction

Infectious bursal disease (IBD) or Gumboro disease was first recognized in Gumboro, Delaware, USA in 1957 (Cosgrove, 1962). Since then, the disease has been reported from most of the major poultry producing countries such as in Europe (Faragher, 1972)and Australia (Firth, 1974). The disease appeared under control with the introduction of proper vaccination programs (Edgar and Cho, 1965). However, in late 1980's, IBD outbreaks due to very virulent IBD virus (vvIBDV) causing high mortality and morbidity in chickens was first reported in England (Chettle *et al.*, 1989) and in Malaysia in 1991 (Hair-Bejo, 1992, 1993 and Phong *et al.*, 2003). IBDV belongs to the family Birnaviridae, members of which have been isolated from fish, infectious pancreatic necrosis virus (Becht, 1980). The target organ for IBDV is the bursa of Fabricius and the high concentration of virus produced by the organ will play a crucial role in the pathogenesis of IBD (Winterfield and Hitchner, 1962; Snedeker and Moulthrop, 1967).

IBDV infection is usually via the oral route, although the upper respiratory tract and conjunctiva can also play a similar role. Following oral infection of classical IBDV (cIBDV), the virus can be detected in the macrophages and

lymphoid cells of the caecum, duodenum and jejunum at 4 to 5 hrs post infection (pi) (McFerran *et al.*, 1980), leading to primary viraemia and the localization of the virus in the bursa of Fabricius as early as 11 hrs pi (Muller *et al.*, 1979). The virus replicate in B-lymphocytes and is released into the circulation causing secondary viraemia and damage to other lymphoid organsand severe haemorrahage either due to viral replication or/and immune mediated response (Weiss and Kaufer, 1994). Death may occur during this period as in the clinical form of IBD, while in subclinical IBD, chickens may die due to secondary infection by other pathogens (Hirai *et al.*, 1981). The incubation period of IBD ranges from 2-4 days (Cosgrove, 1962).

In chickens, prominent collection of lymphoid tissues in the gastrointestinal tract or known as gut associated lymphoid tissues (GALT), some equivalent to mammalian Peyer's patches, occurs at the junction of oesophagus and proventriculus, the junction of proventriculus and gizzard, Meckel's diverticulum, intestinal tract and caecal tonsils (Riddell, 1987). The GALT has evolved with specialized cytological features that reflect the role as the first line of defense in mucosal surfaces and consist of B cells which could support viral replication (Jankovic and Mitrovic, 1966 and Muller, 1986). Little is known on the response of GALT to vvIBDV. It was the objective of the study to determine the response of GALT to vvIBDV of Malaysian isolate.

Materials and Methods

Specific Pathogen Free Chickens: Specific pathogen free (SPF) White Leghorn chickens were rear in a raised floor system with food and water ad libitum. At 28 days of age, the chickens were separated into 2 groups namely; the IBD and the control groups. The chickens in the IBD group were inoculated with 1.0 ml of vvIBDV of Malaysia isolate orally, with virus titre of $10^{4.5}$ EID_{50}/ 1.0ml / chicken. Three chickens each were sacrificed at 0, 1, 2, 3, 4, 5, 6 and 12 hrsand days 1, 2, 3, 4 and 6 post infections (pi). Three chickens each in the control group were sacrificed at days 0, 1, 2, 3, 4 and 6 of the trial period. Any abnormal clinical signs were recorded.

Necropsy: Body weights of the chickens were recorded prior to necropsy. Blood samples were collected for detection of IBD antibody using enzyme linked immunosorbent assay (ELISA). On necropsy, the gross lesions were recorded and the bursa of Fabricius was weights. The bursa of Fabricius and various parts of the gastrointestinal tracts namely, an area at the junction of the oeseophagus and proventriculus, at the junction of the proventriculus and gizzard, Meckel's diverticulum, middle part of ileum and caecal tonsil were fixed in 10% buffered formalin for 24 hours for histological examination. The tissues were stained with hematoxylin and eosin (HE). Immunoperoxidase staning (IPS) was performed in the bursa samples.

Histopathology: The tissues were trimmed to 5mm blocks dehydrated in a series of alcohol, cleared with xylene and embedded in parrffin wax using an automatic tissue processor. Tissues were section with microtome to a thickness of 5 μm, mounted on glass slides and stained with HE (Lillie, 1965). The tissues were then examined under a light microscope using x4, x10 and x40 objectives and the histological changes were recorded.

Immunoperoxidase Staining (IPS): The sections of the bursa of Fabricius were deparafinnised in the oven at 60°C for an hour. It was then rinsed with double distilled water and reacted with 0.5% hydrogen peroxide in absolute methanol for 30 minutes at room temperature to quench endogenous peroxidase activity. Following a wash with phosphate buffered saline (PBS) for 5 minutes the samples were treated with 0.1% trypsin for 15 minutes at 37°C. The samples were again rinsed with three 5 minutes periods in PBS. Blocking was done next with 5% bovine serum albumin (BSA) for 30 minutes following which the samples were incubated with IBD hyperimmune serum from the chickens for 15 hours in a humidified chamber at room temperature. The slides were then rinsed three times for 5 minutes in PBS and reacted with peroxidase conjugated anti-chicken IgG for 30 minutes at room temperature. After another three 5 minute washes in PBS, the slides were incubated with diaminobenzidene (DAB) (DAB reagent set, Kirkrgaard and Perry Laboratories, USA) for 5 minutes at room temperature. The samples were then washed again for the three rinses of PBS for 5 minutes and then counterstained with haematoxylin. The samples were examined using a light microscope for the detection of positive staining.

IBD antibody titre: The enzyme linked immunosorbent assay (ELISA) technique for detection of IBD antibody was carried out according to the methods described by IDEXX Laboratories Incorporation, USA. Briefly, the antigen coated plates and the ELISA kit reagents were adjusted at room temperature prior to the test. The test sample was diluted five hundred folds (1:500) with sample diluent prior to the assay. A 100 μl of diluted sample was then put into each well of the plate. This was followed by 100 μl of undiluted negative control into well A1 and A2, 100 μl of undiluted positive control into well A3 and A4. The plate was incubated for 30 minutes at room temperature.

Each well was then washed with approximately 350 µl of distilled water for 3 times. Goat anti-chicken conjugate (100µl) was dispensed into each well. The plate was incubated at room temperature for 30 minutes, followed by washing each well with 350 µl distilled water for 3 times. TMB solution (100 µl) was dispensed into each well. The plate was then incubated at room temperature for 15 minutes. Finally, 100 µl of stop solution was dispensed into each well to stop the reaction. The absorbance values were measured and recorded at 650nm. IBD antibody titre was calculated automatically, using software by Blankfard and Silk (Blankfard and Silk, 1989).

Statistical analysis: Data was analysed using 2-way analysis of variance (ANOVA), one-way ANOVA, post hoc test with Duncan and Least Significant Difference (LSD)and student independent t-test by using SPSS v.10.0 for Windows (Norusis, 1999).

Results

Clinical signs: No clinical signs of IBDV infections observed in the control group throughout the trial. The chickens in the IBD group showed no clinical illness or mortality at day 1 pi. However, at days 2 and 3 pi, a few chickens showed clinical signs of IBD, such as ruffled feathers, drowsiness, prostration, depression, anorexia and reluctant to move. Three birds found dead at day 3 pi.

Body weight: The body weight of the chickens in the control group increased ($P<0.05$) from 446.7 ± 49.8g at day 0 to 523.3 ± 38.4g at day 6 of the trial period. In the IBD group, decreased of body weight was significantly ($P<0.05$) observed at day 3 pi (256.7 ± 45.6g) when compared to the control group (403.3 ± 51.8g). The body weight in the control and IBD groups were not significantly different ($P<0.05$) at other days.

Bursa weight: The bursa weight of the control group increased ($P<0.05$) throughout the trial from 2.72 ± 0.33g at day 0 to 3.88 ± 0.33g at day 6. For the IBD group, the bursa weight was slightly increased from day 0 to day 2 pi. However, a reduction of bursa weight ($P<0.05$) was recorded at day 3 pi (1.90 ± 0.11g) when compared to the control group (3.22 ± 0.59g).

Bursa to body weight ratio (10^{-3}): The bursa to body weight ratio in the control group increased ($P<0.05$) from 6.11 ± 0.56 at day 0 to 7.53 ± 0.94 at day 6 of the trial period. The ratio in the IBD group was slightly increased from day 0 to day 2 pi, but was significantly ($P<0.05$) reduced at day 3 pi (4.87 ± 0.66) when compared to control (6.70 ± 0.91).

Gross lesions: The carcasses in the control group remained normal throughout the trial. In the IBD group, the lesions were confined at the bursa of Fabricius. The bursa remained normal at day 0 and day 1 pi. However, at day 2 pi, the bursa was enlarged, oedematous and the serosa surface of the organ was covered with yellowish transudate. Moderate to severe petechial and ecchymotic haemorrhages, oedema and caseous materials were observed in the mucosa surface of the organ. Atrophy of the bursa of Fabricius was exhibited from days 3 pi and thereafter. The organ was small, firm and oval or elongated. The caecal tonsil was slightly enlarged, congested or haemorrhagic at days 2, 3 and 4 pi. No obvious lesions were observed in the other GALT. For the dead chickens, at day 3 pi, severe petechial and ecchymotic haemorrhages and oedema of the bursa of Fabricius were observed. The cecal tonsil was slightly enlarged, haemorrhagic or congested. Haemorrhage was also observed at the tight and breast muscles and the junction of the proventriculus and gizzard.

Histopathology: Junction of the oesophagus and proventriculus: No significant lesions were present in the control group throughout the trial. However, at 4, 5, 6 and 12 hrsand days 1 and 2 pi the number of lymphoid cells within the lamina propia were markedly increased in the IBD group when compared to the control. Moderate increased of lymphoid cells were recorded at days 3 and thereafter.

Junction of the proventriculus and gizzard: No significant lesions were present in the control group throughout the trial. In the IBD group, highly increased of lymphoid cells numbers within the lymphoid cells aggregations were observed at 6 and 12 hrsand days 1 and 2 pi when compared to the control group. Mild to moderate increased of lymphoid cells occurred at days 3 and thereafter.

Meckel's diverticulum: No significant changes of the lymphoid tissues observed in the control and IBD groups throughout the trial.

Middle part of ileum: No significant changes observed in the control and IBD groups through the trial, except for mild to moderate degeneration and necrosis of the villus enterocytes noted in some areas at days 3 pi in the IBD group. Lymphoid cells aggregation was not detected in all samples from both the control and IBD groups and thus response of the cells (GALT) at the middle part of ileum to vvIBDV cannot be determined and evaluated.

Caecal tonsil: No significant changes were present in the control group through the trial. In the IBD group, moderate increased of lymphoid cells with some showed degeneration and necrosis occurred at 5 hrs pi to day 2 pi. At days 3 and 4 pi, moderate to severe follicular degeneration, necrosis and mild congestion or haemorrhages were observed.

Bursa of Fabricius: No significant or only mild changes were observed in the control group through the trial. However, the bursa of Fabricius in the IBD group showed mild to moderate lesions at 6, 12 and 24 hrs pi. At day 2 pi moderate to severe cellular degeneration and necrosis occurred in the medulla of the lymphoid follicles. Interstitial connective tissues were oedematous and moderately infiltrated with inflammatory cells. Moderate to severe follicular lymphoid necrosis and degeneration occurred at days 3 pi. Intrafollicular haemorrhages and cysts filled with fibrinous exudate and cells debris were commonly observed in the organ. The interstitial connective tissue was oedematous, haemorrhagic and infiltrated with inflammantory cells. At days 4 and 6 pi, severe follicular lymphoid necrosis and depletion were observed in the organ. The interstitial tissue was heavily infiltrated with heterophils and macrophages. Most of the necrotic cell debris in the follicles had disappeared and replaced by cysts. The dead chickens showed severe lesions with follicular necrosis, haemorrhage, formation of cysts, thickening of the interstitial tissues with oedema and infiltration of moderate amount of inflammatory cells.

Immunoperoxidase staining (IPS): All samples in the control and IBD groups were negative for IPS at 0, 1, 2 and 3 hrs of the trial period. However, at 4, 5, 6 and 12 hrs pi, in the IBD group, the reticular epithelial lining and medullary regions of the bursa follicles showed intense positive staining. At days 1, 2 and 3 pi the cortex of the follicles was progressively more positive staining when compared to the medulla. The cortex and interfollicular connective tissue were highly positive staining at days 4 and 6pi.

IBD antibody titre: IBD antibody titre was not detected in the control group throughout the trial. For the IBD group, the IBD antibody titres was first detected at day 4 pi (552 ± 489) and significantly increased at day 6 pi (1496 ± 137).

Discussion

The study showed that GALT at the upper part of the gastrointestinal tract, at the junction of the oesophagus and proventriculusand junction of the proventriculus and gizzard play an important role in the pathogenesis of IBD following oral route of IBDV infections in addition to the role of the GALT at the duodenum, jejunum and caecum suggested previously (McFerran *et al.*, 1980). The moderate to marked increased number of lymphoid cells in the organs as early as 4 hrs pi at the junction of the oesophagus and proventriculus and 6 hrs pi at the junction of the proventriculus and gizzardand 5 hrs at the caecal tonsil following oral route of IBDV infections may facilitate the primary replication of the virus in the organ. The lymphoid cells aggregations within the lamina propria or GALT of the gastrointestinal tract consist of B cells which could support viral replication and lead to primary viraemia (Jankovic and Mitrovic, 1966; Muller, 1986). The response of GALT at the middle part of ileum could not be determined due to absent of the lymphoid cells aggregation in the samples examined. Surprisingly, no significant responses of the GALT at the Meckel's diverticulum to IBDV observed in the study. The lesions in the caecal tonsil, at days 3 and 4 pi could be due to the secondary viraemia following excessive multiplication of IBDV in the bursa of Fabricius. The caecal tonsil has been reported to contain T cells and B cells and germinal centers (Jankovic and Mitrovic, 1966), which could support viral replication. The virus may also reach the caecal tonsil from the lumen by passing through the surface epithelium or via the vascular system (Olah and Glick, 1979).

It is interesting to note that the increased of bursa weight in the IBD group at day 2 pi could be attributed to the oedema, hyperemia and degeneration of the lymphoid cells in the organ. As the lymphoid cells degeneration and necrosis progressed in the later stage of the infection, the organ undergoes atrophy. Thus, the bursa weight of the IBD group reduced at days 3 pi when compared to the control. It appears that the virus gained entry into the bursa of Fabricius via hematogenous route following oral infections. The virus was first detected at 4 hrs pi within the reticular epithelial lining and medulla regions of the bursa follicles. This was followed by the spread of the virus into the cortex of the follicles. The detection of IBDV in the bursa of Fabricius in the present study is in agreement with

previous report on the replication of the virus in the lymphoid cells (Panisup *et al.*, 1988). Massive viral replication was reported to occur at the bursa as early as 11 hrs pi (Muller *et al.*, 1979). Secondary viraemia occurred following the release of the virus from the bursa which may lead to damages of the other lymphoid organs. Bursa of Fabricius is the central lymphoid organs of chickens and the presence of the bursa is essential to the development of the peripheral lymhpoid tissue and these associated adaptive immune functions. Actively dividing surface immunogloblin M-bearing (Sig-M) cells in the bursa of Fabricius are the major targets for IBDV infection (Hirai *et al.*, 1981). However, the numbers of Sig-M cells are most depressed at day 2 to day 3 pi, whilst the surface IgG-bearing cells are depressed at day 4 pi. Thus, IgM will be produced first and is of short duration, whereas IgG will be produced a slightly laterand gradually increased to a peak concentration. Macrophages play crucial role in the transporting and multiplying the viruses (Van den Berg *et al.*, 1994).

As the IBDV enter the gastrointestinal tract, the T cells within the GALT have been shown to contain mRNA for interferon. These cells also show increased expression of MCH Class II antigen. The T4 react with the MCH II on the macrophage and proliferates into cytokine secretors and memory cells. Interferon will be released which is supported by detection of increased serum interferon levels (Gelb and Kleven, 1979). Interferon is cytokines released from the virus infected cells within a few hours after viral invasion. The interferon stimulates the production of many new proteins, some of which have antiviral activity and also stimulates macrophages and complement mediated virolysis. This will cause releasing of necrotic factors. Antibodies may cause destruction of infected cells not only through complement mediated cytolysis, but also through the activities T cytotoxic cells by antibody dependent cell mediated cytotoxicity (ADCC) (Tizard, 1996). Due to the antiviral activity stimulation of macrophages, T cytotoxic and natural killer cells which is activated by the interferon leading to target cell degeneration and necrosis (Tizard, 1996) and thus the lesions that were seen in the organs due to IBDV infections can be due to the immune mediated responses rather then massive viral replication (Skeeles *et al.*, 1980).

This study has demonstrated that chickens which recovered from severe acute IBD, produced high level of antibody against the virus. The antibody was first detected at day 4 pi and reached higher titre at day 6 pi. The more virulent the IBDV, the much higher and more persistent antibody titre will be stimulated (Naqi *et al.*, 1980). The antibody was not detected during the onset of the clinical signs and severe bursa lesions at days 2 and 3 pi when the virus in the bursa is at the highest concentration. This could be due to much amount of antibody is required for neutralization of the virus (Okoye and Uzoukwu, 1990).

It was concluded that vvIBDV of Malaysian isolate can cause lesions in the GALT. The virus is first replicate in the GALT including in the lymphoid cells aggregation at the upper part of the gastrointestinal tract, at the junction of oesophagus and proventriculusand junction of proventriculus and gizzardand at the ceacal tonsil leading to primary viraemia and replication of the virus in the bursa of Fabricius following oral route of vvIBDV infections. The response of GALT at the middle part of ileum could not determine due to absent of the lymphoid cells aggregation in the samples examined, whilst no significant response of the Meckel's deverticulum to vvIBDV was recorded.

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