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Alloantibodies against MHC Class I: A Novel Mechanism of Neonatal Pancytopenia Linked to Vaccination

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Fetal/neonatal alloimmune thrombocytopenia is a frequent disease in humans where alloantibodies against platelet Ags lead to platelet destruction and hemorrhage. Although a role in the disease for Abs against MHC has been suspected, this has not been formally demonstrated. Since 2007, a hemorrhagic syndrome due to thrombocytopenia and designated as bovine neonatal pancytopenia (BNP) has been recognized in calves in several European countries. An inactivated antiviral vaccine is strongly suspected to be involved in this syndrome because of its highly frequent use in the dams of affected calves. In this study, we show that BNP is an alloimmune disease, as we reproduced the signs by transferring serum Abs from vaccinated BNP dams into healthy neonatal calves. Ab specificity was strongly associated with the presence of allelogeneic MHC class I Abs in the dams. MHC class I staining was also observed on Madin–Darby bovine kidney cells, a cell line related to the one used to produce the vaccine Ag. Our report emphatically demonstrates that alloimmunization against MHC class I is associated with a substantial risk of developing cytopenia-associated syndromes in neonates when a cell line of the same species is used to produce an inactivated vaccine injected into the mother.

Since 2007, an emergent life-threatening disease of neonatal calves has been reported in several European countries (1–4) and has been designated as bovine neonatal pancytopenia (BNP). The disease is characterized by severe thrombocytopenia and leukopenia—essentially a strong neutropenia and a mild lymphopenia—in 2- to 3-wk-old calves and in most cases leads to hemorrhages and a fatal issue. Bone marrow is affected as a profound medullar aplasia is identified in most clinical cases (2). As no infectious agent could be isolated until now and despite the early suspicion of a circovirus cause (2) that was not further confirmed (5), other hypotheses have emerged.

BNP has been produced experimentally in some calves through ingestion of particular colostrums (6, 7), indicating that immune effectors might be involved. Colostrum has a high concentration of IgG (between 50 and 200 g/l) and provides the calf with its sole source of passive immunity in the first hours of life. Indeed, due to the synergistic effect of IgG and the rising maternal IgM, Abs against MHC class I Ags and 2-microglobulin (β2m) are found in the colostrum of most infected dams (8, 9). Abs against these MHC class I Ags and β2m have been identified in the serum of affected calves (10) and it has been proposed that Abs against MHC class I and β2m target bone marrow cells and cause a cytopenia syndrome (11–13).

The aim of this work was to assess the role of Abs in the development of BNP and to identify the specificity of the alloimmune response to clarify the cause of the disease.

In this study, we provide substantial evidences that the alloimmune response is directed against MHC class I Ags and is probably responsible for the pancytopenia observed in bovine neonates after the dam received one or several injections of an inactivated vaccine.

Materials and Methods

Sera, animals, and BNP experimental model

Sera were collected from dams of confirmed BNP calves in Pregsure (Pfizer Animal Health)-vaccinated herds. IgG was precipitated using a final concentration of 40% ammonium sulfate as previously described (12) and then solubilized in NaCl 0.9% before extensive dialysis to remove ammonium sulfate. IgG solutions were 0.2-μm filter sterilized and stored at −80°C until injection.

Neonatal calves without any parental link were collected from herds without BNP history. They were separated from their dams immediately after delivery and before colostrum intake. Each calf was injected i.v. with 2 l of a solution containing ∼100–150 g total IgG. Calves were clinically examined and sampled for the analysis of hematological and biochemical parameters several times during the first 24 h after IgG injection and then every 2–3 d. After IgG injection, total plasma protein concentrations increased from 39.2 ± 6.1 g/l to 56.3 ± 3.1 g/l, corresponding approximately to an increase of 15 g/l in the globulin fraction. This increase is

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consistent with a colostrum intake of 10% of body weight. Bone marrow aspirations were made at the sternum before IgG injection and after 10 d, and smears stained with May–Grünwald–Giemsa were prepared for cytological examination.

All experiments were done in accordance with local and French regulations. The experimental protocol was approved by Comité d’Ethique de Midi-Pyrénées under the agreement number MP03/25/06/10.

**Immunocytochemistry, leukocyte Ab detection by flow cytometry, and ELISA**

Peripheral blood leukocytes (PBLks) were prepared from experimental calves and recovered BNP-calves using EDTA-anticoagulated blood treated with ammonium chloride to lyse RBCs. PBLks were incubated with a titration of pathogenic or control sera, and IgG binding was revealed using DyLight 488-labeled F(ab’)2 goat anti-bovine IgG (Jackson ImmunoResearch). Dead cells were gated out using 7-aminoactinomycin D (BD Biosciences), and a minimum of 20,000 events were acquired on a FACSCalibur (BD Biosciences) prior to analysis with FlowJo software (Tree Star).

For the competitive assay, cells (0.5 × 10⁶), either PBLks or Madin–Darby bovine kidney (MDBK) cells, were incubated with a dilution of serum prior to staining with DyLight 488-labeled anti-bovine IgG (Jackson Immunoresearch) and allophycocyanin-labeled anti-MHC class I W6/32 mAb (BioLegend). After extensive washing, at least 20,000 events were acquired, and data were analyzed with FlowJo software.

For bovine leukocyte Ag (BoLA) MHC class I quantification in biological products, a commercial ELISA was used following the manufacturer’s recommendations (Cusabio Biotech). The range of BoLA MHC detection is 0.04 to 10 ng/ml.

**MHC class I down-expression**

Knockdown of MHC class I was carried out with a small interfering RNA (siRNA) targeting bovine β2-microglobulin (β2-m) or a control siRNA (Eurogentec) at a final concentration of 10 nM using ICAFectine442 siRNA transfection reagent (Eurogentec).

**Immunoprecipitation and visualization of precipitated proteins by SDS-PAGE and blotting**

PBLks were adjusted to 2 × 10⁹ to 3 × 10⁷ cells/ml and were surface labeled with EZ-link Sulfo-NHS-LC-CL-Biotin (GE Healthcare) according to the manufacturer’s instructions. Biotinylated cells were incubated with a 1:20 dilution of BNP-IgG, a negative serum, or PBS (45 min, on ice). After extensive washing to remove unbound and nonspecifically bound Abs, cells were solubilized with lysis buffer (50 mM Tris-HCl, pH 8, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 1% deoxycholate, 0.1% SDS, 1 mM Pefabloc SC, and 0.5 μg/ml leupeptin) on ice for 20 min. After centrifugation (15,000 × g, 20 min), the supernatant was incubated with 20 μl of an anti-bovine IgG (SouthernBiotech) coupled to Adembeads Prot AG (Ademtech). After washing five times with lysis buffer, precipitated proteins were eluted twice with the elution buffer provided with the beads.

Immunoprecipitates were boiled in sample buffer for 5 min and then subjected to SDS-PAGE on NuPAGE 4–12% Bis-Tris gel (Invitrogen). Proteins were transferred onto nitrocellulose membrane (Whatman) for 90 min at 100 V using Tris/glycine buffer. After transfer, the membrane was first blocked with 5% skim milk and 0.1% Tween 20 in TBS and then incubated with HRP-conjugated streptavidin (KPL). Signals were developed using the Immun-Star Western C kit reagent (Bio-Rad) according to the manufacturer’s instructions. Alternatively, blotting for MHC class I was performed with a BoLA class I-specific IL-A88 mAb, a kind gift of S.A. Ellis (Institute for Animal Health, Compton, U.K.), and anti-mouse IgG-HRP for revelation.

**Nano-liquid chromatography and nanospray ionization mass spectrometry analysis**

After Coomassie blue staining of the SDS-PAGE gel, bands corresponding to those detected on the Western blots were excised and digested by the addition of 30 μl of a solution of modified trypsin in 25 mM NH₄HCO₃, 20 ng/μl, sequence grade; Promega). The mixture was incubated at 37°C overnight. The resulting peptide mixtures were analyzed by nano-liquid chromatography and nanospray ionization mass spectrometry (nano-LC-MS/MS) using an Ultimate3000 system ( Dionex) coupled to an LTQ-Orbitrap Velos mass spectrometer (Thermo Fisher Scientific). Five microliters of each sample were loaded on a C18 precolumn (300-μm inner diameter × 5 mm; Dionex) at 20 μl/min in 5% acetonitrile, 0.05% trifluoroacetic acid. After 5 min of desalting, the precolumn was switched online with the analytical C18 column (75-μm inner diameter × 15 cm; packed in-house) equilibrated in 95% solvent A (5% acetonitrile, 0.2% formic acid) and 5% solvent B (80% acetonitrile, 0.2% formic acid). Peptides were eluted using a 5–50% gradient of solvent B during 80 min at a flow rate of 300 nL/min. The LTQ-Orbitrap was operated in data-dependent acquisition mode with the Xcalibur software. Survey scan mass spectrometry spectra were acquired in the Orbitrap on the 300–2000 m/z range with the resolution set to a value of 60,000. The five most intense ion fragments from each scan were selected for CID fragmentation, and the resulting fragments were analyzed in the linear ion trap (LTQ). Dynamic exclusion was used within 60 s to prevent repetitive selection of the same peptide ion for mass spectrometry analysis.

**Database searching and data analysis**

The Mascot Daemon software (version 2.3.2; Matrix Science, London, U.K.) was used to perform database searches in batch mode with all the raw files acquired on each sample. To extract automatically peak lists from Xcalibur raw files, the Extract_msn.exe macro provided with Xcalibur (version 2.0 SR2; Thermo Fisher Scientific) was used through the Mascot Daemon interface. The following parameters were set to create the peak lists: parent ions in the mass range 400–4500, no grouping of MS/MS scans, and threshold at 1000. A peak list was created for each analyzed fraction (i.e., gel slice), and individual Mascot searches were performed for each fraction. Data were searched against all entries in the SwissProt Trembl_20100907 protein database (12,155,553 sequences; 3,930,097,083 residues) Mammalia (338,129 sequences). Oxidation of methionine and carbamidomethylation of cysteine were set as variable modifications for all Mascot searches. Specificity of trypsin digestion was set for cleavage after Lys or Arg except before Pro, and one missed trypsin cleavage site was allowed. The mass tolerances in mass spectrometry and MS/MS were set to 5 ppm and 0.8 Da, respectively, and the instrument setting was specified as “ESI-Trap.” Mascot results were parsed with software developed in-house, Mascot File Parsing and Quantification, version 4.0. Protein hits were automatically validated if they satisfied one of the following criteria: identification with at least one top-ranking peptide of a minimal length of 8 aa and with a Mascot score higher than the identity threshold at p = 0.01 (99.9% probability); or identification with at least two top-ranking peptides each of a minimal length of 8 aa and with a Mascot score higher than the identity threshold at p = 0.05 (95% probability). To calculate the false discovery rate (FDR), the search was performed using the “decoy” option in Mascot and using the same criteria in Mascot File Parsing and Quantification to validate decoy and target hits. The FDR was calculated at the protein level [FDR = (100 × the number of validated decoy hits)/(the number of validated target hits + number of validated decoy hits)] and, using the specified validation criteria, it ranked between 0 and 0.5% for all the samples analyzed with an average value of 0.4%.

**Results**

**Purified IgG from BNP dams induces thrombocytopenia and leukopenia in neonatal calves**

To assess whether BNP is mediated by maternal Abs, we i.v. injected six colostrum-deprived newborn calves with one of four pools of purified IgG prepared from cows that had been vaccinated with the same inactivated BVD vaccine. The pools were from four different herds of three different breeds, and cows (n = 16) had all given birth to calves that developed clinically confirmed cases of BNP. Two calves of six died 2 d after IgG injection, after experiencing intense respiratory distress and severe hemorrhages (data not shown). In the four remaining calves, blood platelet (Fig. 1A) and leukocyte counts (Fig. 1B) were significantly diminished after 10 d (Mann–Whitney U test, n = 4, p < 0.01). In contrast, another calf that received IgG from a nonvaccinated control cow remained healthy without any blood or medullar depletion. Hematological changes resembled that of two other BNP cases that had received colostrums from two of the BNP dams at birth (Fig. 1A, 1B). After 10 d, bloody feces were observed in three of the four surviving BNP-IgG–transferred calves, and petechiae on mucous membranes were observed in all four animals, both of which are indications of clotting disorders (data not shown). A decrease of the cellularity was observed on aspirated bone marrow at day 10 where it was significantly lower than that on day 0, the day of IgG...
Injection (day 0 grade 3.3 versus day 10 grade 1.6 on a scale of 4, n = 4, Mann–Whitney U test, p < 0.01). The proportions of most cell types were reduced, with the exceptions of eosinophils, lymphocytes, and macrophages (Fig. 1C). After 15 and 17 d respectively, two of the four remaining calves were recumbent and were euthanized for ethical reasons. Necropsies revealed hemorrhages throughout the bodies of both calves in the viscera, joints, and muscles. Microscopic examinations of their bone marrow revealed massive cell depletions with no visible megakaryocytes (Fig. 1D), which is consistent with previously described cases of spontaneous BNP (2). The other two calves recovered spontaneously from the disease and developed normally afterward. Their WBC and platelet counts (Fig. 1A, B) and bone marrow composition progressively returned to normal within 1 mo. The clinical and biological signs of the IgG-injected animals that manifested disease were the same as those reported in natural (1, 2) and colostrum-induced BNP cases (6, 7). Our data unequivocally show that IgG collected from BNP dams can induce de novo BNP in unrelated newborn calves.

IgG recognizes MHC class I on blood leukocytes and platelets

Soon after the transfusion of BNP-dam IgG or after the ingestion of colostrum from BNP dams, flow cytometry analysis of calf blood cells using DyLight 488-labeled (Fab′)2-anti-bovine IgG revealed the presence of bound Abs on high proportions of calf granulocytes, monocytes, and lymphocytes (Fig. 2A). We also found that BNP-dam IgG stained blood and bone marrow cells from three recovered cases of spontaneous BNP. IgG binding was also demonstrated on platelets (data not shown). Conversely, when BNP-dam blood cells were assayed, no staining was detected. Altogether, our data indicate that IgG from BNP dams recognizes one or several blood cell surface Ag(s), as has been previously suggested by others in studies of sera from BNP dams (9) or of BNP induced in calves by administration of colostrum from BNP dams (6). Thus, BNP is an alloimmune response in which cows produce Abs against one or several determinant(s), hypothetically of paternal origin, widely expressed on calf blood cells and platelets.

Having characterized a set of pathogenic IgG batches, and further to define the specificity of the IgG associated with BNP development in neonatal calves, we investigated the Ag present on the PBLks of five healed calves (two experimental and three spontaneous cases). To immunoprecipitate the Ag(s), we incubated biotinylated PBLks with dilutions of pathogenic sera or BNP-IgG and released the Ag–Ab complexes by lysing the cells. We precipitated IgG-bound proteins from the supernatant using magnetic beads coated with goat anti-bovine IgG Abs. Next, the precipitated proteins were separated by SDS-PAGE and visualized by silver.
staining (Fig. 2B). Multiple bands were visible on the gel, but we detected no clear, condition-specific differences. By contrast, after the immunoprecipitated proteins were transferred onto a nitrocellulose membrane and the biotinylated surface proteins were revealed using peroxidase-conjugated streptavidin and chemiluminescence, a prominent band of ∼40–45 kDa (Fig. 2C) was observed in the lane containing proteins precipitated by BNP-IgG. In control immunoprecipitations using PBS or a nonpathogenic serum, no such signal was detected.

To identify the putative BNP-IgG Ag(s), the proteins in the gel regions containing the 40- to 45-kDa band identified through blotting were digested in the gel with trypsin, and the resulting peptides were analyzed using online coupling of nano-LC-MS/MS. A search against the mammalian SwissProt database led to the identification of an average of ∼30 proteins in each of the samples analyzed including control samples. The proteins precipitated by pathogenic sera were identified and compared with those precipitated by different combinations of sera/cell sources and control samples (nonpathogenic serum and PBS). This analysis unambiguously identified BoLA MHC class I protein as the Ag recognized by the pathogenic sera, as at least two peptides matched the extracellular domain of this protein for four of the five pathogenic sera analyzed (Table I). The identity of the band was further confirmed using IL-A88, an anti-BoLA class I monomorophic mAb, which identified the expected 45-kDa protein band on an immunoblot (Fig. 2D). Thus, bovine MHC class I protein was recognized as a target of BNP-inducing sera.

To confirm further the specificity of the Ab response on a larger number of samples, PBLks from one BNP-afflicted calf were preincubated with either pooled BNP-IgG or individual sera from 12 BNP dams and 12 non-BNP cows from within the same BVD-vaccinated herds before staining with W6/32, a monomorphic HLA-(A, B, and C)-specific mAb that recognizes MHC class I molecules in several mammalian species including the bovines. Preincubation with pooled BNP-IgG and sera from individual BNP dams partially abolished subsequent staining by W6/32 (Fig. 3A, 3B), indicating that Abs in the BNP-IgG and sera from individual BNP dams partially abolished subsequent staining by W6/32 (Fig. 3A, 3B), indicating that Abs in the BNP-IgG and sera from individual BNP dams competed with W6/32 for a closely related epitope on the BoLA class I molecules. Incubation with non-BNP sera from vaccinated individual cows (Fig. 3B) or with an irrelevant mAb against CD45 did not elicit similar reductions in W6/32 binding (data not shown). This strongly supports the view that these

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*From SwissProt Trembl database.
†The amino acid position in the protein sequence is indicated.
‡The peptide score is given by the Mascot algorithm according to the parameters set for database search as described in Materials and Methods.
Alloantibodies recognize MHC class I on the vaccine parental cell line

To look for the presence of MHC class I in the vaccine and investigate the hypothesis that the Ab response follows vaccination, the antigenic fraction of the vaccine was isolated using 10% butanol as previously described (13). MHC class I was detected on an immunoblot by the IL-A88 mAb (Fig. 4), indicating that the vaccine actually contains BoLA class I. Using a commercial ELISA, the BoLA MHC concentration was estimated to be around 0.5–1 ng/ml in the aqueous phase recovered after butanol treatment, indicating that the vaccine may serve as a source of alloantigen for mounting an Ab response against MHC Ags. Vaccine Ag is produced on a cell line derived from MDBK cells. We observed that not only did BNP-IgG stain the MDBK cells (Fig. 5A), but also that staining with BNP-IgG blocked subsequent MHC class I staining on this cell line (Fig. 5B). Specificity was further confirmed by immunoblotting of MDBK cell lysate with the four pathogenic BNP-IgG, as performed previously for the blood leukocytes (Fig. 5C). Indeed, when MHC class I expression on MDBK cells was partially extinguished using RNA interference targeting β2m-coding mRNA, bovine IgG staining with pooled or individual BNP-sera and MHC class I staining were similarly reduced (Fig. 5D). This again supports the assertion that the vast majority of the allogenic Abs in BNP-dam sera are specific for BoLA class I–β2m complexes.

Discussion

In this study, we provide several compelling lines of evidence that anti-MHC class I Abs are sufficient to induce alloimmune thrombocytopenia and leukopenia in neonates. In humans, MHC class I alloantibodies in individual cases of fetal/neonatal alloimmune thrombocytopenia (FNAIT) have been reported a number of times during the past decades (14–25). In addition to thrombocytopenia, the presence of an associated neutropenia is noticed in a few cases (16–28). These Abs are sometimes detected at the same time as other HPA Abs commonly involved in FNAIT (27–29). Although HLA class I Abs are commonly encountered in the mothers of FNAIT cases (30), their role as the main trigger remains controversial (17, 31, 32).

Several conclusions can be drawn from our study. First, as no other specificity in common among pathogenic sera could yet be identified, an alloresponse against MHC class I is probably the correct explanation for induction of thrombocytopenia and leukopenia in the neonates. However, the pathophysiological mechanism remains unclear. Whether the peripheral consumption of the calf recipient platelets and cells, or the central depletion of stem cells, or both mechanisms are responsible for the development of the disease has to be determined. Whereas MHC class I molecules are widely expressed in the body, the strong impact of MHC alloantibodies on the hematopoietic system, and notably the bone marrow, is perplexing. Higher expression of MHC class I molecules on hematopoietic cells is one possibility. After injection into pregnant mice, paternal MHC class I Abs were transferred to the fetus and accumulated predominantly in the blood, thymus, and liver (33). Moreover, in our situation, all cell types inside the hematopoietic system are not equally affected as some lymphocytes seem to be less sensitive to the depletion and still remain, whereas granulocytes, with a lower intensity of IgG staining, are first to disappear during the course of the leukopenia. Heterogeneous expression of bovine MHC class I, as recently shown in some primates (34) conversely to humans, would explain this observation.

Although all calves developed a thrombocytopenia upon BNP-IgG injection, the disease ending varied among cases in our model. This is also in accordance with previous reports where BNP was induced by ingestion of colostrum (6, 7). In the field, the variability of the Ab concentration in the colostrum, the quantity of ingested colostrum, and the rate of absorption through the digestive tract may modulate the total amount of acquired alloantibodies and expression of the disease. In our study, the BNP-IgG solutions were shown to have very similar titers of alloantibodies when assayed against the MDBK cell line (data not shown), and i.v. injection of IgG diminished the variability of IgG transfer compared with that of colostrum intake. Thus, several other factors are playing a role to modulate incidence and severity of the disease. In humans, a correlation between HPA1a Ab titers in the mother and severity of the thrombocytopenia at birth has been reported, despite several analytical variation factors (35–37). FcRn, the neonatal IgG receptor, has been shown to modulate FNAIT in a mouse model (38). Other genetically determined factors may also exist. It is worth noting that some haplotypes of β2m, which is involved in the assembly of both FcRn and MHC class I mole-
were transfected with to produce experimental BNP cases. D Lanes 1–4 correspond to the different BNP-IgGs used to produce experimental BNP cases. D, MDBK cells were transfected with β2m-specific siRNA. After 12 h, cells were stained with anti-MHC class I W6/32 or with BNP-IgG and DL488–anti-bovine IgG as previously. BNP-IgG staining and MHC class I Ab staining were diminished by similar proportions, indicating that BoLA class I represents most of the specificity present in these BNP-IgG. Data are representative of three experiments performed.

Alloreactive sera recognize MHC class expressed on MDBK cell line. A, MDBK cells, the parental cell line of the one used to produce the vaccine Ag, were incubated with BNP-IgG, and binding was revealed using DyLight488-(Fab′)2 anti-bovine IgG prior to flow cytometry analysis (gray, control serum; black, BNP-IgG; dotted histogram, secondary Ab only). B, Competitive assay for MHC class I binding indicated that MHC class I Ags expressed on MDBK cells are recognized by BNP-IgGs (dotted line, no staining; plain line, PBS; gray, control serum; black, BNP-IgG). C, MDBK cell lysate proteins were separated using SDS-PAGE and blotted with a 1:2000 dilution of BNP-IgG and HRP-labeled anti-bovine IgG or IL-A88 mAb and HRP-anti-mouse IgG, respectively, to confirm BoLA MHC class I specificity. Lanes 1–4 correspond to the different BNP-IgGs used to produce experimental BNP cases. D, MDBK cells, the parental cell line of the one used to produce the vaccine Ag, were incubated with BNP-IgG, and binding was revealed using DyLight488-(Fab′)2 anti-bovine IgG prior to flow cytometry analysis (gray, control serum; black, BNP-IgG; dotted histogram, secondary Ab only). B, Competitive assay for MHC class I binding indicated that MHC class I Ags expressed on MDBK cells are recognized by BNP-IgGs (dotted line, no staining; plain line, PBS; gray, control serum; black, BNP-IgG). C, MDBK cell lysate proteins were separated using SDS-PAGE and blotted with a 1:2000 dilution of BNP-IgG and HRP-labeled anti-bovine IgG or IL-A88 mAb and HRP-anti-mouse IgG, respectively, to confirm BoLA MHC class I specificity. Lanes 1–4 correspond to the different BNP-IgGs used to produce experimental BNP cases. D, MDBK cells were transfected with β2m-specific siRNA. After 12 h, cells were stained with anti-MHC class I W6/32 or with BNP-IgG and DL488–anti-bovine IgG as previously. BNP-IgG staining and MHC class I Ab staining were diminished by similar proportions, indicating that BoLA class I represents most of the specificity present in these BNP-IgG. Data are representative of three experiments performed.

cules, are associated with failure of passive transfer in neonatal calves (39). Whether there is a concentration threshold of MHC class I alloantibodies that can be tolerated by the neonate needs also to be further investigated.

Furthermore, not only the amount of alloantibodies transferred to the calf but also the allospecificity is expected to control severity of the disease. Partial BNP phenotype may be due to the lower avidity of alloantibodies for the MHC molecules expressed by the calf. Because MHC class I genes have the most polymorphic alleles within the mammalian genome and combinatorial haplotypes are numerous, both recognition and affinity may vary. By providing plasma IgG concentration in the range of that permitted by colostrum intake in bovines, we were able to induce thrombocytopenia after all attempts. This high frequency of BNP development contrasts with previous reports (6, 7) and might also be explained by the fact that we used pooled sera from four cattle despite individual sources of Abs from either colostrum or serum and by our possibility to increase the diversity range of IgG specificities. The same phenomenon may have heightened the number of spontaneous cases in regions or herds where the distribution of pooled colostrum to calves is a common practice.

Altogether, the frequency of the disease remains relatively low, as indicated by the results of epidemiological studies; the estimated overall prevalence is probably lower than 0.3% and is usually not more than 10% within a herd (Ref. 11 and G. Foucras, F. Corbière, and F. Schelcher, unpublished observations). Another consequence is the lower than expected prediction of BNP occurrence in the offspring of MHC Ab-positive dams due to a combination of factors that are difficult to evaluate a priori.

The second finding of our study is that the specificity supports the hypothesis of an alloimmunization linked to the vaccination with an inactivated viral vaccine. In humans, allo-MHC immunization is generally observed after the first pregnancy, transfusion of non-cell-depleted blood products, or transplantation. To our knowledge, the development of an alloimmune response after injection of a vaccine produced on a human cell line has never been reported, although never truly investigated. Similarly, all products containing allogeneic cell culture constituents could be considered as possibly immunogenic. The origin of the allospecificity questions the peculiarities of this vaccine, as both Ag and adjuvant may be involved. The fact that we were able to detect MHC Ags within the antigenic fraction of the vaccine may be sufficient to prime an allosresponse in MHC-discordant recipients. Moreover, the adjuvant, by inducing a strong Ab response compared with that of other commercial vaccines against BVD virus (11, 40), may favor the development of high titers of alloreactive Abs in some animals highly responsive to immunization. Because of the large variability of the MHC in mammals, the frequency of the alloresponders is expected to be relatively low, which fits with the low occurrence of the disease as reported by epidemiological studies.

In the current case, if the Ab response was induced by injection of an inactivated BVD vaccine containing bovine MHC class I proteins, the genetic background of the dam and that of the neonate and of the father, which share the recognized epitopes, are determining factors in occurrence of the disease, as described in humans for FNAIT. Even the reported presentation of fetal proteins to the dam by placental leakage could be discussed as a cause of this syndrome for rare cases of the disease in which no BVD vaccination took place (41). Later, this may also enhance the Ab response once priming has occurred.

Third, the approach we developed for the identification of the allospecificity was reported by others during the time of our study (42–44). Our results are one more example that shows the power of detection, no matter whether it is an alloimmune or an auto-
immune disease, for the discovery of Ab specificities. Despite a relatively large number of proteins that are retained by immunorecognition in one sample, comparison of several samples enables identification of the critical Ag. Moreover, preincubation of cell surface proteins with biotin facilitates further isolation of membrane proteins.

At the time when the strategies to reduce alloimmunization are investigated in humans (45), we have identified the possibility that allogeneic proteins may induce the development of an immune response against MHC class I that may later be detrimental to the fetus or the neonate. The possibility that this situation already exists in humans, or will arise in the future, should be considered.

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Disclosures
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References