Upregulation of cytokines is detected in the placentas of cattle infected with Neospora caninum and is more marked early in gestation when fetal death is observed

Citation for published version:
Rosbottom, A, Gibney, EH, Guy, CS, Kipar, A, Smith, RF, Kaiser, P, Trees, AJ & Williams, DJL 2008, 'Upregulation of cytokines is detected in the placentas of cattle infected with Neospora caninum and is more marked early in gestation when fetal death is observed' Infection and Immunity, vol. 76, no. 6, pp. 2352-2361. DOI: 10.1128/iai.01780-06

Digital Object Identifier (DOI):
10.1128/iai.01780-06

Link:
Link to publication record in Edinburgh Research Explorer

Document Version:
Publisher's PDF, also known as Version of record

Published In:
Infection and Immunity

Publisher Rights Statement:
Copyright © 2008, American Society for Microbiology

General rights
Copyright for the publications made accessible via the Edinburgh Research Explorer is retained by the author(s) and / or other copyright owners and it is a condition of accessing these publications that users recognise and abide by the legal requirements associated with these rights.

Take down policy
The University of Edinburgh has made every reasonable effort to ensure that Edinburgh Research Explorer content complies with UK legislation. If you believe that the public display of this file breaches copyright please contact openaccess@ed.ac.uk providing details, and we will remove access to the work immediately and investigate your claim.
Upregulation of Cytokines Is Detected in the Placentas of Cattle Infected with *Neospora caninum* and Is More Marked Early in Gestation When Fetal Death Is Observed\(^\text{\dag}\)\(^\text{\dag\dag}\)

Anne Rosbottom,\(^1\) E. Helen Gibney,\(^1,2\) Catherine S. Guy,\(^1\) Anja Kipar,\(^2\) Robert F. Smith,\(^3\) Pete Kaiser,\(^4\) Alexander J. Trees,\(^1\) and Diana J. L. Williams\(^1\)*

*Veterinary Parasitology, Liverpool School of Tropical Medicine/Faculty of Veterinary Science, University of Liverpool, Pembroke Place, Liverpool L3 5QA, United Kingdom\(^1\); Department of Veterinary Pathology, Faculty of Veterinary Science, University of Liverpool, P.O. Box 147, Liverpool L69 7ZJ, United Kingdom\(^2\); Department of Veterinary Clinical Science, Veterinary Teaching Hospital, Leahurst, Neston CH64 7TE, United Kingdom\(^3\); and Institute for Animal Health, Compton, Berkshire RG20 7NN, United Kingdom\(^4\)

Received 8 November 2006/Returned for modification 30 December 2006/Accepted 12 February 2008

The protozoan parasite *Neospora caninum* causes fetal death after experimental infection of pregnant cattle in early gestation, but the fetus survives a similar infection in late gestation. An increase in Th1-type cytokines in the placenta in response to the presence of the parasite has been implicated as a contributory factor to fetal death due to immune-mediated pathological alterations. We measured, using real-time reverse transcription-PCR and enzyme-linked immunosorbent assay, the levels of cytokines in the placentas of cattle experimentally infected with *N. caninum* in early and late gestation. After infection in early gestation, fetal death occurred, and the levels of mRNA of both Th1 and Th2 cytokines, including interleukin-2 (IL-2), gamma interferon (IFN-\(\gamma\)), IL-12p40, tumor necrosis factor alpha (TNF-\(\alpha\)), IL-18, IL-10, and IL-4, were significantly (\(P < 0.01\)) increased by up to 1,000-fold. There was extensive placental necrosis and a corresponding infiltration of CD4\(^+\) T cells and macrophages. IFN-\(\gamma\) protein expression was also highly increased, and a modest increase in transforming growth factor \(\beta\) was detected. A much smaller increase in the same cytokines and IFN-\(\gamma\) protein expression, with minimal placental necrosis and inflammatory infiltration, occurred after *N. caninum* infection in late gestation when the fetuses survived. Comparison of cytokine mRNA levels in separated maternal and fetal placental tissue that showed maternal tissue was the major source of all cytokine mRNA except for IL-10 and TNF-\(\alpha\), which were similar in both maternal and fetal tissues. These results suggest that the magnitude of the cytokine response correlates with but is not necessarily the cause of fetal death and demonstrate that a polarized Th1 response was not evident in the placentas of *N. caninum*-infected cattle.

\(^\text{\dag}\) Corresponding author. Mailing address: Veterinary Parasitology, Liverpool School of Tropical Medicine, Pembroke Place, Liverpool L3 5QA, United Kingdom. Phone: 44 151 705 3142. Fax: 44 151 705 3373. E-mail: williadj@liv.ac.uk.

\(^\text{\dag\dag}\) Published ahead of print on 24 March 2008.
to placent al malaria infection is associated with intr an terine fetal growth retardation (14, 29).

Thus, it is possible that \textit{N. caninum} infection in pregnant cattle results in a Th1-type immune response at the fetomaternal interface that might precipitate fetal death. Experimental infections have shown that the time of a transplacental infection determines whether or not a cow aborts. An intravenous infection with \textit{N. caninum} tachyzoites in early gestation results in fetal death, but the fetus survives a transplacental infection in late gestation, although it becomes infected (44). We have therefore used this system to compare the expression of Th1 (IL-2, IFN-\gamma, IL-12, TNF-\alpha and IL-18), Th2 (IL-4), and regulatory (IL-10 and TGF-\beta1) cytokines at the fetomaternal interface of cows whose fetuses died after \textit{N. caninum} infection and in cows where transplacental transmission occurred and the fetus survived. We compared the relative contributions of maternal and fetal tissues to cytokine levels and correlated this with pathological alterations and inflammatory infiltration in the placenta. This is the first report describing the levels of both Th1 and Th2 cytokines at the fetomaternal interface of cattle following an experimental \textit{N. caninum} infection.

**MATERIALS AND METHODS**

\textbf{Animals.} Twenty-four Friesian-Holstein maiden heifers were purchased commercially and confirmed negative for evidence of exposure to \textit{N. caninum} by an antibody enzyme-linked immunosorbent assay (ELISA; Mastzyme; Mast Diagnostics, Liverpool, United Kingdom) (43, 45). Cattle were also confirmed negative for bovine viral diarrhea virus antigen and vaccinated against \textit{Leptospira hardjo} (Leptavacoid-H; Schering-Plough Animal Health, Uxbridge, United Kingdom), bovine viral diarrhea virus (Bovidec; Novartis Animal Health, Roysten, United Kingdom), and infectious bovine rhinotracheitis virus (Bovilis IBR marker; Intervet, Milton Keynes, United Kingdom). After testing and vaccination, cattle were housed in dog- and fox-proof accommodations. Estrus was synchronized by using progesterone-releasing intravaginal devices (CEVA Animal Health, Chesham, United Kingdom), and cattle were artificially inseminated by using standard techniques, followed by housing with a bull. Pregnancy was confirmed by transrectal ultrasonography at approximately day 35 of gestation.

\textbf{Parasite infection and postinfection monitoring of fetal viability.} Cattle were divided into two groups of 12, and half of each group (\(n = 6\)) were infected at either day 70 or day 210 of gestation with 10\(^7\) tachyzoites of \textit{N. caninum} Ne Liverpool (3) in phosphate-buffered saline (pH 7.2). Tachyzoites were grown in Vero cells and harvested by passage through a 25-gauge needle to disrupt the Vero cells, followed by filtration through Sephadex PD10 columns (GE Healthcare, Amersham, United Kingdom) to remove Vero cell debris as described previously (45). Control animals (\(n = 6\) per group) were infected with Vero cells treated in the same way as those that were infected with tachyzoites. After infection, fetal viability was monitored by ultrasonographic detection of the fetal heartbeat. Cattle infected on day 70 were ultrasound scanned rectally, twice weekly for the first 2 weeks postinfection and daily thereafter until fetal death; cattle infected on day 210 were scanned twice weekly by ultrasound transabdominally. Peripheral immune responses to \textit{N. caninum} were analyzed as described previously (44, 45). Blood was collected into heparin-treated and plain tubes (Becton Dickinson, Oxford, United Kingdom) for analysis preinfection and 1, 2, and 3 weeks postinfection. PBMC were isolated by using Lymphoprep (density 1.077 g/ml; Axis-Shield, Oslo, Norway) and used to assay \textit{N. caninum} antigen-specific proliferation. IFN-\gamma in the PBMC culture supernatants was measured by ELISA using a Bovigam IFN-\gamma ELISA kit (CSL, Victoria, Australia). Serum \textit{N. caninum}-specific antibodies were measured by using a Mastzyme serum antibody ELISA (Mast Diagnostics).

\textbf{Euthanasia and tissue collection.} Euthanasia was carried out by using a captive bolt pistol, followed by pithing, in accordance with the United Kingdom Home Office License. Cattle infected on day 70 were euthanized 3 weeks after infection, at day 91 \pm 1 of gestation (control animals), or within 24 h of detection of fetal death (infected animals). Cattle infected on day 210 were euthanized 3 weeks after infection, at day 231 \pm 1 of gestation. Placental and fetal tissues were collected within 60 min of euthanasia. For analysis of cytokine mRNA expression by real-time reverse transcriptase PCR, full-thickness pieces of manually sepa-

![FIG. 1. H&E-stained sections of uninfected bovine placenta. (A) Intact placenta. Black arrowheads indicate the maternal epithelial of a crypt containing fetal cotyledonal tissue. (B) Caruncle where the fetal cotyledon has been manually removed. White arrowheads indicate maternal endometrial epithelium surrounding a crypt with little remaining fetal material. (C) Fetal chorionic epithelium that has been manually removed from a placenta.](image-url)
enzyme, a 0.5 mM deoxynucleoside triphosphate mix, 0.5 μg of random primer, 20 IU of RNasin, and 1× reaction buffer, resulting in a final concentration of 50 mM Tris-Cl (pH 8.3), 3 mM MgCl₂, 75 mM KCl, and 10 mM dithiothreitol (all from Promega, Southampton, United Kingdom). RNA was incubated with random primer for 5 min at 70°C and then cooled on ice before the addition of the remaining reagents and incubation for 10 min at 25°C, followed by 60 min at 42°C and a final denaturation for 10 min at 70°C. Reactions were diluted 1:5, and 5 μl was used in the PCRs to analyze cytokine mRNA expression. Where cytokine expression was not detected, repeat reactions were analyzed at a dilution of 1:2. To analyze expression of the housekeeping gene 28S rRNA, reactions were diluted a further 1:100.

**Primer design and real-time PCR.** Primers and probes for 28S, IL-2, IL-4, and IFN-γ were designed by using Primer Express Software (PE Applied Biosystems, Warrington, United Kingdom). Those for TNF-α, IL-12p40, and IL-18 were designed by using Primer-3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/www.cgi). Details of the primers and probes are given in Table 1. For all cytokines, one primer or probe crossed an intron-exon boundary to prevent detection of genomic DNA unless indicated by “†,” where they are for bovine cDNA since the bovine genomic DNA sequences are not available.

**Measurement of TGF-β1 and IFN-γ by ELISA.** A total of 100 μg of frozen, separated cotyledon or caruncle was allowed to thaw in 2 ml of CelllyticMT cell lysis solution (Sigma) containing 5 μl of protease inhibitor cocktail (Sigma) and then homogenized, and the supernatants were collected by centrifugation at 4°C for 10 min at 15,000 × g. The total protein in the supernatants was measured by using a Bio-Rad protein assay (Bio-Rad, Hemel Hempstead, United Kingdom) according to the manufacturer’s instructions. TGF-β1 was measured by using a TGF-β1 ELISA Immunoasay System (Promega, Southhampton, United Kingdom) according to the manufacturer’s instructions for detection of active TGF-β1 (omitting the optional acid activation step). IFN-γ was measured by using a Bovigam IFN-γ ELISA kit as described previously (44).

### Table 1. Sequences of primers and probes used for real-time reverse transcription-PCR

<table>
<thead>
<tr>
<th>Target</th>
<th>Primer orientation or probe</th>
<th>Probe or primer sequence (5'–3')</th>
<th>Exon boundary</th>
<th>Accession no.*</th>
</tr>
</thead>
<tbody>
<tr>
<td>28S</td>
<td>P</td>
<td>AGTAGCTGTGTTCCCTCCGGAAGTTTCCCT</td>
<td>1–2</td>
<td>AF154866†</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>GCCGAAGAAGCTAATCGGACA</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>CGGAGAGCAGCCAGATCTCC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-2</td>
<td>P</td>
<td>AGGTCCTCCAGGATTGACATATTGTCTTAC</td>
<td>1–2</td>
<td>M12791†</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>GGAGAAAGATTTAA[A]AATCTGGAAGACCT</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>TTCTAGTAAACCTTAAGATGTCTTCAATTTCG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-4</td>
<td>P</td>
<td>AGATCTCCGTACGTCGCCAGCGG**</td>
<td>3–4</td>
<td>U14131</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>GCCACACGTGCTTGAACAAA</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>TCATTACAGAAAGACGGCA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-10</td>
<td>P</td>
<td>TGGCAACGCTTGGGAAGATGATCC</td>
<td>2–3</td>
<td>U00799†</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>TGGTGGAATGACCTTTAAGG[AGGT]TGTTACC</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>GGCACTACCTTTCTCGAGTA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IFN-γ</td>
<td>P</td>
<td>TCACTCCAGC[GG]GGAATTGGAATCGAGCTTT**</td>
<td>3–4</td>
<td>Z54144</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>GCAGCTCTGGAAGAAGCTGAGGA</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>TGCGGTTGCGGTGATCT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNF-α</td>
<td>P</td>
<td>CCTCAAGTAAACGCGCGTACTGCCA</td>
<td>2–3</td>
<td>AF011926</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>TCCAAACACCI[AG]GCTCCCTCTC</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>GGAGAGTGGATGCTGC[CTAC]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-12p40</td>
<td>P</td>
<td>ACTCTTAGGTCTGGTGT[CT]TTCTGTACCTCTTT*</td>
<td>2–3</td>
<td>U11815†</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>ATGGAATTTGGTCCTCATGTATT</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>GGTAAGTGTCGAGATAATCTCCTT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-18</td>
<td>P</td>
<td>TCTTCTAACG[GAAGATGAATCTCCTGATAACA</td>
<td>4–5</td>
<td>NM_174091†</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>GTCTACTCTCCTCTTGAGAACA</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>AATATGATGTCATCTTTCTCATTATCA</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>


† Accession numbers are for bovine genomic DNA unless indicated by †, where they are for bovine cDNA since the bovine genomic DNA sequences are not available.

**TABLE 1.** Sequences of primers and probes used for real-time reverse transcription-PCR.
TABLE 2. Standard curve data from six duplicate 10-fold dilutions of 1-µg/ml plasmid stocks

<table>
<thead>
<tr>
<th>Target</th>
<th>Primer concn (µM)</th>
<th>Log dilutions</th>
<th>Mean C&lt;sub&gt;T&lt;/sub&gt;</th>
<th>Mean R&lt;sup&gt;2&lt;/sup&gt;</th>
<th>Mean slope</th>
<th>CV&lt;sub&gt;max&lt;/sub&gt;%</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>28S</td>
<td>0.3</td>
<td>10&lt;sup&gt;-3&lt;/sup&gt; to 10&lt;sup&gt;-6&lt;/sup&gt;</td>
<td>6.65–23.71</td>
<td>0.997</td>
<td>–3.43</td>
<td>3.26</td>
<td>6</td>
</tr>
<tr>
<td>IL-2</td>
<td>0.6</td>
<td>10&lt;sup&gt;-3&lt;/sup&gt; to 10&lt;sup&gt;-8&lt;/sup&gt;</td>
<td>16.18–34.35</td>
<td>0.998</td>
<td>–3.52</td>
<td>2.57</td>
<td>6</td>
</tr>
<tr>
<td>IL-4</td>
<td>0.4</td>
<td>10&lt;sup&gt;-3&lt;/sup&gt; to 10&lt;sup&gt;-8&lt;/sup&gt;</td>
<td>15.96–33.15</td>
<td>0.997</td>
<td>–3.61</td>
<td>3.43</td>
<td>8</td>
</tr>
<tr>
<td>IL-10</td>
<td>0.2</td>
<td>10&lt;sup&gt;-3&lt;/sup&gt; to 10&lt;sup&gt;-8&lt;/sup&gt;</td>
<td>15.65–33.99</td>
<td>0.998</td>
<td>–3.61</td>
<td>4.64</td>
<td>7</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>0.3</td>
<td>10&lt;sup&gt;-3&lt;/sup&gt; to 10&lt;sup&gt;-8&lt;/sup&gt;</td>
<td>14.05–31.77</td>
<td>0.999</td>
<td>–3.54</td>
<td>2.25</td>
<td>8</td>
</tr>
<tr>
<td>TNF-α</td>
<td>0.4</td>
<td>10&lt;sup&gt;-3&lt;/sup&gt; to 10&lt;sup&gt;-8&lt;/sup&gt;</td>
<td>13.23–29.24</td>
<td>0.998</td>
<td>–3.20</td>
<td>3.77</td>
<td>5</td>
</tr>
<tr>
<td>IL-12p40</td>
<td>0.4</td>
<td>10&lt;sup&gt;-3&lt;/sup&gt; to 10&lt;sup&gt;-8&lt;/sup&gt;</td>
<td>15.09–32.47</td>
<td>0.999</td>
<td>–3.45</td>
<td>3.43</td>
<td>5</td>
</tr>
<tr>
<td>IL-18</td>
<td>0.6</td>
<td>10&lt;sup&gt;-3&lt;/sup&gt; to 10&lt;sup&gt;-8&lt;/sup&gt;</td>
<td>15.86–33.52</td>
<td>0.998</td>
<td>–3.51</td>
<td>2.24</td>
<td>4</td>
</tr>
</tbody>
</table>

<sup>a</sup> The mean C<sub>T</sub> (i.e., the threshold value at which the change in reporter dye crosses the significance threshold), R<sup>2</sup> (coefficient of regression), and slope and the maximum coefficient of variation of all points (CV<sub>max</sub>%) for n separate runs of each target are shown. The significance threshold for all runs was set at 0.01. In all reactions, the probe concentration was 100 nM; primer concentrations were individually determined for each gene and are as shown.

**RESULTS**

Fetal death occurs in cattle infected with *N. caninum* at day 70 but not at day 210 of gestation. In animals infected at day 70 of gestation, fetal death occurred 22.7 ± 1.2 days later, and animals were euthanized for tissue collection within 24 h of detection of fetal death. No fetal death occurred prior to euthanasia of the dam in animals infected at day 210 of gestation. Peripheral immune responses to *N. caninum* were as described...
mals proliferated and secreted IFN-\(\gamma\) both days 70 and 210 of gestation. PBMC from infected animals proliferated in response to \(N.\) caninum antigen from 1 week postinfection and seroconverted by 2 weeks postinfection (data not shown). An aliquot of the same parasitic material used for infection of cattle was used to infect Vero cells. Parasite growth was observed after 5 days, confirming parasite viability.

All cytokine mRNA is greatly increased in the bovine placenta after infection with \(N.\) caninum at day 70 of gestation. To investigate the possible role of cytokines in fetal death after experimental infection of cattle with \(N.\) caninum at day 70 of gestation, we measured the levels of IL-2, IFN-\(\gamma\), IL-4, IL-10, IL-12p40, TNF-\(\alpha\), and IL-18 mRNA in bovine placentas. In maternal tissue (caruncles), all cytokine mRNA was significantly increased (\(P < 0.01\)) compared to that in uninfected animals (Fig. 2A). IL-4 and IFN-\(\gamma\) were most highly upregulated by 228 (110 to 1,729)- and 785 (287 to 3,641)-fold, respectively. Also highly upregulated were the IL-12p40, IL-2, and TNF-\(\alpha\) mRNA, whose levels increased by 33 (10 to 215)-, 50 (15 to 221), and 55 (13 to 391)-fold, respectively. IL-10 mRNA increased by 15 (7 to 97)-fold, and IL-18 mRNA was upregulated 7 (5 to 25)-fold. Cytokine mRNA was also significantly (\(P < 0.01\)) upregulated in the fetal cotyledon after \(N.\) caninum infection at day 70 of gestation, with the exception of IL-2 (Fig. 2B). The most highly upregulated were IL-10, IFN-\(\gamma\), and TNF-\(\alpha\) mRNA, which increased by 138 (75 to 479)-, 156 (20 to 10,508)-, and 190 (22 to 3957)-fold, respectively. IL-18, IL-4, and IL-12p40 mRNA were upregulated by 9 (5 to 12)-, 6 (3 to 264)-, and 3 (2 to 49)-fold, respectively. IL-18, IL-4, and IL-12p40 mRNA were upregulated by 9 (5 to 12)-, 6 (3 to 264)-, and 3 (2 to 49)-fold, respectively. In general, cytokine levels in cotyledons were lower and more variable than in caruncles; increases in IL-4 and IL-12p40 mRNA were detected in only two of the six controls, and IL-2 mRNA was detected in only three controls and five infected cotyledons.

In cattle infected at day 70 of gestation, all cytokine mRNA levels except for IL-10 and TNF-\(\alpha\) were significantly higher in the maternal side than in the fetal side of the placenta. Further comparison of the levels of cytokine mRNA in the caruncles and cotyledons after \(N.\) caninum infection at day 70 was undertaken to determine the relative contributions of maternal and fetal tissues to the responses seen. The levels of IL-12p40, IL-2, IL-4, IFN-\(\gamma\), and IL-18 were significantly higher (\(P < 0.01\)) in caruncles than in cotyledons (Fig. 3). Caruncles had 99% ± 0.3%, 94% ± 3%, 93% ± 5%, 92% ± 5%, and 86% ± 3% of the total amounts of each respective cytokine mRNA. In contrast, caruncles had 56% ± 9% and 61% ± 8% of the total IL-10 and TNF-\(\alpha\) mRNA, respectively, and there was no significant difference between the levels in caruncles and the levels in cotyledons.

All cytokine mRNA is modestly increased in the placenta after infection of cattle at day 210 of gestation. To compare placental cytokine levels when \(N.\) caninum infection results in fetal death to that when the fetus survives infection, we examined the quantity of cytokine mRNA at the fetomaternal interface 3 weeks after infection of cattle at day 210 of gestation. All cytokine mRNA levels except for IL-2 were significantly (\(P < 0.01\)) increased in the maternal caruncle, but to a much lesser extent than that seen after infection at day 70 (Fig. 4A). As seen after infection at day 70, IL-4 and IFN-\(\gamma\) mRNA were the most highly upregulated, but by only 10.1 (1.1 to 17.8)- and 16.3 (6.7 to 65.2)-fold, respectively. IL-12p40 and TNF-\(\alpha\) mRNA were upregulated with respective increases of 3.5 (1.8 to 15.4)- and 2.7 (1.6 to 3.4)-fold, and IL-10 and IL-18 mRNA levels increased by 2.0 (1.6 to 3.4)- and 1.4 (1.2 to 2.0)-fold, respectively. Cy-
tokine mRNA in the cotyledons was at the limit of detection and, as such, variable (Fig. 4B). Nevertheless, IL-4 and IFN-γ mRNA levels were significantly (*P < 0.05) increased, and there was a trend toward significance in the increase in TNF-α mRNA in cotyledons. IL-2 and IL-4 mRNA levels were undetectable in three control samples. IL-10 mRNA was undetectable in one control and two infected cotyledon samples, and IFN-γ mRNA was undetectable in one control sample.

A similar Th1/Th2 cytokine ratio is present in the placenta after *N. caninum* infection at days 70 and 210 of gestation. We compared the placental cytokine balance as a ratio of IFN-γ to IL-4 after *N. caninum* infection at day 70 or 210 of gestation. The ratios of IFN-γ to IL-4 in the caruncles were 2.9 ± 0.25 and 2.9 ± 0.77 after infection at days 70 and 210, respectively; there was no significant difference between the two.

Active TGF-β1 is present in the bovine placenta and protein levels increased after infection at day 70 of gestation. Since TGF-β1 protein levels correspond poorly to mRNA levels (24), active TGF-β1 in bovine placentas was measured by ELISA. TGF-β1 was detectable both at days 70 and 210 of gestation (Fig. 5), and the levels increased after infection at day 70; this increase was significant (*P < 0.05) in caruncles (Fig. 5A). At day 70 of gestation, the expression of TGF-β1 was higher in cotyledons than caruncles, but this difference was only significant (*P < 0.05) in control animals (Fig. 5A). The expression of TGF-β1 at day 210 of gestation was similar in cotyledons and caruncles, and there was no increase after infection (Fig. 5B).

The expression of IFN-γ protein is increased in the placenta after *N. caninum* infection at days 70 and 210 of gestation. To determine whether changes in mRNA expression correlated with levels of protein, the levels of placental IFN-γ were measured by ELISA. Very low levels of IFN-γ protein were detectable in uninfected placentas (0.0025 ± 0.0004 and 0.004 ± 0.001 ng/mg protein in caruncles of day 70 and day 210 control cattle, respectively) (Fig. 6). However, the levels of protein increased significantly (*P < 0.01) after infection at both days 70 and 210 of gestation, with fold increases in proportion to those seen for the expression of mRNA. IFN-γ levels in caruncles reached 2.1 ± 0.9 and 0.13 ± 0.07 ng/mg after infection at days 70 and 210, respectively.

*N. caninum* infection of cattle at day 70 of gestation results in extensive necrosis, placental infiltration of CD4+ T cells and macrophages, and expression of IFN-γ in necrotic areas. To determine whether the observed increases in cytokine levels were associated with pathological changes and recruitment of particular cell types into the placenta, histological examination and immunohistological staining for T cells, B cells, and mono-
cytotoxic areas. Sparsely scattered CD79a + B cells were present in placentas collected at both time points, and there were no differences between control and infected animals.

**DISCUSSION**

Here we report for the first time a dramatic increase in the quantities of a number of cytokines at the fetomaternal interface among cattle in which the fetus died after an experimental infection with *N. caninum* tachyzoites in early gestation. In contrast, cytokine mRNA levels were only modestly increased at the fetomaternal interface of cattle infected in late gestation in which the fetuses survived. The cytokines that were upregulated included those implicated as potential abortifacients, such as IFN-γ (20, 23), TNF-α (13, 23), IL-12p40 (46), and IL-2 (40). However, the levels of Th2 and regulatory cytokines, including IL-4, IL-10, and TGF-β1, also increased in the placenta after *N. caninum* infection. The cytokine balance, as indicated by the IFN-γ/IL-4 ratio, was similar after infection in early and late gestation, but the magnitude of expression of all cytokines was very different, with strong cytokine expression occurring in the placentomes of cattle whose fetuses were killed as a result of infection.

These results, showing an increased expression of both Th1 and Th2 cytokines after *N. caninum* infection, contrast with previous suggestions that a Th1-biased response to *N. caninum* occurs in infected cattle (27). However, the data are consistent with studies in *N. caninum*-infected mice for which a mixed systemic cytokine response has been described (19, 26, 35) and recent work from our laboratory showing an increase of both IFN-γ and IL-4 mRNA in PBMC in response to *N. caninum* infection of cattle (37). Moreover, in vitro work suggests that an immune response polarized toward Th1 or Th2 is uncommon in cattle (7). Significantly, we have shown that here that the placental cytokine balance in infected cattle, after infection in early and late gestation, does not support the traditional view that the cytokine environment of pregnancy is biased toward the expression of Th2 type cytokines (36, 42).

There was widespread necrosis and inflammation of the placenta in the day 70 group that was absent in the day 210 group. We have shown elsewhere (15) that this necrosis predominantly affects the cotyledons and is associated with high numbers of parasites. Moreover, the day 70 fetal tissues (notably the heart, liver, and neural tissues) showed evidence of extensive parasitosis, whereas it was minimal in the day 210 group. It is likely that the placental necrosis observed in the day 70 animals contributed to fetal death, but why there is such a difference in the extent of parasite dissemination and necrosis in the day 70 group compared to the day 210 group is not clear, particularly since both groups were infected with the same dose of parasites and killed at the same time (3 weeks) after infection. It is possible that the high levels of IFN-γ produced in the placenta in response to the presence of the parasite are pathogenic (36, 38, 40, 47) or, alternatively, that the necrosis is due directly to parasite mediated cell damage, in which case the immunocompetence of the fetus itself may be significant. Fetal immunocompetence develops gradually through gestation, but *N. caninum* antigen specific fetal responses have only been described from midgestation onward (1, 4). Thus, it is likely that after the parasite has crossed the
placenta and invaded fetal tissues, its multiplication and dissemination is controlled in the immunologically mature day 210 fetus, whereas the day 70 fetus becomes heavily parasitized. We have suggested elsewhere (15) that this results in large numbers of parasites invading the placenta via the fetal circulation in the day 70 group, stimulating a profound inflammatory response in the placenta. This may ultimately cause fetal death either by the direct cytotoxic effects of proinflammatory cytokines and immune CD4+ T cells or by parasite-mediated destruction of fetal trophoblast cells. Both of these mechanisms could potentially lead to placental insufficiency and fetal death in a way similar to that proposed for *Toxoplasma gondii* (8).

By analyzing the levels of cytokines in maternal caruncle
compared to fetal cotyledon tissue we showed that, with the exception of IL-10 and TNF-α, the majority of the cytokines detected were of maternal origin. For IL-12, IL-2, IL-4, and IFN-γ, <8% of the total mRNA detected was in the cotyledons. Nevertheless, for the day 70 group IFN-γ, TNF-α, IL-4, and IL-10 were significantly upregulated in the infected cotyledon compared to the control cotyledon tissue. It is possible that these cytokines were expressed by the fetal trophoblast cells directly or by infiltrating maternal mononuclear cells. We showed by immunohistology that IFN-γ-producing, maternally derived mononuclear cells were present in areas of damaged villous epithelium and so could be the source, particularly of IL-4 and IFN-γ. However, there is evidence that fetal trophoblast cells can express IL-10 and TNF-α (6, 21), and therefore these cytokines could have been either of fetal origin or expressed by infiltrating maternal macrophages, which have also been shown to produce IL-10 and TNF-α (33).

By immunohistology we showed there was an influx of CD4+ T cells, probably of maternal origin, into the caruncle; this influx was most marked in the day 70 group. These cells were probably a significant source of cytokines, and we showed by immunohistology that they expressed IFN-γ, corroborating the work of Maley et al. (27). CD4+ cytotoxic T cells have been described in N. caninum-infected cattle (39). We also show for the first time that IFN-γ was also upregulated in maternal caruncle epithelium. These observations suggest that the increase in cytokine expression and recruitment of macrophages and CD4+ T cells to the placenta were to control parasite multiplication. Similarly, in mice infected with Listeria monocytogenes, upregulation of IL-12, TNF-α, IL-18, and IFN-γ in the placenta is thought to be essential for effective control of the bacterium and in preventing abortion associated with L. monocytogenes infection (2).

Active TGF-β1 protein was present in control and infected fetal and maternal tissue at both days 70 and 210 of gestation, confirming the results of a previous study (30) and the suggestion that it is involved in placental remodeling (12). In the infected cattle at day 70, higher levels of TGF-β1 were detected in the cotyledons in both uninfected and infected cattle compared to the day 210 group, but there was a significant increase in expression of TGF-β in the day 70 caruncles after infection. Regulatory T cells have been implicated in controlling the immunological microenvironment at the materno-fetal interface, and TGF-β is a signature cytokine for this population (48). It is possible that the expression of TGF-β is upregulated to control the recruitment of cytotoxic T cells that could potentially harm the fetus.

In conclusion, N. caninum infection in early gestation, which resulted in fetal death, was associated with a much greater increase in placental cytokine expression, placental necrosis, and inflammatory cell infiltration compared to cattle infected later in gestation when the fetus survived. There was no evidence of a marked polarization of the immune response in the placenta, and the expression of Th1 (including, most markedly, IFN-γ), Th2, and regulatory cytokines increased. Fetal death could have resulted from the fetocytotoxic effects of cytokines such as IFN-γ, TNF-α, or IL-2, or the immune response in the placenta could have been generated to protect the conceptus from direct parasite-mediated cell damage.

ACKNOWLEDGMENTS

We are grateful to the farm staff at the University Animal Husbandry Farm for their dedicated care of the animals used in this study. We thank the technical staff at the Histology Laboratories, Department of Veterinary Pathology, for performing routine processing of tissues. We also thank D. Bainbridge, Department of Veterinary Anatomy, University of Cambridge, for helpful discussions. This study was funded by the Wellcome Trust (grant 0R066095MA), and E.H.G. was supported by a Postgraduate Research Studentship from the University of Liverpool and by Novartis Animal Health, Ltd.

REFERENCES

23. Krishnan, L., J. L. Guilbert, T. G. Wegmann, M. Belosevic, and T. R. Mosmann. 1996. T helper 1 response against Leishmania major in pregnant CS7BL/6 mice increases implantation failure and fetal resorptions. Correla-
tion with increased IFN-γ and TNF and reduced IL-10 production by pla-

Editor: J. F. Urban, Jr.