RELEASE OF ENDOTOXIN FROM RUMEN BACTERIA AND ENDOTOXIN ABSORPTION FROM THE RUMEN

by

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Introduction

It is established that free endotoxins are found in cell-free culture supernatants under growth limiting conditions (Andersen et al., 1979; Bishop and Work, 1965; Ingram et al., 1973; Lindsay et al., 1973). The concensus is that these free endotoxins are a result of cell death and lysis. However, there is increasing evidence that suggests that under normal growing conditions, gram-negative bacteria release endotoxins in the form of cell wall blebs (Devoe and Gilchrist, 1973; Johnson et al., 1975). Recent work by Nagaraja et al. (1978) has shown that endotoxins are present in the cell-free fraction of rumen fluid from cattle. The rumen fluid of grain-fed cattle is about eight times more toxic (on the average) than that of hay-fed cattle. The higher concentration of endotoxin in rumen fluid of grain-fed cattle may be due to more gram-negative bacteria or certain conditions that favor the release of endotoxin from intact gram-negative bacteria. The presence of free endotoxins in the rumen becomes important when considering the role that they may play in the pathogenesis of several diet-induced diseases such as lactic acidosis and the sudden death syndrome (Dougherty and Cello, 1949, 1953; Dougherty et al., 1975; Dougherty, 1976; Huber, 1976; Mullenax et al., 1966; Nagaraja et al., 1978). This study, reported in Part I, was initiated to determine the factors that may influence the release of endotoxin in the rumen.

For endotoxins to take part in the pathogenesis of diseases they must first be absorbed parenterally. The results of studies done on absorption of endotoxin from the intestinal tract of nonruminants and ruminants have been variable. Several researchers have shown that endo-

toxins are absorbed in normal and in disease conditions as well as under in vitro conditions (Gans and Matsumoto, 1974 a,b; Kocsar et al., 1969; Nolan, 1975; Nolan et al., 1977; Ravin et al., 1960; Ravin and Fine, 1962). However, others have been unable to show endotoxin absorption (Huber et al., 1979; Sanford and Noyes, 1958).

Because there is potentially a large pool of endotoxin in the rumen and the rumen epithelium provides a large absorptive area, the study initiated was limited to endotoxin absorption from the rumen.

Under normal conditions, when an animal is fed adequate roughage, the epithelium is healthy. When cattle are fed high grain rations the epithelium may undergo degenerative changes. For example, keratinization, parakeratosis, necrosis and infiltration of hair which may result in tunnels through which rather large molecules and even bacteria may pass (Szemerédy and Raul, 1976; McGavin and Morrill, 1976; Fell et al., 1968; Fell et al., 1972).

Generally, endotoxin absorbed via the blood (ie. portal system) would be detoxified by the liver. Because the endotoxin molecule is partially composed of a lipid core it is possible that it may be absorbed via the lymphatics into the thoracic duct and thereby bypass the detoxification of the liver (Gans and Matsumoto, 1974b).

This study (Part II) used three steers maintained on an all-roughage diet and three steers maintained on an all-grain diet. These two test groups should represent a group of steers with healthy rumen epithelium resulting in little if any endotoxin absorption. The other group should represent steers with diseased rumen epithelium resulting in significantly higher amounts of absorbed endotoxin.

PART I

Experimental Procedure

Effect of volatile fatty acids and lactic acid on release of endotoxin in the rumen.

Experiment 1. Rumen fluid was collected from rumen-fistulated cattle maintained on an 85% grain and 15% hay diet. The grain ration was a 14% crude protein mixture of sorghum grain, corn, soybean meal, Starea (Starea is an extrusion processed mixture of grain and urea--registered trademark 860255, U. S. Patent No. 3642489), dicalcium phosphate, salt and a vitamin A and D supplement. Samples were collected via the rumen fistula 6 hr post feeding and strained through four layers of cheese cloth. Fifty ml of strained rumen fluid was placed in 125 ml Erlenmeyer flasks. A volatile fatty acid (VFA) mixture of acetic (99.8%), propionic (99.9%) and butyric (98.0-100.0%) in a 2:2:1 ratio was used to lower the pH of the samples to the desired level. Phosphate buffer (40.8 g KH_2PO_4; 2.0 g $MgSO_4$; 5.0 g NaCl; 0.5 g $CaCl_2 \cdot 6\ H_2O$ in 10 liters of distilled water and adjusted to pH 7.0 with a saturated solution of Na_2CO_3) was added to the control samples (1:1 ratio of rumen fluid to buffer) to maintain the pH during the 6 hr incubation period. All flasks were flushed with CO_2 and fitted with Bunsen-valve stoppers. The samples were then incubated at 39 C. After incubation, all were adjusted to pH 7.0 using a 10 N solution of NaOH. The samples were then centrifuged at 14,000 x g for 15 min to collect cell-free rumen fluid for the determination of free endotoxin by the chick embryo LD_{50} ($CELD_{50}$) assay. Total bacterial numbers and percentages of gram-positive and gram-negative bacteria were determined on all samples.

Experiment 2. Rumen fluid samples were collected as in Experiment 1.

The samples were concentrated three times by centrifuging 150 ml of rumen fluid at 14,000 x g for 15 min and resuspending the pellet in 50 ml of phosphate buffer. The pHs of the samples were adjusted with acetic, propionic or butyric acids, or with acetic and propionic (1:1), or propionic and butyric (1:1), or with a mixture of acetic, propionic and butyric (2:2:1). The samples were flushed with CO_2, fitted with Bunsen-valve stoppers and incubated at 39 C. (All were adjusted to pH 7.0 with 10 N NaOH after incubation). The samples were then centrifuged at 14,000 x g for 15 min to collect cell-free supernatant for free endotoxin determinations by the mouse LD_{50} (MLD_{50}) assay. Total cell counts and counts of gram-positive and gram-negative bacteria were determined on all samples.

Experiment 3. A pure culture of Megasphaera elsdenii B 159 was grown in medium (NO. 2) as described by Hobson, using Hungate's anaerobic technique. The medium was modified by deleting rumen fluid, cellobiose and maltose, but the concentration of glucose was doubled. Twenty-five ml aliquots of this culture were centrifuged at 14,000 x g for 15 min and then resuspended in anaerobic phosphate buffer solutions with varying pH. The samples were then flushed with CO_2, fitted with Bunsen-valve stoppers and incubated at 39 C. After incubation, pHs were adjusted to 7.0 with 10 N NaOH. The samples were then centrifuged at 14,000 x g for 15 min to collect cell-free supernatant for the mouse LD_{50} assay. Total cell numbers were determined on all samples.

Experiment 4. Rumen fluid was collected, as in Experiment 1, from rumen-fistulated cattle maintained on all-alfalfa. Fifty ml aliquots were placed in 125 ml Erlenmeyer flasks. The pHs were adjusted with lactic acid (85% acid solution of D and L lactic mixture) and controls

were incubated with phosphate buffer. The samples were flushed with CO_2, fitted with Bunsen-valve stoppers and incubated at 39 C. (After incubation all were adjusted to pH 7.0 with 10 N NaOH). The samples were then centrifuged at 14,000 x g for 15 min to collect cell-free rumen fluid for the chick embryo and mouse LD_{50} assays. Total cell counts and counts of gram-positive and gram-negative bacteria were determined on all samples.

Experiment 5. A pure culture of Megasphaera elsdenii B 159 was grown as in Experiment 3. Twenty-five ml aliquots were centrifuged at 14,000 x g for 15 min and the pellets were then resuspended in 25 ml of anaerobic phosphate buffer. The pHs were adjusted with lactic acid as in Experiment 4. Samples were then flushed with CO_2, fitted with Bunsen-valve stoppers and incubated at 39 C. (After incubation all pHs were adjusted to 7.0 with 10 N NaOH). The samples were centrifuged at 14,000 x g for 15 min to collect cell-free supernatant for the mouse LD_{50} assay. Total cell counts were determined on all samples.

Effect of osmotic pressure.

A pure culture of Megasphaera elsdenii B 159 was grown as in Experiment 3. Forty ml aliquots of the culture were centrifuged at 14,000 x g for 15 min and then resuspended in 40 ml of anaerobic saline solutions of varying osmotic pressures (256, 287, 319, 351, 414 or 605 mOs/kg), at a pH of 7.0, in 125 ml Erlenmeyer flasks. The samples were flushed with CO_2, fitted with Bunsen-valve stoppers and incubated at 39 C. After incubation all were adjusted to pH 7.0. Samples were centrifuged at 14,000 x g for 15 min to collect cell-free supernatant for the mouse LD_{50} assay. Total cell counts were determined on all samples.

Effect of volatile fatty acids and osmotic pressure.

A pure culture of Megasphaera elsdenii B 159 was grown as in Experiment 3. Bacteria were collected by centrifuging 40 ml of culture at 14,000 x g for 15 min and resuspending the pellets in 40 ml of anaerobic saline solutions of either 287 or 414 mOs/kg at pH 7.0. The pHs were adjusted with either a VFA mixture (acetic, propionic and butyric in a 1:1:1 ratio) or lactic acid as in Experiment 4. The samples were flushed with CO_2, fitted with Bunsen-valve stoppers and incubated at 39 C. (After incubation, each was adjusted to pH 7.0). The samples were centrifuged at 14,000 x g for 15 min to collect the cell-free supernatant for the mouse LD_{50} assay. Total counts were determined on all samples.

Chick embryo LD_{50}.

Eleven-day-old chick embryos were used to determine lethality of the samples (Smith and Thomas, 1956; Finkelstein, 1966). Twofold serial dilutions of the samples (heated at 100 C for 10 min) were inoculated intravenously (IV) into 30 chick embryos for each dose. Control embryos were injected with sterile normal saline. Embryos were candled at 24 and 48 hr to determine deaths. Those which were dead were observed for hemorrhage and cultured for bacterial contamination. If contaminated, the data were discarded. The LD_{50} was calculated by the procedure of Reed and Muench (1936).

Mouse LD_{50}.

Random-bred albino mice of both sexes and weighing ca. 20 g were maintained on a commercial pelleted feed (Purina Laboratory Chow, Ralston Purina Co., St. Louis, MO). Both feed and water were provided ad libitum. Twofold serial dilutions of the sample (heated at 100 C for 10 min)

were injected intraperitoneally (IP) into groups of mice (15-20 mice per dose) using actinomycin D (20 g/mouse) to potentiate the endotoxin (Cosmogen Lot No. 0234W, 1648W, 1939W or 2690W, Merck, Sharp and Dohme, Westpoint, PA). Deaths were recorded for 48 hr. The LD_{50} was calculated by the procedure of Reed and Muench (1936).

Determination of total number and percentages of gram-positive and gram-negative bacteria.

Total bacterial numbers in samples were determined by diluting samples with 10% formal saline and counting in a Petroff-Hausser bacterial counting chamber (C. A. Hausser and Son, Philadelphia, PA). Percentages of gram-negative and gram-positive bacteria were estimated by counting a minimum of 1000 cells from a gram-stained smear. The smear was amde by spreading 5 l of the diluted sample on a 1 cm^2 area (Warner, 1962).

Results

Effect of volatile fatty acids and lactic acid.

Experiment 1. The results of the Experiment 1 are shown in Table 1. The pH of the control samples dropped approximately one pH unit during incubation while that of the test samples remained relatively constant. In the control samples, there was an initial increase in toxicity followed by a gradual decrease. There was an initial decrease in gram-negative bacterial cell numbers followed by a gradual increase. The initial increase in toxicity is probably due to cell death and lysis of the bacteria. The subsequent decrease in toxicity corresponding to an increase in gram-negative cell numbers suggests that even though these bacteria are growing in number, under these conditions they are not releasing endotoxin. This period is followed by a decrease in gram-negative cell numbers

while the toxicity continues to decrease. The test samples reflect somewhat the same pattern. The gram-negative bacteria are increasing and decreasing in number but are not releasing endotoxin under these conditions.

Experiment 2. Treatment of rumen fluid with individual VFA, various combinations of two VFA and the mixture of all three resulted in a subsequent increase in toxicity (Table 2). Those mixtures containing acetic acid resulted in a slightly more toxic sample with the sample treated with all three VFA being the most toxic. Gram-negative cell numbers remained nearly the same or were increased in practically all samples. Those samples in which cell numbers increased were those containing acetic and some other acid. In this instance it would seem that the growth and increase of gram-negative bacteria corresponds to an increase in toxicity suggesting a release of endotoxin by the bacteria.

Experiment 3. The data in Table 3 suggest that pH has little if any effect on endotoxin release. In the case of a pH of 7.0 and 4.5, there is a decrease in cell number meaning cell death and lysis but the toxicity of the samples decreased slightly. At a pH of 5.5 there was no cell number differences but a slight increase in toxicity denoting that a small amount of endotoxin was released.

Experiment 4. Treatment of rumen fluid, from an animal adapted to all-roughage, with lactic acid resulted in an increase in toxicity to chick embryos (Table 4). The control samples (pH 7.4) however showed the most drastic increase. There was a decrease in gram-negative numbers in the control samples but apparently not sufficiently large enough to account for the increase in toxicity. There was an initial decrease followed by an increase in gram-negative numbers in the test samples suggesting that these growing cells were releasing endotoxin into the rumen

fluid first by lysis and subsequently by release from intact cells. The same experiment repeated in mice yielded somewhat different results (Table 5). Overall the gram-negative bacterial numbers remained relatively constant in the test samples but not in the control (pH 6.9). There was an increase in toxicity of the control sample and the test sample adjusted to pH 5.5. However, there was a slightly higher increase in toxicity of the test sample adjusted to pH 4.5. This suggests that lactic acid is somewhat stimulatory to bacteria in releasing endotoxin.

Experiment 5. The mouse lethalities of the supernatant from the pure culture study somewhat paralled those from Experiment 4 (Table 6). The increase in toxicity of the control sample was comparable to that of the test samples (control at pH 7.0). In this experiment however there was a decrease in the total cell numbers suggesting that the increase in toxicity was due to cell death and lysis rather than to enhancement of release of endotoxin by the lactic acid.

Effect of osmotic pressure.

The effect of osmotic pressure on the release of endotoxin is shown in Table 7. In all the samples there was an increase in toxicity along with an increase in cell number. The increase in toxicity was probably due to the increase in total cell numbers.

Effect of volatile fatty acids and osmotic pressure.

By increasing the osmotic pressure, the level of endotoxin increased. By decreasing the pH with VFA or lactic acid, once again the toxicity increased. The combination of increased osmotic pressure and decreased pH as a result of addition of VFA or lactic acid seemed to increase the endotoxicity of the supernatant (Table 8). There was little change in

total cell numbers, so the increase in toxicity was not due to cell death and lysis but rather the release of endotoxin by the intact bacteria. The pH adjustments by the respective acids were chosen because these were the levels at which they seemed to exert their greatest effect when used by themselves. An osmotic pressure of 287 mOs/kg represents what would be normal and most conducive to cell life. Under normal eating conditions, the osmotic pressure of the rumen may reach a level of 414 mOs/kg. The VFA mixture resulted in a higher toxicity than controls and the lactic acid caused the greatest increase. By increasing the osmotic pressure to correspond to that which occurs in nature, the effect by the acids were increased even more. These results suggest that the acid(s) and the osmotic pressure may exert some synergistic effect on bacteria resulting in an enhancement of endotoxin release.

Discussion

In the fermentation of carbohydrates and proteins by the rumen microflora, VFA and lactic acid are produced and the pH of the rumen contents decreases. Also, the osmotic pressure of the rumen may reach 350-400mOs/kg after eating (Warner and Stacy, 1965). It is for these two reasons that pH and osmotic pressure were chosen for the study. When considering differences between grain-fed cattle and hay-fed cattle, grain-fed animals have a lower rumen pH and a higher osmotic pressure. It was felt that perhaps these two factors may somehow play a role in the fact that grain-fed cattle have more endotoxin in the rumen.

Treatment of bacteria with VFA or lactic acids resulted in release of endotoxin. The increase was not necessarily due to cell death and lysis but rather to the release of endotoxin from intact and live cells.

In some cases the toxicity of the samples increased while the total number of gram-negative bacteria remained the same or increased. Thus the increased endotoxicity could not be due to cell death and lysis but rather to a release of endotoxin from intact cells. The acids appeared to have a pH range at which their effects were more pronounced. This enhancement of release however was due to the acid(s) and was not actually a pH effect. Lactic acid demonstrated the most drastic release of endotoxin. Several other workers have also shown that lactic acid seems to increase endotoxin levels (Dougherty and Cello, 1949; Mullenax et al., 1966; Huber, 1976; Nagaraja et al., 1978). The osmotic pressure also enhanced the release of endotoxin and seemed to accentuate the effects seen from acid treatments. Acid(s) and osmotic pressure both acted synergistically in enhancing the release of endotoxin from gram-negative rumen bacteria.

TABLE 1. EFFECT OF VFA MIXTURE ON THE RELEASE OF ENDOTOXIN FROM RUMEN BACTERIA[a]

Incubation time hr	Control					Test				
	pH	$CELD_{50}$ ml	Total cell counts $x10^9$/ml	Gram-neg. bacteria %	Gram-neg. bacteria Number $x10^9$/ml	pH	$CELD_{50}$ ml	Total cell counts $x10^9$/ml	Gram-neg. bacteria %	Gram-neg. bacteria Number $x10^9$/ml
0	6.65	.0073	6.3	83.7	5.3	4.07	.0044	4.1	83.2	3.4
1	6.34	.0025	1.2	73.0	.88	3.98	.0052	9.0	86.9	7.8
3	5.73	.0064	5.6	83.7	5.0	3.99	.0130	2.2	89.9	2.0
6	5.69	.0108	3.2	84.6	2.7	3.97	.3875	3.1	89.6	2.8

[a]Average of three experiments.

TABLE 2. EFFECT OF LOWERING pH WITH INDIVIDUAL VFA AND VFA MIXTURES ON THE RELEASE OF ENDOTOXIN FROM RUMEN BACTERIA[a]

Incubation time hr	Acid(s)	pH	MLD_{50}[b]	Total cell counts $x10^9$/ml	Gram-negative bacteria	
					%	No. $x10^9$/ml
Control 0	---	7.0	.4447	5.1	77.3	3.9
1	---	7.0	.3051	5.3	77.0	4.1
Test 1	A[c]	5.0	.0914	5.0	71.1	3.5
1	P[d]	5.0	.1165	6.2	66.6	4.1
1	B[e]	5.0	.1157	5.4	70.8	3.8
1	A-P	5.0	.0937	8.0	80.8	6.5
1	A-B	5.0	.1043	9.1	75.1	6.8
1	P-B	5.0	.1174	5.3	79.3	4.2
1	A-P-B	5.0	.0868	5.2	85.3	4.4

[a]Average of three experiments.

[b]Potentiated with 20μg actinomycin D per mouse.

[c]A=acetic.

[d]P=propionic.

[e]B=butyric.

TABLE 3. EFFECT OF pH ON THE RELEASE OF ENDOTOXIN FROM MEGASPHAERA ELSDENII B 159[a]

Incubation time hr	pH	MLD_{50}[b]	Total cell counts $x10^{-8}$/ml
0	7.0	.0625	9
1.5	7.0	.0781	5
0	5.5	.0897	7
1.5	5.5	.0625	7
0	4.5	.0625	5
1.5	4.5	.0913	4

[a]Average of three experiments.

[b]Potentiated with 20μg actinomycin D per mouse.

TABLE 4. EFFECT OF LACTIC ACID ON THE RELEASE OF ENDOTOXIN FROM RUMEN BACTERIA FROM AN ALL-ROUGHAGE FED ANIMAL[a]

Incubation time hr	pH	$CELD_{50}$ ml	Total cell counts $x10^9$/ml	Gram-negative bacteria %	Gram-negative bacteria No. $x10^9$.ml
0	7.4	.0373	3.1	91.4	2.8
3	7.4	.0274	3.0	90.8	2.7
6	7.4	.0031	2.2	91.0	2.0
0	5.5	.0413	4.1	92.2	3.8
3	5.5	.0500	4.0	90.7	3.6
6	5.5	.0157	5.3	92.3	4.9
0	4.5	.0336	3.7	91.8	3.4
3	4.5	.0307	3.2	90.3	2.9
6	4.5	.0250	5.0	91.5	4.6

[a]Average of four experiments.

TABLE 5. EFFECT OF LACTIC ACID ON THE RELEASE OF ENDOTOXIN FROM RUMEN BACTERIA FROM AN ALL-ROUGHAGE FED ANIMAL[a]

Incubation time hr	pH	MLD_{50}[b] ml	Total cell counts x 10^9/ml	Gram-negative bacteria %	Gram-negative bacteria no. x 10^9/ml
0	6.9	.1771	4.1	90.5	3.7
3	6.9	.0725	2.0	95.3	1.9
6	6.9	.1042	2.5	89.9	2.2
12	6.9	.0430	3.2	91.4	2.9
0	5.5	.4423	3.0	93.2	2.8
6	5.5	.1704	3.1	91.3	2.8
0	4.5	.2573	3.4	95.0	3.2
3	4.5	.0745	3.3	94.4	3.1
6	4.5	.0781	2.5	91.7	2.3
12	4.5	.0470	3.2	93.4	3.0

[a]Average of three experiments.

[b]Potentiated with 20 μg actinomycin D per mouse.

TABLE 6. EFFECT OF LACTIC ACID ON THE RELEASE OF ENDOTOXIN FROM MEGASPHAERA ELSDENII B 159[a]

Incubation time hr	pH					
	7.0		5.5		4.5	
	MLD_{50} ml[b]	Total cell cts $x10^8$/ml	MLD_{50} ml[b]	Total cell cts $x10^8$/ml	MLD_{50} ml[b]	Total cell cts $x10^8$/ml
0	.0441	3	.0601	5	.0775	4
1.5.	.0621	5	.0370	5	.0665	5
3	.0080	1	.0140	1	.0140	1

[a]Average of three experiments.

[b]Potentiated with 20μg actinomycin D per mouse.

TABLE 7. EFFECT OF OSMOTIC PRESSURE ON RELEASE OF ENDOTOXIN FROM MEGASPHAERA ELSDENII B 159[a]

	Incubation time hr	Osmotic pressure[b] mOs/kg	MLD_{50}[c] ml	Total cell counts $x10^8$/ml
Control	0	287	.1607	5
	3	287	.0625	7
Test	3	256	.0313	8
	3	319	.0381	6
	3	351	.0313	7
	3	414	.0409	7
	3	605	.0504	7

[a]Average of three experiments.

[b]Values determined from Handbook of Chemistry and Physics.

[c]Potentiated with 20μg actinomycin D per mouse.

TABLE 8. EFFECT OF OSMOTIC PRESSURE AND LACTIC ACID ON THE RELEASE OF ENDOTOXIN FROM *MEGASPHAERA ELSDENII* B 159[a]

Incubation hr	pH					
	7.0-no acid		5.5-VFA mixture		4.5-lactic acid	
	MLD_{50}[b] ml	Total cell cts $x10^8$/ml	MLD_{50}[b] ml	Total cell cts $x10^8$/ml	MLD_{50}[b] ml	Total cell cts $x10^8$/ml
287 mOs/kg						
0	.2863	2	.2197	3	.1094	3
3	.1137	3	.1443	4	.0524	3
6	.0972	3	-----	-	.0824	2
414 mOs/kg						
0	.2513	3	.1681	3	.1000	3
3	.1275	2	.1141	3	.0245	2
6	.0313	2	-----	-	.0526	3

[a]Average of three experiments.

[b]Potentiated with 20 µg actinomycin D per mouse.

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PART II

Experimental Procedure

Experimental Animals and Rations. The experimental animals consisted of six grade Holstein steers aged seven months to one year and ranging from 200 to 400 kg body weight. Three steers were maintained on an all-roughage diet of alfalfa and/or prairie hay for ca. 5 months. Three months prior to the experiment the other steers were fed a 12.98% protein ration consisting of 92.34% rolled sorghum grain, 4.10% soybean, 1.00% urea, 1.90% dicalcium phosphate, 0.47% trace mineralized salt and 0.19% vitamin A and D supplement.

Surgical Preparation. The steers were rumen-fistulated. The portal vein and right ruminal vein were cannulated according to the method of Parker et al. (1963). The thoracic duct was cannulated according to the procedure outlined by Hartmann and Lascelles (1966).

Endotoxin Preparation and Administration. Escherichia coli endotoxin (Lipopolysaccharide W, E. coli 0128:B12 Difco Laboratories, Detroit, MI) was labeled with ^{51}Cr (sodium chromate, ICN Pharmaceuticals, Inc., Chemical and Radioisotope Division, Irvine, CA) according to the method described by Chedid (1962). Ten millicuries of label was added to 875 mg of toxin. The mixture was incubated at 39 C for 48 hr. The solution was then dialyzed against deionized water for 4 days and centrifuged at 14,000 x g. It was then resuspended in sterile normal saline. The endotoxin was administered intraruminally.

Blood Sampling and Analyses. Blood samples were collected from jugular and portal veins just prior to endotoxin administration and at 5, 15 and 30 min and at 1, 2, 3, 4, 5, 6, 12 and 24 hr after admini-

stration. Lymph was collected by continuous flow for the first 6 hr and again at 12 and 24 hr. Blood and lymph samples were subjected to counting in an 1185 Series Gamma Counter (Nuclear-Chicago, Des Plaines, IL) along with feces and urine samples. Each sample was duplicated and each duplicate counted twice for 10 min. Endotoxin was assayed in serum collected from the portal blood and in lymph by the biological assay procedure using actinomycin D. Serial twofold dilutions were made of each serum sample in pyrogen-free saline and actinomycin D (Am D, Cosmegen R Lot No. 0240B, Merck, Sharpe and Dohme, Westpoint, PA) added at a rate of 20 μg per mouse. Each dilution was injected intraperitoneally into a group of five mice (ca. 20 g random bred albino mice fed a commercial pelleted feed: Purina Laboratory Chow, Ralston Purina Co., St. Louis, MO) and deaths recorded up to 48 hr. The mean lethal dose (mouse LD_{50}) was calculated by the method of Reed and Muench (1938). Jugular blood samples were subjected to total and differential white blood cell count, platelet, calcium and glucose determinations. White blood cell and platelet counts were performed on samples with ethylenediaminetetraacetate (EDTA) as an anticoagulant. Total leukocyte counts were made with a Coulter counter (Coulter Electronics Inc., Hialeah, FL), and platelet counts were with unopettes (Becton Dickinson and Co , Rutherford , NJ) and Neubauer counting chamber. Stained blood smears were prepared from each sample and a total of 200 leukocytes counted. Calcium was measured by SMA 12 Autoanalyzer determination (Technicon Corp., Tarreytown, NY). Glucose was measuree by Abbott Laboratories reagent kit (Abbott Laboratories, Pasadena, CA).

One week after the absorption experiment, E. coli endotoxin was

injected into the portal vein cannula at the rate of 1 μg per kg body weight. Jugular blood samples were collected and hematology and blood chemistry determination were performed as before.

Results

Out of six steers, four were rumen fistulated, four had a functional thoracic duct cannula, four had a functional portal vein cannula and one had a functional right ruminal vein cannula. Following administration of the endotoxin, none of the steers showed clinical signs of endotoxemia (ie. dyspnea, hypersalivation, predominantly abdominal breathing, severe diarrhea, etc).

Absorption in Roughage-fed steers. The MLD_{50} and radioactive count of each sample collected from those steers on the all-roughage ration are shown in Table 1. Throughout the 24 hr period the radioactive counts remained only slightly above background. This suggests that no endotoxin or even any free label was absorbed. The LD_{50} of jugular blood or portal blood or lymph from the thoracic duct did not change following intraruminal administration of endotoxin. Also total and differential leukocyte counts, platelet counts, calcium and glucose concentration in blood did not change suggesting there was no absorption of intraruminally administered endotoxin (Table 2).

Absorption in grain-fed steers. The MLD_{50} and radioactive counts of each serum sample of the steers adapted to an all-grain ration are shown in Table 3. The serum samples from grain-fed steers were more toxic to mice than those of the all-roughage animal. But there was generally no change in toxicity when compared to the sample obtained prior to endotoxin administration. Once again the radioactive counts were only slightly

above background suggesting that endotoxin and/or free label were not absorbed through the rumen. The total and differential white blood cell counts, platelets, calcium and glucose concentration did not change following intraruminal administration (Tables 4, and 7).

Response of steers following administration of endotoxin into the portal vein. When endotoxin was administered via the portal cannula the animals showed clinical signs of endotoxemia and exhibited the classic hematological responses (leukopenia followed by a leukocytosis, lowered serum calcium and hyperglycemia) as shown in Tables 5, 7 and 8.

Discussion

The idea of endotoxin absorption from the GI tract is a very controversial one. Those individuals who have used labeled endotoxin found in the blood as an indication of absorption have been able to show that indeed endotoxins are absorbed. On the other hand, those workers who have relied on strictly the hematological responses as a sign of absorption have been unable to show endotoxin absorption. It is for this reason that this study was conducted in such a way as to analyze for endotoxin absorption by labeling the endotoxin prior to administration and assaying for radioactivity in blood and lymph, analyzing blood samples for the classic hematological responses, and finally injecting serum samples into mice to determine if endotoxin was absorbed.

The rumen, being a highly absorptive area of the GI tract and a pool of a potentially substantial quantity of endotoxin, was singled out as a source of absorbed endotoxin. It was thought for some time that the acid of the abomasum would detoxify the endotoxin molecule as it passed from the rumen on down the GI tract. Huber (1979) has shown however that abomasal

fluid does not detoxify the endotoxin molecule. The logic that endotoxin must be absorbed from the rumen in order for it to take part in the pathogenesis of certain diseases may no longer be valid. However, it still remains that the vast absorptive surface of the rumen epithelium provides an ideal site for absorption. The endotoxin could be absorbed through the blood. In this instance the toxin would enter the portal vein and pass to the liver for possible detoxification. But endotoxin is lipid in nature and so it may be absorbed via the lymphatic system. In this instance it would enter the thoracic duct and pass to the heart bypassing the detoxification of the liver.

It would appear however that in the instance of a healthy animal, regardless of its diet, endotoxin is probably not absorbed. The epithelium of the roughage-fed cattle was healthy. Upon inspection of the rumen epithelium of the grain-fed cattle, it was noted that the papillae were darkened and some were keratinized but no lesions or misshapen papillae could be found. This lack of lesions may partially explain why no endotoxin absorption was found in these particular steers. This is not to say however that under no circumstances are endotoxins absorbed. There may be those instances in which an animal's resistance and general health may be compromised. Something may happen to change the absorptive qualities of the rumen epithelium. Under these conditions, diet-induced diseases for example, endotoxins may be absorbed and their absorption may play a role in the pathophysiology of these diseases.

TABLE 1. RADIOACTIVE COUNTS AND MOUSE LETHALITIES OF SERUM AND LYMPH FROM ROUGHAGE-FED STEERS FOLLOWING INTRARUMINAL ADMINISTRATION OF ENDOTOXIN[a]

Sample time min	Jugular blood[b] cts/ml/min	Jugular[c] MLD_{50}	Portal serum[b] cts/ml/min	Portal[c] MLD_{50}	Thoracic lymph[b] cts/ml/min	Thoracic[c] MLD_{50}
0	10.1	.47	5.1	.35	7.5	.37
5	41.7	.50	15.9	.50	8.7	.40
15	13.7	.39	5.7	.50	4.5	.48
30	9.3	.37	10.6	.44	7.2	.41
60	18.1	.45	12.1	.45	5.4	.35
120	36.8	.41	8.0	.50	7.7	.45
180	59.7	.50	18.8	.50	28.7	.50
240	7.8	.50	4.3	.50	16.1	.48
300	8.9	.50	6.2	.50	24.3	.50
360	19.7	.50	18.0	.50	26.2	.37
720	55.9	.50	3.3	.50	5.9	.50
1440	7.2	.50	10.9	.50	8.8	.50

[a]Average of three experiments.

[b]Counts above background.

[c]Potentiated with 20 μg actinomycin D per mouse.

TABLE 2. TOTAL AND DIFFERENTIAL WHITE BLOOD CELL COUNTS, PLATELET COUNTS AND SERUM CALCIUM AND GLUCOSE CONCENTRATIONS OF ROUGHAGE-FED STEERS FOLLOWING INTRARUMINAL ADMINISTRATION OF ENDOTOXIN[a]

Sample time min	Total white blood cell counts per μl	Neutrophils %	Lymphocytes %	No. platelets x$10^3/\mu$l	Calcium mg/dl	Glucose mg/dl
0	11,333	43	51	860.0	8.62	73
5	18,750	49	47	905.0	8.77	82
15	10,733	40	55	943.3	8.64	84
30	13,800	59	37	888.3	8.57	83
60	10,567	47	47	771.6	8.68	85
120	8833	43	53	655.0	9.81	76
180	10,900	55	40	746.6	9.55	80
240	9700	34	63	1010.0	9.60	83
300	9600	22	78	965.0	9.10	81
360	10,700	46	50	898.3	9.60	80
720	9600	48	44	801.6	9.00	87
1440	9733	39	52	821.6	9.03	82

[a]Average of three experiments.

TABLE 3. RADIOACTIVE COUNTS AND MOUSE LETHALITIES OF SERUM AND LYMPH FROM GRAIN-FED STEERS FOLLOWING INTRARUMINAL ADMINISTRATION OF ENDOTOXIN[a]

Sample time min	Jugular blood[b] cts/ml/min	Jugular[c] MLD_{50}	Portal serum[b] cts/ml/min	Portal[c] MLD_{50}	Rt. Ruminal serum[b] cts/ml/min	Thoracic lymph[b] cts/ml/min	Thoracic[c] MLD_{50}
0	3.9	.05	7.3	.08	4.4	4.5	.50
5	4.0	.13	4.6	.08	4.9	3.9	.50
15	4.5	.05	5.0	.08	4.7	3.6	.50
30	3.9	.05	4.1	.08	4.5	4.5	.50
60	3.4	.05	5.1	.08	5.2	4.6	.50
120	3.8	.05	4.5	.05	4.2	2.3	.50
180	4.3	.04	4.5	.05	5.2	3.9	.50
240	4.4	.03	4.7	.06	4.0	2.9	.50
300	4.6	.03	4.5	.06	4.5	4.1	.50
360	4.1	.03	5.2	.06	4.5	4.4	.50
720	4.7	.03	4.6	.06	4.5	4.9	.50
1440	4.7	.03	4.9	.06	4.5	3.6	.50

[a]Average of three experiments.

[b]Counts above background.

[c]Potentiated with 20μg actinomycin D per mouse.

TABLE 4. TOTAL AND DIFFERENTIAL WHITE BLOOD CELL COUNTS, PLATELET COUNTS AND SERUM CALCIUM AND GLUCOSE CONCENTRATIONS OF GRAIN-FED STEERS FOLLOWING INTRARUMINAL ADMINISTRATION OF ENDOTOXIN[a]

Sample time min	Total white blood cell counts per μl	Neutrophils %	Lymphocytes %	No. platelets x$10^3/\mu$l	Calcium mg/dl	Glucose mg/dl
0	9308	31	61	519.0	8.08	77
5	8867	42	57	718.0	8.61	73
15	7933	34	65	570.0	8.82	75
30	6417	26	67	521.0	8.93	80
60	6042	17	68	504.0	8.81	73
120	6458	26	65	482.0	8.95	66
180	4775	13	69	494.0	8.76	68
240	4817	10	71	436.0	8.75	76
300	7367	32	61	503.0	8.82	65
360	6292	18	71	490.0	8.30	69
720	13,792	27	54	471.0	8.71	77
1440	10,875	14	57	584.0	8.83	76

[a]Average of three experiments.

TABLE 5. TOTAL AND DIFFERENTIAL WHITE BLOOD CELL COUNTS, PLATELET COUNTS AND SERUM CALCIUM AND GLUCOSE CONCENTRATIONS OF STEERS FOLLOWING ADMINISTRATION OF ENDOTOXIN INTO THE PORTAL VEIN[a]

Sample time min	Total white blood cell counts per μl	Neutrophils %	Lymphocytes %	No. platelets x$10^3/\mu$l	Calcium mg/dl	Glucose mg/dl
0	10,713	35	59	640.0	8.91	80
5	8312	37	57	566.1	9.30	83
15	5225	39	54	518.3	9.54	104
30	3500	15	70	371.2	9.25	182
60	2537	3	91	368.7	8.81	171
120	2963	11	72	345.5	8.17	193
180	2650	13	79	350.4	7.29	109
240	3413	21	75	308.6	7.94	76
300	3725	16	76	228.3	7.78	59
360	6975	31	61	342.5	7.34	55
720	13,787	61	28	281.6	7.58	51
1440	18,350	70	25	625.5	8.13	69

[a]Data from four steers.

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RELEASE OF ENDOTOXIN FROM RUMEN BACTERIA AND ENDOTOXIN ABSORPTION FROM THE RUMEN

by

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Abstract

It has been determined that there are endotoxins in the rumen fluid of cattle. These endotoxins may play a role in certain diet-induced diseases such as lactic acidosis and the sudden death syndrome. For these endotoxins to play a role in the pathogenesis of these diseases, they must first be released and subsequently be absorbed into the circulatory system.

Rumen bacteria, mixed and pure cultures, were subjected to acid and osmotic pressure treatments. In the fermentation of carbohydrates and proteins, these two values are increased. The acid treatments consisted of lowering the pH with volatile fatty acids (acetic, propionic and/or butyric) or lactic acid. The pH range tested was 4.5 to 7.0. Those mixtures which contained acetic acid or lactic acid exhibited the greatest increase in endotoxicity. The osmotic pressure effect was acheived by collecting and resuspending bacterial cells in salt (NaCl) solutions of varying concentrations. The osmotic pressure range observed was 287 mOs to 605 mOs/kg. An increase in osmotic pressure resulted in a sample with increased endotoxicity. The data show that lowering pH and/or increasing osmotic pressure enhances endotoxin release and the two factors may interact synergistically.

Six steers (three on all-roughage and three on all-grain) were given 875 mg of ^{51}Cr-labeled E. coli endotoxin per rumen. Total and differential white blood cell counts, platelet counts, calcium, glucose, radioactive counts and serum and/or lymph LD_{50} were performed on all samples. Samples were collected at 0, 5, 15, 30 min, 1, 2, 3, 4, 5, 6, 12 and 24 hr. The epithelium of the roughage-fed cattle was healthy. Upon inspection of the rumen epithelium of the grain-fed cattle, it was noted that the papillae were darkened and some were keratinized but no

lesions or misshapen papillae could be found. No endotoxin absorption was found in either the roughage-fed or the grain-fed steers. The lack of lesions may partially explain why no endotoxin absorption was found. One week later each steer was infused with 1 µg/kg body weight of E. coli endotoxin per portal vein. In this instance, when the endotoxin was administered intravenously, the classic signs of endotoxemia developed (dyspnea, hyperventilation, predominantly abdominal breathing, diarrhea, hypersalivation, etc). This further determined that no endotoxin absorption had occured in the previous experiments. It was determined that under normal health conditions endotoxins are not absorbed from the rumen.