Experimental Fetal Infection with Bovine Viral Diarrhea Virus
I. Virological and Serological Studies

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ABSTRACT

The serological and virological results of an experimental infection of bovine fetuses with bovine viral diarrhea virus are presented. Four fetuses, 120-165 days gestational age, were inoculated *in utero* with a second passage virus strain. Two fetuses received a sham-inoculum.

A humoral immune response in the virus-inoculated fetuses, was demonstrated three weeks later. In three fetuses only IgM and IgG_1 were detectable. The serum from the fourth fetus also contained IgG_2 and IgA. Bovine viral diarrhea virus-neutralizing antibodies were detected in two fetuses. These two fetuses inoculated at 135-150 days gestational age, represent the youngest reported bovids, giving a specific response in three weeks following an experimental infection with bovine viral diarrhea virus. The fetal sera did not contain heat-labile factors, which could mediate the neutralization.

The virus was not reisolated from any of the fetuses, but viral antigen was nevertheless demonstrated by immunocytochemical methods in sections of several of the fetal organs, primarily lymphoid tissues.

RÉSUMÉ

Les auteurs rapportent les résultats sérologiques et virologiques d'une infection expérimentale de foetus bovins avec le virus de la diarrhée à virus bovine. Ils en inoculèrent quatre, *in utero*, entre 120 et 165 jours de gestation, avec une souche du virus qui avait subi deux passages. Deux autres foetus reçurent par ailleurs une inoculation simulée.

Trois semaines plus tard, ils démontrèrent la présence d'anticorps sériques, chez les foetus auxquels ils avaient injecté le virus. Chez trois d'entre eux, ils ne détectèrent que des IgM et des IgG_1, tandis que le sérum du quatrième contenait aussi des IgG_2 et des IgA. Ils détectèrent aussi des anticorps neutralisants chez deux de ces foetus qui avaient reçu l'inoculation entre 135 et 150 de gestation et qui, de ce fait, représentent les plus jeunes bovins à avoir donné une réponse spécifique, trois semaines après avoir subi une infection expérimentale avec le virus de la diarrhée à virus bovine. Le sérum de ces foetus ne contenait toutefois pas de facteurs thermolabiles susceptibles d'agir à titre de médiateurs dans les épreuves de séroneutralisation.

Les auteurs ne réussirent pas à recouvrer le virus, chez ces foetus; ils démontrèrent cependant la présence de l'antigène viral, par des méthodes immunocytochimiques, dans des sections de plusieurs organes de ces foetus, en particulier dans celles de leurs organes lymphoïdes.

INTRODUCTION

The frequent detection of either specific neutralizing antibodies to bovine viral diarrhea virus (BVDV) in fetal and precolostral bovine serum or detection of BVDV in tissues from apparently normal bovine fetuses and newborn animals suggest that a subclinical, nonfatal infection is a common outcome of congenital BVDV-infection (11, 13, 24). The bovine fetus gains immune competence to BVDV around day 180 of gestation (3, 4, 14). Kendrick and Braun (14) suggested that an infection before the development of immune competence may result in viral persistence. After this time neutralizing antibodies are assumed to be produced and the virus eliminated.

According to Done *et al* (8), the early fetal infection with BVDV does not always generate a later production of antibodies. Early attempts at experimental induction of immune tolerance to BVDV were unsuccessful (2, 5). Recently however, a persistent BVDV-infection in calves following congenital invasion has been reported. The state was characterized as specific immune tolerance (6).

The aim of the present investigation was to study the interactions

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TABLE I. The Cow and Fetal Number, Estimated Gestational Age of the Fetuses at the Time of Inoculation and the Infection Dosis

Cow No.	Fetus No.	Gestational Age Day 0 in the Experiment	Inoculum
H1	V1	160-180 days	control-inoc.
H2	V2	135-150 days	$10^{5.4}$ $TCID_{50}$
H3	V3	135-150 days	$10^{5.4}$ $TCID_{50}$
H4	V4	120-130 days	$10^{5.4}$ $TCID_{50}$
H5	V5	120-130 days	control inoc.
H6	V6	150-165 days	$10^{5.4}$ $TCID_{50}$

between BVDV and the immature bovine immune system — elucidated by functional and morphological studies as well as by determination of BVDV localization in the lymphoid tissues.

In this communication we report results of the virological and serological investigations. The morphological aspects of the fetal infection will be dealt with in a subsequent paper (18).

MATERIALS AND METHODS

EXPERIMENTAL ANIMALS

Six pregnant cows of different breed, originating from various herds, were used. The periods of pregnancy were stated to be between four and six months and this was confirmed by palpation per rectum (Table I). All but one of the cows had neutralizing antibodies against BVDV prior to the inoculation (Fig. 1).

Twenty-seven fetuses, four and one half to six and one half months gestational age, were collected at an abattoir and functioned as additional controls for the serological and bacteriological investigations, virus isolation and morphological studies (17).

THE VIRUS INOCULUM

A cytopathogenic BVDV was isolated from a calf suffering from mucosal disease ("chronic virus diarrhea"). The supernatant of a 10% suspension of ground tissues was inoculated into Roux bottles (200 cm^2) with a monolayer of one day old secondary bovine kidney cells. Following absorption for one hour the cultures were washed and incubated with fresh medium (Eagle's minimum essential medium with 7% fetal bovine serum), five days at 37°C. Control cultures were treated similarly, thus replacing the tissue inoculum with medium.

The cultures were passaged once to bovine kidney cells. The inoculum was prepared from this second passage and stored in portions of 5 mL at -80°C. The BVDV specificity of the inocula (in the following designated BVD HO) and the absence of BVDV in sham-inocula were demonstrated by indirect immunofluorescence. The virus suspension was titrated by microplate technique and the titer found to be $10^{5.2}$ $TCID_{50}$/mL.

INOCULATION PROCEDURE

The cows were used in the experiment as shown in Table I. Access

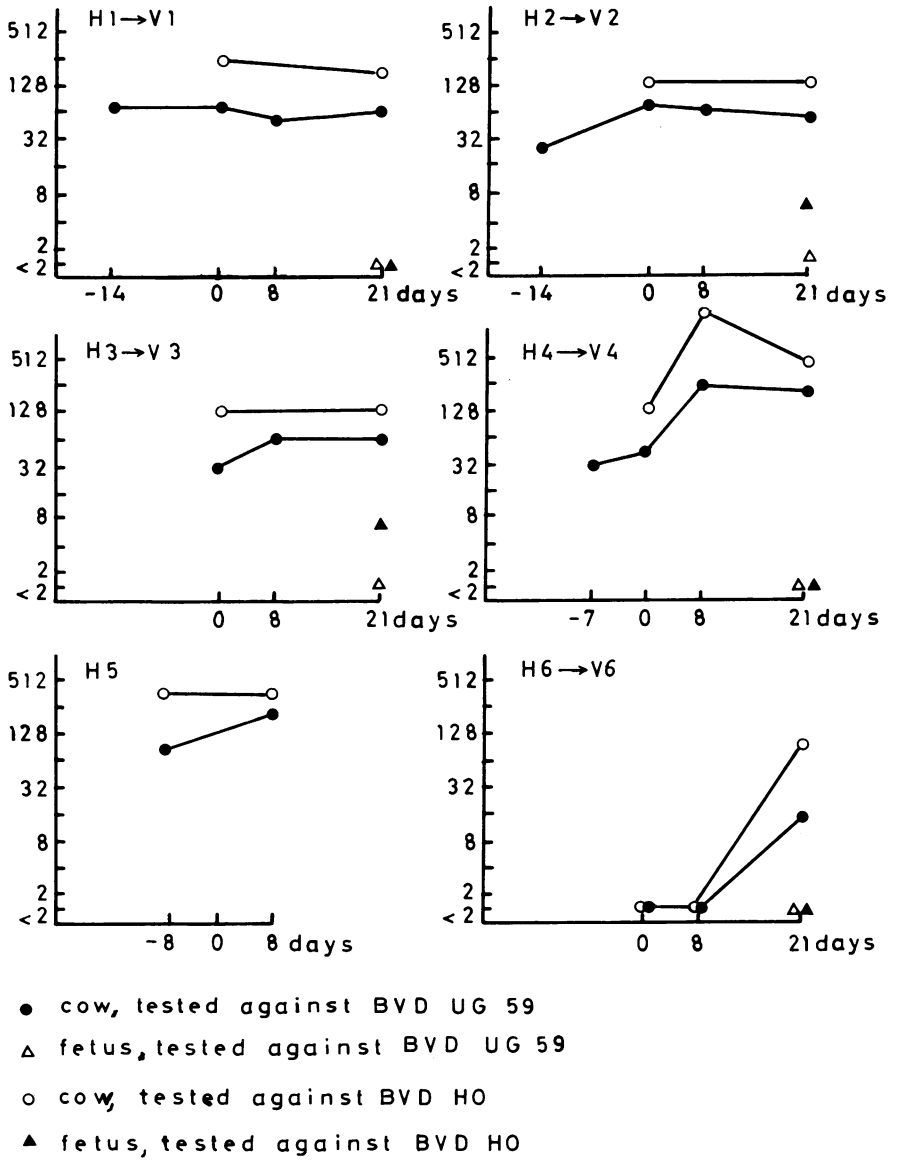

Fig. 1. Results of the serum-neutralizing test on cow and fetal sera. The inoculation was carried out on day 0. Cows H1 and H5 bore control-fetuses. Cow H5 aborted on day 8 pi and was excluded from the experiment.

to the gravid uterus was gained by a standing right flank laparotomy. The inoculum, 1½ mL of either virus or sham-suspension, was injected through the uterine wall, into the muscle of the right pelvic limb of the fetus. During the three week experimental period, the cows were followed by daily clinical examinations. Blood was drawn for serological and virological examinations on days -14, -7(8), zero, three, eight, 14 and 21 postinfection (pi).

COLLECTION OF SAMPLES

Three weeks pi, a standing left flank caesarian section was performed. Amniotic fluid was collected for bacteriological, virological and serological studies. Blood was obtained from the umbilical artery prior to tearing of the cord. Tissues were collected for bacteriological and virological examinations and for morphological studies (18).

BACTERIOLOGICAL EXAMINATIONS

A routine examination was performed on samples from the spleen, liver, lung, placenta and the amniotic fluid.

VIROLOGICAL EXAMINATIONS

Tissues investigated included thymus, spleen, ileum, colon, cervical, mesenteric and popliteal lymph nodes, liver, lung, brain, tonsil, tongue, skin and muscle at the site of inoculation and placentomes. Further, blood samples from fetuses and cows were tested for virus. Virus isolation was performed according to Sorensen and Askaa (27) using primary bovine kidney cell cultures. The tissues concerned were tested separately. All samples were passaged three times before the final evaluation by microscopic observation for cytopathic effect and examination for the presence of BVDV by indirect immunofluorescence (20). A tissue pool was also prepared from each fetus and tested similarly.

The procedures for demonstration of BVDV-antigen in tissue sections by the indirect immunofluorescence technique using $F(ab')_2$-fragments of antisera and the immunoperoxidase technique have been described previously (19, 20). The studies were performed on cryostat sections fixed in 20% phosphate buffered acetone.

SEROLOGICAL INVESTIGATIONS

Rocket Immune Electrophoresis

A method outlined by Dalsgaard *et al* (7) was followed, modified by the use of phosphate buffer, pH 7.0 (21) in the gel. The rabbit antibovine-IgG was prepared according to Harboe and Ingild (10). A similarly prepared reagent from DAKO Immunoglobulins (Copenhagen, Denmark) was also used.

The IgM was detected by incorporating specific rabbit antibovine-IgM (Miles Lab. Ltd., Slough, United Kingdom) into the gel.[1]

Quantitative Radial Immunodiffusion Tests

Test kits, commercially available from Miles Lab. Ltd., (Slough, United Kingdom) were used for quantitation of the IgG (low level-kit), IgM, IgG_1, IgG_2 and IgA. The kits were evaluated after 48 hours. The immunoglobulin concentrations of the fetal sera were determined by reference to a standard curve, produced from standards included in the kit sets. All sera were tested in triplicate and the results are presented as the average.

SERUM-NEUTRALIZATION TEST

Sera from cows and fetuses were tested for neutralizing antibodies against the Danish strain BVD UG 59 (11th passage on bovine kidney cells and bovine testicle cells) and the virus used for inoculation, BVD HO (second bovine kidney cell culture passage) using the "chessboard neutralization assay", previously described by Jensen (12).

The assay was performed on both heat-inactivated (56°C, 30 minutes) and neat serum samples for the purpose of demonstrating possible mediating activity of heat-labile serum-factors.

RESULTS

The cows remained healthy throughout the experiment. Virus was neither isolated from blood samples nor from the placentomes. One cow (H5), the fetus sham-inoculated, aborted eight days pi. The fetal tissues were altered by sterile autolysis as established from gross and microscopic as well as microbiological examinations. The abortion was attributed to surgical manipulation. The bacteriological examinations gave negative results in both control and experimental fetuses.

The serum neutralizing antibody titer against BVDV increased in two cows (H4, H6) following intrafetal inoculation (Fig. 1). This was demonstrated eight days pi in H4 and 21 days pi in H6, with no demonstrable response eight days pi in the latter. It was notable, that no specific response was demonstrated in the fetuses of these cows, whereas the inverse situation was present in cow/fetus H2/V2 and H3/V3, respectively. In all six cows, the neutralization titer was higher tested against strain BVD HO than against BVD UG 59.

The samples from abattoir fetuses and control fetuses V1 and V5 were negative for BVDV and other cytopathic bovine viruses. All attempts of viral reisolation from the experimental fetuses were unsuccessful. However, the BVDV-antigen was detected by immunocytochemical techniques in several fetal organs (Table II). Generally each focus of infection involved very few cells and was sharply limited.

All four BVDV-inoculated fetuses had responded with marked IgG- and IgM-production, whereas no IgG was detected in serum from the sham-inoculated fetus or in sera from abattoir fetuses of the

[1]A reagent was also kindly provided by Dr. T. Newby, Department of Animal Husbandry, University of Bristol, Langford, United Kingdom.

TABLE II. The Results of the Virological Investigations, Including Isolation and Immunocytochemical Methods, of Tissues from Bovine Fetuses Inoculated *in utero* with Bovine Viral Diarrhea Virus (BVDV)

		BVDV-antigen detection by IFT[d]-IPT[e]												
Fetus	Virus isolation	Thymus	Spleen	Cervic ln	Mesent ln	Proxim colon	Ileum	Palat tonsil	Popliteal ln (right)	Lungs	Tongue	Skin	Cere-bellum	Muscle at inoc site
V1[a]	—[f]	—	—	—	—	—	—	—	—	—	—	—	—	—
V2[b]	—	—	+[g]	+	—	—	—	—	+	+	—	—	+	—
V3[b]	—	+	+	+	+	—	—	+	+	+	—	—	—	—
V4[b]	—	—	+	+	—	—	—	—	NT[h]	NT	—	—	+	—
V5[a,c]	—	NT	—	—	NT	NT	NT	NT	NT	—	NT	NT	NT	NT
V6[b]	—	+	+	+	+	—	—	—	+	+	—	—	(+)	—

[a]Sham-inoculated control fetuses
[b]Inoculated with BVDV
[c]Aborted eight days after inoculation
[d]IFT = Indirect immunofluorescence technique (19)
[e]IPT = Indirect immunoperoxidase technique (20)
[f]Negative in virological examinations
[g]BVDV antigen detected
[h]NT = Not tested

same age (Fig. 2, Table III), (17). In three fetuses (V2, V4, V6) IgG_1 made up all the detectable IgG, whereas in fetus V3 IgG_2 was also present. Moreover, IgA was present in the serum from this fetus (Table III). The fetal IgG differed from the postnatal IgG by the absence of a cathodically moving and precipitating fraction in the former (Fig. 2).

When testing the fetal sera for neutralizing antibodies against BVD HO, used for inoculation, a titer was demonstrated in fetuses V2 and V3 (Fig. 1), whereas no neutralizing activity against BVDV-strain UG 59 was detected in any of the fetal sera. In fetuses as well as in cows similar results were obtained with both neat and heat-inactivated serum.

TABLE III. Results of the Immunoglobulin-Quantitation, by Radial Immuno-diffusion,[a] in BVDV-Inoculated and Control Bovine Fetuses[b]

	mg/100 mL			
Fetus	IgG_1	IgG_2	IgM	IgA
V1	0	0	0	0
V2	23	0	10	0
V3	57	28	240	18
V4	21	0	21	0
V6	11	0	21	0
Average of fetuses from abattoir, 4½ -5½ months gestational age (17)	0	0	0	0

[a]Immunoglobulin Test-kits, Miles Lab. Ltd. (Slough, United Kingdom)
[b]Serum was not available from a control fetus (V5) aborted eight days after sham-inoculation

DISCUSSION

Although virus was not reisolated from four experimentally inoculated fetuses, three weeks pi, the specific detection of BVDV-antigen in the fetal tissues by previously improved immunocytochemical methods (19, 20), the presence of pathological lesions, and an immunological reaction reflected functionally as well as morphologically (18) renders it probable, that the fetuses had suffered from an active infection.

The immunocytochemical methods do not permit a determination of the state of the viral antigen. The negative results in attempts of reisolation may be ascribed to the presence of only minute amounts of infectious material. This presumption is corroborated by the preferential localization of the antigen in

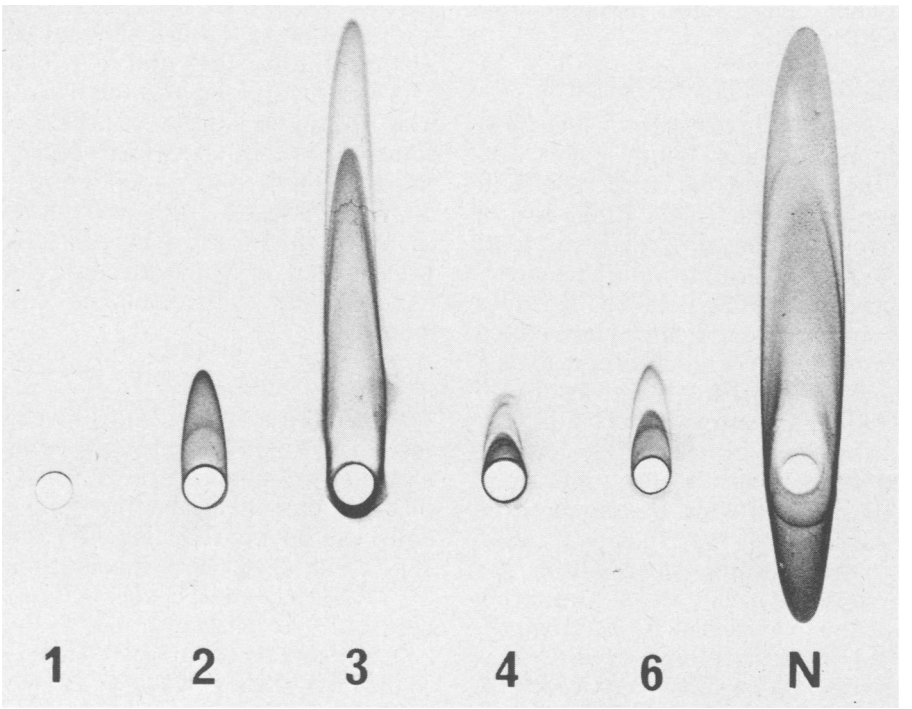

Fig. 2. Detection of IgG by rocket immune electrophoresis of sera from five experimental bovine fetuses and a serumpool from healthy, BVDV-free calves (N). 1: V1, sham-inculated fetus. 2-6: V2, V3, V4, V6, BVDV-inoculated fetuses. The calf serum was diluted 1:4 whereas fetal sera were tested undiluted. A characteristic difference in the precipitation pattern for calf and fetal serum, respectively, is noted.

macrophages and other cells of the mononuclear phagocyte system (18). Or, the presence of neutralizing antibodies and other viral inhibitors may interfere with the isolation of virus (2, 24). Interferon have been reported to be produced in several tissues and to be present in serum up to four weeks following BVDV infection of 150 days old bovine fetuses (23).

The selective occurrence of IgM and IgG_1 in three viral inoculated fetuses is compatible with a primary humoral response (26). The demonstration, by rocket immune electrophoresis, of a difference in the precipitation pattern between the fetal and postnatal serum-IgG indicates that the immunoglobulin is of fetal origin and the production assumed to be induced by the viral antigen. This is also supported by the results of investigations in fetuses from abattoirs. By the serological measures used in this investigation, IgG was not normally detected in the serum of fetuses younger than six months gestational age (17).

The presence of IgA and IgG_2 in conjunction with substantial quantities of IgM and IgG_1 in the serum of fetus V3 might be due to placental transfer of maternal immunoglobulins. This was determined by Brown *et al* (4) to be the explanation for a similar detection of serum IgA and IgG_2 in a bovine fetus following experimental BVDV infection. However, specific antibodies against Chlamydia were demonstrated in the serum of the cow, H3, but could not be detected in the serum of fetus V3 (L. Ronsholt, unpublished data). This seems to exclude a maternal origin of the immunoglobulins, as a placental transfer is expected to be nonselective. Instead the presence of serum IgA may be correlated with the precocious development of Peyer's patches and presence of plasma cells in the intestinal submucosa (18). Thus, in ruminants the intestine is the major source of serum IgA (1).

The two fetuses with BVDV-neutralizing antibodies represent the youngest individuals, hitherto reported, giving a specific response following experimental infection three weeks previously. Both were 135-150 days gestational age at the time of inoculation. Neutralizing antibodies to BVDV have previously been detected in sera from 120-180 days old fetuses collected at an abattoir (11). However, the source of the antibodies i.e. whether of maternal or fetal origin, was not determined. Casaro *et al* (5) found a low BVDV-neutralizing titer in a 190 days old fetus 22 days after inoculation, but no titer in a 180 day old fetus 28 days pi. They, as well as others concluded that the bovine fetus gains immune competence to BVDV close to the 180th gestational day. Neutralizing antibodies are then produced within 20-30 days (5, 24).

The difference in neutralizing activity against the strain UG 59 and the strain used for inoculation (BVD HO) in fetal and cow serum, respectively, may reflect the differences in passage number and not only differences between the native strains (M.H. Jensen, unpublished observations; 16). Antigenic modulation may occur during continued passage, thus rendering the virus more difficult to neutralize (15). The neutralizing antibody-titers found in cow-sera seem to corroborate this interpretation. In all six cows the neutralizing titer against BVD HO and BVD UG 59 showed a constant difference of one to two serum-dilutions, the titer against strain UG 59 always being the lower.

Early immune sera may require cofactors (complement and/or other heat-labile serum-factors) to neutralize the inducing viral agent. This is ascribed to low avidity and/or affinity of early antibodies, especially the IgM-fraction (15). We were not able to detect such mediating activity of heat-labile serum-factors.

The bovine fetus can produce immunoglobulins without detectable specificity in response to a BVDV-infection before obtaining immune competence to the virus (2, 4, 14, 22). But even after that time immunization will lead to production of not only specific but also antigen-dependent, nonspecific immunoglobulins (25). This process may operate long before a specific response can be raised. It has not been possible to correlate the differences in specific immune competence against BVDV with specific morphological characteristics (18) — a phenomenon which may be typical for many antigens and animal species (9, 26).

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