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Brief report

Pregnancy boosts vaccine-induced Bovine Neonatal Pancytopenia-associated alloantibodies

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ABSTRACT

Although maternal vaccination is generally considered to be safe, the occurrence of Bovine Neonatal Pancytopenia (BNP) in cattle shows that maternal vaccination may pose a risk to the offspring. Pregsure© BVD-induced maternal alloantibodies cause BNP in newborn calves. The occurrence of BNP years after last Pregsure© BVD vaccination indicates that alloantibody levels may remain high in dams. Since pregnancy induces alloantibodies we hypothesized that pregnancy boosts the vaccine-induced alloantibody response. Alloantibody levels in Pregsure© BVD-vaccinated dams increased from conception towards the end of gestation and declined after parturition. In parallel, BVDV-antibody levels remained constant, indicating that there is specific boosting of alloantibodies. Since the rise in alloantibodies coincides with pregnancy and other alloantigen sources were excluded, we concluded that fetal alloantigens expressed during pregnancy boost the alloimmune response in the dam. These results help explain why BNP cases occur even years after Pregsure© BVD has been taken off the market.

1. Introduction

Immunization of the mother to protect the offspring from infections is used increasingly both in humans [1,2] and animals [3]. Although maternal vaccination is generally considered to be safe [1–3], the occurrence of Bovine Neonatal Pancytopenia (BNP) in cattle shows that maternal vaccination may pose a risk to the offspring. BNP, a fatal disease of neonatal calves, is strongly associated with use of the currently discontinued vaccine Pregsure© BVD (Pfizer Animal Health) in the dam [4]. The disease is characterized by internal and external bleeding, lymphocytopenia, thrombocytopenia and bone marrow aplasia [5,6]. Pregsure© BVD vaccination induces alloantibodies [7] and the subsequent absorption of these alloantibodies from the cow's colostrum causes BNP in the calf [8,9]. The dominant target of the vaccine-induced BNP-associated alloantibodies is major histocompatibility complex class I (MHC I) [10]. Although alloantibodies can be detected in almost all Pregsure© BVD-vaccinated dams, those that have had a BNP calf have significantly higher alloantibody levels than non-BNP dams [7,11] and the occurrence of BNP most likely depends on the alloantibody dose ingested by the calf [10,11]. Surprisingly, BNP cases still occur more than 5 years after Pregsure© BVD has been taken off the market (A.J.G. Smolenaars; GD Animal Health, personal communication), indicating that alloantibody levels may remain high in the dam without Pregsure© BVD vaccination.

In cattle, MHC I is expressed on the fetal membranes [12] and pregnancy can induce MHC I-specific alloantibodies in the dam [13]. We hypothesized that pregnancy boosts the Pregsure© BVD induced alloimmune response, leading to high alloantibody levels and the occurrence of BNP, even a prolonged time after the last vaccination of the dam. To test this hypothesis, alloantibodies in Pregsure© BVD-vaccinated dams were measured longitudinally and linked with reproductive status to assess the effect of pregnancy on alloantibody levels.

2. Materials and methods

2.1. Animals

In the period from April 2013 until October 2014 blood was sampled six to eight times from dams ($n = 8$) at a farm with reoccurring BNP cases. The dams were of the Holstein-Friesian breed

Abbreviations: BNP, Bovine Neonatal Pancytopenia; BVDV, Bovine Viral Diarrhea virus; MDBK, Madin Darby Bovine Kidney cells; b2m k/o MDBK, Beta-2-microglobulin knock-out MDBK cells; GMFI, Geometric Mean Fluorescent Intensity; S/P, sample to positive ratio.

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and were aged between 4, 5 to 11 years (median 6) at the first sampling and were serviced by different sires. Collected serum was stored at −20 °C. All dams were vaccinated multiple times with Pregsure© BVD, with the last Pregsure© BVD vaccination being in February 2010. BNP dams ($n = 3$) had previously given birth to a calf that developed BNP following feeding of their colostrum. The occurrence of BNP was confirmed via hematology and/or pathology. Non-BNP dams ($n = 5$) had given birth to calves that didn't show any signs of BNP. During the sampling period dams were not vaccinated with any vaccine. The herd was free of Bovine Viral Diarrhea virus (BVDV), which was checked regularly as part of a BVDV-free monitoring program (GD Animal Health) by measuring BVDV antibodies in calves aged 8 to 12 months.

This study was approved by the Animal Ethical Committee of Utrecht University.

2.2. Flow cytometry

Alloantibody levels in serum were assessed by determining antibodies specific for Madin Darby Bovine Kidney cells (MDBK; ATCC-CCL22), the cell line used for production of Pregsure© BVD. Beta-2-microglobulin knock-out MDBK cells (b2m k/o MDBK) [10], that do not express MHC I on the cell surface, were used to measure non-MHC I alloantibodies. Cells were incubated in serum diluted 1:20 and binding of bovine IgG was detected using polyclonal sheep anti-bovine IgG antibodies (Abd Serotec). Flow cytometry was performed on a FACSCanto™ (BD Biosciences) and data analyzed using Flowjo software (TreeStar Inc.). Sera from dams not vaccinated with Pregsure© BVD ($n = 5$) served as (isotype) controls. Data, representative of two independently performed experiments, are depicted as Geometric Mean Fluorescent Intensity (GMFI) corrected for isotype controls.

2.3. Bovine Viral Diarrhea virus ELISA

To measure antibody levels against BVDV over time and to confirm that animals were neither vaccinated against BVDV, nor naturally infected with BVDV during the sampling period, total serum antibodies against BVDV were measured using a commercial ELISA (BVDV Antibody Test Kit, Idexx), according to the manufacturer's instructions. Sample to positive ratio (S/P) was calculated from duplicate measurements and data are representative for two independent experiments.

2.4. Statistical analysis

The parturition to conception interval varied from 133 to 252 days. To compare the moment of sampling between animals, for each animal this interval was transformed to a standard interval of 220 days, while the conception to parturition interval was set at 280 days. To compare changes in antibody levels over time between animals, relative antibody levels were calculated by dividing individual time points within one animal by the average antibody level specific for MDBK, b2m k/o MDBK or BVDV of that particular animal. To analyze the effect of gestation on antibody levels, average relative antibody levels in the first, second and third trimester of gestation were compared using a one-way repeated measures ANOVA with Greenhouse-Geisser correction and differences in relative antibody levels between the first and third trimester were compared using Holm-Sidak's post-hoc test (GraphPad Software).

3. Results

The level of total alloantibodies changed over time, declining after parturition and rising from conception towards parturition (Fig. 1). When looking at absolute antibody levels these changes were most apparent in BNP dams, which have markedly higher alloantibody levels than non-BNP dams (Fig. 1A). Relative antibody levels showed a similar change over time in all Pregsure© vaccinated dams and alloantibody levels were significantly higher in the third trimester of gestation than in the first (Fig. 1D). As expected, non-MHC I-specific alloantibody levels were lower than total alloantibody levels (Fig. 1B). The changes in non-MHC I alloantibodies showed a pattern similar to the changes in total alloantibodies and levels were also significantly higher in the third trimester than in the first (Fig. 1E). Total antibodies against BVDV were relatively constant over time and there was no relation with reproductive status (Fig. 1C and F). Antibody levels of individual dams (Fig. 1G–I) also show that within individuals, both BNP and non-BNP dams, alloantibody levels change over time, whereas BVDV antibody levels remain relatively constant.

4. Discussion

The occurrence of Bovine Neonatal Pancytopenia in newborn calves depends on the dose of Pregsure© BVD-induced maternal alloantibodies ingested by the calf [10,11,14]. Although antibody levels normally decline over time, the incidence of BNP is not affected by the time since last Pregsure© BVD vaccination of the dam [11] and BNP cases still occur years after the vaccine has been taken off the market (A.J.G. Smolenaars; GD Animal Health, personal communication). This indicates that alloantibody levels remain high in dams without Pregsure© BVD vaccination. We hypothesized that fetal alloantigens boost the vaccine-induced alloimmune response during pregnancy. Longitudinal measurements of alloantibody levels in Pregsure© BVD-vaccinated dams showed that alloantibody levels changed over time, increasing from conception towards the end of gestation and declining after parturition (Fig. 1). In contrast, BVDV antibody levels remained relatively constant. This shows that there is specific boosting of alloantibodies, whereas antibodies specific for BVDV, the major antigen included in the Pregsure© BVD vaccine, or antibodies in general are not boosted. Since dams were not vaccinated during the sampling period and an autoimmune response can be ruled out as alloantibodies do not bind autologous cells [10], we conclude that fetal alloantigens boost the alloantibody response during pregnancy. The quick drop in alloantibody levels after parturition may be the result of the receding maternal alloimmune response due to the removal of fetal antigens in combination with the massive translocation of IgG to colostrum, which leads to a decrease in total serum IgG levels [15]. Translocation of alloantibodies from serum to colostrum is a critical step in the pathogenesis of BNP, since alloantibodies absorbed from colostrum cause the disease in the calf.

In cows, naturally occurring alloantibodies against fetal alloantigens usually peak around parturition and can be boosted by subsequent pregnancies [13,16]. Although this is similar to the kinetics of BNP-associated alloantibodies, alloantibodies against MDBK cells, used for production of Pregsure© BVD, are (virtually) undetectable in dams not vaccinated with Pregsure© BVD [11]. Therefore, the alloantibody response measured in this study is unique to the BNP problem and shows a specific boost of the Pregsure© BVD induced alloimmune response.

MHC I-specific alloantibodies are believed to mediate pathogenicity in BNP [10]. MHC I is expressed on fetal trophoblasts, beginning in the second trimester and rising towards the end of pregnancy [12,17], and the increase in alloantibodies in Pregsure© BVD-vaccinated dams corresponds with the exposure to fetal MHC I. Non-MHC I-specific alloantibodies also rise towards the end of gestation. We've previously shown that the major

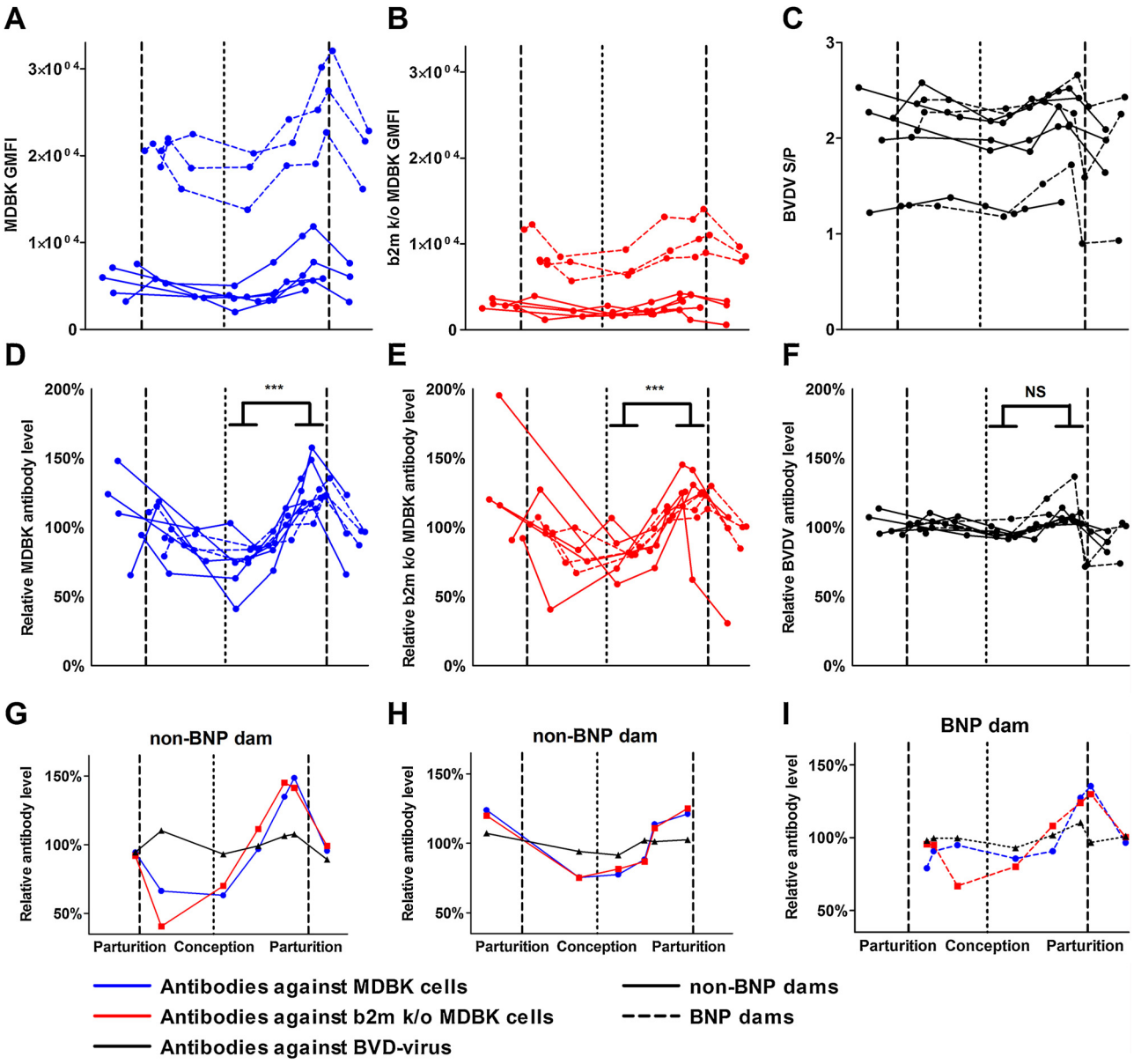

Fig. 1. Alloantibody and BVDV antibody levels over time in Pregsure© BVD-vaccinated dams. (A) Total serum IgG alloantibody binding of MDBK cells was measured using flow cytometry. (B) As in A, but using b2m knock-out (k/o) MDBK cells to measure non-MHC class I specific alloantibodies. (C) Total serum antibodies against Bovine Viral Diarrhea Virus (BVDV) measured using ELISA. Data are expressed as sample to positive ratio (S/P) calculated from duplicate measurements. (D–F) Data from (A)–(C) were transformed to express relative antibody levels by dividing individual time points within one animal by the average antibody level specific for MDBK, b2m k/o MDBK or BVDV of that particular animal. (G, H and J) For three individual dams relative antibody levels against MDBK cells, b2m k/o MDBK cells and BVDV are shown together in one graph. GMFI = Geometric Mean Fluorescent Intensity. The horizontal-axis is relative to the time of parturition and conception in all graphs. Differences in relative antibody levels between the first and third trimester were compared using Holm-Sidak's post-hoc test. *** $P \leq 0.001$. NS = not significant. The results of one representative experiment out of two independently performed experiments are shown.

non-MHC I target of BNP alloantibodies is Very Late Antigen-3 [10], an integrin α3/β1 heterodimer. Both integrin α3 and β1 are also expressed on fetal trophoblasts [18–20]. Furthermore, fetal microchimerisms frequently occur in bovine pregnancies [21,22]. Via the expression of alloantigens on trophoblasts and through fetal microchimerisms the maternal immune system can encounter a wide array of fetal alloantigens, which may boost the non-MHC I-specific alloantibody response. BNP dams are repeatedly exposed to alloantigens, through multiple Pregsure© BVD vaccinations and pregnancy, which has been shown to broaden the alloantibody specificity [13]. Indeed, BNP alloantibodies have a very broad MHC I specificity [10,14] and this may explain why there is little change in MHC class I specificity following sequential parturitions [14].

Although maternal vaccination is generally considered to be safe [1–3], there is a potential risk to the offspring, underlined by the occurrence of BNP. BNP was not detected during the licensing of Pregsure© BVD; the incidence of BNP following vaccination was low, and depends on multiple factors such as the genetic background of the dam [11], and the adverse effects presented themselves in the calf after pregnancy, rather than in the dam or during pregnancy. The example of BNP shows that registering adverse vaccine effects associated with maternal vaccination can be difficult and emphasizes the importance of post-licensure data.

We show that the BNP-associated alloantibody response in dams is boosted without Pregsure© BVD vaccination. Since boosting of the alloantibody response in the dam coincides with pregnancy and all other sources of alloantigens were excluded, we conclude

that fetal alloantigens expressed during pregnancy trigger this boost. The results from this study help explain why BNP-associated alloantibody levels remain high in dams and why BNP cases continue to occur years after Pregsure© BVD has been taken off the market.

Authors' contributions

LB collected the samples, carried out the experiments, the data collection and analysis and prepared the manuscript. All authors contributed to the design of the study, revised the manuscript, and approved the final version of the manuscript.

Conflict of interest statement

The authors declare that there are no conflict of interests.

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