Cell-mediated response in cattle experimentally infected with bluetongue virus serotype 2

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Summary

Cell-mediated immunity in cattle infected with bluetongue virus serotype 2 was examined using the 3-(4,5, dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) lymphocyte proliferation assay and the enzyme-linked immunosorbent assay (ELISA) kit for γ-interferon quantification in serum. Although infection induced the production of neutralising antibodies, no significant statistical differences were observed between the infected and the control animals when tested with the MTT assay. Constant levels of γ-interferon were detected in the serum infected animals during the trial but again no significant statistical differences were recorded. The results of the study are discussed.

Keywords

Bluetongue, Cattle, Cell-mediated immunity, Gamma interferon, MTT assay.

Introduction

Bluetongue (BT) is an infectious, non-contagious, arthropod-borne disease transmitted by various species of biting midges of the genus *Culicoides* and affects domestic and wild ruminants. Clinical symptoms occur primarily in sheep. The causative agent of the disease is a virus of the genus *Orbivirus*, family *Reoviridae*. To date, twenty-four serotypes have been identified worldwide (5, 14).

Since BT is a vector-borne disease, the duration and the intensity of viraemia in the vertebrate host are essential for the transmission of the virus (15). During infection, bluetongue virus (BTV) locates itself between the invaginations of the cell membrane of erythrocytes and hence it establishes a close relationship with the erythrocytes of the susceptible species (1, 3, 4, 21).

Whilst this may protect the virus from the immune system response, it also renders the virus strongly dependent on the half-life of the erythrocytes. In cattle, erythrocytes have a long half-life of up to 120 days (22).

Recent studies on more than 500 cattle naturally and experimentally infected with BTV showed that detectable viraemia persists for up to 63 days post infection (pi) with a probability exceeding 99% (2, 31). Goats and sheep experimentally infected with BTV may also exhibit a prolonged viraemia lasting 47 and 54 days pi, respectively (19).

Like most viral infections, BTV infection is also able to stimulate the immune response, thereby conferring total protection from re-infection with the same serotype. The humoral immune response

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plays an important role in conferring this protection. When an animal with neutralising antibodies is re-infected with the same serotype, it does not exhibit either clinical symptoms or viraemia. However, in instances where a viraemia is detected, the titres will be low but not epidemiologically significant (28, 33, 34).

The role of cell-mediated immune response to BTV infection is not well understood. A cell-mediated immune response, induced after the administration of live BTV, has been detected in mice (17). It is thought to be present in sheep and in cattle when protection against infection has been demonstrated despite the absence of neutralising antibodies (9, 13, 16, 18, 32). Some studies suggest that the cell-mediated immune response may explain the absence of clinical signs in cattle infected with BTV (6, 7, 9, 13).

The aim of this study is to assess the cell-mediated immune response in cattle experimentally infected with BTV serotype 2 (BTV-2).

Materials and methods

Virus

A wild strain of BTV-2 recovered from the spleen of an infected sheep during the outbreaks in 2000 in Sardinia, Italy, was used for experimental infection. Antigen used for the stimulation test was obtained from the South African live-attenuated BTV-2 strain provided by the Onderstepoort Veterinary Institute (OVI) in South Africa, a BT reference laboratory of the OIE (*Office International des Épizooties*: World Organisation for Animal Health).

Animals

Five serologically negative cows, aged between 6 and 9 years, were kept in a stable protected from *Culicoides*. Four were infected subcutaneously with 2 ml of BTV-2 containing 2x $10^{5.8}$ $TCID_{50}$ (23) while the fifth was used as the negative control. Heparinised blood samples (40 ml) were collected from each animal prior to inoculation and once a week for eleven weeks following experimental infection; all samples were processed without delay.

Serum samples (10 ml) were collected immediately following inoculation, once every three hours for the next nine hours and thereafter, twice a week for the following eleven weeks. Sera were stored at –20°C until tested.

In vitro antigenic stimulation test

Lymphocyte isolation

Peripheral blood was diluted 1:4 in lysing buffer pH 7.2 (TRIS 0.017M and ammonium chloride 0.144M) and centrifuged at 200 g for 10 min at 4°C. The supernatant was discarded and the pellet resuspended in 45 ml of RPMI medium (Roswell Park Memorial Institute) (RPMI-1640 medium modified). The cells were washed twice by centrifugation at 200 g for 10 min at 4°C and then resuspended in 5 ml of RPMI complete medium (20% foetal bovine serum, glutamine 2 mM, amphotericin B, penicillin and streptomycin 100x [25 µg/ml amphotericin B, 10 000 IU/ml penicillin, 10 mg/ml streptomycin] and gentamycin 50 mg/ml [gentamycin sulphate solution]). A total of 250 000 mononuclear cells per well were laid onto 96 flat-bottomed well microtitre plates and incubated for 2 h at 37°C with 5% CO_2.

Antigen preparation

Vero monolayer cells were infected with ≥$10^6$$TCID_{50}$/ml BTV-2 viral suspension; virus was adsorbed for 1 h at 37°C and then modified Eagle's medium (MEM) with 10% foetal bovine serum was added. Once the cytopathic effect (CPE) had affected approximately 90% of the monolayer cells, the supernatant was harvested and centrifuged at 500 g for 30 min at 4°C. The pellet was suspended

in phosphate buffered saline (PBS) (pH 7.2) and sarcosyl 30% (N-lauroylsarcosine sodium salt solution). The viral suspension underwent three sonication cycles in an ice bath using a 300 W probe; the sonicated suspension was then centrifuged at 10 000 g for 10 min at 4°C. The supernatant was stratified on a 40% sucrose gradient and centrifuged at 100 000 g for 2 h at 4°C; the pellet was suspended in PBS (pH 7.2), sonicated at 300 W and stored at –70°C.

The protein concentration of the antigen was determined using the bicinconinic-acid assay.

MTT cell proliferation assay

Reduction by living cells of tetrazolium salt [3,(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] or MTT assay was performed, as described by Mosmann (23) and modified by Denizot and Lang (8). Lymphocyte samples on all plates were stimulated with viral antigen at the optimal concentration of 2.2 mg/ml and with a suspension of 10 mg/ml ConA (Concanavalin A) (8, 23). Five replicates for each sample and for the control were plated. Non-stimulated mononuclear cells were used as negative controls. After incubation for 24 h at 37°C and 5% CO_2, a solution of 3 mg/ml MTT salt was added to the cell cultures and the plates incubated for a further 3 h in the same conditions. After centrifugation at 67 g for 5 min, an MTT salt solution was obtained in the cell cultures by adding absolute isopropanol and after agitation of the plates for a further 5 min.

The test was read using a spectrophotometer at 595 nm. The results were expressed as the difference between the mean optical densities (OD) of the stimulated mononuclear cell replicates and the negative control replicates.

Statistical analysis

The statistical analysis was based on the OD values. A comparison was made between the mean OD values of the ConA-stimulated and non-stimulated mononuclear cells of each animal. A further comparison was made between the mean OD values of the antigen-stimulated and the non-stimulated mononuclear cells of each animal. The Wilcoxon non-parametric test for dependent groups was applied to both comparisons.

The Mann-Whitney non-parametric test for independent groups was also used to compare the mean OD values between the antigen-stimulated and the non-stimulated mononuclear cells of each infected animal against those of the control animal. The same test was performed to compare OD mean values of ConA-stimulated mononuclear cells between each infected animal and the control animal (30).

γ-interferon quantitative assay

The γ-interferon quantitative assay on serum samples was performed using the BioX Gamma Interferon ELISA quantitative assay kit in accordance with the instructions of the manufacturer.

Virus neutralisation assay

Serum samples were tested using the virus neutralisation assay (VN) (12, 29). Positive and negative controls were provided by the OVI.

Fifty µl of eight serum dilutions (1:10-1:1 280), were mixed with an equal volume of reference BTV-2 (100 $TCID_{50}$) in a microtitre plate and incubated at 37°C and 5% CO_2. After incubation for 1 h, a 100 µl suspension of 10^4 Vero cells in MEM with antibiotics (penicillin 100 IU/ml, streptomycin 100 µg/ml, gentamycin 5 µg/ml and nystatin 50 IU/ml) and 3% foetal bovine sera were added to each well. After 4 to 6 days of incubation at 37°C and 5% CO_2, the wells were monitored for CPE using an inverted microscope. A sample was considered positive when it neutralised at least 50% of CPE at the lowest dilution (1:10). The highest serum dilution capable

of neutralising over 50% of CPE in the cell culture provided the serum titre.

Virus isolation

Virus isolation was performed on heparinised blood samples using intravenous inoculation of embryonated chicken eggs followed by repeated passage in Vero cells in accordance with the OIE *Manual of diagnostic tests and vaccines for terrestrial animals* (10, 11, 25, 29, 36).

Blood samples collected from infected animals were washed three times with PBS (pH 7.2) containing antibiotics (penicillin 100 IU/ml, streptomycin 100 µg/ml, gentamycin 5 µg/ml and nystatin 50 IU/ml) and then centrifuged at 230 g for 15 min. Blood cells were resuspended in buffered lactose peptone (BLP) and were sonicated at 300 W to disrupt the cell membrane. The sonicated product was centrifuged at 1 250 g for 15 min; 0.1 ml supernatant was inoculated intravenously into five 9-12 day-old embryonated chicken eggs. The eggs were incubated at 34°C and candled daily. Embryo deaths that occurred within the first 24 h post inoculation were regarded as non-specific. Embryos that died between days 2 and 7 post inoculation were kept at 4°C and embryos that were still alive on day 7 were sacrificed. The brain, heart, liver and spleen from infected embryos were homogenised with sterile quartz powder and centrifuged at 1 250 g for 20 min at 4°C. The supernatant was added to Vero monolayer cells maintained in an antibiotic medium and incubated at 37°C with 5% CO_2.

If no CPE was detected during the following 7 days, the monolayer was scratched and centrifuged at 203 g for 15 min and the supernatant used for the infection of a new monolayer cell. The presence and identification of virus in the cells was assessed by immunofluorescence (IF) using monoclonal antibody (IgG_{2a} anti-K) to BTV core protein VP7 and fluorescein isothyocianate (FITC)-labelled anti-mouse IgG. In positive samples, the viral serotype was identified using a virus typing assay in accordance with the OIE *Manual* (25).

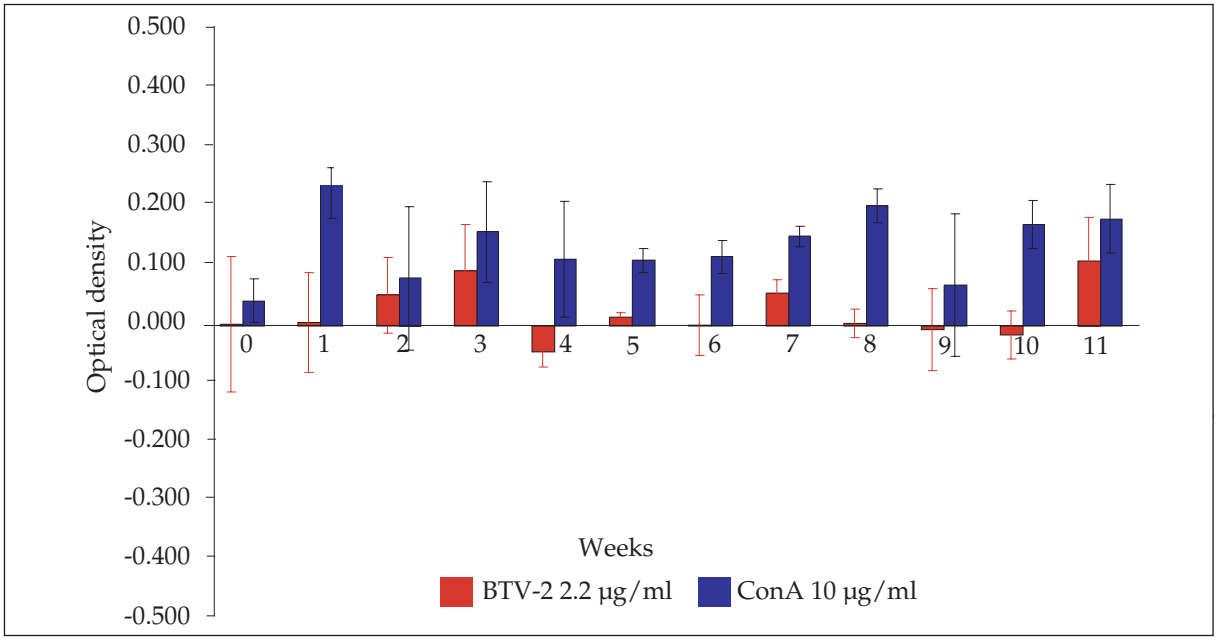

Figure 1
Mean values of optical density in the MTT assay and standard deviation in ConA and antigen-stimulated mononuclear cells. Results are expressed as the difference between the mean values of optical density of stimulated and non-stimulated mononuclear cells: animal No. 1.

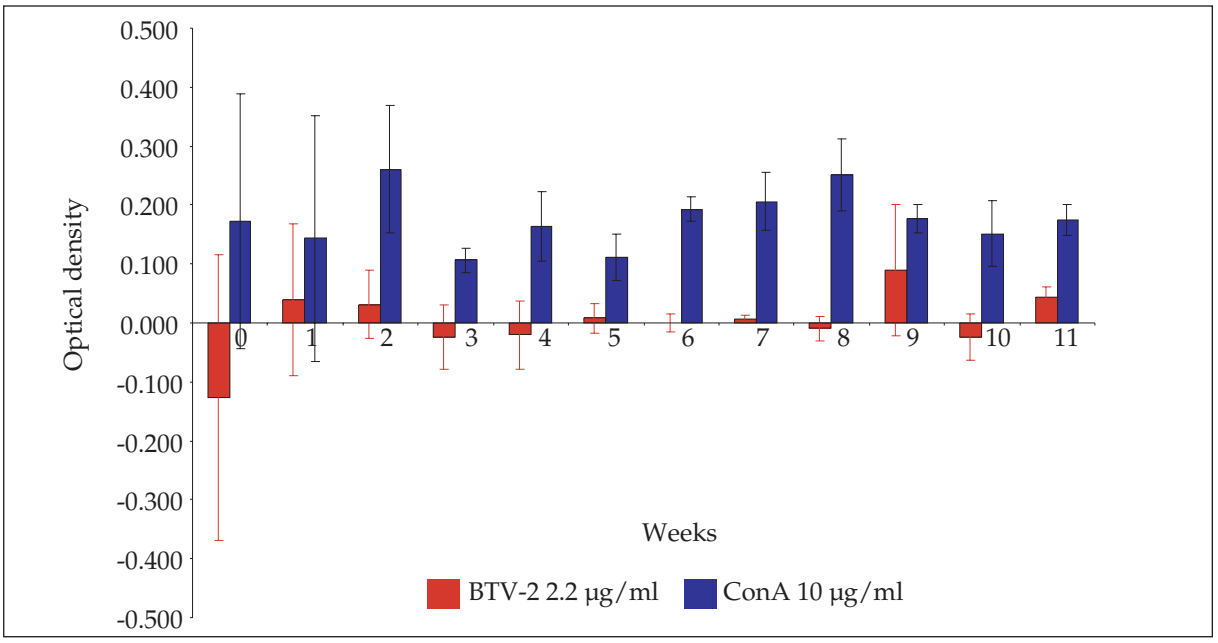

Figure 2
Mean values of optical density in the MTT assay and standard deviation in ConA and antigen-stimulated mononuclear cells. Results are expressed as the difference between the mean values of optical density of stimulated and non-stimulated mononuclear cells: animal No. 2.

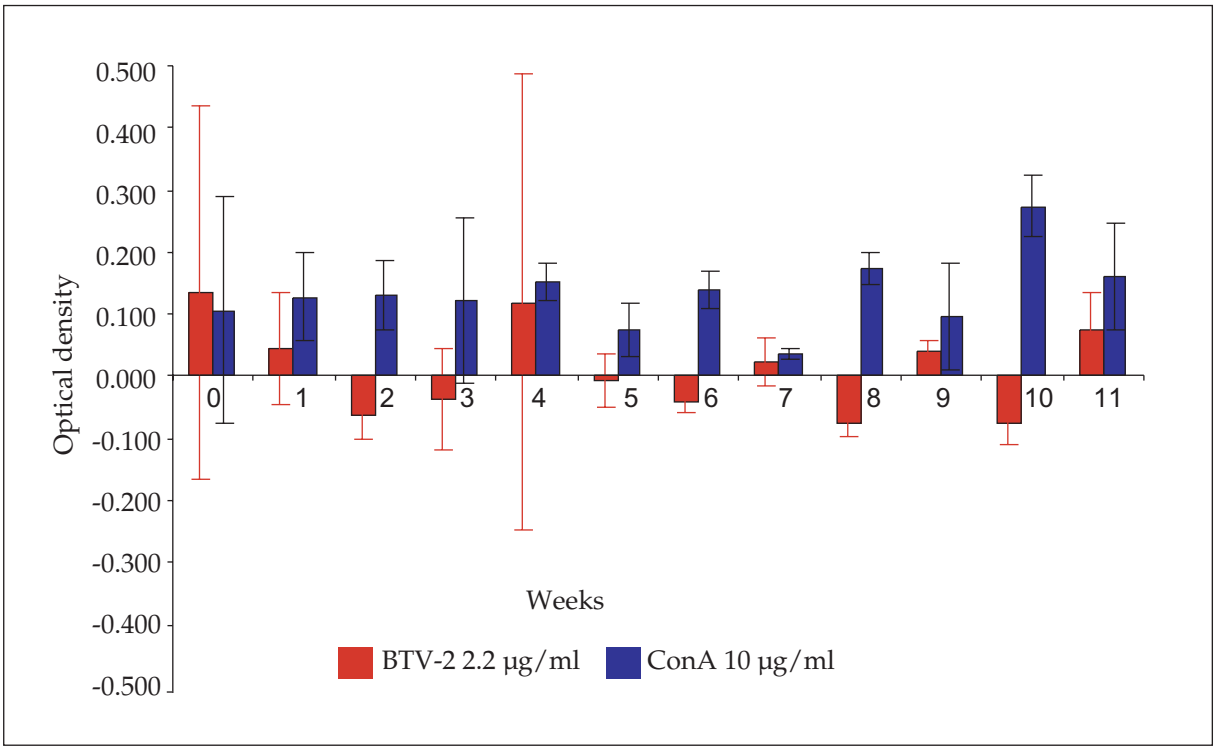

Figure 3
Mean values of optical density in the MTT assay and standard deviation in ConA and antigen-stimulated mononuclear cells. Results are expressed as the difference between the mean values of optical density of stimulated and non-stimulated mononuclear cells: animal No. 3.

Results

Evaluation of mononuclear cells stimulation using MTT assay

The results of the ConA- and antigen-stimulated mononuclear cells are presented in Figures 1, 2, 3, 4 and 5. Table I gives the results of the Wilcoxon test between the ConA-stimulated and the non-stimulated mononuclear cells for each animal during the trial. A statistically significant difference can be observed for each animal.

As far as stimulation with antigen is concerned, the results of the Wilcoxon test did not show significant statistical differences between the antigen-stimulated and the non-stimulated mononuclear cells for each animal (Table II).

The comparison of the mean OD values of the ConA-stimulated mononuclear cells between each infected animal and the control animal is given in Table III; none of the animals showed significant statistical differences.

The results of the Mann-Whitney test are given in Table IV which compares the mean OD values of the antigen-stimulated mononuclear cells for each infected animal and for the control. No significant statistical differences were noted.

Levels of γ-interferon cytokines circulating in blood

The results of the γ-interferon quantitative assay are shown in Figure 6.

Infected animals showed a constant production of γ-interferon of about 2.5 UA/ml commencing from day 0 until day 15 pi; subsequently, the levels of cytokines declined to less than 0.5 UA/ml.

The control animal showed the same trend in γ-interferon levels despite much higher values ranging between 0.8 and 5.2 UA/ml.

Virus neutralisation assay

The VN results are shown in Figure 7. Antibody titres were detected by the third week pi in all infected animals. A peak titre of 1:1 280 was

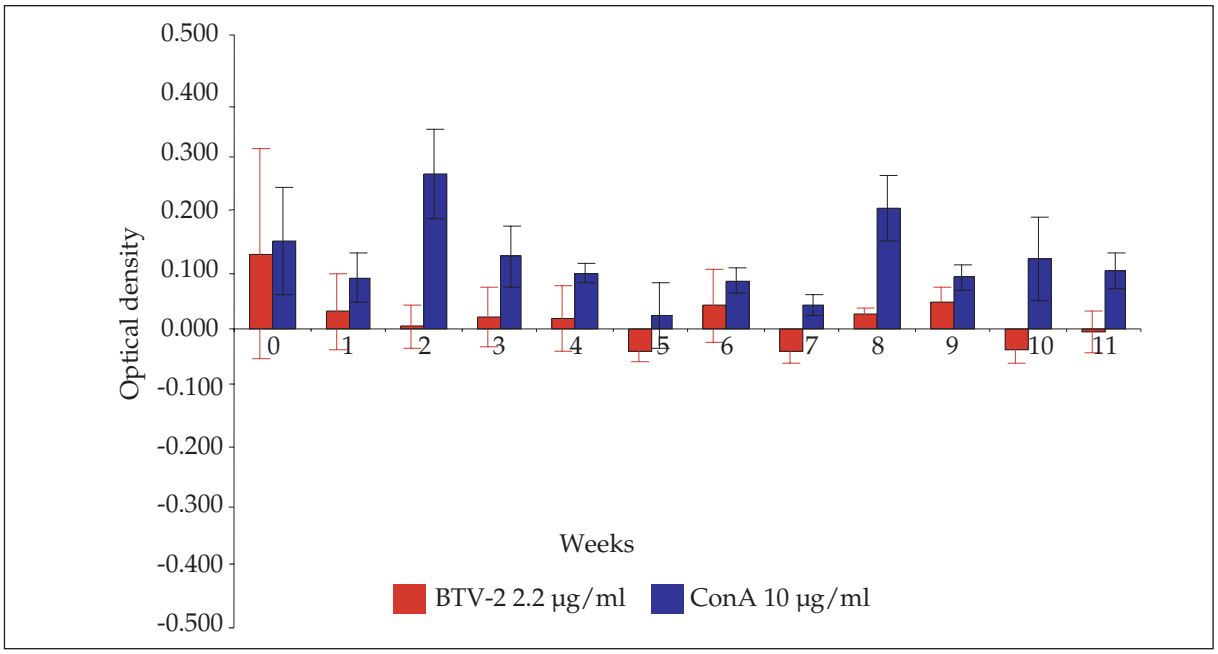

Figure 4
Mean values of optical density in the MTT assay and standard deviation in ConA and antigen-stimulated mononuclear cells. Results are expressed as the difference between the mean values of optical density of stimulated and non-stimulated mononuclear cells: animal No. 4.

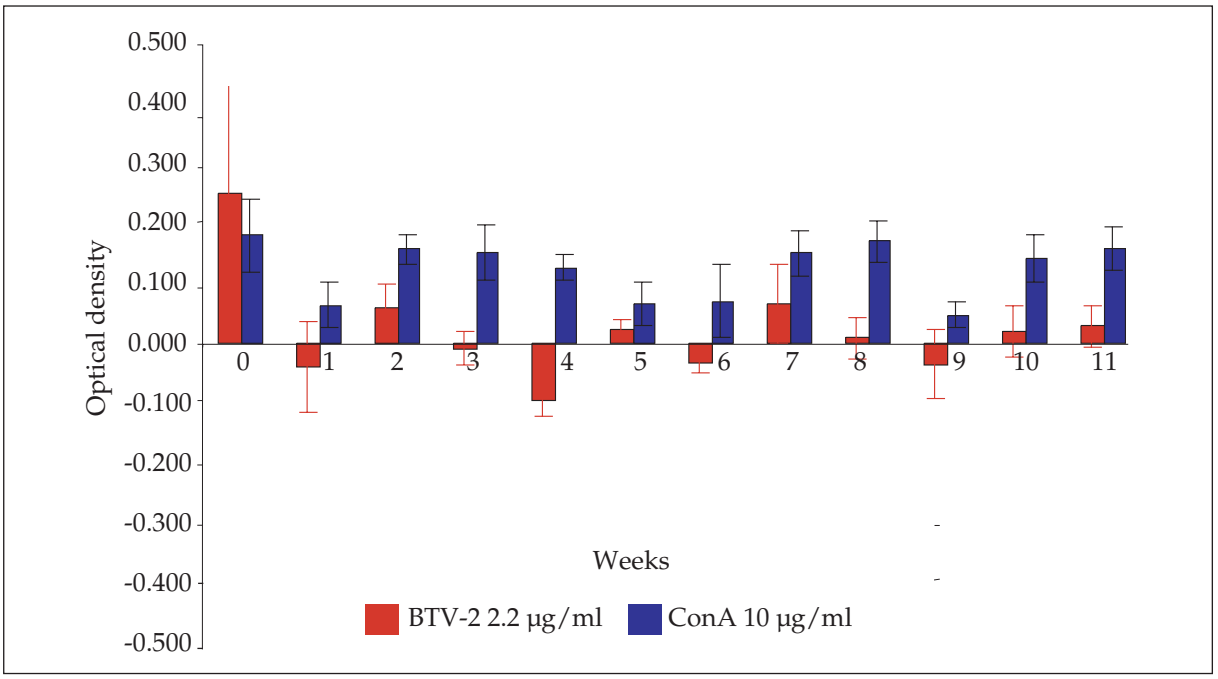

Figure 5
Mean values of optical density in the MTT assay and standard deviation in ConA and antigen-stimulated mononuclear cells. Results are expressed as the difference between the mean values of optical density of stimulated and non-stimulated mononuclear cells: control animal.

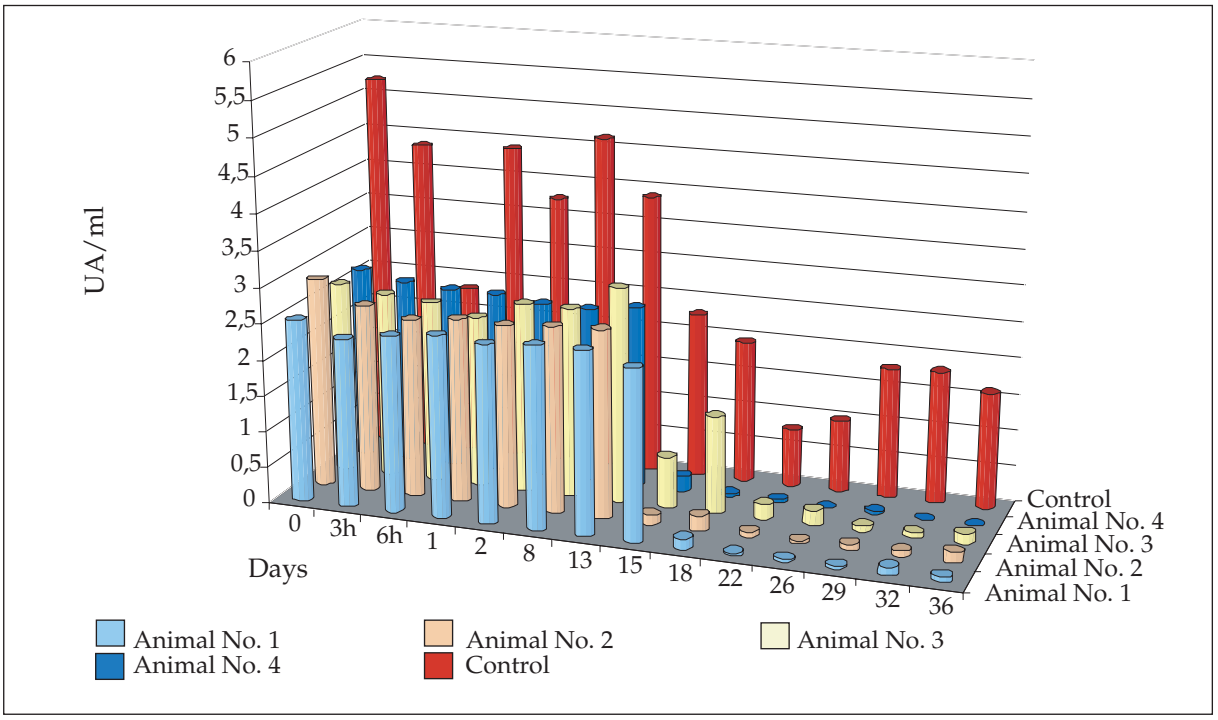

Figure 6
Levels of γ-interferon for serum samples for each animal

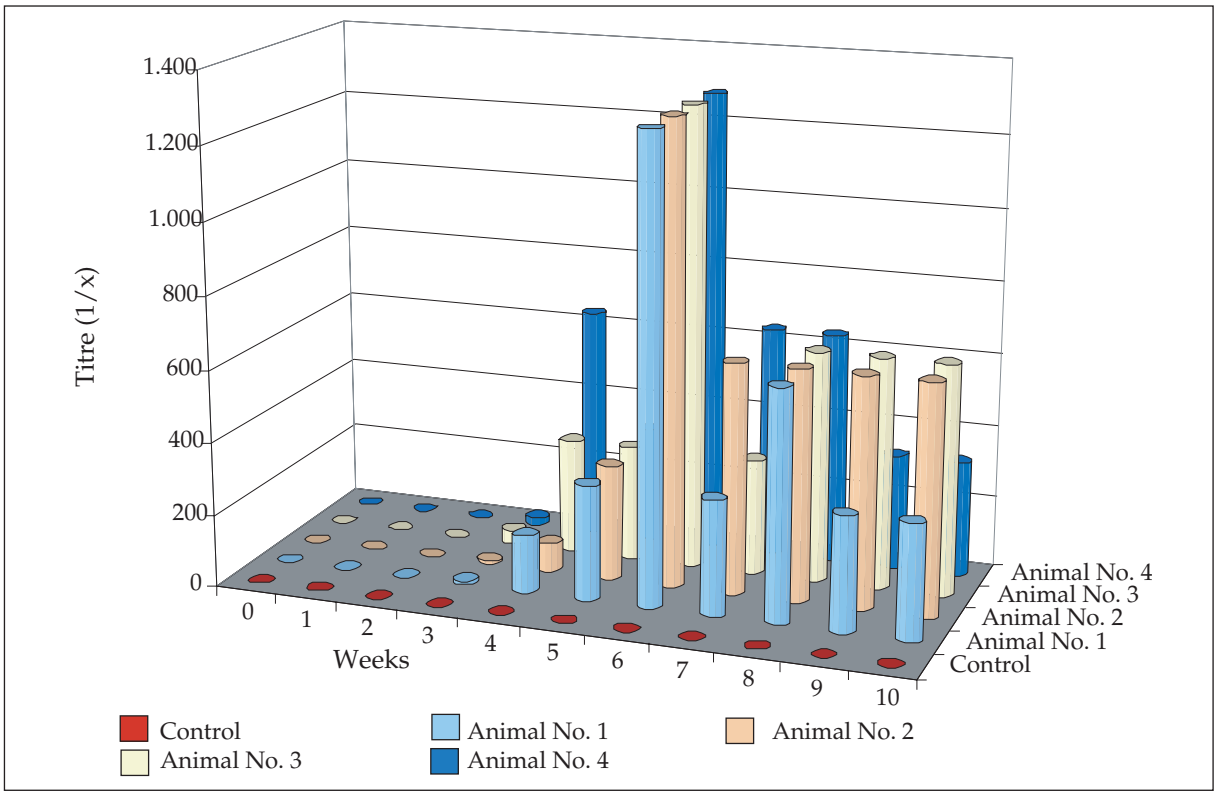

Figure 7
Results of the virus neutralisation assay until the tenth week post-infection for each animal

recorded during the sixth week pi in all animals. No antibody production was detected in the control animal.

Virus isolation

Viraemia in infected animals reached a peak of $10^{4.6}$ $TCID_{50}$/ml during the second week pi and declined at the end of the fifth week.

The control animal did not display any viraemia throughout the trial; the results are shown in Figure 8.

Discussion and conclusions

Cell-mediated immunity against BTV-2 was monitored in four experimentally infected cattle using the lymphocyte stimulation test and the γ-interferon quantitative assay.

After infection, neutralising antibodies were detected; however, no significant statistical differences in the lymphocyte response to BTV-2 were observed between infected and control animals. This may be due to the specific method used for lymphocyte stimulation. The MTT test is a colorimetric non-radioactive assay for measuring cellular proliferation through increased metabolisation of tetrazolium salt (23) offering major advantages in speed, simplicity and cost compared to radioactive methods (8).

However, several studies indicate that the MTT test is less sensitive than radioactive methods, probably because of the small amounts of mitochondrial dehydrogenase in lymphocytes required to metabolise tetrazolium salt (8, 35). Moreover, the variability observed between the replicates of each sample indicates inaccuracy in the measurement of the colorimetric reaction.

Furthermore, lymphocytes were stimulated by the ConA but not by the BTV-2 antigen. This may

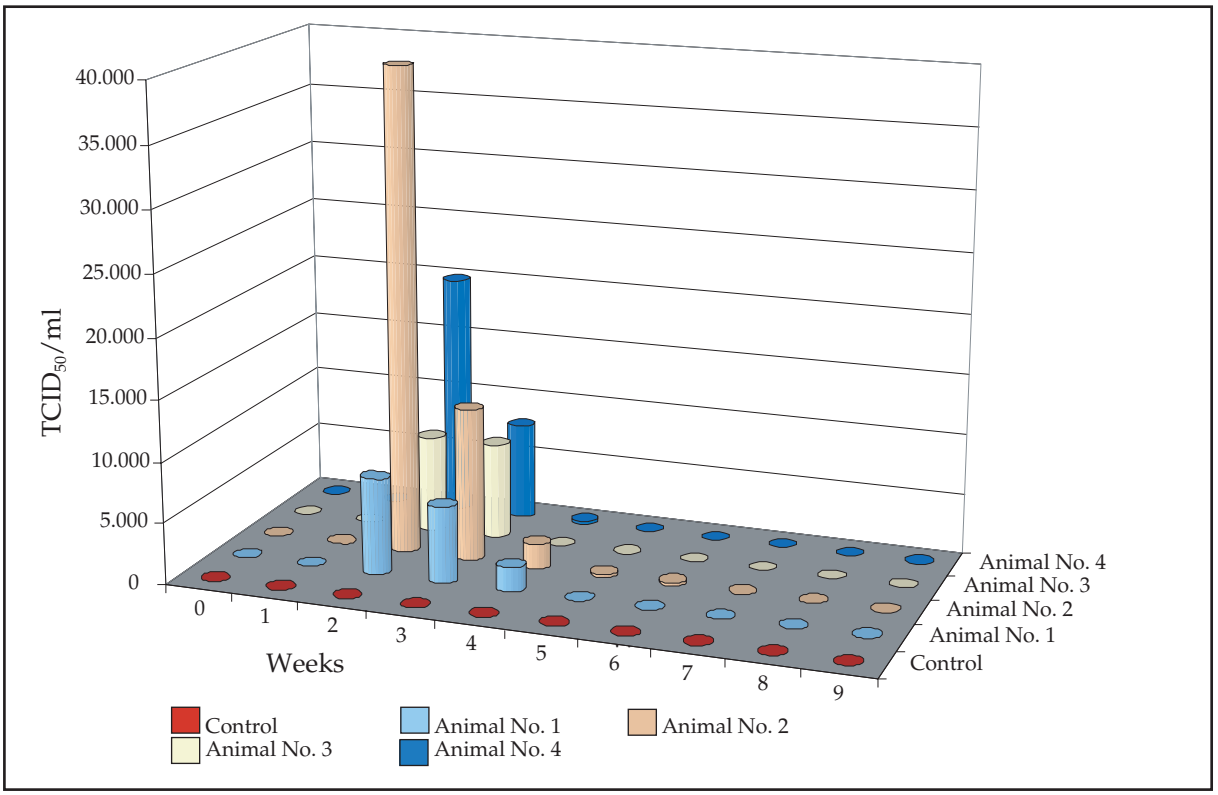

Figure 8
Virus isolation: $TCID_{50}$/ml in each animal

be due to the dose and preparation method of the antigen, or due to insufficient exposure to the antigen. Nevertheless, in previous studies (20, 24), BTV has been shown to inhibit lymphocyte proliferation during experimental infection. This suppressive response may contribute to the prolonged viraemia observed in cattle even in the presence of neutralising antibodies (9, 13). The reduced response of cell-mediated immunity to

Table I
Comparison of optical density values between ConA-stimulated mononuclear cells and non-stimulated mononuclear cells for each animal.
Results of the Wilcoxon test and significance value.

Animal	Wilcoxon test	p
No. 1	-3.59	<0.05
No. 2	-3.59	<0.05
No. 3	-3.59	<0.05
No. 4	-3.59	<0.05
Control	-3.59	<0.05

Table II
Comparison of optical density values between antigen-stimulated mononuclear cells and non-stimulated mononuclear cells for each animal.
Results of the Wilcoxon test and significance value.

Animal	Wilcoxon test	p
No. 1	-1.766	>0.05
No. 2	-0.392	>0.05
No. 3	-0.549	>0.05
No. 4	-0.471	>0.05
Control	-0.746	>0.05

Table III
Comparison of optical density values of Con-A-stimulated mononuclear cells in infected and control animals.
Results of Mann-Whitney test and significance value.

Animal	Mann-Whitney test	p
No. 1 vs control	69	>0.05
No. 2 vs control	50	>0.05
No. 3 vs control	49	>0.05
No. 4 vs control	65.5	>0.05

Table IV
Comparison of optical density values of antigen-stimulated mononuclear cells in infected and control animals. Results of Mann-Whitney test and significance value

Animale	Mann-Whitney test	p
No. 1 vs control	62	>0.05
No. 2 vs control	64	>0.05
No. 3 vs control	58	>0.05
No. 4 vs control	59.5	>0.05

BTV antigen may also explain the lack of γ-interferon production observed in this study.

Unlike previous studies (20, 27), the γ-interferon production remained constant immediately after infection and during the days that followed. A decline in the serum γ-interferon level was observed from day 15 to day 36 pi. However, the results do not indicate a link between the interferon response and viral infection. In conclusion, further experiments on a larger number of animals are required to better define the role of the cell-mediated immunity in the pathogenesis and protection of cattle against BTV infection.

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