# Effect of fetal bovine serum and heat-inactivated fetal bovine serum on microbial cell wall-induced expression of procoagulant activity by equine and canine mononuclear cells in vitro

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**Objective**—To determine the effect of fetal bovine serum (FBS) and heat-inactivated FBS (HI-FBS) on lipopolysaccharide (LPS)- and zymosan-induced procoagulant activity of equine and canine mononuclear cells.

**Sample Population**—Mononuclear cells from 18 horses and 3 dogs.

**Procedures**—Cells were incubated with various concentrations of FBS, HI-FBS, LPS, zymosan, polymyxin B, and anti–LPS-binding protein monoclonal antibody or combinations of these constituents. A 1-stage recalcification assay was used to determine procoagulant activity.

**Results**—Addition of FBS to media significantly increased procoagulant activity; equine and canine cells were stimulated by 1% and 10% FBS, respectively. Coincubation of cells with FBS and polymyxin B did not reduce this effect, suggesting that the response was not attributable to LPS contamination. Addition of HI-FBS to media did not stimulate procoagulant activity of equine or canine cells, and the sensitivity of the equine cells to LPS was significantly increased by HI-FBS. This increased LPS sensitivity was reduced 40% with monoclonal antibody directed against human recombinant LPS-binding protein. Increasing concentrations of HI-FBS significantly increased LPS– and zymosan-induced procoagulant activity of canine cells.

**Conclusion and Clinical Relevance**—Procoagulant activity production in equine and canine mononuclear cells was significantly increased by addition of FBS, whereas heat inactivation of FBS eliminated this effect. Heat inactivation did not eliminate the function of serum proteins involved in enhancement of LPS- and zymosan-induced procoagulant activity. Results suggest that HI-FBS can be used as a source of serum proteins that increase the sensitivity of mononuclear cells to bacterial and yeast cell wall components. (*Am J Vet Res* 2006;67:1020–1024)

Received September 27, 2005.
Accepted December 5, 2005.
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**Abbreviations**

| | |
|---|---|
| LPS | Lipopolysaccharide |
| TLR | Toll-like receptor |
| FBS | Fetal bovine serum |
| HI | Heat inactivated |
| DPBS | Dulbecco PBS |

Tissue factor is a 47-kd transmembrane glycoprotein that belongs to the cytokine receptor superfamily. Tissue factor, the main physiologic initiator of coagulation in vivo, is not normally expressed on cells within the circulation. However, monocytes and endothelial cells express tissue factor when exposed to factors that induce the tissue factor gene and synthesis of tissue factor glycoprotein, such as bacterial cell wall components. The most commonly studied of these factors are gram-negative bacterial LPS, tumor necrosis factor, interleukins, phorbol myristate acetate, and immune complexes.[1] Furthermore, coincubation of LPS-activated lymphocytes or platelets with monocytes induces expression of tissue factor by the monocytes.[2-4]

Tissue factor, also termed procoagulant activity, reflects the ability of the stimulated cells to shorten the coagulation time of plasma. Results of previous studies[2,3,5-8] performed in bovids, dogs, horses, and humans indicate that most of the LPS-induced procoagulant activity in peripheral blood is attributable to the activation of monocytes. Furthermore, activation of coagulation via tissue factor initiates other pathologic responses, including the development of disseminated intravascular coagulation, which is important in the pathogenesis of sepsis-associated organ injury.[9,10]

In addition to LPS, other microbial cell wall components can strongly induce inflammatory responses. In many species, monocytes and macrophages are activated by peptidoglycan or glycans of fungal origin. Zymosan, which comprises the cell wall of yeast, contains a variety of oligosaccharides and glycans from the yeast cell wall that stimulate inflammatory responses in leukocytes. Whereas LPS stimulates inflammatory responses through TLR 4, peptidoglycan and fungal glycan appear to induce inflammatory responses through several other receptors, including TLR 2-TLR 1, TLR 2-TLR 6, and nucleotide oligomerization domain (NOD).[11] Zymosan was included in the present study to allow comparison between LPS- and glycan-induced inflammatory responses in monocytes.

To date, studies regarding expression of tissue factor by equine monocytes have been performed under

serum-free conditions. Although the results of these studies[12,13] have provided insight into the role of tissue factor expression in conditions such as endotoxemia and colic, the experimental conditions do not reflect the physiology of this system. For example, serum-free conditions fail to provide sources of either LPS-binding protein or opsonin. Results of in vitro studies[6,14] performed with cells from other species have indicated that the presence of LPS-binding protein and opsonin markedly increase the sensitivity of the cells to endotoxin and zymosan, thereby reflecting one of the roles of these proteins in vivo. We are unaware of any studies regarding expression of tissue factor by canine monocytes.

As a readily available source of LPS-binding protein and opsonin for in vitro studies, many investigators include FBS in the culture medium, often at concentrations ranging from 1% to 10%. Because of the lack of commercial sources of either purified or recombinant equine or canine LPS-binding proteins, our intent was to determine the effects of FBS and HI-FBS on LPS- and zymosan-induced procoagulant activity of equine and canine mononuclear cells.

## Materials and Methods

**Animals and isolation of mononuclear cells**—Eighteen clinically healthy horses (Thoroughbred and Quarter Horse geldings and mares) from the University of Georgia's Equestrian Team and 3 clinically healthy male large-breed dogs owned by the University of Georgia were used in this study. The use of these animals was reviewed and approved by the Institutional Animal Care and Use Committee of the University of Georgia. Before inclusion in the study, the dogs were determined to be free of heartworm infection. Blood samples were obtained from the dogs at weekly intervals and identified by donor; experiments were repeated for each donor. Peripheral blood samples were obtained from horses and dogs via venipuncture after sterile preparation of the skin over a jugular vein. All blood samples were processed immediately after collection. Mononuclear cells were isolated via density gradient centrifugation involving a radiopaque contrast medium consisting of polysucrose and sodium diatrizoate.[a] Briefly, the syringes containing the blood from the horses were left undisturbed in an upright position for 20 to 30 minutes to allow settling of the erythrocytes. The leukocyte-rich plasma was then transferred to a pyrogen-free polypropylene tube, diluted 1:1 with DPBS solution without $Ca^{2+}$ or $Mg^{2+}$,[b] and gently layered over the radiopaque contrast medium. Each blood sample from the dogs was centrifuged at 1,200 × *g* for 10 minutes, after which the buffy coat was transferred to a pyrogen-free polypropylene tube. The buffy coat, diluted 1:1 with DPBS solution, was gently layered over the radiopaque contrast medium by use of a sterile 60-mL syringe barrel and an 18-gauge, 1.5-inch needle. All tubes containing either diluted equine leukocyte-rich plasma or canine buffy coat cells were centrifuged at 400 × *g* for 30 minutes, after which the mononuclear cell band was removed by aspiration and the cells were washed twice in DPBS solution. As a final step, the mononuclear cells were suspended in RPMI 1640[b] at a final concentration of $4 \times 10^6$ cells/mL. For both species, this isolation procedure consistently yielded > 98% mononuclear cells with > 95% viability, as determined from results of microscopic examination and exclusion of trypan blue dye.

**Cell incubation, sample handling, and assay for procoagulant activity**—For each species, triplicate sterile microfuge tubes containing the mononuclear cells were incubated for 5 hours at 37°C in 5% $CO_2$. At the end of the incubation, the cells were pelleted by centrifugation and the supernatants were removed; 250 µL of DPBS solution containing 0.1% Tween 20 was then added to each tube. The tubes were frozen at –80°C until assayed for procoagulant activity.

Immediately prior to determining procoagulant activity, the tubes were thawed and sonicated to lyse the cells. Procoagulant activity was determined by assessing the ability of cellular lysates to shorten the recalcification time of pooled equine plasma in a 1-stage clotting time assay.[14] Clotting times were determined in triplicate photoelectronically.[c] Procoagulant activities of samples were determined by reference to a standard curve generated by known dilutions (0.09% to 100%) of equine brain thromboplastin prepared in our laboratory.

**Preliminary experiments**—In preliminary experiments, either equine or canine mononuclear cells were incubated for 5 hours at 37°C in 5% $CO_2$ in RPMI alone or in RPMI containing FBS.[d] Cell lysates were obtained as described, and procoagulant activity in each sample was determined. Unexpectedly, inclusion of FBS markedly increased pro coagulant activity; the response was evident at concentrations as low as 1% FBS for equine mononuclear cells and 10% FBS for canine mononuclear cells.

**Effects of FBS and HI-FBS on procoagulant activity**—After completion of the preliminary experiments, further experiments were performed to determine whether heat inactivation would alter the procoagulant effect of FBS. Fifty milliliters of FBS was incubated for 30 minutes at 56°C, after which aliquots were placed in pyrogen-free tubes and stored frozen at –80°C until used. Equine and canine mononuclear cells were incubated for 5 hours at 37°C in 5% $CO_2$ in RPMI alone or RPMI containing FBS or HI-FBS (1% FBS or 1% HI-FBS for equine cells and 1% FBS, 10% FBS, 1% HI-FBS, or 10% HI-FBS for canine cells). To determine whether the cellular response to FBS might be a result of LPS contamination, additional experimental tubes containing polymyxin B (10 µg/mL)[a] were processed in parallel.

**Effects of HI-FBS on LPS-induced procoagulant activity of equine mononuclear cells**—Having determined that 1% HI-FBS alone did not increase procoagulant activity of equine mononuclear cells, an experiment was performed to determine whether 1% HI-FBS contained LPS-binding activity (ie, increased the sensitivity of equine mononuclear cells to LPS). Separate suspensions of mononuclear cells were incubated for 5 hours at 37°C in 5% $CO_2$ in RPMI alone, RPMI containing LPS, or RPMI containing LPS and 1% HI-FBS. Lipopolysaccharide (*Escherichia coli* O55:B5)[e] concentrations from 0.1 pg/mL to 1 ng/mL were used. At the end of the incubation, the samples were handled as described and procoagulant activity was determined.

After obtaining results suggesting that HI-FBS contained LPS-binding activity, the effects of an MAb directed against human recombinant LPS-binding protein on LPS-induced procoagulant activity of equine mononuclear cells in RPMI containing 1% HI-FBS were determined. This MAb has been shown to block binding of LPS to LPS-binding protein. Separate suspensions of mononuclear cells were incubated for 5 hours at 37°C in 5% $CO_2$ in RPMI alone; RPMI containing LPS (100 pg/mL) and 1% HI-FBS; RPMI containing LPS, 1% HI-FBS, and MAb (0.5, 5, or 50 pg/mL)[f]; or RPMI containing 1% HI-FBS and MAb (0.5, 5, or 50 pg/mL). At the end of the incubation, the samples were handled as described and procoagulant activity was determined.

**Effects of different concentrations of HI-FBS on LPS- and zymosan-induced procoagulant activity by canine**

**mononuclear cells**—In canine mononuclear cells, the effect of HI-FBS concentration on procoagulant activity induced by *E coli* O55:B5 LPS (1 ng/mL) or zymosan[g] (10 µg/mL) was investigated. Separate suspensions of mononuclear cells were incubated for 5 hours at 37°C in 5% $CO_2$ in RPMI alone, RPMI containing LPS or zymosan, or RPMI containing LPS or zymosan and different concentrations of HI-FBS (1%, 5%, or 10%). At the end of the incubation, the samples were handled as described and procoagulant activity was determined.

**Statistical analysis**—All data are expressed as means ± SE. Statistical analysis of the data was performed by use of a 1-way ANOVA followed by a post hoc Bonferroni multiple-comparison test. A value of $P < 0.05$ was considered to be significant. Statistical analysis of data was performed with statistical software programs.[h]

## Results

Incubation of equine mononuclear cells with 1% FBS alone caused a dramatic and significant ($P < 0.01$) increase in procoagulant activity (mean ± SE, 982.6 ± 264.6%), compared with procoagulant activity of cells incubated in RPMI alone (1.5 ± 0.3%). Coincubation of cells with 1% FBS and polymyxin B (a cationic antibiotic that avidly binds the lipid A region of LPS) did not alter this response (1,031.7 ± 277.6%). This finding suggests that the stimulatory effect of 1% FBS was not attributable to LPS contamination of the serum. In contrast, incubation of cells with 1% HI-FBS resulted in procoagulant activity (13.2 ± 5.4%) that was indistinguishable from values obtained with cells incubated in medium alone.

In medium alone, the procoagulant activity of canine mononuclear cells was 1.6 ± 0.1%. Compared with this value, incubation of canine mononuclear cells with 10% FBS, but not 1% FBS, resulted in a significant ($P < 0.01$) increase in procoagulant activity (316.4 ± 177.4% and 6.4 ± 1.8%, respectively); the response to 10% FBS was not altered by coincubation with polymyxin B (224.8 ± 129.9%). Incubation of the cells with either 10% or 1% HI-FBS resulted in procoagulant activity values (1.6 ± 0.1% each) similar to that obtained for cells incubated in medium alone.

Compared with values obtained for equine mononuclear cells incubated in RPMI without LPS (0 pg/mL), incubation of these cells with *E coli* O55:B5 LPS in RPMI resulted in a significant increase in procoagulant activity only at a concentration of 1 ng/mL (**Table 1**). In contrast, incubation of cells with *E coli* O55:B5 LPS in RPMI containing 1% HI-FBS resulted in significant ($P < 0.01$) increases in procoagulant activity that were evident at LPS concentrations as low as 10 pg/mL; with *E coli* O55:B5 LPS at concentrations of 10 pg/mL, 100 pg/mL, and 1 ng/mL in RPMI containing 1% HI-FBS, procoagulant activity was significantly higher than the value obtained for cells incubated with the same concentration of LPS in RPMI without HI-FBS. Coincubation of equine mononuclear cells with the MAb directed against human recombinant LPS-binding protein, 1% HI-FBS, and *E coli* O55:B5 LPS reduced the response obtained with HI-FBS and *E coli* O55:B5 LPS by approximately 40%, suggesting part of the effect of HI-FBS was attributable to LPS-binding protein.

Increasing concentrations of HI-FBS significantly increased LPS- and zymosan-induced procoagulant activity of canine mononuclear cells (**Table 2**). With LPS (1 ng/mL) or zymosan (10 µg/mL) as a stimulus, procoagulant activity in cells incubated in RPMI and 10% HI-FBS was significantly higher than values obtained in similar incubation conditions with 1% HI-FBS. Also, with LPS (1 ng/mL) as a stimulus, procoagulant activity in cells incubated in RPMI and 10% HI-FBS was significantly higher than the value for cells in RPMI without HI-FBS. These findings suggest that heat inactivation of FBS does not impair the functions of LPS-binding protein or opsonin.

Table 1—Mean ± SE procoagulant activity (%) of equine mononuclear cells (isolated from 5 horses) after 5 hours of incubation in RPMI with various concentrations of LPS with or without 1% HI-FBS.

| | Incubation condition | |
|---|---|---|
| **LPS concentration** | **RPMI** | **RPMI and HI-FBS** |
| 0 pg/mL | 1.1 ± 0.2 | 5.9 ± 2.6 |
| 0.1 pg/mL | 1.0 ± 0.0 | 8.8 ± 2.7 |
| 1 pg/mL | 0.9 ± 0.0 | 13.7 ± 5.2 |
| 10 pg/mL | 1.1 ± 0.2 | 211.0 ± 89.4[b] |
| 100 pg/mL | 16.4 ± 4.1 | 622.2 ± 154.0[b] |
| 1 ng/mL | 388.3 ± 45.2[a] | 688.6 ± 115.3[b] |

[a]Value significantly ($P < 0.01$) different from the value for cells in RPMI with no LPS (0 pg/mL). [b]Value significantly ($P < 0.01$) different from the value for cells in RPMI with no HI-FBS at the same concentration of LPS.

Table 2—Mean ± SE procoagulant activity (%) of canine mononuclear cells after 5 hours of incubation in RPMI with LPS (1 ng/mL) or zymosan (10 µg/mL) with or without HI-FBS (1%, 5%, or 10%) in independent experiments. The value for cells incubated in RPMI without stimulant in the LPS and zymosan stimulation experiments was 1.5 ± 0.1% and 1.4 ± 0.0%, respectively.

| | Stimulant | |
|---|---|---|
| **Incubation condition** | **LPS*** | **Zymosan†** |
| RPMI | 1.7 ± 0.3 | 4.68 ± 2.2 |
| RPMI and 1% HI-FBS | 12.9 ± 4.9 | 30.6 ± 27.9 |
| RPMI and 5% HI-FBS | 111.4 ± 35.4 | 174.7 ± 45.0 |
| RPMI and 10% HI-FBS | 347.9 ± 111.0[a,b] | 308.0 ± 90.8[c,d] |

*Results obtained from 2 samples collected from each of 3 dogs in 2 independent experiments. †Results from samples collected from 3 dogs.

[a]Value significantly ($P < 0.01$) different from values for cells in RMPI alone; RMPI and LPS; and RMPI, 1% HI-FBS, and LPS. [b]Value significantly ($P < 0.05$) different from the value for cells in RMPI, 5% HI-FBS, and LPS. [c]Value significantly ($P < 0.01$) different from values for cells in RMPI alone or RMPI and zymosan. [d]Value significantly ($P < 0.05$) different from the value for cells in RMPI, 1% HI-FBS, and zymosan.

## Discussion

Stimulation of monocytic phagocytes by pathogen-associated components, such as LPS, involves interactions among a group of proteins. These include LPS-binding protein in plasma and cell surface receptor proteins, such as CD14, TLR 4, and MD2.[15] In the sequence of events that occurs during cellular activation, LPS-binding protein acts as a transfer protein, shuttling LPS monomers from aggregates that form in aqueous environments to CD14 on the cell surface.[5,6,16] This function of LPS-binding protein substantially increases the sensitivity of the cells to LPS, allowing them to respond to lower concentrations (ie,

picograms rather than nanograms or micrograms/milliliter).[6,17] Consequently, in vitro studies designed to simulate these interactions should include a source of LPS-binding protein. Currently, there are no readily available sources of either purified or recombinant LPS-binding protein for inclusion in in vitro studies involving equine or canine cells.

To address this problem, we evaluated FBS as a potential source of LPS-binding protein for experiments with equine and canine mononuclear cells; FBS has been used successfully for this purpose in studies[5,6] with murine and human cells. In contrast to what has been reported for several other species, inclusion of FBS in the cell culture medium resulted in strong expression of procoagulant activity in the present study. In our initial studies, this response was striking when 1% FBS was used with equine mononuclear cells and when 10% FBS was used with canine cells. In fact, expression of procoagulant activity in response to FBS equaled or exceeded the response initiated by high concentrations of LPS in serum-free medium. Because no decrease in FBS-induced expression of procoagulant activity by either equine or canine mononuclear cells via coincubation with polymyxin B (to bind any LPS that might be contaminating the serum) was detected, we concluded that factors other than LPS were responsible for initiating the response. In an effort to reduce this stimulatory effect of FBS on the mononuclear cells, we heated the FBS for 30 minutes at 56°C and determined that this treatment eliminated the stimulatory effect of FBS on cells of both species. This approach has been used for years to destroy heat-labile components of the complement system and reduce contamination of serum by microorganisms.[18] However, in recent years, improvements in the filtering processes used to prepare commercially available FBS have reduced or eliminated the need to heat inactivate FBS before use.[19]

In the present study, we did not attempt to determine which components of FBS were reduced or eliminated by the heat-inactivation process. The point of interest was whether the heat-inactivation process would yield a product that had LPS-binding protein effects. Consequently, experiments were performed in parallel to determine whether inclusion of HI-FBS in the culture medium would increase the sensitivity of the mononuclear cells to LPS. Indeed, the equine mononuclear cells expressed significant amounts of procoagulant activity in the presence of 10 pg of LPS/mL when 1% HI-FBS was included in the medium, compared with the response to the same concentration of LPS in medium alone. Furthermore, the 40% reduction in procoagulant activity induced by LPS in the presence of an MAb directed against human recombinant LPS-binding protein suggests that at least some of the increase in cellular sensitivity to low concentrations of LPS associated with HI-FBS was attributable to LPS-binding protein.

The capacity of HI-FBS to facilitate activation of canine mononuclear cells by zymosan, a preparation of yeast cell wall, was also evaluated in the present study. Cell wall components from fungi and gram-positive bacteria appear to drive inflammatory responses in a similar fashion via cell surface receptors (including TLR 2) and by NOD family member–mediated signaling responses.[20-22] The transfer of these cell wall components is enhanced by factors in the serum that may include CD14 and LPS-binding protein.[23-25] In our study, the addition of HI-FBS enhanced the cellular response to zymosan in a concentration-dependent manner. Although the breakdown of C3 in serum may also act to opsonize zymosan, heat inactivation of the serum was sufficient to destroy complement activity, suggesting that other serum factors were important in facilitating the zymosan-induced induction of procoagulant activity in these experiments.[26]

a. Histopaque 1077, Sigma-Aldrich, St Louis, Mo.
b. Mediatech Inc, Herndon, Va.
c. ACL 1000, Instrumentation Laboratory, Lexington, Mass.
d. HyClone, Logan, Utah.
e. List Biologics, Campbell, Calif.
f. Cell Sciences, Canton, Mass.
g. Molecular Probes Inc, Eugene, Ore.
h. Graphpad Prism, version 3.0, Graphpad Software Inc, San Diego, Calif.

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