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Bovine Neonatal Pancytopenia: Is this alloimmune syndrome caused by vaccine-induced alloreactive antibodies?

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ABSTRACT

Bovine Neonatal Pancytopenia (BNP) is a new emerging disease observed since 2007 in Germany and neighbouring countries. The syndrome affects newborn calves and is characterized by pancytopenia, severe bleeding and high lethality. So far, a causative role of infectious or toxic agents has been ruled out. Instead, the syndrome is induced after ingestion of colostrum, the first milk that supplies the calf with maternal antibodies. In analogy to similar diseases in humans it has therefore been postulated that BNP is caused by alloreactive, maternal antibodies. There is a striking association between BNP and a previous vaccination of the respective dams with a particular vaccine against Bovine Virus Diarrhoea (BVD). This association has led to a suspension of the marketing authorisation for the vaccine, by the European Commission. The current study investigates the role of this vaccine in the pathogenesis of BNP. By flow cytometry we were able to demonstrate that sera of BNP dams (dams that gave birth to a BNP calf) harbour alloreactive antibodies binding to surface antigens on bovine leukocytes. A significantly weaker alloreactivity was observed with sera of non-BNP dams that have been vaccinated with the same vaccine but delivered healthy calves. No binding was seen with non-BVD-vaccinated control cows and animals that were vaccinated with other inactivated BVD vaccines so far not associated with BNP. The binding is functionally relevant, because opsonization of bovine leukocytes with alloantibodies led to an elevated cytophagocytosis by bovine macrophages. To test whether the vaccine induces alloreactive antibodies two strategies were employed: Guinea pigs were vaccinated with a panel of commercially available BVD-vaccines. Only the incriminated vaccine induced antibodies binding surface antigens on bovine leukocytes. Additionally, two calves were repeatedly vaccinated with the suspected vaccine and the development of alloreactivity was monitored. In dependence of the number of booster immunizations the induction of alloreactive antibodies could be observed. Finally, by affinity purification we were able to directly demonstrate that BNP associated alloantibodies cross react with the bovine kidney cell line used for vaccine production. Together this provides strong evidence that this particular BVD vaccine has the potential to induce BNP associated alloantibodies.

1. Introduction

Thrombocytopenia in calves is a well-known but rare disease which can have a variety of different causes [1]. In 2007 an accumulation of cases with severe haemorrhagic presentation was noted in Bavaria but consecutively also in other parts of Germany and in neighbouring countries. A Satellite Symposium of the European Buiatric Congress was dedicated to this new syndrome, henceforward termed *Bovine Neonatal Pancytopenia* (BNP). By end of December 2010 more than 4000 cases were reported for the EU with nearly 3000 cases in Germany (Cussler, unpublished observation). Clinical symptoms usually develop with the age of 10–20 days and comprise cutaneous bleeding, petechiae, and melena. In general 5 days after onset of clinical symptoms the patients succumb to blood loss and secondary infections [2]. The case fatality rate reaches up to 90% [3]. Haematologically, the syndrome is characterized by a marked pancytopenia, including thrombo- and leukocytopenia, and the pathognomonic finding at post-mortem is an aplasia of the bone marrow (panmyelophthisis) accompanied by extensive internal and/or external bleeding [3]. So far, most of the known causes of haemorrhagic symptoms in bovines such as toxins [1] or infectious agents [4,5] in particular Bovine

Abbreviations: BNP, *Bovine Neonatal Pancytopenia*; BVD, Bovine Virus Diarrhoea; MFI, Median fluorescence intensity; PEI, Paul-Ehrlich-Institute; SNT, Sero-Neutralization-Test.

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Viral Diarrhoea Virus (BVDV) and bluetongue virus have been ruled out [2,3]. Kappe et al. discuss the involvement of Porcine Circovirus 2 (PCV-2) [6] but this has not been confirmed by others [7]. In addition, a small-scale study that we performed in collaboration with the Friedrich-Loeffler-Institute, the German Federal Institute for Animal Health, on samples from BNP calves and non-BNP calves from different regions of Germany provided no evidence for a causative role of PCV-2 (Schirrmeier, personal communication). The syndrome develops upon ingestion of colostrum and it can experimentally be reproduced when newborn calves are fed with colostrum of BNP dams, i.e. cows that gave birth to a BNP calf [8]. This is not strictly dependent on the genetic relationship between colostrum donor and calf, i.e. calves from other dams can develop symptoms upon ingestion of colostrum from a BNP dam and – vice versa – calves from BNP dams remain healthy if they receive foreign colostrum [9]. As a first measure against BNP it has therefore been suggested to discard the colostrum of BNP dams [10].

In cattle maternal antibodies are not transferred *in utero* due to the particular anatomy of the bovine placenta. Instead the supply with maternal antibodies is achieved via colostrum during the first hours of life. It has therefore been hypothesized that maternal antibodies "toxic" for blood and bone marrow cells of the calf are the decisive component that induces BNP. Alloimmune phenomena are well described in humans and other species: one example is Neonatal Alloimmune Thrombocytopenia (NAIT). Women homozygous for a certain single nucleotide polymorphism may develop anti Human Platelet Antigen 1 antibodies when they are pregnant with a heterozygous foetus. During a subsequent gravidity these antibodies traverse the placental barrier and cause thrombocytopenia and subsequent intracranial haemorrhage in the foetus. The syndrome occurs with a frequency of about 1 in 1000 pregnancies [11]. In contrast to NAIT, which is known since decades [12], the first cases retrospectively recognised as BNP occurred in 2005 in Germany and in Belgium the first cases were traced back to 2006 (Cussler, unpublished observation). It is therefore unlikely, that BNP is due to natural sensitisation, i.e. through contact with foetal blood cells as is the case for NAIT, because then the syndrome should have been observed much earlier. The occurrence of BNP after 2005 and the spreading to some, but not all neighbouring states was a remarkable feature of the disease.

First epidemiological investigations revealed that the farms affected by BNP regularly performed vaccinations against BVD [2]. This was confirmed by pharmacovigilance investigations in Germany and also at the EU level. These investigations demonstrated that the vast majority of BNP cases is associated with one particular BVD vaccine, PregSure®BVD, which was further corroborated by the observation, that the occurrence of BNP is restricted to EU member states where the vaccine was marketed. Taking the growing evidence for an involvement of the vaccine into account, the Marketing Authorisation Holder announced a marketing stop for PregSure®BVD in April, 2010 for Germany [13]. In July 2010 the European Medicines Agency (EMA) recommended to suspend the marketing authorisation for PregSure®BVD "until scientific evidence is available to demonstrate that the administration of the vaccine ... does not lead to an increased risk of *Bovine Neonatal Pancytopenia* or that risk mitigation measures ... can be implemented" [14]. As the National Competent Authority of the Reference Member State that issued the first marketing authorisation [15] the Paul-Ehrlich-Institute (PEI) set out to investigate the role of PregSure®BVD in the pathogenesis of BNP. In particular, we wanted to address: (i) whether alloreactive antibodies could account for the induction of BNP, (ii) whether such alloantibodies could be functionally relevant, and (iii) whether PregSure®BVD could be involved in the induction of such antibodies.

2. Materials and methods

2.1. Field cases and clinical material

The majority of BNP cases were identified by M.H. of the Tiergesundheitsdienst (Cattle Health Service), North Rhine Westfalia. BNP diagnosis based on clinical findings was confirmed by haematology and bone marrow biopsy or post-mortem. All cases have been reported to and reviewed by the national pharmacovigilance system. Sera from BNP dams were obtained by venipuncture and transferred within 24 h for further investigation to the PEI. All BNP dams had received at least two vaccinations with PregSure®BVD according to the recommended vaccination scheme and most of them received booster vaccinations. Additional sera from BNP dams were provided by a veterinary practice in the northern part of Hesse and by the Clinics of Internal Medicine and Surgery of Ruminants of the Veterinary Faculty in Munich. The same case definitions as described above apply to these samples. Control sera were sampled from a dairy herd that had not been vaccinated against BVD, from a herd that had been vaccinated with a different inactivated BVD vaccine and from a herd that had been vaccinated with PregSure®BVD but had so far no history of BNP cases. The complete vaccination history of all animals included in this study is available to the authors. For leukocyte preparations whole blood samples were taken from healthy calves up to the age of 10 weeks. For individual experiments blood was obtained from adult cows. Where relevant the age of the individual animal is stated in the figures legend.

2.2. Cell preparation and cell culture

Leukocytes were prepared from whole blood by ammonium-chloride lysis. Briefly, 20 ml EDTA blood from calves were centrifuged for 10 min at $400 \times g$. Erythrocytes were lysed for 10 min by the addition of a buffer containing 0.15 M NH_4Cl, 10 mM $NaHCO_3$, 0.1 mM EDTA. The resulting leukocyte pellet was then washed with PBS. In many instances it was additionally purified by Ficoll Paque (1.077 g/ml; GE Healthcare) gradient centrifugation: Leukocytes were re-suspended in 5 ml PBS containing 0.5% FCS, carefully layered on 5 ml Ficoll Paque and centrifuged for 20 min at $400 \times g$ with low deceleration rate. The interphase was recovered and the resulting PBMC pellet was washed twice with PBS containing 0.5% FCS. For individual experiments short-term T cell lines were obtained by Phytohaemagglutinin (PHA) stimulation. To this end PBMCs were re-suspended in complete medium, i.e. RPMI 1640 (Gibco) supplemented with L-Glutamine, Penicillin-Streptomycin and 10% FCS and stimulated at 1×10^6 cells/ml with 0.1 μg/ml purified PHA (Oxoid) and a 1:20 dilution of a hybridoma supernatant containing human IL-2. The resulting polyclonal T cell lines are referred to as PHA blasts.

For individual experiments permanent cell lines such as MDBK, a bovine kidney cell line, BHK-21, a hamster kidney cell line or RK13, a rabbit kidney cell line from the cell culture stock of the PEI were used. Additionally, three cell lines used for BVD vaccine production kindly provided by manufacturers were tested. All permanent cell lines were maintained according to manufacturer's instruction. The bovine kidney cell lines used in our experiments were tested to be free of BVDV.

2.3. Sero-Neutralization-Test (SNT)

SNT was performed according to OIE guidelines [16]: briefly, serial dilutions of bovine sera were incubated for 2 h with 100 $CCID_{50}$ of cytopathogenic BVD virus strain, NADL. Preincubated virus was transferred to microtiter-plates that had been seeded over night with 4×10^4 MDBK cells per well. After 3–4 days the development of a cytopathic effect (CPE) was assessed by