Identification of a Cell Population That Produces Alpha/Beta Interferon In Vitro and In Vivo in Response to Noncytopathic Bovine Viral Diarrhea Virus


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Received 13 June 2004/Accepted 15 February 2005

In vitro infection of bovine cells of many origins with the cytopathogenic bovine viral diarrhea virus (cpBVDV) results in the induction of alpha/beta interferon (IFN-α/β), whereas noncytopathogenic BVDV (ncpBVDV) isolates have been shown not to induce IFN-α/β in vitro. Similarly, cpBVDV induces IFN-α/β in the early bovine fetus, but ncpBVDV does not. However, acute infection of naïve cattle with ncpBVDV results in IFN-α/β production. In this study, we identified and characterized a minor population of cells, present in lymph nodes that produce IFN-α in response to ncpBVDV. These cells expressed the myeloid markers CD14, CD11b, and CD172a but did not express CD4 and CD45RB. We also established that these cells produced IFN-α in the absence of detectable productive infection.

The pestiviruses—bovine viral diarrhea virus (BVDV), classical swine fever virus, and border disease virus of sheep—are closely related members of the Flaviviridae, with a single-stranded, positive-sense RNA genome of approximately 12.5 kb (17).

Bovine viral diarrhea virus (BVDV) exists as two biotypes differentiated by their cell culture phenotype. In vitro infection of bovine cells of many origins with the cytopathogenic (cp) isolate results in the induction of alpha/beta interferon (IFN-α/β) (1). Noncytopathogenic (ncp) BVDV isolates have been shown not to induce IFN-α/β in vitro (1, 7, 20) and to block the induction of IFN-α by double-stranded RNA (dsRNA) or infection with other viruses (21, 22). Recent studies have shown that noncytopathogenic (ncp) isolates prevent IFN induction by inhibiting the binding of interferon regulatory factor 3 to DNA, following its nuclear translocation (2). Similarly, cpBVDV induces IFN-α/β in the early bovine fetus, but ncpBVDV fails to do so (5). In contrast, infection of postnatal cattle with ncpBVDV results in elevated circulating IFN-α/β levels for up to 8 days postchallenge (6). The cellular source of IFN-α/β in response to ncpBVDV has yet to be determined, although it was identified that blood leukocytes were not producing IFN-α/β. Also, we have shown that acute infection of calves with cpBVDV does not result in detectable quantities of circulating IFN-α/β, which contrasts with the potent induction of IFN-α/β in vitro (1). In this study, we have reconciled these apparently contrasting effects of ncp and cp virus on the induction of IFN-α/β in vitro and in vivo. To further explain these differences, we have used a subcutaneous challenge model to identify in which local anatomical sites IFN-α/β is produced by ncpBVDV and cpBVDV isolates. These investigations were extended to identify the IFN-α/β-producing cells, the phenotype of which has not been previously described in cattle. Furthermore, we have established that ncpBVDV can stimulate IFN-α/β production from a partially purified population of cells in vitro.

MATERIALS AND METHODS

Cattle. Calves (Bos taurus) were conventionally reared British Holstein Friesians bred at the Institute for Animal Health. Each animal was shown to be BVDV seronegative and virus negative by previously described assays (11). All experiments were approved by the Institute’s ethical review process and were in accordance with national guidelines on animal use.

Interferon assay. IFN-α/β biological activity was measured by using a chloramphenicol transferase reporter assay that detects but does not distinguish between IFN-α and IFN-β (12).

Viruses. The experiments utilized a homologous pair of ncp and cp viruses (Pe515ncp and Pe515cp) originally isolated from a case of mucosal disease (4). A type 2 ncpBVDV isolate designated KE13, a gift from Knut Elbers, Boehringer-Ingleheim, Germany, was also used for acute challenge studies. The viruses were propagated in with a primary cell line derived from calf testis (Cte) cultured in tissue culture medium and titers were determined, as previously described (4). These virus preparations have been shown to have a differential capacity to induce IFN-α/β on a number of different cell types (5, 6); consistently, Pe515ncp and KE13 failed to induce IFN-α/β, but Pe515cp induced IFN-α/β.

BVDV challenge of lymphatic cunnulated animals. Using surgically prepared animals (16), we investigated the capacity of the two biotypes of BVDV to stimulate IFN-α/β production in the lymph-draining subcutaneous sites in vivo. Pseudoafferent cunnulated animals were infected subcutaneously with either cpBVDV or ncpBVDV (5 × 10^6 PFU in 2 ml of medium). Samples of lymph and blood were collected at time points pre- and postchallenge. Three surgically prepared animals were challenged with each biotype. A similar surgical procedure was used to collect effluent lymph from animals with intact lymph nodes. After cunnulation, cpBVDV (5 × 10^6 PFU in 2 ml of medium) was injected subcutaneously directly over the lymph node. IFN-α/β was not detected in the lymph of mock-challenged animals.

Isolation of cell populations from bovine lymph nodes and culture procedure. To obtain a cell preparation enriched for low-density cells, such as monocytes and dendritic cells (DCs), present in the organized lymphoid tissue that had the potential for IFN-α/β production, we isolated single-cell suspensions from bovine lymph nodes and enriched for low-density cells using an iodosixan (Optiprep, Nycomed, Denmark) gradient, following the manufacturer’s instructions. After the cells were harvested from the gradient, they were resuspended in TCM, which is RPMI 1640 with Glutamax-I and 25 mM HEPES (Life Technologies,
Ltd., Paisley, Scotland) containing 10% heat-treated (56°C) fetal calf serum, 1 mM sodium pyruvate, 50 μg/ml gentamicin, and 5 × 10^{-3} M 2-mercaptoethanol. Into each well of a 96-well plate (Costar, Corning, United Kingdom), 5 × 10^{5} cells of each population were added in 100 μl of TCM. Virus preparations (multiplicity of infection of 1 PFU/cell), mock-infected cells lysates (with the number of cells equal to those in virus preparations), dsRNA [poly(I:C), 10 μg/ml; Sigma, United Kingdom], or CpG DNA (0.1 μM, type A oligodeoxynucleotides 2216; Invivo- gen, United Kingdom) was added to each well in 100 μl of TCM. All reagents were shown to be endotoxin free (Coatest Chromo-LAL; Quadratech, United Kingdom).

Preparation of sorted cell populations. To further characterize the cells that produced IFN-α/β in response to ncpBVDV, density gradient-purified cells from lymph nodes were subjected to further rounds of purification by magnetic cell sorting. Cells were incubated with a mixture of anti-CD3 (MM1A; VMRD), anti-CD21 (CC21) (19), and anti-immunoglobulin (anti-Ig) light-chain (ILA 59) antibodies (23). Positive and negative sorted cells were tested separately (5 × 10^{5} cells per well) for their ability to produce IFN-α/β in response to ncpBVDV, cpBVDV, dsRNA, and CpG DNA. Similarly, peripheral blood mononuclear cells (PBMC) were purified, and sorted cell populations were tested for their ability to produce IFN-α/β as described above.

Production of an anti-recombinant bovine IFN-α monoclonal antibody. To characterize the cells producing IFN-α/β in response to ncpBVDV, we produced a monoclonal antibody (MAb) against recombinant bovine IFN-α. Young adult BALB/c mice were inoculated subcutaneously with 50 μg rbo IFN-α (recombinant bovine interferon IFN-α-1, a gift from CIBA-GEIGY, Ltd., Basel, Switzerland) mixed with an equal volume of Titermax Gold (Stratech, Cambridge, United Kingdom) three times over a 3-month period. Serum was taken and tested by enzyme-linked immunosorbent assay (ELISA) for antibody to rbo IFN-α.

Eight weeks after the final subcutaneous inoculation, a mouse with a high antibody titer was inoculated intravenously with 25 μg of rbo IFN-α in phosphate-buffered saline 4 days before being killed, and the spleen was removed for fusion with SP2/0 cells (13). Supernatants from wells containing colonies of cells were tested by both the rbo IFN-α ELISA and immunohistochemical analysis of cryosections from bovine lymph nodes of an animal challenged with ncpBVDV 4 days previously. Wells positive for the presence of specific MABs were identified, and colonies were cloned by limiting dilution, combining a visual check that single colonies were present in the wells.

The ELISA used to screen mouse sera and supernatants from hybridomas to detect secretion of rbo IFN-α-specific MABs was essentially as previously described (14). One monoclonal antibody was selected based on a positive ELISA result and the ability to specifically stain clusters of large dendrite-like cells in the paracortex and medulla of BVDV-infected lymph nodes. The antibody, designated BF2, was shown to be IgM isotype; although it showed strong specific staining of cryosections, it did not stain a population of cells specifically by fluorescence-activated cell sorter analysis (data not shown).

Phenotypic characterization of IFN-α/β-producing cells by immunofluorescent staining. Cryostat sections of lymph nodes from either control animals, mock infected with cell lysate, or animals that had been infected subcutaneously with PeS15ncp (5 × 10^{5} PFU) directly over the lymph node either 2 or 7 days previously were used. Prescaphal lymph node cryosections were also prepared from a calf 3 days after challenge with a type 2 ncpBVDV, strain KE13. The detection of infected cells in histological sections by immunofluorescence (IF) was only possible after challenge with KE13, which grows to higher titers in lymphoid tissue than PeS15ncp. Sections were examined by indirect IF and stained for BF2-positive cells and the cellular phenotypic markers listed in Table 1 (3, 18; http://www.iah.bbsrc.ac.uk/leukocyte/bovsite.html). Sections were also stained with BVDV nonstructural protein NS3 with monoclonal antibodies WB112 and WB103 (VLA, Weybridge, United Kingdom) before analysis with confocal laser scanning microscopy (Leica TCSPN).

RESULTS

IFN-α/β production in pseudoafferent lymph after either ncp- or cpBVDV challenge. Using surgically prepared animals (16), we investigated the capacity of the two biotypes of BVDV to stimulate IFN-α/β production at subcutaneous sites and in the draining lymph in vivo. The results shown are from one animal challenged with each biotype. A consistent pattern of IFN-α/β production was measured in all the calves challenged with each biotype, although the magnitude of the response varied. After ncpBVDV challenge, small quantities of IFN-α/β were detectable in the pseudoafferent lymph between 27 and 44 h postchallenge (Fig. 1A). Similarly, small quantities of IFN-α/β were detectable in the blood during the same time period. In contrast, large quantities of IFN-α/β (>90 IU/ml) were detectable within 2 h in the lymph of the animals inoculated with cpBVDV, yet there was no detectable IFN-α/β in the blood of these animals (Fig. 1B). Total pseudoafferent lymph cell preparations (5 × 10^{5} cells) did not produce IFN-α/β in response to either mock antigen or ncpBVDV (Fig. 1B).

IFN-α/β production in efferent lymph after ncpBVDV challenge. After ncpBVDV challenge of animals with the draining lymph node still present, approximately 30 IU/ml of IFN-α/β was detectable in the efferent lymph between 24 and 26 h postchallenge (Fig. 1C). Also, 6 IU/ml, 18 IU/ml, and 16 IU/ml of IFN-α/β were detected after 48, 72, and 96 h postchallenge, respectively. A similar pattern of IFN-α/β production was measured in two cannulated/challenged calves; data from one animal are shown in Fig. 1C.

IFN-α/β production from lymph node cell preparations. The quantity of biologically active IFN-α/β that was induced from either total cells or density gradient purified lymph node cells after the addition of either dsRNA, PeS15ncp, PeS15cp, or control antigen is shown in Fig. 2. As expected, only dsRNA and PeS15cp stimulated IFN-α/β production from total cell

<table>
<thead>
<tr>
<th>Antibody</th>
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<tr>
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<td>CD1b</td>
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</tr>
<tr>
<td>CC43</td>
<td>CD1w3</td>
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a The phenotype was determined by examining cryosections of bovine lymph nodes postinfection with ncpBVDV by indirect immunofluorescence and confocal microscopy. Interferon-producing cells were identified by positive staining with the monoclonal antibody BF2 (Fig. 4) and characterized by dual staining with cell phenotype markers (3, 18; http://www.iah.bbsrc.ac.uk/leucocyte/bovsite.html).

b Partially characterized antibody recognizing monocyte/macrophage/NK cells.
populations. However, when the enrichment for low-density cells dsRNA was used, Pe515cp and Pe515ncp stimulated IFN-α/β production in vitro. These results are representative of six separate experiments, although the absolute values between experiments varied. We concluded that the density gradient had enriched for a population of cells that were able to produce IFN-α/β in response to ncpBVDV. During the development of these assays, we realized that larger quantities of

FIG. 1. (A) IFN-α/β biological activity detected in the lymph of a pseudoafferent cannulated calf at time points after inoculation of $5 \times 10^6$ PFU ncpBVDV subcutaneously into the drainage area of the cannulated vessels. The results represent the mean value of triplicate wells, error bars are ± standard deviation. A total of 5 IU of IFN-α/β biological activity was detected in the plasma of this animal between 27 and 44 h after inoculation of ncpBVDV; samples taken at other time points were negative. (B) IFN-α/β biological activity detected in the plasma and lymph of a pseudoafferent cannulated calf at time points after inoculation of $5 \times 10^6$ PFU cpBVDV subcutaneously into the drainage area of the cannulated vessels. The absence of IFN-α/β production from total afferent lymph cells stimulated in vitro with either mock antigen or ncpBVDV is also shown. The results represent the mean values of triplicate wells; error bars are ± standard deviation. (C) IFN-α/β biological activity detected in the efferent lymph draining the prescapular lymph of a cannulated calf. Lymph was collected at time points after inoculation of $5 \times 10^6$ PFU ncpBVDV subcutaneously directly over the prescapular lymph node. The results represent the mean values of duplicate wells.

FIG. 2. IFN-α/β biological activity detected in the supernatant of single-cell suspensions ($5 \times 10^5$ cells/well) of bovine prescapular lymph nodes after 48 h in culture. Total cell preparations or cells purified over an Iodixanol (Nycomed, Denmark) gradient were cultured with either mock antigen (Mo), double-stranded RNA (pL-C), noncytopathic BVDV (ncp), or cytopathic BVDV (cp). The results represent the mean values of duplicate wells.
IFN-$\alpha/\beta$ were consistently produced after in vitro stimulation, when lymph nodes were harvested from an animal recovering from adventitious infection or after being immunized with Mycobacterium bovis BCG (10$^6$ CFU of BCG Pasteur injected subcutaneously) (data not shown). These observations suggest the responding cells either increase in number in the lymph node or increase their capacity to produce IFN-$\alpha/\beta$ during an inflammatory process.

**IFN-$\alpha/\beta$ production from sorted lymph node cell preparations.** Large quantities of IFN-$\alpha/\beta$ were produced from negatively sorted populations (cells lacking CD3, CD21, or Ig) when stimulated with either dsRNA (149 IU/ml), Pe515cp (120 IU/ml), or Pe515ncp (47 IU/ml) (Fig. 3A). Small quantities (<5 IU/ml) of IFN-$\alpha/\beta$ were induced only by dsRNA and Pe515cp from the original density gradient-purified cells. However, IFN-$\alpha/\beta$ production was readily detected in a similar number of negatively sorted cells when stimulated with dsRNA, cpBVDV, or ncpBVDV. Addition of mock lysate to negatively sorted cells induced <5 IU/ml of IFN-$\alpha/\beta$. The results of these studies are shown in Fig. 3A and are expressed as the mean of measurements from duplicate wells. These results are representative of three separate experiments, although the absolute values between experiments varied. We assume the variability of the results is due to the different percentages of the responding cells present in the lymph nodes used for each assay.

The response of cells lacking CD3, CD21, or Ig purified from blood to dsRNA and CpG DNA are shown in Fig. 3B. Lymph node cells also produce equivalent quantities of IFN-$\alpha/\beta$ in response to CpG DNA and dsRNA (data not shown). Clearly, there are cells present in these sorted populations from blood and lymph nodes that produce IFN-$\alpha/\beta$ in response to dsRNA and CpG DNA; however, only ncpBVDV induced IFN-$\alpha/\beta$ from lymph node-derived cells.

Detailed phenotypic analysis by fluorescence-activated cell sorter of the CD4$^+$ CD3$^-$ cells from lymph nodes did not
identify cells that had a phenotype consistent with the phenotype of high IFN-α-producing cells, plasmacytoid DCs (pDCs), identified in other species (data not shown). Therefore, phenotypic characterization of the IFN-α/β producing cells was performed by indirect IF and analyzed with confocal laser scanning microscopy.

**Phenotypic characterization of interferon-producing cells in lymph node cryosections.** The interferon-producing cells were

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**FIG. 4.** (A to F) Cattle were infected with ncpBVDV (5 × 10⁶ PFU) subcutaneously adjacent to the prescapular lymph node. Two days later, the lymph nodes were harvested postmortem, and cryosections were stained by indirect immunofluorescence, followed by confocal microscopic analysis. (A) BF2⁺ cells localize in the regions surrounding B-cell follicles in lymph nodes from ncpBVDV-infected calves. Lymph node sections were stained with CD21 (fluorescein isothiocyanate [FITC], green) and BF2 (tetramethyl rhodamine isocyanate [TRITC], red). BF2-positive cells were shown to localize in the paracortex around B-cell follicle areas but were not CD21⁺. (B) Sections were stained with BF2 (FITC, green) and CD4 (Alexa Fluor 633, blue). The absence of pale blue staining indicates BF2-stained cells are CD4 negative. (C) Sections were stained with CD2 (TRITC, red), CD3 (Alexa Fluor 633, blue), and BF2 (FITC, green). Dual staining of cells for CD2 and CD3 is shown as purple. The absence of white staining indicates BF2-staining cells are CD2⁺ and CD3⁺. (D) Sections were stained with BF2 (FITC, green) and CD11c (Alexa Fluor 633, blue). The absence of pale blue staining indicates BF2-staining cells are CD11c negative. (E) Sections were stained for BF2 (TRITC, red), and CD172a (FITC, green). The presence of yellow staining indicates colocalization of BF2 and CD172a. (F) Sections were stained for BF2 (TRITC, red) and CD11b (FITC, green). The presence of yellow staining indicates colocalization of BF2 and CD11b. (G) Cattle were infected with KE13 ncpBVDV, and lymph node sections were analyzed 3 days postchallenge. Nuclei were stained with To-Pro3 (Molecular Probes, Cambridge Bioscience, United Kingdom) (blue), antibody WB112 (BVDV protein NS3) (FITC, green), and BF2 (IFN-α) (TRITC, red).
present in the T-cell region of the paracortex in foci of approximately 20 to 30 cells Fig. 4A. The number, distribution, and phenotype of these cells were consistent in the sections obtained either 2 or 7 days after challenge with either Pe51Sncp or 3 days after challenge with KE13ncp. Examples of analyzed confocal images are shown in Fig. 4B to F, and the phenotypic characterization of these interferon-producing cells is summarized in Table 1. Interestingly, the number of BF2 positively stained cells was greatly increased in infected cells compared to control nodes (data not shown).

Interferon-producing cells in lymph nodes are not infected with ncpBVDV. Three days after acute infection with a type 2 ncpBVDV (strain KE13), it was possible to detect cytoplasmic staining for the nonstructural protein NS3 in cyrosections prepared from lymph nodes (Fig. 4G). Even though the antibodies WB112 and WB103 (NS3) and BF2 (IFN) produced strong specific cytoplasmic staining in discrete cells, there was no colocalization of the two signals, indicating that the IFN-producing cells were not detectably infected with BVDV.

DISCUSSION

We have shown that ncpBVDV does not induce IFN-α/β from pseudoafferent lymph cells or from PBMC either in vivo or in vitro. After in vivo challenge with ncpBVDV, we detected small quantities of IFN-α/β in pseudoafferent lymph when it was also detectable in blood. It is most likely the IFN-α/β detected in the blood and lymph between 27 and 44 h is being produced by specific cells in solid lymphoid tissue and at other sites by virus that has disseminated from the original site of inoculation. Indeed, our observation that approximately 30 IU/ml of IFN-α/β is detectable in efferent lymph within 24 h of localized challenge also suggests that the cells producing IFN-α/β in response to ncpBVDV are present in solid lymphoid tissue.

During the course of the present study, we identified the cells producing IFN-α in response to ncpBVDV infection in vivo. Using the knowledge gained of the phenotype of these cells, we enriched lymph node cell preparations for these cells in vitro and demonstrated that ncpBVDV can stimulate IFN-α/β production in vitro.

The cells that produce IFN-α/β in response to ncpBVDV are non-T, non-B, and major histocompatibility complex class II-positive cells. Overall, the phenotypic characterization of these IFN-α/β-producing cells (Table 1) suggests they cannot be defined as bovine pDCs, in particular, because of the absence of CD4 and CD45RB staining and the presence of the myeloid cell markers SIRPs (CD172a) and CD14. However, the precise phenotypic nature of bovine plasmacytoid DC remains to be defined.

In studies with humans and mice, pDCs have been characterized in detail (9). One of the features of pDCs is that they do not respond to dsRNA. However, other cell types have recently been shown to produce large quantities of IFN-α/β in response to appropriate signals, e.g., dsRNA (8). Interestingly, more IFN-α/β was produced from the gradient-purified (Fig. 2) and negatively sorted (Fig. 3) cells than from total cells in response to dsRNA. This suggests that we have enriched for a population of cells that produce large quantities of IFN-α/β in response to dsRNA, similar to those described by Diebold et al. (8). However, the phenotype of the cells producing IFN-α/β in response to ncpBVDV is most probably not the bovine equivalent of the IFN-producing CD11c dendritic cells identified by Diebold et al. (8). Monocytes and B cells are also capable of producing IFN-α; in particular, viral infection of monocytes has been shown to induce IFN-α production (10).

The surface phenotype of the cells producing IFN-α/β in response to ncpBVDV and the lower quantities of IFN-α/β produced compared to that expected from pDCs suggest that the population of cells identified are indeed myeloid. However, the IFN-α/β-producing cells we have identified are clearly different functionally from blood-derived monocytes, which do not produce IFN-α/β in response to ncpBVDV. Moreover, the low level of CD14 expression suggests that these cells are probably not monocytes.

A key question is whether the IFN-α-producing cells are infected with BVDV. Immunohistochemical analysis of lymph node sections after acute infection with a type 2 BVDV isolate showed that the IFN-α-producing cells do not express the viral nonstructural protein NS3. Based on this observation, it is possible to speculate that IFN-α is induced from these specialized cells by extracellular engagement of virus particles.

Despite the difficulties in isolating highly purified populations of the ncpBVDV-containing cells, further detailed studies are planned using in situ hybridization. These further studies will include examining the expression of IFN-α/β subtypes, Toll-like receptors, and interferon regulatory factors.

Our findings improve our understanding of the different outcomes after acute infection of naïve animals with either ncp- or cpBVDV. We conclude that the rapid induction of IFN-α/β at the site of BVDV entry limits the dissemination of virus and accounts for the failure to detect viremia postchallenge (15; B. Charleston, unpublished data), whereas the short delay to induce IFN-α/β in vivo in response to ncpBVDV allows the dissemination of virus and the establishment of viremia, a crucial step in the life cycle of ncpBVDV if the early fetus is to be infected. Also, by identifying a cell type within solid lymphoid tissue capable of producing IFN-α/β in response to ncpBVDV, we provide an explanation for the differential capacity of ncpBVDV to induce IFN-α/β in vitro and in vivo.

REFERENCES