Hematologic and serum biochemical changes in *Salmonella* ser Typhimurium-infected calves

Renato L. Santos, DVM, PhD; Renée M. Tsolis, PhD; Andreas J. Bäumler, PhD; L. Garry Adams, DVM, PhD

Objective—To evaluate hematologic and serum biochemical changes in *Salmonella* ser Typhimurium-infected calves.

Animals—16 male 3- to 4-week-old dairy calves.

Procedure—13 calves were experimentally infected with *S* Typhimurium (strains IR715 and CS401, which are derivatives of ATCC 14028), and 3 calves were uninfected controls. Several hematologic and serum biochemical parameters were measured.

Results—Hematologic changes included increases in PCV, RBC count, and hemoglobin concentration, associated with a transitory leukopenia characterized by neutropenia and lymphopenia. Biochemical findings included hypoglycemia, increased BUN, creatinine, and fibrinogen concentrations, and decreased sodium, total CO_2, calcium, total protein, and albumin concentrations. Increased total bilirubin concentration associated with decreased conjugated bilirubin concentration was also observed. No significant changes in aspartate aminotransferase, γ-glutamyltranspeptidase, alkaline phosphatase, and creatinine kinase activities were detected.

Conclusions and Clinical Relevance—Experimental salmonellosis of calves results in marked to severe dehydration, accompanied by metabolic acidosis, hypoglycemia, and an acute inflammatory response associated with increased fibrinogen concentrations and severe neutropenia immediately after inoculation. (*Am J Vet Res* 2002;63:1145–1150)

Salmonella ser Typhimurium is a major cause of calf sickness and death in the United States.[1] A survey performed in Britain revealed that *Salmonella* serotypes are associated with 12% of diarrhea outbreaks among calves.[2] In 1994, bovine deaths due to digestive diseases in the United States totaled 666,000 cattle, resulting in a financial loss of approximately $316.5 million.[3] *Salmonella* infection accounts for a substantial part of these costs. In addition to the economic impact on the animal industry, *Salmonella* infection is 1 of the most common food-borne infections in humans in the United States with an estimated 1.41 million cases and more than 500 human deaths.[4] Approximately 95% of the human *Salmonella* infections are foodborne, corresponding to approximately 30% of deaths caused by food-borne infections in the United States[4] Importantly, *S* Typhimurium, which is 1 of the serotypes most frequently isolated from sick cattle in the United States[5] is also 1 of the serotypes with the highest ability to cause human food-borne illness.[6]

Although knowledge of *Salmonella* virulence factors has increased substantially during the past few years,[7,8] the host response to infection is still poorly understood. In addition to its potential impact on animal health, a better understanding of the bovine host response to *Salmonella* infection is of extreme relevance for experimental medicine, because the calf is a valuable model for human non-typhoidal *Salmonella* infections.[8,9] Mice have been extensively used for the study of *Salmonella* pathogenesis, but they do not develop diarrhea when infected with *S* Typhimurium. Murine infections with *S* Typhimurium result in a disease similar to typhoid fever in humans, which is caused by the host specific serotype *S* Typhi.[8,9]

Most natural *Salmonella* infections in cattle are due to *S* Typhimurium and *S* Dublin. These infections result in a variety of clinical manifestations, which depend on the serotype and age of the host.[6,10] In calves < 6 to 8 weeks of age, the infection usually causes acute diarrhea associated with dehydration[6] and death that is inversely proportional to age.[11] With oral infection, *S* Typhimurium is able to invade intestinal epithelial cells, cross the epithelial layer, and reach the lamina propria, where it is found mostly within phagocytic cells.[12] *Salmonella*-induced diarrhea in calves is associated with a severe fibrinopurulent and necrotizing enteritis that tends to be more severe in the ileal Peyer's patches.[13-15] These lesions result in severe loss of electrolytes, protein, and fluid that causes severe fluid and acid-base imbalance.[16,17] The most important hematologic changes during acute experimental infection includes changes in the leukocytes, although controversial results have been reported with either neutropenia or neutrophilia occurring after experimental infection.[11,16] Under experimental conditions, oral infections with 10^4 to 10^7 **colony forming units (CFU)** cause transient diarrhea that persists for 48 to 192 hours, whereas death may be caused at doses between 10^8 and 10^{11} CFU.[11,13,14,18]

The scarcity of comprehensive data on the clinical pathologic changes during *Salmonella* infection of calves prompted us to more fully characterize blood cellular and chemical parameters to possibly improve

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From the Department of Veterinary Pathobiology, College of Veterinary Medicine, Texas A&M University, College Station, TX 77843-4467 (Santos, Adams) and the Department of Medical Microbiology and Immunology, College of Medicine, Texas A&M University Health Science Center, College Station, TX 77843-1114 (Tsolis, Bäumler).
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Address correspondence to Dr. Adams.

our understanding of clinical disease and death caused by this infection. The purpose of the study reported here was to evaluate the dynamics of hematologic and serum biochemical changes in *S* Typhimurium-infected calves.

Materials and Methods

Bacterial strains and growth conditions—Bacterial strains used in this study were *S* Typhimurium strain IR715,[19] which is a spontaneous nalidixic acid-resistant derivative of the strain **ATCC (American Type Culture Collection)** 14028, and strain CS401, which is a spontaneous streptomycin-resistant derivative of ATCC 14028 containing a transposon insertion in *phoN* (14028 *phoN*::Tn*10*d(Cm) Str^r).[20] CS401 is fully virulent in calves.[21] Bacteria were cultured aerobically under agitation at 37 C in **Luria-Bertani (LB)** broth or on LB agar plates. If appropriate, nalidixic acid was added at the concentration of 50 mg/L.

Experimental calves and infection—Milk-fed male Friesian-Holstein calves (n = 16), aged 3 to 4 weeks, were obtained from a commercial dairy calf rearing operation. The weight of the calves ranged between 45 and 52 kg. The calves were cared for according to the American Association for Accreditation of Laboratory Animal Care guidelines. Calves were fed antibiotic-free milk replacer twice daily and were given water ad libitum. Prior to their use for experiments, the calves were evaluated to ensure CBC and selected blood chemistry values were within reference intervals, and fever and fecal excretion of *Salmonella* serotypes were absent. All experimental calves were screened for fecal excretion of *Salmonella* serotypes by use of fecal swab specimens, enrichment of tetrathionate broth,[a] and plating on brilliant agar,[b] and were found to have culture results prior to experimental exposure. The optical density of overnight cultures at 600 nm was determined, and calves were orally infected with approximately 10^{10} CFU of *S* Typhimurium strains. The appropriate CFU numbers were added to 50 ml of a suspension of 5% magnesium trisilicate, 5% sodium bicarbonate, and 5% magnesium carbonate buffer. This preparation was added to 950 ml of milk replacer and fed orally to calves. Eleven calves were inoculated with strain IR715, 2 calves with strain CS401, and the remaining 3 calves were not inoculated and were kept under identical experimental conditions. Serial 10-fold dilutions of the inoculum were spread on LB plates to determine the exact challenge dose per calf. All calves inoculated with either strain developed diarrhea, and the clinical and pathologic findings have been reported.[14]

Clinical evaluation and blood collection—Calves were monitored clinically twice daily. Shedding of *S* Typhimurium was monitored by taking daily fecal swab specimens, subsequently enriching them in tetrathionate broth,[a] and plating them on brilliant green agar[b] When calves developed anorexia or were unable to stand, they were euthanatized for humane reasons as described.[22] Blood was collected from the jugular vein into evacuated tubes for harvesting of serum, and further samples were collected into tubes containing disodium EDTA as anticoagulant to evaluate CBC and chemistry profiles, respectively, from individual calves prior to inoculation. Subsequent samples were obtained from the calves 24, 48, 72, and 96 hours after inoculation.

Hematologic and serum biochemical analyses—Data regarding CBC, including total RBC, WBC, and platelet counts, were obtained from anticoagulated blood by use of an automated hematology analyzer.[c] Packed cell volumes were obtained manually from blood by use of 50-µl microhematocrit tubes after centrifugation for 5 minutes at 1,520 × g. White blood cell differential counts were performed manually by 1 clinical pathologist and were based on a percentage of 200 WBC counted on a blood smear. Absolute values of the various WBC were obtained by multiplying the percentage of the specific cell type by the total WBC count. Serum biochemical values were by use of a chemistry analyzer.[d] All values were compared with established reference range values (**Table 1**).

Table 1—In-house derived reference ranges for hematologic and serum biochemical parameters in 25 healthy 6-week-old calves

Variable	Reference range
PCV(%)	24–46
RBC (× 10^6/µl)	5–10
Hemoglobin (g/dl)	8–15
WBC (cells/µl)	4,000–12,000
Mature neutrophils (cells/µl)	600–4,000
Band neutrophils (cells/µl)	0–120
Metamyelocytes (cells/µl)	0
Lymphocytes (cells/µl)	2,500–7,500
Monocytes (cells/µl)	25–840
Eosinophils (cells/µl)	0–2,400
Basophils (cells/µl)	0–200
Thrombocytes (× 10^3/µl)	100–800
Fibrinogen (mg/dl)	300–700
Glucose (mg/dl)	49–132
Urea nitrogen (mg/dl)	4–23
Creatinine (mg/dl)	0.6–1.6
Sodium (mmol/L)	129–146
Potassium (mmol/L)	3.04–4.99
Chloride (mmol/L)	92.1–106
Total CO_2 (mmol/L)	22.9–35.3
Calcium (mg/dl)	7.4–11.5
Phosphorus (mg/dl)	3.9–9.2
Anion gap	5–12
Total protein (g/dl)*	5.0–7.1
Albumin (g/dl)*	2.0–2.9
Total bilirubin (mg/dl)	0–0.6
Conjugated bilirubin (mg/dl)	0.04–0.44
Aspartate aminotransferase (U/L)	53–173
γ-glutamyltranspeptidase (U/L)	0–100
Alkaline phosphatase (U/L)	27–130
Creatinine kinase (U/L)	55 – 392

Statistical analyses—Analyses of the data were performed in a paired *t*-test,[23] in which the mean values for each parameter obtained from each calf before infection were compared with mean values obtained at each time point after infection, and differences were considered significant when $P < 0.05$. After 96 hours **post-inoculation (PI)**, the small number of survivors (3 survivors among 13 infected calves) precluded further statistical analysis.

Results

Data from the 3 uninoculated control calves, which were kept under the same experimental conditions throughout the time course of infection without being exposed to *S* Typhimurium, were used to ensure that environmental factors other than infection did not contribute to substantial changes in the studied parameters. No significant variation was detected in data from the control calves. Calves infected with either strain IR715 or CS401 had similar findings for the various parameters studied and were therefore analyzed as a single group.

Hematologic findings—The PCV, RBC count, and hemoglobin concentration were increased significantly 24 hours after inoculation and remained significantly higher than the pre infection values throughout the study (**Fig 1A-C**). Fibrinogen concentrations were significantly increased at 48 and 72 hours PI. Total leuko-

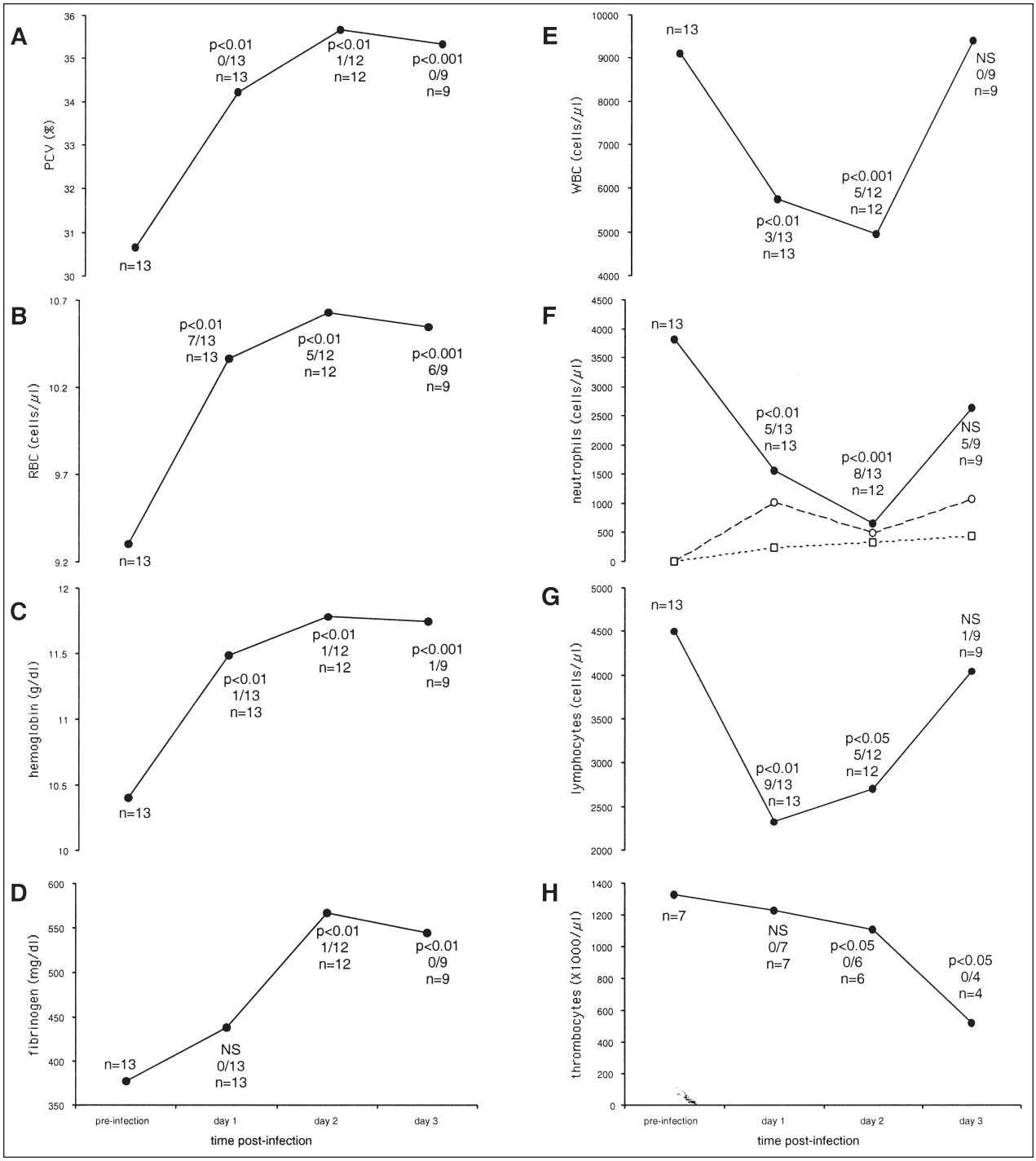

Figure 1—Hematologic findings (mean values) in calves inoculated with *Salmonella* Typhimurium. *P* values indicate comparison with value obtained before inoculation. Ratios indicate the proportion of calves with values outside of reference range. Sample size is indicated by n. In graph F, closed circles represent mature neutrophils, open circles represent band neutrophils, and open squares represent metamyelocytes.

cyte counts, as well as neutrophil and lymphocyte counts, were significantly decreased during the first 48 hours after infection but returned to pre inoculation concentrations by 72 hours PI. The decrease in the number of mature neutrophils was associated with increased numbers of band neutrophils and metamyelocytes, which was indicative of depletion of the bone marrow reserve of mature neutrophils, suggesting extensive or severe inflammatory lesions with heavy demand and use. Mean number of band neutrophils and metamyelocytes (491 and 319 cells/µl, respectively) at 48 hours PI became higher than the number of mature neutrophils (661 cells/µl). Mean number of thrombocytes was decreased significantly at 48 and 72 hours PI, but in none of the calves in which this parameter was measured were values less than reference

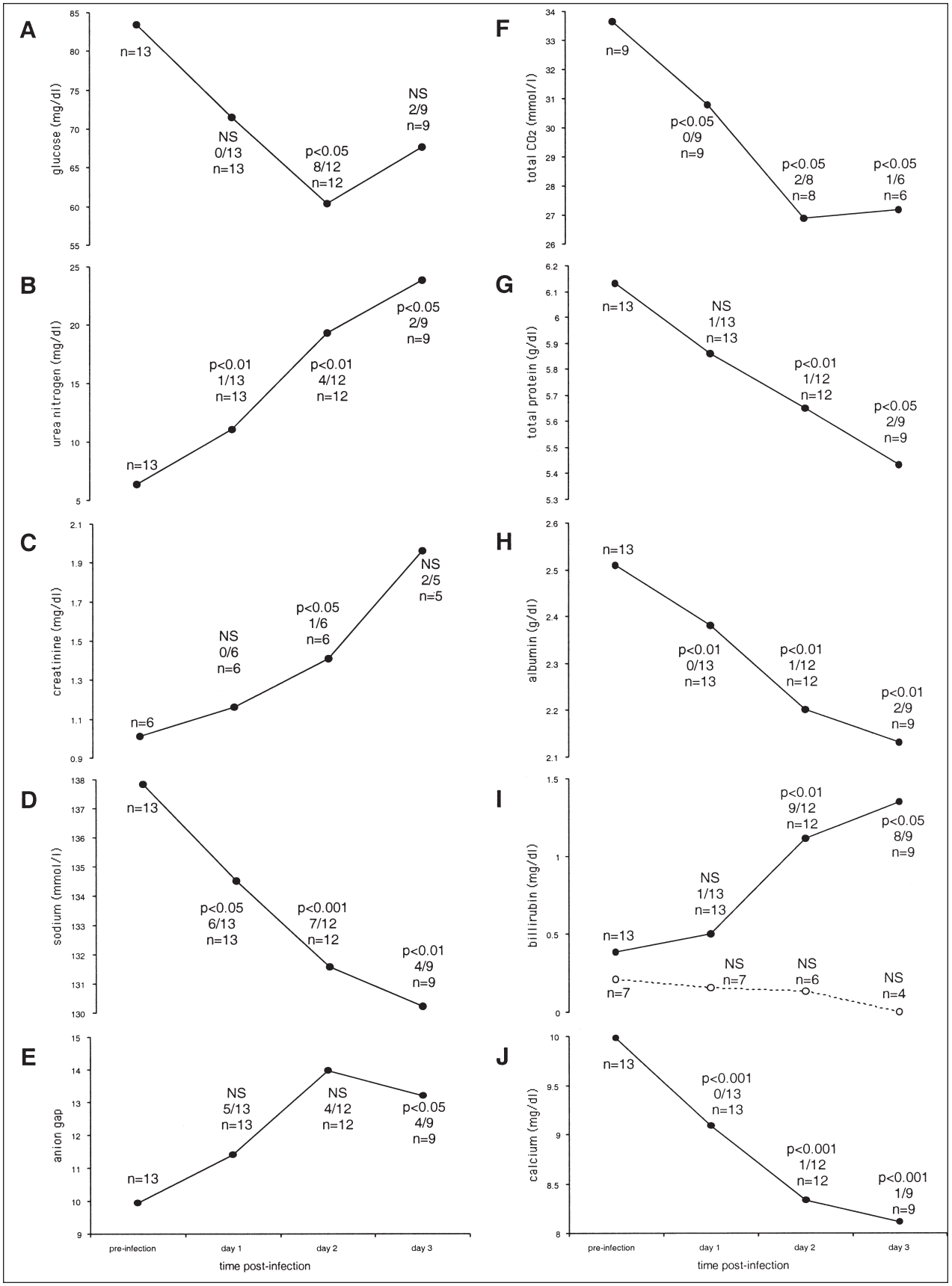

Figure 2—Serum biochemical findings (mean values) in calves inoculated with *S*Typhimurium. In graph I, closed circles represent total bilirubin concentration and open circles represent conjugated bilirubin concentration. *See* Figure 1 for key.

range. Mean number of monocytes increased continuously throughout the study (509, 597, 851, and 1,202 cells/μl, before infection and at 24, 48, and 72 hours PI, respectively). However, mean number of monocytes after inoculation was not significantly different from values determined before inoculation. The numbers of eosinophils and basophils decreased sharply after inoculation; however, because of the low reproducibility of the differential count of these cells attributable to their low concentration,[24] the data were not subjected to statistical analysis.

Serum biochemical findings—A significant decrease in glucose concentration was detected at 48 hours PI and most of the infected calves (8/12) had hypoglycemia at that time point (**Fig 2**). Urea nitrogen concentration increased steadily throughout the study. Creatinine concentration had a similar profile. Concentrations of sodium, total CO_2, calcium, total protein, and albumin decreased significantly after inoculation, which resulted in an anion gap that was significantly higher than values before inoculation and at 72 hours after inoculation. Concentration of chloride decreased after inoculation in a pattern similar to that observed for sodium, but there were no significant differences between pre- and post inoculation values. No significant changes in phosphorus concentrations were detected after inoculation. A significant increase in total bilirubin was detected at 48 and 72 hours PI, which was associated with decreased conjugated bilirubin concentration. No significant changes were detected among activities of aspartate aminotransferase, γ-glutamyl transpeptidase, alkaline phosphatase, or creatine kinase after inoculation.

Discussion

Although some of the parameters we investigated have been studied and reported in previous publications,[11,16,17] those studies did not cover a wide variety of parameters, and the variation in methodology makes it hard to compare results of different experiments. For instance, previous reports indicate that *S* Typhimurium infection in calves may result in either leukopenia or leukocytosis. In our study, paired analysis allowed a clearer evaluation of these parameters over the time course of infection, decreasing the influence of individual variation on the final interpretation of the data.

Although few calves were inoculated with strain CS401, the response to inoculation with this strain was similar to that detected with the wild type. This finding is in agreement with previous observations on clinical and pathologic aspects of infection with this strain.[21] Hemoconcentration (increased PCV, hemoglobin concentration, and relative polycythemia) was detected in virtually all the calves after inoculation. These data indicated that a high degree of dehydration had developed in the infected calves. Estimation of the plasma volume[25] on the basis of the PCV indicated that infected calves typically had a 15 to 20% decrease in plasma volume at any time point after inoculation. The degree of dehydration may be underestimated by hematologic parameters depending on the amount of blood lost in the feces as a consequence of the acute enteritis caused by *S* Typhimurium infection in calves.[15] Severe loss of body fluid has been reported in calves experimentally infected with *S* Typhimurium.[17] In sharp contrast, the concentration of total plasma protein, which is another indicator of plasma volume, decreased significantly and continuously after inoculation. Since the infected calves developed severe dehydration with mild to moderate hemoconcentration, this finding may seemed paradoxical, but may be explained by severe intestinal protein loss associated with the profound fibrinopurulent necrotizing enteritis that develops after *S* Typhimurium infection in calves, in which a large amount of protein-rich effusion enters the intestinal lumen.[14,15] A decrease in concentration of albumin parallel to that of total protein was detected after inoculation, indicating that the loss of protein was nonselective. Dehydration likely resulted in inadequate renal perfusion, which could explain the increase in BUN and creatinine concentrations observed after inoculation. Increased BUN in calves experimentally infected with *S* Typhimurium has been reported.[16]

Another important hematologic change that occurred during the first 48 hours immediately after inoculation was a marked leukopenia with neutropenia and lymphopenia, which was due to the small granulocyte reserve in the bovine bone marrow.[24] A regenerative response with increased numbers of band neutrophils and metamyelocytes was detected as early as 24 hours PI. The early neutropenia followed by neutrophilia explained the controversial reported data in which neutropenia and neutrophilia were observed after experimental infection with *S* Typhimurium.[11,16] Interestingly, the dramatic decrease in WBC in the blood occurs at the same time that a massive infiltration of neutrophils in the intestinal mucosa occurs.[14,15] This massive neutrophilic infiltration correlates with intestinal fluid secretion.[26]

Fibrinogen, a marker of acute-phase response in cattle,[27] was increased after inoculation. Increased fibrinogen concentration and slightly decreased thrombocyte concentration after inoculation are more consistent with a localized infection such as fibrinopurulent enteritis than with systemic sepsis and endotoxemia in which a marked decrease in fibrinogen and thrombocytes are expected when consumptive coagulopathy develops.[28]

In addition to dehydration, infection with *S* Typhimurium was associated with a significant acid-base imbalance, as indicated by increased anion gap and especially by the decreased total CO_2, which reflects the bicarbonate concentration.[25] These data indicate that the calves developed metabolic acidosis associated with the acute diarrhea.

Increase in total bilirubin concentration and decrease in conjugated bilirubin concentration is most likely the result of diminished uptake of unconjugated bilirubin from plasma by hepatocytes and decreased reflux of conjugated bilirubin from hepatocytes into plasma, which are effects of dehydration and lack of food intake.

Our results should be interpreted by taking into consideration that a high, standardized dose of bacteria was used. This is in contrast to the course of a natural outbreak in which affected animals are exposed to a wide range of infectious doses. Clinical manifestations

are dependent on the age of the host and infectious dose. Usually, oral inoculations with 10^4 to 10^7 CFU cause transient diarrhea that persists for 48 to 192 hours, whereas death may result from doses between 10^8 and 10^{11} CFU.[11,13,14,18] Furthermore, morbidity and mortality rates are inversely proportional to age.[11] Therefore, our results may not necessarily reflect those detected under field conditions in older calves or in calves challenged with a lower infectious dose. In addition to their clinical importance, these data should provide background information for experimental evaluation of genetically modified strains of salmonellae.

Our data suggest that acute salmonellosis in young calves results in dehydration and metabolic acidosis, hypoglycemia, and an acute inflammatory response associated with increased fibrinogen concentration and severe neutropenia immediately after inoculation. These data provide evidence for the roles that dehydration and metabolic dysfunction play in the acute illness and death associated with perinatal salmonellosis and diarrhea.

[a]Difco, Detroit, Mich.
[b]BBL, Sparks, Md.
[c]Abbott Cell Dyne 3500, Chicago, Ill.
[d]VITROS 250, Ortho Clinical Diagnostics, Johnson & Johnson, Raritan, NJ.

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