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Immunosuppression in sheep induced by cyclophosphamide, bluetongue virus and their combination: Effect on clinical reaction and viremia

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ABSTRACT

The main purpose of this work was to establish an experimental model for immunosuppression in sheep, and evaluate its possible effects on bluetongue viremia. Animals were allocated in 4 groups: Cy (cyclophosphamide), BT (bluetongue), CyBT (combined Cy and BT) and Co (control), and underwent clinical evaluations, virological testing, peripheral blood immunophenotyping and determination of antiviral humoral immune responses. Intravenous administration of cyclophosphamide (37.5 mg/kg body weight) resulted in immunosuppresion induction, as significant drops were observed in blood leukocytes and lymphocyte subset counts ($CD2^+$, $CD4^+$, $CD8^+$, $CD19^+$), lasting 3–10 days after its administration. Reduction in B-cell ($CD19^+$) counts was more pronounced than in T-/NK-cell ($CD2^+$) counts (92% and 59%, respectively). BTV-9 inoculation resulted in pronounced lymphocytopenia observed from day 1 post-inoculation. Their combined administration resulted in a more intense immunosuppressive effect, as indicated by the greater reduction in lymphocyte, granulocyte, $CD4^+$ and $CD8^+$ cell counts. In group CyBT, earlier initiation of fever by one day (day 6 p.i.) compared to group BT (day 7 p.i.), and delay in antibody responses by one day was observed, compared to group BT. Neutralizing antibodies in both groups (BT, CyBT) were detectable from day 10 p.i., but no significant titer differences were observed. Infectious virus titers were detected from day 4 p.i. in group BT and from day 3 in group CyBT. Statistical significances in virus titers were also observed (greatest mean titer difference: 1.4 $\log_{10}$ $CEID_{50}$/ml RBCs at day 5 p.i., $P < 0.001$), indicating possible impact of immunosuppression on virus transmission and epidemiology of bluetongue.

1. Introduction

Immunosuppression is defined as the reduced activation or efficacy of the immune system, due to either self-mechanisms or exogenous factors. Various conditions can induce immunosuppression in animals, including stress, viral infections and pharmacologically active substances. Acute or chronic stress can consequently lead to immunological impairment. Immunosuppression is also observed in several cases of infectious diseases. Characteristic examples of virus-induced immunosuppresion in sheep are those of *Border disease virus* [1], of small ruminant lentiviruses [2], as well as of *Bluetongue virus* (BTV), a dsRNA virus, member of the *Reoviridae* family (genus *Orbivirus*) [3]. Over twenty-seven BTV serotypes have been identified [4]. The virus is transmitted by *Culicoides* biting midges, affects all ruminants and causes bluetongue, an OIE-listed infectious disease. After a susceptible host is bitten by an infected midge, the virus replicates in the draining lymph node [5,6]. Then it spreads further to other organs, via efferent lymph vessels and blood circulation [7]. During viremia, BTV promiscuously associates with all blood cells, resulting in a proportionate relationship between the virus titer in each fraction and the numbers of each cell type. Adsorption of virus particles on

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red blood cells (RBCs) facilitates prolonged viremia and infection of midges that feed on viremic animals. Additionally, due to this association to RBCs, infectious BTV can co-circulate in blood with high neutralizing antibody (NAb) titers for several weeks [5–8].

Clinical manifestations in BTV-affected animals range depending on the virus strain, the breed of the animals and their immunological status. Clinical signs include fever, facial edema, oral lesions, emaciation and lameness [6]. BTV replicates in monocytes, with formation of viral inclusion bodies and virus-specific tubules [8]. It is also suggested that activated lymphocytes and γ/δ T-cells can be infected [2,7,9,10]. BTV can induce pan-leukopenia at the peak of the infection, leading to transient immunosuppression and predisposition to several secondary bacterial infections [3,11–13]. Additionally, BTV infection results in lower numbers of T-cells and an increase of the $CD4^+$:$CD8^+$ ratio, during the period of transient leukopenia [14].

Besides the aforementioned viral effects, suppression of the immune system can be also achieved via administration of immunosuppressive drugs [15]. Cytotoxic drugs, which comprise a category of immunosuppressive agents, interfere in nucleic acid activity, thus inhibiting cell division [16]. The most important of these agents is cyclophosphamide, which is especially toxic for dividing immunocompetent cells, impairing both T-cell and B-cell responses, as well as suppressing macrophage function. Among its toxic effects, suppression of bone marrow is of major importance, given that it leads to leukopenia [15]. Although several studies with successful induction of immunosuppression have been performed in bovine, limited publications for suppression of the immune system of sheep exist [2,17].

The aim of the present experimental study is to evaluate: i) the effect of administration of cyclophosphamide at immunosuppressive dose, on the peripheral blood leukocyte counts, as well as on lymphocyte subset counts of sheep, ii) the immunosuppressive effect of BTV-9 experimental inoculation, and iii) the combined effect of immunosuppression by cyclophosphamide and BTV-9 infection in sheep, on bluetongue viremia and clinical reaction.

2. Materials and methods

2.1. Virus isolate

BTV-9 (strain GR199/98RS, GenBank acc. Nr. AY677629.1), isolated from sheep [18] was used. The strain underwent 2 passages in embryonated chicken eggs (ECEs), 9 passages in BHK-21 cells (clone 13; ECACC Nr. 85011433), 3 passages in Vero cells (ECACC Nr. 84113001), as well as a final passage in C6/36 cells (ECACC Nr. 89051705). Cell culture supernatant from the last passage (i.e. in C6/36 cells) was aliquoted and stored at −80 °C, until it was used for the experimental infections (virus stock).

The infectious virus titer of the cell culture supernatant of the passage in C6/36 cells was determined by end-point dilution assay. Briefly, 100 μl of 10-fold serial dilutions (10^{-1} to 10^{-11}) of the supernatant were inoculated onto Vero cells, previously seeded on 96-well cell culture plates (8 wells per dilution). Subsequently, plates were incubated for 1 h at 37 °C in 5% CO_2 environment. After incubation, 100 μl of Dulbecco's Modified Eagle's Medium (DMEM; Invitrogen-Gibco, Groningen, The Netherlands) supplemented with 2% Fetal Bovine Serum (FBS; Invitrogen-Gibco, Groningen, The Netherlands) and 1% antibiotics (Invitrogen-Gibco, Groningen, The Netherlands) were added per well. Plates were incubated at 37 °C in a 5% CO_2 environment, and were being checked over the following 7 days under an inverted microscope, for evidence of viral cytopathic effect (CPE). The virus titer was calculated using the Reed-Muench method [19]. Based on this titration, the virus stock was diluted appropriately, to achieve a titer of $10^{6.5}$ $TCID_{50}$ (50% tissue culture infective dose) per ml, which was used for the experimental infections.

2.2. Animals

Twenty-three female 1.5-year-old Karagkouniko sheep (a Greek breed) were used. All animals were ascertained for their BTV-free status by serological testing (as described in section 2.8). Additionally, all animals were seronegative for maedi-visna, brucellosis, paratuberculosis and were de-wormed with *per os* administration of 7.5 mg/kg body weight (BW) albendazole (Provet, Athens, Greece) [20]. A mixed ratio comprised of alfalfa hay and pellets was being administrated *ad libitum* to the sheep, along with water.

2.3. Drug-induced immunosuppression

Cyclophosphamide (Endoxan®; Baxter Oncology, Halle/Künsebeck, Germany) was used as immunosuppressive agent, at a dose of 37.5 mg/kg BW. The selection of this dose was based on preliminary experiments, where dexamethasone, azathioprine and cyclophosphamide were evaluated in Karagkouniko sheep, alone or in combination, in different doses and schemes (data not shown). In parallel, an injectable suspension containing procaine benzylpenicillin and dihydro-streptomycin (Ilcocillin® PS; Premier Shukuroglu, Athens, Greece) was administrated, along with tylosine (Tylan®; Elanco Animal Health, Greenfield, IN), to prevent bacterial infections due to immunosuppression.

2.4. Experimental design, clinical evaluations and blood samplings

Sheep were randomly allocated into 4 groups. Group Cy ($n = 3$) received cyclophosphamide intravenously, at the aforementioned dose. Group BT ($n = 8$) received 2 ml of BTV-9 ($10^{6.5}$ $TCID_{50}$/ml) divided in two; 1 ml in the neck region and 1 ml, on the lateral surface of the left thigh, subcutaneously. Group CyBT ($n = 7$) received cyclophosphamide, and after 3 days BTV-9, as described above. Group Co ($n = 5$) was used as untreated controls.

Physical examination, along with rectal temperature monitoring were being performed on each sheep, from day −2, and until day 29 post-inoculation (p.i.) (Fig. 1). Blood was being collected at specific time-points in EDTA-anticoagulated vacuum tubes, as well as in plain serum tubes. The time-points where each test was performed is shown in Fig. 1.

2.5. Hematology

For the evaluation of hemogram parameters, EDTA-anticoagulated whole blood samples were analyzed using an automated hematology analyzer (Scil Vet ABC; Scil Animal Care Company, Holtzheim, France). Additionally, blood smears were prepared within 2 h of collection, stained with Giemsa (Accustain® Giemsa Stain, Modified; Sigma-Aldrich Chemie GmbH, Steinheim, Germany), washed, air-dried and microscopically examined to determine differential leukocyte counts.

2.6. Flow cytometry (FC)

Lymphocyte subset counts were determined with flow cytometry, after immune labeling with specific monoclonal antibodies (mAbs). An anti-CD2 antibody (isotype IgG2a, cell line 36F-18; VMRD Inc., Pullman, WA) was used for the identification of T-/NK-cells. An anti-CD4 fluorescein isothiocyanate (FITC)-conjugated antibody (isotype IgG2a, clone 44.38; AbD Serotec, Oxford, UK) was used for the identification of helper T-cells, and an anti-CD8 R-phycoerythrin (RPE)-conjugated antibody (isotype IgG2a, clone