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IMMUNOSUPPRESSIVE EFFECT OF BOVINE VIRAL DIARRHEA VIRUS (BVDV) ON PERSISTENTLY INFECTED CATTLE

(With one Table and 9 Figures)

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التأثير المناعي المثبط لفيروس الإسهال الفيروسي البقري في الأبقار مستمرة الإصابة

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لدراسة الحالة المناعية للأبقار مستمر ة الإصابة بفيروس الإسهال الفيروسي البقـــري تـم فحص هذه الحيوانات دمويا و مناعيا. فحص الدم اشتمل على العد الكلي و العـد المقـارن لكرات الدم البيضاء بينما تم الفحص المناعي بإجراء تقنية المناعة النسيجية باستخدام أجسام مضادة للأجسام و الخلايا المناعية التالية(IgA,secretory IgA,IgG1,S-100,CD4+ and CD8+).استخدمت هذه التقنية للوقوف على حالة الاسـتجابة المناعيـة للحيوانـات المصابة. كل الحيوانات المختبرة أظهرت نقص في عدد كرات الدم البيضـاء. الحيوانـات المختـبرة مـا عـدا العجـول أوضحـت نقـص شـديد فـي (IgA, secretoryIgA, IgG1 and S-100) بينما لم يكن هناك نقـص فـي الأجسـام والخلايا المناعية المختبرة في العجول المصابة و ذلك بمقارنتها بـالعجول السـليمة التـي استخدمت كضوابط كما لم يظهر فارق في $CD4^+$ and $CD8^+$ بين الحيوانـات المصابـة والحيوانات السليمة التي استخدمت كضوابط. هذه النتائج أوضحت أن فـيروس الإسـهال الفيروسي البقري له تأثير مثبط للمناعة في الحيوانات مستمرة الإصابة خلال تاثيره علـى المناعة الموضعية و لكن هذا التأثير لم يثبت في العجول الصغـيرة المختـبرة فـي هـذه الدراسة. هذه أول دراسة تتناول تأثير فيروس الإسهال الفيروسي البقـري علـى المناعـة الموضعية كما أنها أول دراسة تتناول S-100 كخلايا مناعية لها دور في عمليـة المناعـة وتأثير الإصابة بالفيروس عليها.

SUMMARY

In order to study the immunological state of cattle persistently infected with BVDV, the animals were examined hematologically and immunohistochemically. Hematological examination included total and differential leukocytic count while immunohistochemical assay was carried out using Avidin Biotin Complex (ABC) immunoperoxidase method applying monoclonal antibodies to bovine IgA, Secretory IgA, IgG1 , S-100 (antigen presenting cells , investigated for the first time in the present work) , $CD4^+$ ($T_{helper/inducer}$ -cells) and $CD8^+$ ($T_{cytotoxic/suppressor}$-cells) was used for detection of immune response to BVDV. All examined animals showed leukopenia characterized by neutropenia and lymphopenia. The examined animals except calves exhibit reduction in IgG1, IgA, Secretory IgA and S-100 whereas there was no difference between examined immunoglobulins of persistently infected calves and healthy uninfected control calves were observed.Also, there was no difference between $CD4^+$, $CD8^+$ in persistently infected and healthy control animals. These results suggest that noncytopathic BVDV seems to cause impairment of immunological state of persistently infected cattle through its effect on local immunity but this effect has not been proved in calves examined in the present study.This is the first study to investigate the effect of BVDV on local immunity and the first study of S-100 as immunological cells.

Key words: *BVDV- Persistent Infection- Local Immunity- Immunohistochemistry ABC technique- Immunosuppression- Monoclonal antibodies- Pestiviruses-Immunology- Intestine - Thymus - Molecular technique*

INTRODUCTION

BVDV is the type species of the Pestivirus genus and classified in the family Flaviviridae (Francki et al., 1991; Horner et al., 1995)). Based on the cytopathogenicity in cell culture, BVDV has been divided into two biotypes cytopathic and noncytopathic (Gillespie et al., 1960). The noncytopathic biotype of BVDV predominates over cytopathic BVDV in nature (Bolin et al., 1994; Collen et al., 2000).

Infection with BVDV is associated with a spectrum of syndromes (Baszler et al., 1995; Bitsch et al., 2000) and known to cause multiple

problems in cattle. The most serious consequence of infection occurs after in utero infection of the fetus (Yu et al., 1994), cows infected with BVDV during the first four months of gestation (before the fetus is immunocompetent) may give birth to calves that immunotolerant and persistently infected with BVDV (Bolin, 1990). Furthermore, the progeny of such persistently infected animals are likewise persistently infected and immunotolerant (Sopp et al., 1994). After birth, the calves will remain persistently infected with BVDV but unable to produce any significant quantity of neutralizing antibodies (Larsson et al., 1995)

Immunotolerance and persistent infection with BVDV appears to be associated with infections with noncytopathic biotype (Kamstrup et al., 1991)). However, the mechanism by which BVDV induce and maintain the tolerant state and the kind of interaction that takes place between the virus and cells of immune system are still unclear (Coulibaly et al., 1986). The occurrence of clinically inapparent and persistent form of BVDV has been reported in many countries allover the world (Shimizu and Satou, 1987).

BVDV has been demonstrated to be immunosuppressive agent which infect the cells of immune system affecting the function of several cell types resulting in a reduction in the defense mechanisms (Bolin et al., 1985b; Atluru et al., 1990; Baker, 1990 and Brown et al., 1991) and many studies have shown that BVDV caused immunosuppression of both humoral and cell-mediated functions (Atluru et al., 1979;Roth et al., 1986)

In persistently infected animals, it was shown that BVDV infect B and T lymphocytes,monocytes as well as null cells, so immunotolerant state involves the antibody response and cell-mediated mechanisms (Ellis et al.,1988) which could lead to defective immune responses and play a role in the tolerance (Sopp et al.,1994).

Leukopenia and lymphopenia have previously been observed following experimental inoculation of cattle with BVDV (Corapi et al., 1989), the lymphopenia reflects a decrease in B cells as well as in T cells subset (Bolin et al., 1985a; Ellis et al., 1988). Moreover, lymphocytes and neutrophils obtained from persistently infected animals have an altered function (Roth et al., 1986). Persistently infected cattle may have a permanent impaired immune response (Muscoplat et al., 1973; Roth et al., 1986) with extensive damage of the immune system (Howard, 1990) and seems to have an increased susceptibility to other infections (Houe and Heron, 1993). It was clearly demonstrated that cattle persistently infected with BVDV have a depressed antibodies to bovine leukemia

virus, this depressed response probably resulted from a decrease in IgG1 synthesis (Roberts et al., 1988) and both persistently infected animals and animals with mucosal disease had a reduced level of IgG2 (Coulibaly et al., 1986). In vitro infection of bovine splenic lymphocytes with BVDV significantly inhibited plasma cell development and immunoglobulin synthesis, thus indicating hampered function of B cells or the cells that regulate the B cells (Atluru et al., 1979; Atluru et al., 1990).

Much work has been done in developing methods for study of BVDV using monoclonal antibodies, this will have an important place in research studies (Mignon et al., 1991). There is a need to perform immunohistochemical technique for further study of BVDV (Haines et al., 1992)

Unfortunately, there has been no documented research about the effect of BVD viral infections on the local immune response and a little is known about the local immunological change of apparently healthy cattle persistently infected with BVD virus. The purpose of the present study was to inquire into the immunological effect of BVDV possibly induced in the local immunity of persistently infected animals using immunohistochemical procedures.

MATERIAL and METHODS

Hematological examination:

Five ml EDTA blood were collected weekly from fourty animals persistently infected with BVDV. 5 ml EDTA blood were collected from 5 healthy control animals for 6 months.Total leukocytic count was performed on an automated cell counter .Differential leukocytic count was determined by examination of Giemsa stained blood films.

Immunohistochemical assay:

Tissues examined immunohistochemically were collected from small,large intestine and thymus. These tissues are kept in 10% neutral buffered formalin.

Avidin Biotin Complex immunoperoxidase (ABC) method:

The collected tissues were examined for the detection of IgA,secretory IgA, IgG1,S-100,CD4+ and CD8+ using Avidin Biotin complex (ABC) immuno- peroxidase method as described by (Hsu et al.,1981;Hewicker et al.,1995) as follow. Paraffin sections from examined tissues were cutted at 4 ul and picked up from warm water (40°C) on poly-L-lysine coated slides and dry overnight in an oven at 37°C.Sections were deparaffinized and rehydrated by sequential

immersion of the slides in xylem followed by graded concentrations of ethanol and then tap water. The sections were treated for proteolysis with phosphate buffered saline (PBS 0.1M,pH7.6 prewarmed to 37°C) containing 0.25% trypsin (139 Units/mg; Fluka,Buchs, Switzerland) and 0.02% CaCl2 for 60 min at 37°C.After proteolytic tretment,the sections washed three times each for 5 min in Tris-PBS (0.05M, pH7.6). Endogenous peroxidase activity was blocked by immersion of slides in a solution of 0.5% H2O2 in methanol for 30 min at room temperature (RT). Before incubation with primary specific monoclonal antibodies against IgA and IgG1, the sections were overlaid with normal rabbit serum diluted 1:10 in PBS for 20 min at RT while in case of using monoclonal antibodies against secretory IgA, S-100, CD4+ and CD8+ as primary antibodies, the sections were incubated with normal swine serum diluted 1:10 in PBS for 20 min at RT to inhibit non-specific immunoglobulin binding. The monoclonal mouse antibodies against IgA and IgG1 were applied diluted 1:500 and 1:100in PBS while monoclonal rabbit antibodies against secretory IgA, S-100, CD4+ and CD8+ were applied diluted 1:500,1:1000,1:500 and 1:500 respectively in PBS. Primary antibodies were incubated with the sections for 18 hours in humid chamber at 4°C, the slides were then washed three times in Tris PBS for 5 min. The biotinylated secondary antibodies (Amersham,Little Chalfont,Buckinghamshire,UK) rabbit anti-mouse immunoglobulin diluted 1:200 or swine anti-rabbit immunoglobulin diluted 1:100 in PBS were applied for 30 min at RT. After washing in Tris-PBS three times for 5 min, the sections were incubated with ABC complex which prepared according to the manufacturer's direction (Vectostain Elite ABC Vector laboratories,Burlingame,CA, USA) for 30 min at RT. Antibody binding was demonstrated with a solution of 0.05% 3,3`-diaminobenzidine tetrahydrochloride (DAB, Fluka) in Tris PBS containing 0.01% H2O2 (prepared immediately before use) as peroxidase substrate for 5 min at RT. All sections were counterstained with Mayer's hematoxylene, dehydrated with a series of alcohol ,cleared with xylem and a cover slip was applied with Entellan (Merck, Darmstadt, Germany). The specificity of the immunohistochemical reactions was checked by replacing the primary antisera with non-immune sera or by omitting the secondary antibodies or the ABC solution. For comparison, sections from control animals were stained with the same monoclonal antibodies.

All data concerned primary monoclonal antibodies used in the present study are shown in Table (1).

Table 1: Data of used monoclonal antibodies

Mab Design	Specificity	Reference	Source
CC42	IgA	Howard and Morrison (1991)	Birmingham, UK
Big312 D3	SIgA	Baldwin et al., (1988)	VMRC, Pullman, USA
CC21	IgG1	Naessen et al., (1990)	Birmingham, UK
IL-A58	S-100	Lowe (1990)	Tiburg,The Netherlands
CC8	CD4	Bensaid and Hadlam (1991)	Tiburg,The Netherlands
CC63	CD8	MacHugh and Sopp (1991)	Tibur,The Netherlands

MAb = Monoclonal antibodies sIgA = Secretory IgA

RESULTS

Hematological examination revealed leukopenia with lymphopenia and neutropenia in persistently infected animals. Immunohistochemical assay proved a reduction in IgA, Secretory IgA, IgG1 and S-100 in all examined animals except calves which showed no reduction in the examined immunoglobulins.No reduction observed in CD4+ and CD8+ in all examined animals (the results of immunohistochemistry are shown in Figures (1-9).

DISCUSSION

In the present study, monoclonal antibodies-based immunohistochemistry as molecular biology based technique was used in study of the local immunological response of persistently infected cattle.Bielefeldt Ohmann (1987) reported that monoclonal antibodies-based immunohistochemistry has now become an essential part of studies aimed at elucidating immunological reactions caused by virus infection. The production of bovine monoclonal antibodies should increase the

understanding of the immune response of cattle to BVD virus (Onisk et al., 1991).

Permanent leukopenia with lymphopenia and neutropenia was detected in animals examined in this study. Leukopenia with lymphopenia and neutropenia have been reported in cattle infected with BVD virus (Bolin et al., 1985a; Roth et al., 1986; Ellis et al., 1988; Sopp et al.,1994 and Spagnuolo et al., 1997), however these authors observed transient and not permanent leukopenia as they investigated acutely infected cattle but in the present study I investigated animals persistently infected with BVD virus.Several explanations for the cause of leukopenia accompanied BVD viral infection were discussed. The chemotactic response of monocytes is depressed in the presence of BVD virus (Ketelsen et al., 1979) therefore, absence or dysfunction of monocytes as a result of BVD viral infection could be responsible for the depressed proliferation of lymphocytes (Larsson, 1988). Atluru et al. (1992) assumed that BVD virus may enter T-cells or monocytes ,thus, may inhibit the early activation of signal transduction enzymes involved in lymphocyte proliferation.

Immunosuppressive effect of BVD virus was evidenced in the present study through the effect of persistent virus on local immunity. BVD virus considered immunosuppressive in cattle (Coulibaly et al., 1986; Ellis et al., 1988; Baker, 1990 and Brown et al., 1991). Several explanations for this apparent paradox were reported. Immunosuppressive properties of BVD virus resulted from depression of lymphocytes activity and immunoglobulin synthesis (Muscoplat et al., 1973 and Atluru et al., 1979). Roberts et al., (1988) claimed that BVD virus suppressed interferon production and impairs lymphocytes function,monocyte chemotaxis and humoral antibody production. BVD virus inhibited bovine interleukin-2 production and inhibited an important enzymes that regulate mononuclear cell proliferation (Atluru et al., 1990). Immunosuppressive effect of BVD virus could be through T-cell mediated destruction (Liebler et al., 1995). From the result of the present work, I can add that one of the most important mechanisms concerned immunosuppressive effect of BVD virus in persistently infected cattle is the effect of virus in the local immunity.

In this study it was found that four major immunoglobulins (IgA, Secretory IgA, IgG1 and S-100) were reduced in persistently infected cattle. This finding is interesting in the context of the apparent tolerance to the persistent virus as defined by complete absence of neutralizing antibody production which characterize these animals (Bielefeldt

Ohmann et al. 1987). Atluru et al. 1979) concluded that BVD virus caused a consistent decrease in IgG synthesis and the result of work of Coulibaly et al. (1986) recorded a reduction of IgG level in persistently BVD viral infected cattle. It was clearly demonstrated that cattle persistently infected with BVD virus have a depressed antibody response to Bovine Leukemia Virus (BLV), this depressed response probably resulted from a decreased IgG1 synthesis (Roberts et al., 1988). Great reduction in S-100 was detected in the present study. Lack of response of cattle infected with BVD virus could be due to an indirect effect of BVD virus on B-cells functions caused by a defect in virus antigen presentation (Bielefeldt Ohmann et al., 1987) but these authors did not investigate specific antigen presenting cells (S-100) which was investigated for the first time in the present work.

Reduction in the examined immunoglobulins was not observed in calves examined in this study, this may be explained by the presence of colostral antibodies. Persistently infected animals are often seronegative (Duffell and Harkness, 1985) but calves may be tested seropositive because of the presence of maternal immunity (Meyling and Jensen, 1988). Shanon et al. (1992) have concluded that persistently infected calves which tested negative for BVD virus in the first weeks of life, subsequently tested positive when retested at 15 weeks of age, this is due to presence of colostral antibodies to BVD virus in the first weeks of life.The presence of colostral antibodies to pestiviruses has been reported in calves (Nettleton and Entrican, 1995).

The results of the present study revealed that there are no change in CD4+ and CD8+ in persistently infected cattle. Sopp et al. (1994) and Nettleton and Entrican (1995) observed no change in the percentage of CD4+ and CD8+ in persistently infected cattle.However, many authors (Atluru et al., 1979; Roth et al.,1986 and Ellis et al.,1988) reported that immunosuppressive effect of BVDV include both humoral and cell mediated response, but these authors studied this effect on blood and not locally as done in the present work

One of the most important clinical and practical findings in this study was the presence of permanent leukopenia in persistently infected animals, so, great attention should be directed toward animals that have leukopenia for long time, these animals may be persistently infected with BVD virus

and disseminate the virus during the entire life without detection as they are apparently healthy.

In conclusion, the immunosuppression caused by BVD virus in persistently infected cattle is a complex multifactorial syndrome. One explanation of this syndrome which was not investigated before this study is the effect of BVD virus on the local immunity. This finding may increase our understanding of the cause of maintenance of virus in persistently infected animals and subsequently its transmission to other animals. This fact must be put in consideration before planning of any control program for BVD virus.

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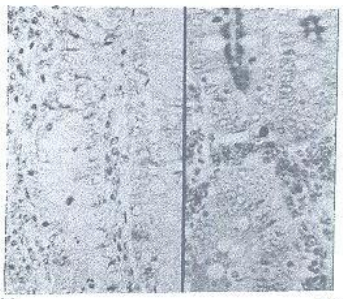
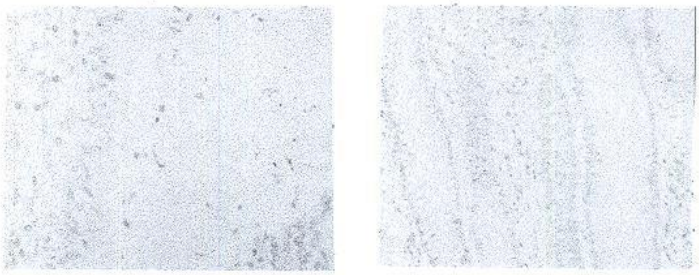


(a) (b)

Fig.(1): IgA in persistently infected (a) and healthy control (b) animals. ABC technique

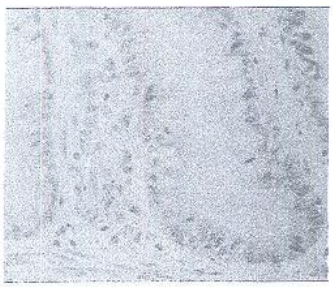
(2) (3)

Fig(2,3): Secretory IgA in persistently infected (2) and healthy control (3) animals. ABC technique.

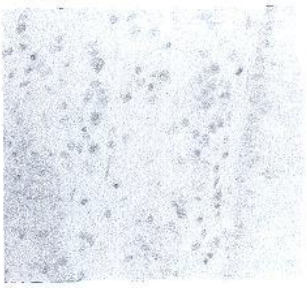


(4) (5)

Fig.(4,5): IgG1 in persistently infected (4) and healthy control (5) animals. ABC technique

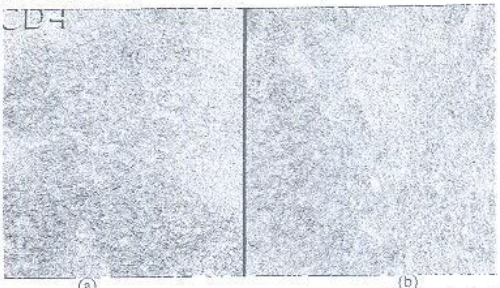


(6)



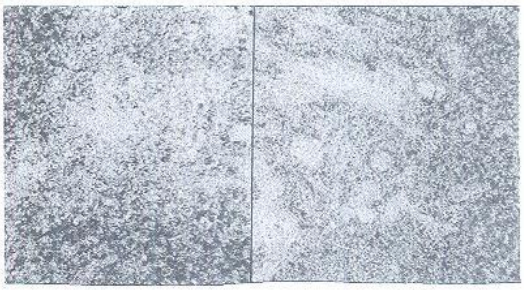
(7)

Fig.(6,7): S-100 in persistently infected (6) and healthy control (7) animals. ABC technique



(a) (b)

Fig.(8) CD4+ in healthy control (a) and persistently infected (b) animals.ABC technique



(a) (b)

Fig.(9) CD8+ in healthy control (a) and persistently infected (b) animals.ABC Technique