AJRI 2000; 43:305–311
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American Journal of Reproductive Immunology
ISSN 8755-8920

Acute Intrauterine Infection Results in an Imbalance between Pro- and Anti-Inflammatory Cytokines in the Pregnant Rabbit

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Leslie KK, Lee SL, Woodcock SM, Davies JK, McDuffie RS, Hirsch E, Sherman MP, Eskens JL, Gibbs RS. Acute intrauterine infection results in an imbalance between pro- and anti-inflammatory cytokines in the pregnant rabbit. AJRI 2000; 43:305–311 © Munksgaard, Copenhagen

PROBLEM: Intrauterine infection results in an increase in cytokines. This study compared the time courses for the pro- and anti-inflammatory cytokine responses in 33 pregnant rabbits at 70% gestation. Pro-inflammatory markers were activated nuclear factor-kappa B (NF-κB) in placenta and tumor necrosis factor-alpha (TNF-α) in amniotic fluid. These were compared to the anti-inflammatory cytokine, interleukin-1 receptor antagonist (IL-1ra), in placenta and uterus.

METHOD OF STUDY: Does were endoscopically inoculated with *Escherichia coli* through their cervices and sacrificed at six intervals between 0 and 30 hr post-inoculation.

RESULTS: Activated NF-κB, determined by electromobility gel shift assay, increased significantly 16 hr after bacterial inoculation ($P \leq 0.05$). This was directly mirrored by TNF-α concentrations, determined by bioassay, in the amniotic fluid. However, IL-1ra levels, determined by enzyme-linked immunosorbent assay, did not increase in response to infection.

CONCLUSIONS: Intrauterine infection results in an imbalance between pro- and anti-inflammatory cytokines that may potentiate infection-induced preterm delivery.

Key words:
Interleukin-1 receptor antagonist, nuclear factor-κB, tumor necrosis factor-α

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Submitted June 30, 1999; accepted October 11, 1999.

INTRODUCTION

Preterm birth is the leading perinatal problem in the developed world, accounting for up to 80% of all perinatal deaths.[1] The causes of preterm birth are diverse; however, intrauterine infection plays a significant role.[2] In animal models, introduction of lower genital tract bacteria or bacterial products, such as lipopolysaccharide (LPS), into the uterus or cervix prior to term leads to reproducible pregnancy loss accompanied by histologic and microbiologic evidence of infection.[3,4] Prostaglandin $F_{2\alpha}$ and E_2, important mediators of uterine contractility and cervical dilatation, are induced by infection in response to cytokines that are produced in fetal and maternal tissues,[5] including the placenta and the uterus.

Cytokines are polypeptides that serve as inter- and intracellular messengers regulating, among other processes, host defense. Pro-inflammatory cytokines, such as interleukin-1 (IL-1) and tumor necrosis factor-alpha (TNF-α), are induced in response to infection.[6] The principal transcription factor that up-regulates cytokine genes involved in the inflammatory response is nuclear factor-kappa B (NF-κB), composed of at least two peptide subunits, p50 and RelA (also known as p65). These subunits combine to form the homo- or heterodimeric complexes p50/50 or p50/RelA, respectively.[7]

NF-κB is constitutively expressed in all cells (for reviews, see references[6,7]). In the normal, uninfected state, nearly all of the NF-κB is bound in the cytoplasm in an inactive form by the inhibitor, IkappaB (IκB). When retained within the cytoplasm and bound by the inhibitor, NF-κB is incapable of activating gene transcription leading to the inflammatory response. With infection, IκB is rapidly degraded and activated NF-κB is released to enter the nucleus. There, it binds to specific NF-κB DNA response elements in the promoters of genes encoding cytokines, such as IL-1, IL-6, and TNF-α, leading to the synthesis of new transcripts.[7] IL-1 and TNF-α, in turn, participate in a positive feedback loop, whereby they further enhance the activation of NF-κB.[8,9] However, a negative feedback loop also exists because NF-κB induces IκB, thereby limiting the pro-inflammatory response.[10] Previous studies have shown that activated, nuclear NF-κB is present principally in cells involved in ongoing infection or inflammation and can be detected by its ability to bind to NF-κB oligonucleotide response element probes, such as those employed in electromobility gel shift assays.[11] Such probes are not bound by NF-κB when it is in a complex that contains IκB.[10] Therefore, activated NF-κB is a marker for the pro-inflammatory cascade and can be assayed by electromobility gel shift assay.

Endogenous anti-inflammatory cytokines, such as interleukin-1 receptor antagonist (IL-1ra), are also produced in many tissues.[12] IL-1ra competes with IL-1 for receptors on the cell surface. IL-1ra-bound receptors fail to induce the downstream signal transduction cascade necessary to maintain the cell's inflammatory state; therefore, IL-1ra is an anti-inflammatory cytokine and a competitive inhibitor of IL-1.

This study was undertaken in the pregnant rabbit model to evaluate the hypothesis that infection ascending from the lower genital tract into the uterine cavity triggers an over-exuberant pro-inflammatory cytokine response linked to preterm delivery. The objective was to determine the time course for activation of the pro-inflammatory response using activated NF-κB in the placenta and TNF-α in the amniotic fluid as markers, and to compare these to the expression of the anti-inflammatory cytokine IL-1ra in the rabbit placenta and uterus. NF-κB was chosen for study because it represents the central transcription factor involved in the inflammatory response. TNF-α was chosen because we have a reliable assay to detect it, and because it is one of the primary pro-inflammatory mediators, and IL-1ra was chosen for study because it is considered to be a primary endogenous anti-inflammatory cytokine. Although we wished to measure additional cytokines, such as IL-1, no assays deemed reliable in the rabbit were available.

MATERIALS AND METHODS

Inoculation of Rabbit Does and Tissue Collection
This protocol was approved by the Animal Use and Care Committee of the University of Colorado Health Sciences Center. Under anesthesia with 25 mg/kg ketamine and 5 mg/kg xylazine, New Zealand White rabbit does (Myrtle's Rabbitry, Inc., Thompson Station, TN) were endoscopically inoculated with 10^5 colony-forming units of *Escherichia coli* (ATTC 12014) at the level of the cervix on day 21 of pregnancy (70% gestation). This procedure has been described previously.[3] Rabbits were anesthetized a second time, euthanized with an intracardiac injection of 130 mg/kg sodium pentobarbital, and a laparotomy was performed at the following time points after cervical bacterial inoculation: 0 hr ($n = 5$), 4 hr ($n = 5$), 8 hr ($n = 5$), 16 hr ($n = 7$), 24 hr ($n = 7$), and 30 hr ($n = 4$). Time points were chosen based upon previous studies with this model.[13] Amniotic fluid was aspirated after exposure of the membranes by hysterotomy. Placental and full-thickness uterine tissues were snap frozen in liquid nitrogen and stored at $-80°C$.

Protein Extraction

Frozen placental and uterine tissues were placed on ice in a sterile petri dish. One gram of each sample was minced in 5.0 mL ice-cold ACK lysing buffer (Biofluids, Inc., Rockville, MD) for 10 min to remove blood contaminants, then centrifuged at 1000 rpm for 5 min. The pellet was collected and washed with 10 mL ice-cold 10 mM phosphate-buffered saline via centrifugation at 1000 rpm for 5 min. Each pellet was resuspended in 2 mL homogenization buffer (250 mM sucrose, 15 mM Tris, pH 7.9, 60 mM KCl, 15 mM NaCl, 2 mM EDTA, pH 8.0, 0.5 mM EGTA, 0.5 mM Spermidine, 0.15 mM Spermine, 0.1% Triton X-100, 1 mM dithiothreitol (DTT), 0.5 mM phenylmethylsulfonyl fluoride, 1 mg/mL Pepstatin, 2 μM Aprotinin). The cells were lysed with 10 strokes of a hand-held homogenizer (Kontes Glass Company, Vineland, NJ). Each sample was then centrifuged at 10,000 rpm at 4°C for 25 min. The supernatant from each sample was collected and the protein concentration determined by colorimetric analysis.

Radiolabeling of Double-Stranded NF-κB Oligonucleotide

1.75 pmol of double-stranded NF-κB response element oligonucleotide probe (Promega, Madison, WI) was radiolabeled with 1 μL γ-^{32}P-ATP (7000 Ci/mmol, 2 mCi/12 μL; ICN, Costa Mesa, CA) using T4 polynucleotide kinase (Gibco BRL, Grand Island, NY). The unincorporated label was removed with STE SELECT-D G-25 columns (5Prime-3Prime, Inc., Boulder, CO) and the labeled NF-κB probe was stored at -20°C.

Electromobility Gel Shift Analysis

Each binding reaction contained 65 μg of total protein from control and test tissues, labeled NF-κB response element probe (2×10^4–2×10^5 cpm), 5% glycerol, and ddH_2O to a total volume of 10 μL. The negative control contained no protein, and the positive control contained LPS-activated 264 cell extract, a gift from G. Feldman (Food and Drug Administration, Bethesda, MD). Specificity of bands for NF-κB was proven using competition by tenfold excess unlabeled NF-κB response element probe and previously with supershifting using antibodies directed against the RelA and p50 NF-κB subunits (gifts from N. Rice, National Institutes of Health, Bethesda, MD). Each sample was incubated for 20 min at room temperature to allow binding to the radiolabeled NF-κB response element probe.

Samples were run over 5% 29:1 (acrylamide:bisacrylamide) gels in 1 × TAE at 20 mA constant current per gel for 1.75 hr and dried. Autoradiography was performed overnight at -80°C and the autoradiograms were quantified using a densitometer (BioRad Model GS 670, Life Science Division, Hercules, CA).

The p50/50 homodimer and p50/RelA heterodimer bands on the autoradiogram were quantified with negative control regions of each gel subtracted as background. The intensity data for the gels were normalized using ratios obtained from the control lanes. The mean p50/50 and p50/RelA band intensities for all animals from each time point were calculated.

Bioassay for TNF-α in Amniotic Fluid

TNF was determined with a well-established bioassay, which is a modification of a reported method[14] using a mouse fibrosarcoma cell line (WEHI-13VAR, American Type Culture Collection, item no. CRL-2148, Manassas, VA). These cells show both stability and high sensitivity towards recombinant TNF-α and TNF-β.

Cells were grown in flasks containing RPMI-1640 (Irvine Scientific, Santa Ana, CA). The RPMI-1640 was supplemented with 10% fetal bovine serum (Atlanta Biologicals, Atlanta, GA), 2 mM l-glutamine, 10 mM HEPES, dextrose (final concentration 4.5 g/L), 1.0 mM sodium pyruvate (Life Technologies, Grand Island, NY), penicillin, streptomycin, and amphotericin B (1% final concentration of Fungi-Bact, Irvine Scientific). Cells were incubated at 37°C in 5% CO_2 until confluent, then dissociated and with 0.25% trypsin and 0.03% EDTA in Hank's balanced salt solution (Irvine Scientific). A portion of the cell suspension was counted using a hemacytometer, and the cells were diluted to a density of 1×10^6 cells/mL using fresh growth media. Twenty microliters (20,000 cells) were seeded into each well of 96-well plates. The final volume of each well was increased to 100 μL by adding fresh media, which was renewed in each well at intervals of 24 hr until the cells became confluent. Thereafter, media was aspirated and replaced with 90 μL of RPMI-1640 containing 25 mM HEPES, 3% fetal bovine serum, 1% Fungi-Bact solution, and actinomycin D at a final concentration of 0.5 μg/mL (Sigma Chemical Co., St. Louis, MO). The final 10 μL of volume per well was either (1) recombinant human TNF-α (Life Technologies) diluted to different concentrations in the aforementioned assay media or (2) amniotic fluid from individual pregnant rabbits. The bioassay was performed without knowledge of the treatment received by the pregnant rabbits, i.e., when the rabbits were euthanized after bacterial inoculation. After 24 hr of incubation with TNF standards or unknown samples, 10 μL of 3-[4,5-dimethylthiozol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT, Sigma no. CGD-1) solution was added to the wells. The solution contained 5 mg/mL of MTT in RPMI-1640

without phenol red. After 3 hr of incubation, MTT solvent was added to the wells and incubated at 37°C in the dark until all reduced MTT had been dissolved (24 hr). An enzyme-linked immunosorbent assay (ELISA)-type plate reader was then used to measure the absorbance at 550 nm, and a background absorbance at 650 nm was subtracted from these readings. The standard curve was constructed using TNF concentrations from 7.5 to 500 pg/mL. The best curve fit for the TNF standards was a semi-log plot. If an unknown specimen was higher than the peak concentration of the standard, the amniotic fluid was diluted and re-analyzed. Goat anti-human TNF-α antibody totally reversed cytotoxicity observed in cells incubated with different concentrations of recombinant human TNF-α. To prove that the cytotoxicity was caused by TNF, and not mediated by endotoxin in a sample, amniotic fluid specimens showing TNF activity were re-analyzed in the presence and absence of polymyxin B (10 μg/mL). This antibiotic is known to bind and inactivate endotoxin.[15] The activity of the unknown specimen in the presence of polymyxin B was used for determining differences between groups. Inter- and intra-assay variances are less than 5%. A direct comparison of the bioassay with an immunoassay for TNF-α (R&D Systems, Minneapolis, MN) in the rat revealed less than 10% variance between assays.

ELISA for IL-1ra in Protein Extracts

An ELISA was employed for IL-1ra, as previously described.[16] This assay uses a monoclonal rat anti-mouse capture antibody, a polyclonal goal anti-mouse detection antibody, and murine IL-1ra as a standard (R&D Systems). The murine ELISA was used because neither rabbit IL-1ra protein nor antibodies to rabbit IL-1ra are available. The extent of cross-reactivity of the anti-mouse antibodies with the rabbit protein is unknown definitively; however, the cDNA sequences are 76% identical between the mouse and the rabbit, indicating a high degree of homology. Whole cell protein extracts from the rabbit placentas and uteri were standardized to the total protein content of each extract and assayed for IL-1ra. The amount of IL-1ra was reported as ng IL-1ra per mg total protein. The sensitivity of the test in the mouse is reported to by 0.3 ng. The inter-assay variances are between 6 and 8%.

Statistical Considerations

A prestudy power analysis was performed based upon previous preliminary data for TNF-α levels in amniotic fluid.[13] The number of animals at the later intervals was increased to reflect the greater variability of TNF-α levels as time progresses after bacterial inoculation. Using this calculation, it was determined that studying 50 animals would provide 90% power and 33 animals would provide 80% power to determine a significant difference between animals at 0 hr compared to later time points, with $P \leq 0.05$ considered to be significant. Because of the clear trend in our data, demonstrating a rising pro-inflammatory response in the absence of changes in IL-1ra, we studied 33 instead of 50 animals.

Data were analyzed by a one- or two-tailed homoscedastic t-test to identify differences between the 0-hr animals and the rabbits from later time points, and reported as mean $\pm$ SEM. Statistical significance was defined as $P \leq 0.05$.

RESULTS

NF-κB Activation in the Rabbit Placenta in Response to Ascending Bacterial Infection

Fig. 1 illustrates an electromobility gel shift experiment demonstrating active NF-κB from placentas of three representative rabbits from each time point (0, 4, 8, 16, 24, and 30 hr) after cervical bacterial inoculation. These data indicate that activated NF-κB is present in very low amounts until after 8 hr post-inoculation and peaks at 16 hr post-inoculation. NF-κB exists in at least two active forms in the rabbit placenta, a p50/RelA heterodimer and a p50/50 homodimer. These bands are indicated as p50/RelA and p50/50, respectively, on Fig. 1.

Fig. 2 illustrates the identical data from Fig. 1 in graphic form, showing the relative densitometric units of the bands. Compared to time 0, an induction of more than 20-fold is noted for individual NF-kB dimers and for total NF-kB, where total NF-κB equals the additive densitometric readings for p50/50 and p50/RelA. When analyzed statistically compared to 0 hr, p50/50, p65/RelA, and total NF-κB demonstrate a significant increase at 16 hr using a one-tailed homoscedastic t-test, $P = 0.02$ for p50/50, $P = 0.04$ for p50/RelA, and $P = 0.02$ for total NF-κB.

TNF-α Levels in the Amniotic Fluid

With intrauterine infection caused by *E. coli*, mean amniotic fluid TNF-α concentrations at 0 hr increased 30-fold, from approximately 50 pg/mL to more than 1700 pg/mL (Fig. 3). Interestingly, maximal concentrations of TNF-α in amniotic fluid were measured at 16 hr; this was the time interval that coincided with peak NF-κB binding to DNA (Fig. 2). However, the variability in the data was large, possibly due to low bacterial growth in some animals, and the magnitude of the induction did not reach statistical significance

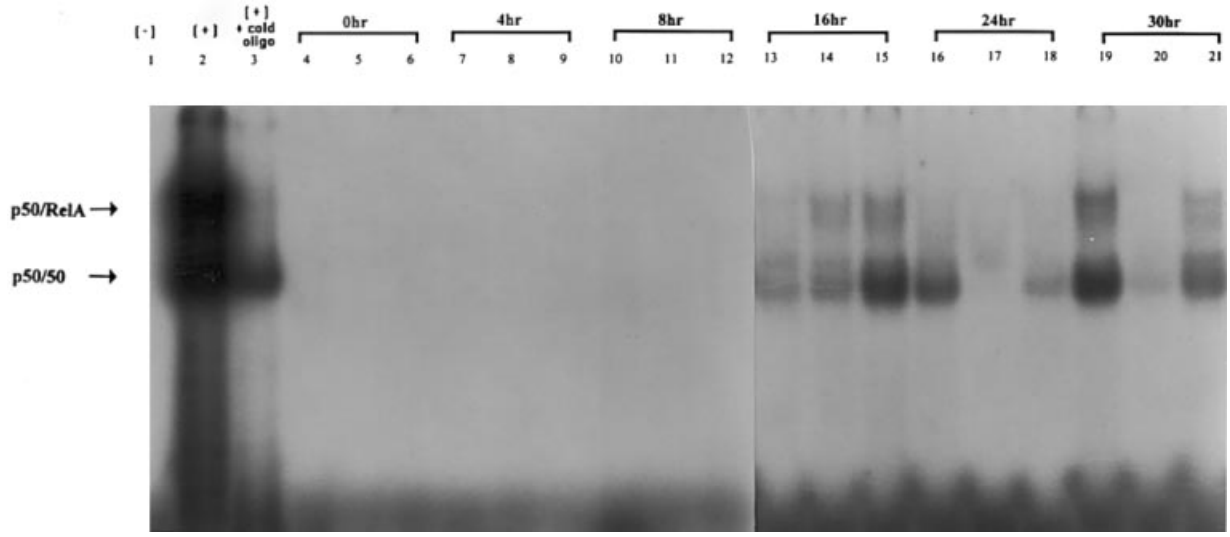

Fig. 1. Electromobility gel shift analysis of protein extract obtained from rabbit placentas at varying time points after cervical bacterial inoculation. Lane 1 = negative control without protein extract, 2 = LPS-activated 264 cells as a positive control, 3 = 264 cell extract with addition of cold NF-κB oligonucleotide probe demonstrating diminished intensity of the bands. Lanes 4–21 show placental extracts from three representative rabbits from each time point post-inoculation: lanes 4–6 = 0 hr, lanes 7–9 = 4 hr, lanes 10–12 = 8 hr, lanes 13–15 = 16 hr, lanes 16–18 = 24 hr, and lanes 19–21 = 30 hr. The top band represents the p50/RelA heterodimer, and the lower band represents the p50/50 homodimer.

using a one-tailed homoscedastic t-test ($P = 0.07$). When stratified for the bacterial colony count in the amniotic fluid as determined on plates seeded at necropsy, TNF-α levels clearly correlated with bacterial growth: the mean TNF-α level for fluid with ≤ 10 colonies was 84.15 ± 53.32 (SEM) compared to a mean of 1231.89 ± 508.81 (SEM) for fluid with more than 10 colonies ($P = 0.018$).

IL-1ra in the Placenta and the Uterus After Bacterial Inoculation

IL-1ra was assayed in protein extracts from placentas (Fig. 4) and uteri (Fig. 5). In general, the mean values were 1.5-fold higher in the placenta compared to the uterus. However, no significant change in IL-1ra occurred over time in response to infection. In fact, the IL-1ra levels remained remarkably constant over time: placental mean values were 23.98 ± 3.52 (SEM) ng/mg total protein at 0 hr, 28.08 ± 5.23 (SEM) ng/mg total protein at 16 hr ($P = 0.56$ compared to 0 hr), and 31.05 ± 4.50 (SEM) ng/mg total protein at 30 hr ($P = 0.30$ compared to 0 hr). Uterine extracts were similarly assayed, and again, no significant increase was noted. The mean value at time 0 was 9.71 ± 5.37 (SEM) ng/mg total protein compared to 16.15 ± 6.06 (SEM) ng/mg at 16 hr ($P = 0.47$) and 17.03 ± 3.85 (SEM) ng/mg at 24 hr ($P = 0.29$). All P-values were derived from two-tailed homoscedastic t-tests.

DISCUSSION

In a pregnant rabbit model of ascending intrauterine infection employing the virulent pathogen *E. coli*, the onset of infection is rapid and results in preterm delivery within 24–48 hr. This represents a severe model of infection in which an inflammatory response, under the primary control of the transcription factor NF-κB, is rapidly mobilized in reproductive tissues.

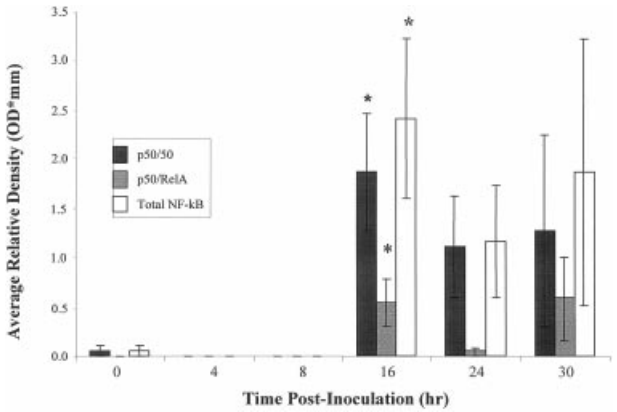

Fig. 2. Quantitation of the representative autoradiogram from Fig. 1 by densitometry. NF-κB isoforms p50/50 and p50/RelA as well as total NF-κB increased significantly with time, peaking at 16 hr after cervical bacterial inoculation. Values are reported as the mean ± SEM, * denotes $P \leq 0.05$ compared to 0 hr values.

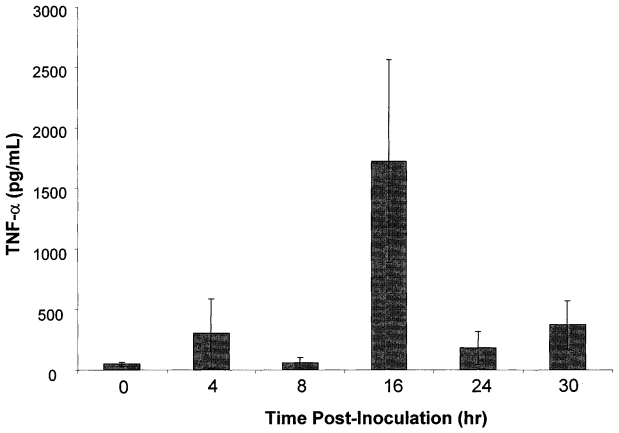

Fig. 3. TNF-α in amniotic fluid. TNF-α levels in the amniotic fluid reached a maximum at 16 hr post-cervical bacterial inoculation. Values are mean ± SEM, $P = 0.07$ for 16 hr compared to 0 hr.

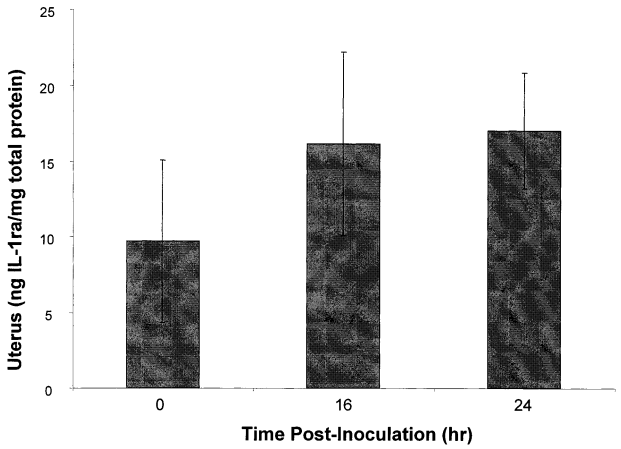

Fig. 5. IL-1ra in uterine protein extracts. Values are means ± SEM and are normalized for the total protein content of the tissue.

This study provides novel data on the time course of placental NF-κB p50/50 homodimer and p50/RelA heterodimer activation, which peak at 16 hr after cervical bacterial inoculation. The statistically significant rise in activated NF-κB is mirrored by an increase in mean TNF-α levels in the amniotic fluid, although the variability in the data precluded a statement of statistical significance for TNF-α induction at 16 hr compared to 0 hr ($P = 0.07$).

Unlike NF-κB, our data indicate that IL-1ra, a principal anti-inflammatory cytokine, does not increase in response to infection. These data, in which IL-1ra was assayed from placental and uterine protein extracts (and are corrected for the total protein content of the tissues), are similar to those reported in a murine model by Hirsch,[16] but differ from those reported from amniotic fluid in women by Romero[17] and in monkeys by Witkin.[18] For example, Witkin reported a significant rise in the concentration of IL-1ra from the amniotic fluid of monkeys by 30 hr after direct intra-amniotic group B streptococcal injection. Differences between the experimental paradigms, the bacterial organisms used, the species of animal, the site of assay (placental and uterine protein extracts versus amniotic fluid), and correcting for total protein content of tissues instead of measuring the absolute IL-1ra values in amniotic fluid may explain our differing findings. However, using our rabbit model of rapidly ascending infection, IL-1ra production from the placenta and from the uterus apparently cannot keep up with the overwhelming pro-inflammatory cascade initiated by NF-κB. It should be pointed out that use of a murine ELISA to measure rabbit IL-1ra, as was necessary in our study due to the unavailability of anti-rabbit antibodies, does not allow quantitation of absolute levels of cytokine within the tissue samples. However, it seems reasonable to assume that relative differences in tissue expression would be demonstrated by corresponding differences in assay results.

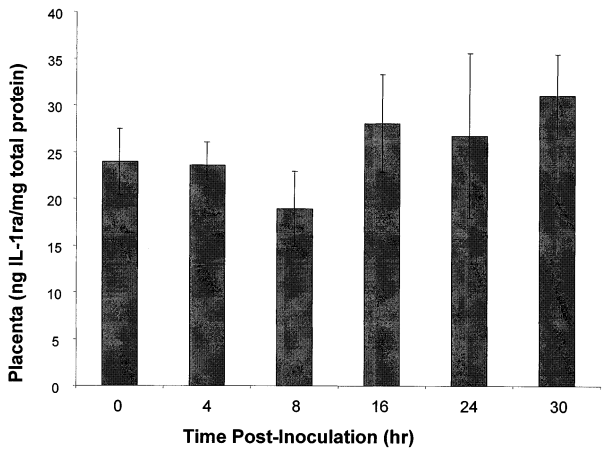

Fig. 4. IL-1ra levels in placental protein extracts. Values are means ± SEM and are normalized for the total protein content of the tissue.

Our data suggest that, particularly in severe infections, the induction of activated NF-κB and TNF-α outstrips the relatively constant levels of anti-inflammatory cytokines in the placenta and the uterus of the rabbit. The extensive literature documenting the role of cytokines in prostaglandin production provides a likely link between uterine contractility and an exuberant inflammatory response in animal models and in humans.[19,20] Therefore, we propose that an imbalance between pro- and anti-inflammatory cytokine production occurs in reproductive tissues. These data were derived from the rabbit; however, our findings may have implications for women in preterm labor as well. In particular, our findings regarding NF-κB activation are novel and provide an attractive "upstream" target for potential therapeutic interventions in the treatment of infection-mediated preterm labor.

Acknowledgments

The study was supported by the March of Dimes, Grant # 99a-0688-A, a contract from NICHD for the study of cytokines in fetal tissues, # 263-MD-731641-1, and NIH grants R29 CA67313-05 and R21 CA76508-02. Dr. Dennis Lezotte assisted with the statistical analyses.

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