Effects of Melengestrol Acetate on L-selectin and β2-integrin Expression of Polymorphonuclear Leukocytes from Heifers Challenged with Lipopolysaccharide

M. E. Corrigan[1]
C. R. Wyatt[2]
E. R. Loe[1]
B. E. Depenbusch[1]
M. J. Quinn[1]
F.-P. Wang[2]
J. J. Higgins[3]
J.S. Drouillard[1]

[1]*Department of Animal Sciences and Industry*
Kansas State University, Manhattan 66506

[2]*Department of Diagnostic Medicine and Pathobiology*
Kansas State University, Manhattan 66506

[3]*Department of Statistics*
Kansas State University, Manhattan 66506

Contribution No. 06-232-J from the Kansas Agricultural Experiment Station, Manhattan.

KEY WORDS: Melengestrol acetate, polymorphonuclear leukocyte, lipopolysaccharide

ABSTRACT

Sixteen crossbred heifers were used in completely randomized design to determine the effects of melengestrol acetate (MGA) on polymorphonuclear leukocyte (PMN) L-selectin and β2-integrin expression and leukocyte numbers in heifers following Escherichia coli endotoxin (LPS) injection. On d 0, cattle were stratified by weight and randomly assigned, within strata, to diets they were fed throughout the trial. The diets consisted of 53.4% concentrate, 46.6% alfalfa hay, and either 0 or 0.5 mg MGA per heifer daily. On d 24, 1.0 µg/kg BW E. coli LPS was injected intravenously via jugular venipuncture. Blood samples were taken on d 0, 21, and 24 (4 h after LPS injection) and subjected to differential leukocyte counts as well as flow cytometric analysis of CD11b, CD11c CD18, CD62L, MHC1, and CD45 expression on PMN. Rectal temperatures were also recorded on d 24 (0 and 4 h after LPS injection). Following LPS injection, heifers in both treatment groups experienced decreases in circulating concentrations of all leukocyte populations (effect of sampling time $P < 0.01$), except eosinophils, which increased (effect of sampling time $P < 0.06$). Heifers fed MGA had higher overall circulating concentrations of neutrophils

Table 1. *Experimental diets (% of DM) Fed to Heifers Throughout the 20-d Experiment.*

Item	Treatment	
	MGA	No MGA
Basal diet (%)		
Steam-flaked corn	44.5	44.5
Alfalfa hay	46.6	46.6
Steep liquor	5.0	5.0
Urea	0.5	0.5
Soybean meal	0.3	0.3
Salt	0.3	0.3
Limestone	0.3	0.3
Premix[a]	2.5	2.5
MGA supplement, g/d[b]	113	
Control supplement, g/ day[b]		113

[a] *Formulated to provide 2293 IU/kg vitamin A, 11 IU/ kg vitamin E, 0.1 mg/kg Co, 10 mg/kg Cu, 0.7 mg/kg I, 6 mg/kg Mn, 0.3 mg/kg Se, 6 mg/kg Zn, 10 mg/kg tylosin, and 33 mg/kg monensin.*

[b] *Supplements consisted of ground corn with and without 0.5 mg/animal daily of MGA and were topdressed onto ration at feeding and mixed in by hand.*

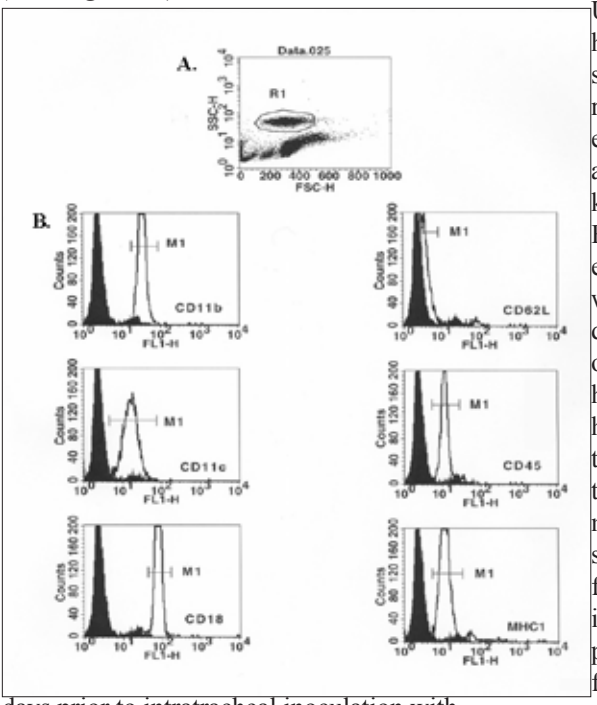
($P < 0.05$), but concentrations of other leukocytes were not different between treatments ($P > 0.13$). Treatment and sampling time differences in cells staining positive for various cell surface antigens appeared to be the result of nonspecific binding of isotype control antibodies. Mean fluorescence intensity (MFI) of isotype controls tended to be higher in heifers fed MGA ($P < 0.07$ and $P < 0.06$ for IgM and IgG, respectively) and sampling time effected MFI of isotype controls ($P < 0.01$ for both). Sampling time effected MFI of all cell surface antigens ($P < 0.01$) with values for CD11b, CD11c, CD18, MHC1, and CD45 increasing, and values for CD62L decreasing following LPS injection on d 24 compared to d 21. The only effect of treatment on MFI was for MHC1 with heifers fed MGA having overall lower values ($P < 0.03$) compared to controls. Lower rectal temperature in heifers fed MGA ($P < 0.02$) appeared to be the result of a more rapid return to normal levels following LPS challenge in those heifers compared to controls. These observations imply that MGA has effects on the febrile response to LPS in heifers and may affect antigen presentation of PMN to T-cells, but does not appear to effect cell adhesion molecule expression.

INTRODUCTION

Migration of neutrophils into inflamed tissues is initiated by weak interactions between L-selectin (cluster of differentiation 62L; CD62L) on the cell surface, and E-selectin and P-selectin on endothelial cells. Activation of β2-integrins on the neutrophil surface is caused by chemoattractants such as IL-8, thereby allowing them to interact with cellular adhesion molecules (CAM) expressed on activated endothelial cells, resulting in firm adhesion. β2-integrins consist of an alpha subunit associated with a CD18 beta subunit. Ligands for various β2-integrins include ICAM-1, ICAM-2, fibrinogen, and the complement protein iC3b (Seely et al., 2003).

Melengesterol acetate is a synthetic progestin commonly used to suppress estrus in feedlot heifers. Previous research from our lab has shown that heifers fed MGA for 34 days prior to Escherichia coli lipopolysaccharide (LPS) injection exhibited increases in circulating neutrophils of 8.4 and 90% 2 and 4 h after challenge, respectively. In contrast, control heifers experienced decreases in circulating neutrophils of 43.6 and 4.6% 2 and 4 h post-challenge, respectively. In another experiment, heifers fed MGA for 14

***Figure 1**. Dot plot (A.) and fluorescence intensity histograms (B.) of blood samples analyzed by flow cytometery for CD11b, CD11c, CD18, CD62L, CD45, and MHC1. A: Polymorphonuclear leukocytes were gated out (Region 1; R1) based on their forward scatter (x-axis; FSC-H) and side scatter (y-axis; SSC-H) characteristics. B: Expression of cell surface molecules was determined by creating histograms of log fluorescence intensity (x-axis; FL1-H) and frequency (y-axis; Counts) of isotype control antibody labeled cells (dark peaks). Cell surface antigen antibody labeled peaks (transparent peaks) where overlayed onto the histograms and the left boundary for region of positive fluorescence was set where isotype control and surface antigen peaks separated and the right boundry was set where the surface antigen peak ended (M1; Figure 1B).*



days prior to intratracheal inoculation with Mannheimia haemolytica experienced 142 and 149% increases in circulating neutrophils 12 and 24 h post-challenge, while control heifer experienced increases in circulating neutrophils of 91 and 84% 12 and 24 h post-challenge, respectively (Corrigan et al., 2005).

Kontula et al. (1983) found that some synthetic progestins bind to the glucocorticoid receptor on human mononuclear leukocytes. Administration of glucocorticoids causes elevation of circulating neutrophils that is partially mediated through reduced expression of CD62L and CD18 (Burton et al., 1995). The study presented herein was conducted to examine effects of MGA on L-selectin and β2-integrin expression on peripherial blood polymorphonuclear leukocytes (PMN) from heifers following LPS challenge.

MATERIALS

Animals and Experimental Design

Procedures for this study were approved by the Kansas State University Institutional Animal Care and Use Committee. Sixteen crossbred heifers were purchased from a local sale barn and used in a completely randomized design to determine the effects of MGA on PMN L-selectin and β2-integrin expression and leukocyte numbers in heifers following E. coli LPS injection. On d 0 of the experiment, cattle were individually weighed, stratified by weight, and randomly assigned, within strata, to diets of 53.4% concentrate, 46.6% alfalfa hay, and either 0 or 0.5 mg MGA per heifer daily (Table 1) fed for the duration of the experiment. Cattle were then randomly assigned to 1.5 m × 3.7 m individual pens in a metal barn with slatted concrete floors. Diets were fed once daily at approximately 0900, in amounts that led to only traces of previous food being present at the next feeding time. On d 24, 1.0 μg/kg BW E. coli LPS (Serotype 055:B5; Alexis Biochemical Corp., San Diego, CA) was injected intravenously via jugular venipuncture. Rectal temperatures were recorded on d 24 (immediately prior to and 4 h after LPS injection). Blood samples, taken on d 0, 21, and 24 (4 h after LPS injection), were used for determination of fibrinogen concentration, differential leukocyte counts, and flow cytometric analysis of CD11b, CD11c CD18, CD62L, major histocompatibility

***Table 2**. Circulating leukocyte and fibrinogen concentrations of heifers fed 0 or 0.5 mg of melengestrol acetate daily for 24 d and injected with 1.0 µg/kg BW E. coli (Serotype 055:B5) lipopolysaccharide on d 24.*

		Day			*P-value*	
Item/Treatment[a]	0	21	24[b]	TRT[a]	Day	TRT × Day
WBC, per µL[c]				0.32	< 0.01	0.66
No MGA	8310	9440	2480			
MGA	9280	1031	2430			
Lymphocytes, per µL[c]				0.88	< 0.01	0.27
No MGA	5925	6813	1993			
MGA	6438	6363	1681			
Monocytes, per µL[c]				0.13	< 0.01	0.27
No MGA	261	380	24			
MGA	456	386	103			
Seg. neutrophils, per µL[c]				0.04	< 0.01	0.27
No MGA	2075	2111	168			
MGA	2188	3313	378			
BAND neutrophils, per µL				-	-	-
No MGA	0	0	0			
MGA	0	0	6.25			
Eosinophils, per µL[d]				0.58	0.06	0.39
No MGA	59	138	349			
MGA	173	233	250			
Basophils, per µL[e]				0.78	< 0.01	0.22
No MGA	25	86	10			
MGA	64	38	5			
Fibrinogen, mg/dL				0.09	0.55	0.46
No MGA	463	338	388			
MGA	500	525	450			

[a]Heifers fed 0 or 0.5 mg/d melengestrol acetate
[b]Sample taken 4 h following intravenous injection of E. coli lipopolysaccharide.
[c]Days 0 and 21 > d 24 (P < 0.01).
[d]Day 0 < d 24 (P = 0.02).
[e]Day 21 > d 24 (P < 0.01).

complex-1 (MHC1), and CD45 expression on PMN.

Blood Sample Collection and Leukocyte Isolation

All blood samples were taken via jugular venipuncture and collected in evacuated tubes (Becton Dickenson Vacutainer Systems, Franklin Lakes, NJ). Blood for flow cytometry was collected in tubes containing acid citrate dextrose as an anticoagulant and diluted 3:1 with 0.87% tris-NH4CL (8.7 g per L NH4CL, 1.21 g per L tris base) to lyse erythrocytes. Next, leukocytes were washed

three times, counted in a hemocytometer chamber, and diluted to a final concentration of between 1× 106 and 1 × 107 cells per mL. Whole blood for differential leukocyte counts was collected in tubes containing EDTA as an anticoagulant.

Monoclonal Antibodies and Immunostaining Procedure

Monoclonal antibodies (Mabs) to bovine leukocyte differential molecules used to evaluate granulocyte population included: mouse anti-CD11b (cell line MM12A, IgG1; concentration used = 10 µg/mL; VMRD, Pullman, WA), mouse anti-CD11c (cell line BAQ153A, IgM; concentration used = 20 µg/mL; VMRD, Pullman, WA), mouse anti-CD18 (cell line BAQ30A, IgG1; concentration used = 10 µg/mL; VMRD, Pullman, WA), and mouse anti-CD62L (cell line DU1-29, IgG1; concentration used = 20 µg/mL; VMRD Pullman, WA). Monoclonal antibodies used for leukocyte verification were mouse anti-bovine CD45 (clone CC1, IgG1; concentration used = 500 µL/mL; Serotec, Kidlington, Oxford, UK) and mouse anti-ovine MHC1 (clone 41.17, IgG; concentration used = 15 µg/mL; Serotec, Kidlington, Oxford, UK). Monoclonal antibodies used as isotype controls were

Table 3. *Percent of peripherial blood polymorphonuclear cells positive for cell surface antigens in heifers fed 0 or 0.5 mg of melengestrol acetate daily for 24 d and injected with 1.0 µg/kg BW E. coli (Serotype 055:B5) lipopolysaccharide on d 24.*

		Day			P-value	
Item/Treatment[a]	0	21	24[b]	TRT[a]	Day	TRT × Day
CD11[bc]				0.05	< 0.01	0.06
No MGA	95.90	86.05	91.50			
MGA	94.96	92.32	95.42			
CD11[cd]				< 0.01	< 0.01	< 0.05
No MGA	84.94	71.62 [i]	28.07 [i]			
MGA	86.78	86.50 [j]	44.44 [j]			
CD18[e]				< 0.10	< 0.01	0.08
No MGA	98.36	85.99	95.09			
MGA	97.39	92.32	96.64			
CD62L[f]				0.55	< 0.01	0.59
No MGA	24.09	24.88	0			
MGA	17.96	21.28	1.85			
CD45[g]				0.68	0.02	0.05
No MGA	90.60	77.09	44.42			
MGA	72.87	78.2	70.41			
MHC1[h]				0.23	< 0.10	0.40
No MGA	84.21	73.04	57.46			
MGA	64.88	66.58	58.98			

[a]Heifers fed 0 or 0.5 mg/d melengestrol acetate
[b]Sample taken 4 h following intravenous injection of E. coli lipopolysaccharide.
[c]Day 0 > d 21 (P < 0.01); d 21 < d 24 (P < 0.10).
[d]Days 0 and 21 > d 24 (P < 0.01); d 0 > d 21 (P < 0.05).
[e]Days 0 and 24 > d 21 (P < 0.01); d 0 > d 24 (P < 0.05).
[f]Days 0 and 21 > 24 (P < 0.01).
[g]Day 0 > d 21 (P < 0.01); d 21 > d 24 (P < 0.05).
[h]Day 0 > d 24 (P < 0.05)
[ij]Means in column representing same surface antigen differ (P < 0.01).

mouse anti-canine B lymphocyte subpopulation (cell line CADO34A, IgM; concentration used = 20 µg/mL; VMRD, Pullman, WA) and mouse anti-canine CD45-like (cell line CADO19A, IgG1; concentration used = 15 µg/mL; VMRD, Pullman, WA). The secondary antibody used was FITC conjugated goat F(ab')2 anti-mouse IgG + IgM (H+L; product code M35201; concentration used = 10 µg/mL; Caltag Laboratories, Burlingame, CA). Mouse anti-human CD11a (clone HI111, IgG1; concentration used = 20 µg/mL; Biolegend, San Diego, CA) was also used, but there was an apparent lack cross-reactivity of the antibody in cattle, as null values were recorded for every an-

Table 4. *Mean fluorescence intensity of peripherial blood polymorphonuclear cells labeled for cell surface antigens in heifers fed 0 or 0.5 mg of melengestrol acetate daily for 24 d and injected with 1.0 µg/kg BW E. coli (Serotype 055:B5) lipopolysaccharide on d 24.*

		Day			*P-value*	
Item/Treatmenta	0	21	24b	TRT[a]	Day	TRT × Day
IgM isotype controlc				0.05	< 0.01	0.06
No MGA	4.47	13.50	37.91			
MGA	5.73	10.22	28.00			
IgG isotype controld				< 0.01	< 0.01	< 0.05
No MGA	4.28	16.40	20.23			
MGA	4.59	10.31	15.21			
CD11be				< 0.10	< 0.01	0.08
No MGA	32.13	53.80	83.63			
MGA	27.98	50.15	90.74			
CD11ce				0.55	< 0.01	0.59
No MGA	15.93	28.67	42.78			
MGA	15.51	28.95	36.18			
CD18e				0.68	0.02	0.05
No MGA	74.64	135.15	170.26			
MGA	66.53	127.11	189.92			
CD62Lz				0.23	< 0.10	0.40
No MGA	4.63	5.00	0			
MGA	8.74	4.71	0.92			
CD45e						
No MGA	10.31	18.46	25.27			
MGA	8.18	15.69	25.69			
MHC1e						
No MGA	10.20	21.23	51.06			
MGA	7.74	12.85	40.56			

[a]Heifers fed 0 or 0.5 mg/d melengestrol acetate.
[b]Sample taken 4 h following intravenous injection of E. coli lipopolysaccharide.
[c]Day 0 and 21 < d 24 (P < 0.01); d 21 < d 24 (P < 0.01).
[d]Day 0 < d 21 and d 24 (P < 0.01).
[e]Day 0 and 21 < d 24 (P < 0.01); d 0 < d 21 (P < 0.01).
[f]Day 0 > d 24 (P < 0.10); d 21 > d 24 (P < 0.01).

Table 5. *Rectal temperatures of heifers fed 0 or 0.5 mg of melengestrol acetate daily for 24 d and injected with 1.0 μg/kg BW E. coli (Serotype 055:B5) lipopolysaccharide on d 24.*

	Hours After LPS Injection			P-value	
Treatmenta	0	4	TRTa	Hour	TRT × Hour
			0.02	0.15	0.17
No MGA	39.5	39.5			
MGA	39.6	40.1			

imal at every observation point.

Cells were labeled for flow cytometry in 96-well micro plates. Briefly, 100 μL of the cell suspension and 50 μL of the primary Mabs dilutions were added to wells and incubated on ice for 30 min. Cells were washed three times and incubated on ice with the 100 μL of the diluted FITC conjugated secondary antibody in the dark for 30 min. Cells were again washed three times and fixed in 4% formalin phosphate buffered solution.

Flow Cytometry

Flow cytometry and data analysis were performed using a FACSCalibur flow cytometer and CellQuest software (Beckton Dickenson Immunocytometry Systems, San Jose, CA). A minimum of 10,000 events were acquired per sample. Dot plots were used to gate out PMN from other cells based on their forward and side scatter characteristics (Figure 1A). Histograms of the labeled PMN peaks were created and over-layed onto isotype control peaks. The left boundary of the region of positive cells was set where isotype control Ab and surface antigen Ab peaks separated, and the right boundry was set where the labeled cell peak ended (M1; Figure 1B). The percentage of cells from isotype control samples in M1 was subtracted from the percentage of cells labeled with antibodies to cell surface antigens with the same isotype in M1 to determine percentage of positive cells (+). Mean fluorescence intensity (MFI) was also calculated for cells in the positive region.

Differential Leukocyte Counts and Fibrinogen

Differentiated leukocyte counts were performed on smear slides stained with a modified Wright stain. Fibrinogen was measured with a refractometer using methods described by Jain (1986).

Statistical Analysis

Differential leukocyte counts, fibrinogen, cell surface antigens, and temperature were analyzed as repeated measures (completely randomized design) using the PROC MIXED procedure of SAS (SAS Inst. Inc., Cary, NC). The model effects included treatment, sampling time, and treatment × sampling time. An unstructured variance-covariance matrix was assumed for the repeated measurements.

RESULTS

Differential leukocyte counts and Fibrinogen

Data for differential leukocyte counts and fibrinogen are presented in Table 2. Data for band neutrophils did not meet the criteria for a repeated measures analysis in SAS. This is likely do to high number of null values recorded for this variable, as positive values were only observed in three animals in the MGA treatment group (20, 20 and 10 cells per μL) following the LPS challenge on d 24. Consequently, data for band neutrophils were excluded from the final analysis. There was an effect of sampling time on white blood cells, segmented neutrophils, lymphocytes, monocytes, and basophils ($P < 0.01$ for each). Sampling time tended to effect eosinophil concentrations ($P = 0.06$). Concentrations of all white blood cell populations except for eosinophils and basophils were lower on d 24 compared to d 0 and 21 ($P < 0.01$). Concentrations of eosinophils were higher on d 24 compared to d 0 ($P = 0.02$) and concentrations of basophils were lower on d 24 compared to d 21 ($P < 0.01$) but not d 0. The effect of treatment on segmented neutrophils ($P = 0.04$) was observed as heifers fed MGA having higher concen-

trations compared to controls. However, no effect of treatment was observed for any other leukocyte population. No of treatment × sampling time was observed for any of the leukocyte populations.

There were no effects of sampling time or treatment × sampling on fibrinogen concentrations. However, there was a tendency for treatment to effect fibrinogen concentrations with heifers fed MGA having higher overall concentrations ($P = 0.09$).

Cell Surface Antigens

Data for percentage of PMN staining positive for cell surface antigens are presented in Table 3. There were effects of sampling time on the percentages of CD11b+, CD11c+, CD18+, CD62L+ ($P < 0.01$ for each), and CD45+ ($P = 0.02$) PMN. Sampling time tended to affect percentage of MHC1+ ($P < 0.10$) PMN. On d 24, percentages of CD11c+ ($P < 0.01$), CD62L+ ($P < 0.01$), and CD45+ ($P < 0.05$) PMN were lower compared to d 0 and 21. The lowest values for percent of CD18+ PMN were recorded on d 21 ($P < 0.01$ when compared to d 0 and 24). There was an effect of treatment on percentage of CD11c+ ($P < 0.01$) PMN, with heifers fed MGA having higher overall values compared to controls. Heifers fed MGA also tended to have a higher percentage of CD11b+ ($P = 0.05$) and CD18+ ($P < 0.10$) PMN compared to controls. Treatment × sampling time affected the percentage of CD11c+ ($P < 0.05$) PMN, and heifers fed MGA had higher percentages of CD11c+ PMN on d 21 and 24 ($P < 0.01$) compared to controls. There also tended to be effects of treatment × sampling time on the percent of CD11b+ ($P = 0.06$), CD18+ ($P = 0.08$) and CD45+ ($P = 0.06$) PMN.

Data for MFI of surface antigens are presented in Table 4. Effects of sampling time were observed for MFI of all cell surface antigens ($P < 0.01$), with values being higher on d 24 compared to d 21 and 0 for CD11b ($P < 0.01$), CD11c ($P < 0.01$), CD18 ($P < 0.01$), MHC1 ($P < 0.01$), and CD45 ($P < 0.05$). CD62L MFI values were lower on d 24 compared to d 21 ($P < 0.01$) and tended to be lower on d 24 compared to d 0 ($P < 0.01$). The only effect of MGA level observed for MFI was on MHC1, with heifers fed MGA having overall lower values ($P = 0.02$) compared to controls. No effects of treatment × sampling time on MFI were observed for any of the cell surface antigens.

Rectal Temperature

Rectal temperatures were recorded on d 24, 0, and 4 h after LPS injection (Table 5). The treatment effect on rectal temperature was that heifers fed MGA had lower overall temperatures ($P = 0.02$) compared to controls. However, no effects of sampling time, or treatment × sampling time were observed. The more remarkable observation is the absence of a difference in rectal temperatures in heifers fed MGA 0 and 4 h after LPS injection (39.5 °C for both time points). Rectal temperatures in heifers not fed MGA were elevated 0.5 °C, 4 h after LPS injection when compared to 0 h levels (39.6 and 40.1° C, 0 and 4 h after LPS injection, respectively).

DISCUSSION

In this experiment, PMN were not separated into neutrophil, basophil, and eosinophil populations for flow cytometric analysis of surface antigens. Based on data from previous trials in our laboratory, it was expected that segmented neutrophils would represent a high percentage (approximately 95%) of PMN following LPS challenge. In this trial, however, post-challenge segmented neutrophil concentrations represented 59.7% and 31.9%. Eosinophils represented 39.5% and 66.2% of the post-challenge peripherial blood PMN in heifers fed MGA versus no MGA, respectively. There is a possibility that differences in cell surface antigen expression by neutrophils and eosinophils following activation by LPS may have influenced treatment means. This must be considered, as eosinophils are cells that are typically associated with allergic responses and parasitic infection. Higher percentages of eosinophils in the total PMN population observed in this trial were due to decreases in neutrophil concentrations following LPS

injection. They were not the result of large increases in circulating eosinophil concentrations. Heifers in both treatment groups experienced marked leucopenia 4 h after LPS challenge as a result of decreases in circulating neutrophil concentrations. In our previous study utilizing an LPS challenge model, overall neutrophil concentrations increased following injection. Considerable decreases in neutrophil concentrations in this study indicate that activation of the endothelium and subsequent neutrophil attachment was higher than in the previous study.

Heifers fed MGA had higher overall levels of neutrophils compared to controls (Table 2). As discussed in the introduction, we have previously observed higher concentrations of neutrophils in peripheral blood samples taken from heifers fed MGA and subjected to either LPS injection or M. haemolytica challenge when compared to control heifers. In one previous study, levels of neutrophils following LPS challenge were substantially increased for heifers fed MGA, while levels were slightly decreased for control heifers (our unpublished observations). In another study in which heifers were subjected to M. haemolytica challenge, both MGA and control heifers experienced an increases in peripheral blood neutrophils, but the increase was numerically greater for heifers fed MGA compared to control heifers (Corrigan et al., 2005). This may indicate a reduced expression of CAM on the endothelium, as a reduction of E-selectin expression on endothelial cells stimulated with IL-1 in the presence of progesterone has been observed (Aziz and Wakefield, 1996). Additionally, treatment with medroxyprogesterone actate in postmenopausal women receiving estrogen caused a reduction in blood concentrations of soluble VCAM-1, intracellular adhesion molecule-1 (ICAM-1), and E-selectin. Lower blood concentrations of these molecules indicate lower expression on the endothelium since they are shed from endothelial cells within 24 h (Wakatsuki et al., 2002). These reductions may be the indirect result of decreased inflammation that occurs with administration of progesterone and some synthetic progestins, as it has been reported that progesterone has no effect and some synthetic progestins increase ICAM-1 and VCAM-1 expression (Tatsumi et al., 2002). It is conceivable that greater neutrophil concentrations in heifers fed MGA are indicative of altered recruitment of cells to the endothelium. However, results from this study suggest that it is not likely due to an effect on L-selectin and β2-integrin expression on PMN. Alternatively, progesterone has been shown to delay apoptosis in neutrophils (Molloy et al., 2003), potentially explaining the observations of higher levels of neutrophils in heifers fed MGA.

Antibodies to MHC1 and CD45 were included in this experiment for verification of cells, as all leukocytes express both surface antigens. The fact that the percentage of CD45+ and MHC1+ was not 100% at every sampling point indicates that there was a deficiency in methods used to detect the actual percentage of PMN positive for these surface antigens. Any observed differences between treatments or sampling times may be due to nonspecific binding of isotype control antibodies, as MFI tended to be lower in heifers fed MGA ($P = 0.07$ and $P = 0.06$ for IgM and IgG, respectively; Table 3). There was a significant effect of sampling time on MFI for both isotype controls ($P < 0.01$). With this in mind, the decrease in percentage of cells staining positive for CD45 following LPS challenge does not appear to be accurate. Increases in MFI of MHC1 and CD45 on PMN following LPS challenge would be expected, however. Although no reports of increases in CD45 expression on bovine PMN when activated with LPS were found, increases in CD45 expression on human neutrophils following activation with formyl-methyl-leucyl-phenylalanine (fMLP) plus platelet-activating factor or fMLP plus cytochalasian B have been reported (Kuijpers et al., 1991). MHC1 is important in antigen presentation to CD8(+) T cells and it has been demonstrated that neutrophils have the ability to present bacterial antigens to CD8(+) T cells via MHC-1 (Potter and Harding, 2001; Tvinnereim et al., 2004).

The shift in pregnant women from a T helper type 1 (Th1), cell mediated response, to a T helper type 2 (Th2), humoral response, is thought to be mediated by progesterone. Miyaura and Iwata (2002) observed that culturing T-cells with progesterone led to an increase in the development of IL-4 and IL-10 producing cells. Interleukin-4 down-regulates IL-1, IL-6, and tumor necrosis factor-α (TNF-α) production in macrophages (Tizard, 2004), and causes IgG1 production that is associated with a Th2 type response (Estes and Brown, 2002). Interleukin-10 is known to inhibit production of IL-12, a Th1-promoting factor (Moore et al., 2001). Lower MFI of MHC1 on neutrophils from heifers fed MGA may point to another mechanism through which progestins mediate the shift to a Th2 type response.

The percentages of cells staining positive for β2-integrins again bring to question the ability of methods used to detect the actual percentage of cells positive for cell surface antigens. Burton et al. (1995) found 100% expression of CD18 on bovine neutrophils, even following a severe reduction in MFI of CD18 by administration of glucocorticoids. The observations of the lowest percentage of CD11c+ on d 24 and the lowest percentage of CD18+ on d 21 again may reflect changes in nonspecific binding of the isotype controls. Overall higher percentages of CD11b+, CD11c+, and CD18+ PMN in samples taken from heifers fed MGA may also reflect higher nonspecific binding of isotype controls to cells taken from those animals. Another possible explanation could be an indirect effect of decreased expression of receptors for those cell surface molecules on the endothelium in those heifers, but this is unlikely as MFI for CD11b, CD11c, and CD18 were not different between treatments.

Increases in MFI of CD11b, CD11c, and CD18 on d 24, following LPS injection, were expected. Diez-Fraile et al. (2003) observed higher MFI of CD11b and CD18 on peripheral blood neutrophils taken from dairy cows following injection with E. coli LPS in the left front and rear quarters of the mammary gland. Loike et al. (1991) observed that higher adhesion of human neutrophils to fibrinogen coated culture plates caused by stimulation with TNF-α was significantly decreased by addition of anti-CD11c Mab. This indicates that the increase in adhesion was the result of greater expression of CD11c caused by TNF-α. Increased circulating TNF-α concentrations following LPS challenge in calves have been reported (McMahon et al., 1998; Sartin et al., 2003). These findings are a possible explanation for the increases in MFI of CD11c on PMN on d 24 following LPS injection. CD11b/CD18 and CD11c/CD18 are both receptors for fibrinogen (Seely et al., 2003), but it is not likely that fibrinogen levels had any effect on MFI of these surface antigens, as no effect of sampling day on fibrinogen levels was observed.

Decreases in the percentage of CD62L+ PMN and MFI of CD62L on d 24 following LPS challenge were not surprising since proteolytic cleavage of CD62L from activated leukocytes is well documented (Chen et al. 1995; Kahn et al. 1994). Shedding of CD62L is thought to mediate rolling of leukocytes to sites of inflammation. Hafezi-Moghadam and Ley (1999) found that inhibition of CD62L shedding on leukocytes caused a significant reduction in rolling velocity of leukocytes in mice. Pre-challenge values for percent of CD62L+ PMN appear to reflect a failure of the methods used to provide accurate estimates since other reports of unstimulated bovine neutrophils and PMN positive for CD62L are near 100% (Burton et al., 1995; Diez-Fraile; 2003).

An increase in rectal temperature would be expected following LPS (Sartin et al., 2003), but no effect of sampling time on rectal temperature was observed. It is conceivable that this was due to a deficiency in the observational protocol to detect the peak in rectal temperature post-challenge. The lack of a numerical difference of rectal temperatures taken 0 and 4 h after LPS injection in heifers fed MGA is likely the result of rectal temperatures in those heifers

returning to normal levels more quickly than in control heifers, rather than the absence of an increase in rectal temperature following LPS challenge. Sartin et al. (2003) found that calves implanted in the ear with a slow-release formulation of 200 mg of progesterone and 20 mg of 17 β-estradiol benzoate returned to normal temperatures more rapidly following intravenous injection of 0.6 μg/kg of BW of E. coli LPS when compared to control calves subjected to LPS injection. We have also observed that heifers fed MGA for 34 d prior to intravenous injection of LPS tended to have a reduced change in temperature post-challenge when compared to controls (our unpublished observations).

Medroxyprogesterone acetate and megesterol acetate have been shown to have a higher relative binding affinity for the glucocorticoid receptor of human mononuclear leukocytes than cortisol (Kontula et al., 1983). Zavy (1991) reported decreases in blood cortisol levels following administration of high daily dosages (10 mg/steer and 100 mg/steer) of MGA, indicating a negative feedback mechanism possibly mediated through binding of the glucocorticoid receptor. Melengesterol acetate fed at the labeled dose does not appear to have comparable effects to dexamethasone and cortisol on MFI of cell adhesion molecules observed by Burton et al. (1995). However, this does not rule out the possibility of glucocorticoid actions of MGA. Plasma levels of MGA in heifers fed 0.5 mg/d have been shown to be approximately 30 pg/mL (Hageleit et al., 2000). In the Burton et al. study, on the other hand, blood cortisol levels peaked at 62 ng/mL in cortisol treated cows, and dexamethasone was injected at a dose of 0.04 mg/kg BW/d for three consecutive days. Alternatively, dissociative glucocorticoid activity of medroxyprogesterone acetate in normal human lymphocytes has been reported (Bamberger et al., 1999), and thus the lack of an effect of MGA on L-selectin and β2-integin expression does not rule out the possibility of other glucocorticoid-like effects.

Implications

Treatment and sampling time differences in cells that stain positive for various cell surface antigens observed in this trial appear to be the result of nonspecific binding of isotype control antibodies, rather than an actual effect of treatment or sampling time. No treatment differences in MFI of CD11b, CD11c, CD18, CD62L, or CD45 were observed, indicating that higher circulating neutrophil concentrations in heifers fed MGA seen in this and previous trials are not attributable to L-selectin or β2-integrin expression on polymorphonuclear leukocytes. Lower rectal temperatures in heifers fed MGA are likely the result of a more rapid return to normal temperature level following LPS challenge. This observation along with lower overall MHC1 MFI in heifers fed MGA, implies that MGA has effects on the febrile response to LPS and may affect antigen presentation of PMN to T-cells.

REFERENCES

1. Aziz KE, Wakefield D. Modulation of endothelial cell expression of ICAM-1, E-selectin, and VCAM-1 by beta-estradiol, progesterone, and dexamethasone. Cell. Immunol.1996;167:79-85.
2. Bamberger CM, Else T, Bamberger A-M, Bell FU, Schulte HM. Dissociative glucocorticoid activity of medroxyprogesterone acetate in normal human lymphocytes. J Clin Endocrinol. 1999;84:4055-4061.
3. Burton, JL, Kehrli, Jr. ME, Kapil S, Horst RL. Regulation of L-selectin and CD18 on bovine neutrophils by glucocorticoids: effects of cortisol and dexamethasone. J Leukocyte Biol. 1995;57:317-325.
4. Chen A, Engel P, Tedder TF. Structural requirements regulate endoproteolytic release of the L-selectin (CD62L) adhesion receptor from the cell surface of leukocytes. J Exp Med. 1995;182:519-530.
5. Corrigan ME, Drouillard JS, Spire MF, Mosier DA, Minton JE, Higgins JJ, Loe ER, Depenbusch BE, Fox JT. Effects of melengestrol acetate on the inflammatory response in heifers challenged with Mannheimia haemolytica. J Anim Sci. 2007;85:1770-1779.
6. Diez-Fraile A, Meyer E, Duchateau L, Burvenich C. L-selectin and β2-integrin expression on circulating bovine polymorphonuclear leukocytes during endotoxin mastitis. J Dairy Sci. 2003;86:2334-2342.
7. Estes DM, Brown WC. Type 1 and type 2 responses in regulation of Ig isotype expression in cattle. Vet Immunol Immunop. 2003;90:1-10.
8. Hafezi-Moghadam A, Ley K. Relevance of L-selectin shedding for leukocyte rolling in vitro. J Exp Med. 1999;189:939-947.

9. Hageleit, M, Daxenberger A, Kraetzl W-D, Kettler A, Meyer HHD. Dose dependent effects of melengestrol acetate (MGA) on plasma levels of estradiol, progesterone, and luteinizing hormone in cycling heifers and influences on oestrogen residues in edible tissues. APMIS. 2000;108:847-854.

10. Jain NC. Schalm's Veterinary Hematology. 4th ed. Lea and Febiger. Philadelphia, PA. 1986.

11. Kahn J, Ingraham RH, Shirley F, Migaki GI, Kishimoto TK. Membrane proximal cleavage of L-selectin: identification of the cleavage site and a 6-kD transmembrane peptide fragment of L-selectin. J Cell Biol. 1994;125:461-470.

12. Kontula K, Paavonen T, Luukkainen T, Andersson LC. Binding of progestins to the glucocorticoid receptor. Biochem Pharmacol. 1983;32:1511-1518.

13. Kuijpers TW, Tool ATJ, van der Schoot CE, Ginsel LA, Onderwater JJM, Roos D, Verhoeven AJ. Membrane surface antigen expression on neutrophils: A reappraisal of the use of surface markers for neutrophil activation. Blood. 1991;78:1105-1111.

14. Loike JD, Sodeik B, Cao L, Leucona S, Weitz JI, Detmers PA, Wright SD, Silverstein SC. CD11c/CD18 on neutrophils recognizes a domain at the N terminus of the Aα chain of fibrinogen. Cell Biol. 1991;88:1044-1048.

15. McMahon CD, Elsasser TH, Gunter DR, Sanders LG, Steele BP, Sartin JL.Estradiol/progesterone implants increase food intake, reduce hyperglycemia, and increase insulin resistance in endotoxic steers. J Endocrinol. 1998;159:469-478.

16. Miyaura H, Iwata M. Direct and indirect inhibition of Th1 development by progesterone and glucocorticoids. J Immunol. 2002;168:1087-1094.

17. Molloy EJ, O'Neill AJ, Grantham JJ, Sheridan-Pereira M, Fitzpatrick JM, Webb DW, Watson RG. Sex-specific alteration in neutrophil apoptosis: the role of estradiol and progesterone. Blood. 2003;102:2653-2659.

18. Moore KW, Malefyt RW, Coffman RL, O'Garra A. Interleukin-10 and interleukin-10 receptor. Annu Rev Immunol. 2001;19:683-765.

19. Potter NS, Harding CV. Neutrophils process exogenous bacteria via an alternate class 1 MHC processing pathway for presentation of peptides to T lymphocytes. J Immunol. 2001;167:2538-2546.

20. Sartin J L, Elsasser TH, Kahl S, Baker J, Daniel JA, Schwartz DD, Steele B, Whitlock BK. Estradiol plus progesterone treatment modulates select elements of the proinflammatory cytokine cascade in steers: Attenuated nitric oxide and thromboxane B2 production in endotoxemia. J Anim Sci. 2003;81:1546-1551.

21. Seely AJE, Pascula JL, Christou NV. Science review: Cell Membrane expression (connectivity) regulates neutrophil delivery, function, and clearance. Critical Care. 2003;7:291-307.

22. Tatsumi H, Kitawaki J, Tanaka K, Hosoda T, Honjo H. Lack of stimulatory effect of dienogest on the expression of intracellular adhesion molecule-1 and vascular cell adhesion molecule-1 by endothelial cells as compared with other synthetic progestins. Maturitas. 2002;42:287-294.

23. Tizard IR. Veterinary Immunology, An Introduction. 7th ed. W. B. Saunders Co., Philadelphia, PA. 2004.

24. Tvinnereim AR, Hamilton SE, Harty JT. Neutrophil involvement in cross-priming CD8+ T cell responses to bacterial antigens. J Immunol. 2004;173:1994-2002.

25. Wakatsuki A, Okatani Y, Ikenoue N, Fukaya T. Effect of medroxyprogesterone acetate on vascular inflammatory markers in postmenopausal women receiving estrogen. Circulation. 2002;105:1436-1439.

26. Zavy MT. Method to decrease cortisol secretion by feeding melengestrol acetate. United States Patent 5,252,564. 1993.