Pathophysiology of experimental *Aeromonas hydrophila* infection in mice

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Summary. A virulent fish strain of *Aeromonas hydrophila* was inoculated intramuscularly into laboratory mice (B10.G strain). Histological, biochemical and haematological changes during the first 36 h of the infection were measured. Inoculation led to septicaemia, tissue damage, endotoxic shock and death. Histological examination revealed: (1) severe muscle necrosis at the injection site; (2) oedema, haemorrhage and neutrophil infiltration of the lung; and (3) focal parenchymal necrosis in the liver. Significant increases in aspartate aminotransferase, alanine aminotransferase, intestinal bilirubin and blood urea nitrogen were noted in blood and intestinal samples; decreased plasma glucose and haematological changes were also recorded. Ketones, increased protein, glucose, bilirubin and blood were detected in the urine. Endotoxaemia was demonstrated as early as 2 h after inoculation and persisted for more than 36 h. The changes resembled those described for certain other experimental infections in laboratory animals. Our results suggest that endotoxin contributed to the pathogenesis of aeromonas infection in mice.

Introduction

Aeromonas hydrophila, a gram-negative bacillus, is commonly isolated from natural waters and from the organs of healthy and diseased fish (Shotts *et al.*, 1972; Hazen *et al.*, 1978). It has been found with increasing frequency as an opportunistic pathogen in human wounds sustained in aquatic environments (Deepe and Coonrod, 1980). These infections may remain localised and mild, or cause extensive muscle necrosis, or lead to septicaemia and infection in other sites. There may be skin lesions, fever, chills, and hypotension (Trust and Chipman, 1979). Furthermore, aeromonas infections frequently occur in immunocompromised hosts (Ketover *et al.*, 1973; Sirinavin *et al.*, 1984) and a mortality rate exceeding 50% has been reported in infected patients with neoplastic diseases (Ketover *et al.*, 1973).

Natural and experimental aeromonas infections in fish, amphibia and reptiles are associated with haematological (Roberts, 1978) and histopathological changes (Huizinga *et al.*, 1979). The pathogenesis of infections in poikilotherms and man are

incompletely understood but may be associated with the release of toxins (Ketover *et al.*, 1973). *In vitro*, *A. hydrophila* produces two haemolysins with different membrane-damaging effects on fibroblasts (Thelestam and Ljungh, 1981), and acyltransferase and phospholipase which deacylate human-erythrocyte membrane glycerophospholipids (MacIntyre and Buckley, 1978). The effects of *A. hydrophila* toxins produced *in vitro* have been studied in rats (Ljungh and Kronevi, 1982), rabbits (Wretling *et al.*, 1971) and mice (Rigney *et al.*, 1978), but it is not known whether such toxins are elaborated *in vivo*.

Because aquatic environments are a frequent source of infection for man, we investigated pathophysiological changes in mice given intramuscular inocula of a virulent fish strain (AM3) of *A. hydrophila*. Most, but not all, of our observations were made 12–36 h after inoculation because 50% of the deaths that occurred took place during that period.

Materials and methods

Bacterial strain

A virulent strain (AM3) of *A. hydrophila* from a diseased fish was provided by D. H. Lewis, Texas A&M University. Its virulence was maintained by occasional

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passage through goldfish, and the LD50 for mice was 4.5×10^6 cfu/mouse (Brenden, 1984).

Animals

Inbred male B10.G mice aged 2–3 months and weighing 20–30 g were used. Two weeks earlier they had been treated for 3 days with 0.25% Panacure anthelmintic (Fenbendazole, Hoechst-Roussel Pharmaceuticals, Inc., Sommerville, NJ).

Preparation of bacterial suspensions

An 18-h culture of strain AM3 on sheep-blood agar (Difco) was subcultured in 3 ml of Brain Heart Infusion (BHI) Broth (Difco) for 18 h at 37°C. The culture was centrifuged and the pelleted cells were resuspended in phosphate buffered saline (PBS) to 40% transmittance at 435 nm (Spectronic 20 Spectrophotometer, Bausch and Lomb). Viable counts made in triplicate on BHI agar showed that such suspensions contained $c. 3 \times 10^8$ cfu/ml.

Animal inoculation

The bacterial suspension (0.1 ml; 3×10^7 cfu) was injected intramuscularly in the left hindquarter of each test mouse. This inoculum produced 75% mortality within 6 days (144 h) and 50% mortality within 36 h (Brenden, 1984). Histological, biochemical, urinological, haematological and toxicological (endotoxin) studies were made with groups of 126, 130, 100, 130 and 25 infected mice, respectively.

Pathological examination

A total of 126 mice were given injections of *A. hydrophila*. Four were killed by cervical dislocation each hour for the first 11 h. Six were killed at 12, 24 and 36 h. Control mice given 0.1 ml of PBS were also killed on each occasion. Gross pathological changes, body weight and spleen weight were recorded. Samples of spleen, lung, liver, kidney and duodenum, and muscle from the injection site were then removed and fixed in 10% neutral buffered formalin for 24 h, embedded in paraffin and sectioned at 5–7 μ m. Sections were stained with haematoxylin and eosin, and by the McManus method for glycogen (Luna, 1968), the Gomori method for iron (Luna, 1968), and Taylor's modification of Gram's method (Bartholomew, 1980). Additional tissue samples were collected from survivors killed on day 6 (144 h) and immediately after death from those that succumbed to infection.

Biochemical examination

One hundred and thirty mice received injections of *A. hydrophila*, 80 received 0.1 ml of PBS, and 45 remained untreated. Three infected mice were killed each hour for the first 11 h and 15–20 mice at 12, 24 and 36 h. PBS-

treated control mice were sampled at 12, 24 and 36 h. Untreated mice were sampled at the beginning of the experiment ("0 h").

Mice were given an intraperitoneal 0.1-ml injection of ammonium heparin (100 units) 10 min before slaughter. The major blood vessels in the opened thorax were severed and blood was collected from the thoracic cavity with a syringe. The plasma was separated and frozen at 0°C. The abdominal cavity was also opened and a length of small intestine measuring 7 cm from the pyloric sphincter was excised and the contents were expressed into 2 ml of PBS. The intestinal samples were placed in a light-tight container and refrigerated (4°C) overnight.

Plasma samples were diluted 1 in 10 in 0.9% saline for assay of aspartate aminotransferase (ASAT), alanine aminotransferase (ALAT), blood urea nitrogen (BUN) and glucose (GLU) on a KDA Analyzer (American Monitor Corp., Indianapolis, IN). Plasma was also analysed for sodium and potassium with a Model 143 Flame Photometer (Instrumentation Laboratory Inc., Boston, MA), and for total protein with a Refractometer (American Optical Co., Buffalo, NY). Undiluted intestinal contents were analysed for total and conjugated bilirubin with an ACA III Clinical Analyzer (Dupont Co., Wilmington, DE).

Urine analysis

One hundred mice were given *A. hydrophila* and groups of six were killed at 1–12, 24 and 36 h. Forty-five PBS-treated control animals were also killed, in groups of three on each occasion. Urine was collected by needle and syringe from the urinary bladder after death. The analysis included tests for protein, blood, ketones, glucose, bilirubin, urobilinogen and nitrite by means of N-Multistix (Ames Chemical Co., Ames, IA). Visual colour and pH were recorded and specific gravity was measured with a refractometer.

Haematological examination

Fifteen mice received 0.1 ml of ammonium heparin (100 units) intraperitoneally. Blood was then collected from the cut tail for the measurement of microhaematocrit (HCT) values and preparation of blood smears for white blood cell (WBC) differential counts. When, 2 days later, the same animals were given an injection of *A. hydrophila*, similar examinations were made from tail blood on single mice at hourly intervals from 1 to 11 h.

Another 115 mice were given *A. hydrophila* and blood samples were collected from the tail of groups of 10–15 at 12, 24 and 36 h. Sixty untreated mice were sampled at the beginning of the experiment ("0 h") to show the normal blood picture. Samples were collected from the tails of sixty further mice, which had received an injection of PBS, in groups of 20 at 12, 24 and 36 h. Mice received 0.1 ml of ammonium heparin (100 units) intraperitoneally before blood collection. Infected animals surviving beyond 36 h were sampled from the tail at 48, 60, 72 and 144 h, 2–8 mice being examined on each occasion. Blood

collected at 12–144 h was examined for total red blood cell (RBC) and WBC counts, haemoglobin (HGB), HCT values, and differential WBC count. Blood to be examined for RBC and WBC counts and HGB was collected from the tail of each mouse into two 10- μ l capillary tubes. The content of each capillary tube was expelled into a tube containing 10 ml of PBS (0.9%). The diluted blood was examined with a Coulter Counter S Sr (Coulter Electronics Inc., Hialeah, FL). The Coulter data for WBC, RBC, and HGB were multiplied by five to compensate for a five-fold difference in dilution factors required for Coulter analysis of mouse blood over that required for human blood (1 in 1000 dilution of mouse blood versus 1 in 200 dilution of human blood). A hospital Coulter Counter S Sr was employed in this study and the upper and lower limits of analysis were therefore based upon human RBC concentrations. Mouse blood, having a higher concentration of RBCs (Altman and Dittmer, 1974), required a greater initial dilution before analysis on the Coulter Counter. The average WBC, RBC and HGB values of two dilution tubes were recorded and analysed statistically. Blood intended for HCT determinations was collected into heparinised capillary tubes, which were then sealed with clay and centrifuged for 3 min at 13460 *g* in an IEC MB Microhaematocrit Centrifuge (International Equipment Co., Needham Heights, MA). Blood smears were stained by the Wright-Giemsa method for differential white blood cell counts.

Limulus amoebocyte lysate test

Twenty-five mice were given *A. hydrophila* and 15 received PBS. One mouse/h (1–12, 24 and 36 h) from each group was given 0.1 ml of ammonium heparin (100 units) intraperitoneally, anaesthetised (sodium pentobarbital; Fort Dodge Laboratories, Fort Dodge, IA), and bled by cardiac puncture. The plasma was treated with chloroform as specified by Sigma Technical Bulletin (Sigma Chemical Co., St Louis, MO) for use in the E-toxate Test.

Statistics

The *t*-test was used to analyse spleen weights. A two-way analysis of variance (ANOVA) was used to analyse biochemical and haematological data. When no significant differences between untreated (control) and PBS-treated (control) mice appeared, these uninfected animals were regarded as comprising a single control group. Data showing statistical differences ($p < 0.05$) by ANOVA were analysed with the Newman Keul's test (Kirk, 1968) to locate the treatment effects.

Results

Clinical and pathological changes

Clinical signs. Two hours after infection, all mice exhibited huddling, raised fur, and shaking, and, as death approached, laboured breathing and diarr-

hoea. Ocular discharge and occasionally opacity were observed.

Gross pathology. Severe haemorrhage and swelling were observed within 24 h at the site of injection. By 144 h the lesion covered an area of 15–20 mm², with necrosis extending into the musculature.

Experimental mice necropsied at 12, 24 and 36 h revealed congestion of the liver, spleen and kidney, and the heart was enlarged and dark in colour. There was a bright orange discolouration of the intestinal tract. Spleens from infected animals killed at 144 h (0.337 ± 0.13 g) were significantly larger ($p < 0.05$) than those of control animals (0.043 ± 0.04 g).

Histopathology. All organs examined were congested, and the injection site showed severe necrosis of skin and muscle. Pulmonary congestion and WBC infiltration were noted as early as 1 h after inoculation. Liver pathology was variable. Congestion, swollen Kupffer cells and enlarged hepatocytes represented mild changes observed as early as 8 h after infection, and focal necrosis was seen later (24 and 36 h). There was no depletion of glycogen but iron was present in all samples taken from 8–36 h.

At 2 h the spleen was congested, and loss of definition of the red and white pulp and an increase in the number of megakaryocytes were observed at 24 h. Iron was present in samples taken at 8–36 h.

The kidneys and small intestine showed tissue oedema. Gram's stain failed to reveal bacteria in any tissue, but bacteria were cultured from all internal organs.

Biochemical changes

Glucose analysis. The plasma glucose values fluctuated but they were significantly lower ($p < 0.0001$) than those of the controls at 12 and 24 h (table I).

ASAT and ALAT analysis. Plasma ASAT values rose steadily in infected mice, significant differences ($p < 0.0001$) from controls being recorded at 11, 12, 24 and 36 h. Plasma ALAT values fluctuated but were significantly elevated ($p < 0.0001$) at 12 and 24 h. The surviving animals sampled at 144 h had ASAT and ALAT values similar to those of the controls (table I).

BUN analysis. BUN values were within one standard deviation from the mean of the control group for the first 10 h of the study and significantly raised ($p < 0.0001$) at 11, 12, 24 and 36 h. Survivors had, at 144 h, values similar to those of the controls (table I).

Intestinal bilirubin. Intestinal bilirubin concentrations were significantly raised ($p < 0.0001$) in all

Table I. Biochemical analysis of plasma and intestinal contents from *A. hydrophila*-inoculated mice

Hours after infection	Biochemical values (mean $\pm$ SEM)				
	Plasma GLU (mg/dl)	Plasma ASAT (u/L)	Plasma ALAT (u/L)	Plasma BUN (mg/dl)	Intestinal bilirubin (mg/dl)
0†	167.5 $\pm$ 5.1	429.3 $\pm$ 20.7	79.3 $\pm$ 2.8	28.4 $\pm$ 0.8	0.52 $\pm$ 0.03
1	*246.7 $\pm$ 19.4	307.0 $\pm$ 90.0	104.7 $\pm$ 7.7	21.3 $\pm$ 7.6	0.48 $\pm$ 0.06
2	188.0 $\pm$ 6.4	692.0 $\pm$ 292.3	148.0 $\pm$ 32.2	20.0 $\pm$ 2.0	0.71 $\pm$ 0.11
3	147.3 $\pm$ 27.3	741.7 $\pm$ 361.4	267.3 $\pm$ 113.3	18.4 $\pm$ 5.9	0.61 $\pm$ 0.12
4	183.0 $\pm$ 45.7	550.7 $\pm$ 71.3	142.3 $\pm$ 6.9	24.7 $\pm$ 2.9	1.08 $\pm$ 0.29
5	*219.3 $\pm$ 92.9	522.0 $\pm$ 30.0	120.3 $\pm$ 12.2	22.0 $\pm$ 1.0	*1.51 $\pm$ 0.37
6	152.0 $\pm$ 17.0	607.7 $\pm$ 130.0	138.0 $\pm$ 21.7	29.3 $\pm$ 6.7	*1.85 $\pm$ 0.89
7	151.7 $\pm$ 20.9	1020.0 $\pm$ 605.7	186.3 $\pm$ 79.0	18.5 $\pm$ 5.5	*2.58 $\pm$ 0.77
8	157.3 $\pm$ 25.7	944.3 $\pm$ 140.6	177.0 $\pm$ 16.7	26.5 $\pm$ 1.5	*4.27 $\pm$ 1.38
9	149.3 $\pm$ 14.9	1025.0 $\pm$ 92.9	139.7 $\pm$ 17.3	22.5 $\pm$ 2.5	*3.09 $\pm$ 1.04
10	123.3 $\pm$ 24.9	949.0 $\pm$ 75.5	125.7 $\pm$ 12.6	21.0 $\pm$ 3.0	*5.33 $\pm$ 1.71
11	133.7 $\pm$ 21.8	*1814.5 $\pm$ 939.5	157.5 $\pm$ 52.5	*64.0 $\pm$ 0.0	*4.21 $\pm$ 0.73
12	*107.2 $\pm$ 9.9	*2416.1 $\pm$ 337.9	*327.9 $\pm$ 66.1	*53.7 $\pm$ 5.4	*3.03 $\pm$ 0.59
24	*81.5 $\pm$ 7.5	*3407.3 $\pm$ 375.9	*338.9 $\pm$ 39.7	*95.0 $\pm$ 5.7	*5.77 $\pm$ 1.34
36	143.2 $\pm$ 8.5	*1809.2 $\pm$ 137.6	200.4 $\pm$ 11.6	*66.7 $\pm$ 5.7	*3.19 $\pm$ 0.66
144	134.2 $\pm$ 12.1	321.6 $\pm$ 45.4	87.7 $\pm$ 6.5	19.6 $\pm$ 1.4	0.69 $\pm$ 0.08

GLU = glucose; ASAT = aspartate aminotransferase; ALAT = alanine aminotransferase; BUN = blood urea nitrogen. N = 3 for samples taken at 1–11 and 144 h; N = 12–18 for samples taken at 12–36 h.

* Significantly different from the control group (see below) by ANOVA ($p < 0.0001$) and Newman Keul's test ($p < 0.05$).

† Control group (see *Materials and Methods, Statistics*); N = 108–119.

samples taken from 5 to 36 h. Survivors killed at 144 h had bilirubin values near to those of controls (table I).

Other biochemical parameters. Plasma sodium, potassium, and total protein concentrations did not differ significantly from those of the controls, provided that the plasma samples were obtained from unhaemolysed blood.

Urine analysis

Infected mice sampled at 5, 6, 10 and 11 h yielded insufficient urine for analysis; samples taken at 1, 2, 3, 4, 7, 8 and 9 h had specific gravity, colour, pH, protein, glucose, ketones and urobilinogen values similar to those of control mice, but bilirubin and blood were detected. At 12, 24 and 36 h, urine from infected mice was abnormal in colour (yellow, green, or brown) and contained high concentrations of protein (100–300 mg%). Of the 12 and 24 h urine samples, 50–100% gave positive tests for glucose, ketones, bilirubin and blood, whereas 0–56% were positive in 36 h samples.

Haematological changes

Infected mice showed slightly increased HCT

values (52–60%; $p > 0.05$) between 2 and 8 h (table II), but by 24 h there was no difference from the control (pre-infection) values (mean 50.6%). There was a significant decrease ($p < 0.001$) thereafter. By day 6 (144 h), the RBC values of infected mice were higher than at 72 h but significantly lower ($p < 0.001$) than control values (table III). Visible haemolysis was present in some samples. Significantly reduced values ($p < 0.001$) for RBC and HGB were recorded at 36, 48, 60, 72 and 144 h.

White blood cell counts measured at 12 h and later were higher than the control values, but significant differences ($p < 0.001$) occurred only at 24 and 144 h (table III). Differential WBC counts showed a significant increase in polymorphonuclear leukocytes (PMN) and decrease in lymphocytes (LYM) in the 12, 24 and 36 h samples (table III). This shift towards a predominance of PMNs was observed as early as 5 h after infection (table II).

Endotoxin assay

Plasma from experimental mice sampled at 2–36 h after inoculation was positive for endotoxin by the amoebocyte lysate test. The gelation pattern for samples taken at 2–7 h was one of ‘starchy

Table II. Haematological examination of mice 1–11 h after inoculation of *A. hydrophila*

Mouse no.	Time (h) after infection at which post-infection samples taken	Pre-infection values for					Post-infection values for				
		HCT (%)	WBC differential count				HCT (%)	WBC differential count			
			PMN (%)	LYM (%)	MON (%)	EOS (%)		PMN (%)	LYM (%)	MON (%)	EOS (%)
1	1	54	5	95	...	...	48	27	64	9	...
2	2	45	18	76	6	...	53	42	56	2	...
3	3	52	10	90	...	...	57	39	54	5	2
4	4	49	12	86	2	...	53	32	64	4	...
5	5	48	18	80	2	...	53	52	44	4	...
6	6	50	8	88	4	...	60	52	46	...	2
7	7	50	18	79	...	3	59	62	34	4	...
8	8	49	13	87	...	...	54	55	39	6	...
9	9	50	24	76	...	...	52	50	44	6	...
10	10	51	8	89	3	...	55	66	32	2	...
11	11	52	4	96	...	...	50	62	34	4	...

HCT = microhaematocrit; WBC = white blood cells; PMN = polymorphonuclear leukocyte, LYM = lymphocyte, MON = monocyte, EOS = eosinophil.

Table III. Haematological examination of mice 12–144 h after inoculation of *A. hydrophila*

Time (h) after inoculation	Haematological values (mean $\pm$ SEM)							
	WBC ($10^3/\text{mm}^3$)	RBC ($10^6/\text{mm}^3$)	HGB (mg/dl)	HCT (%)	WBC differential count			
					PMN (%)	LYM (%)	MON (%)	EOS (%)
0†	14.5 $\pm$ 0.3	11.3 $\pm$ 0.1	18.6 $\pm$ 0.1	50.6 $\pm$ 0.2	14.8 $\pm$ 2.1	83.0 $\pm$ 2.6	2.0 $\pm$ 0.5	0.2
12	23.4 $\pm$ 1.8	10.4 $\pm$ 0.4	19.5 $\pm$ 0.4	51.8 $\pm$ 1.2	*73.0 $\pm$ 1.4	*16.0 $\pm$ 2.4	11.0 $\pm$ 2.2	0.0
24	*26.7 $\pm$ 3.3	10.4 $\pm$ 0.4	18.9 $\pm$ 0.4	50.7 $\pm$ 1.4	*70.0 $\pm$ 4.0	*16.0 $\pm$ 1.0	13.0 $\pm$ 2.0	1.0
36	23.5 $\pm$ 1.9	*8.5 $\pm$ 0.4	*15.5 $\pm$ 0.4	*42.1 $\pm$ 1.0	*65.0 $\pm$ 7.8	*30.0 $\pm$ 8.2	5.0 $\pm$ 0.8	0.0
48	16.9 $\pm$ 1.5	*8.3 $\pm$ 0.3	*14.2 $\pm$ 0.5	*35.4 $\pm$ 1.8	...	...	...	...
60	18.7 $\pm$ 1.6	*7.7 $\pm$ 0.2	*13.9 $\pm$ 0.4	*35.0 $\pm$ 1.0	...	...	...	...
72	23.8 $\pm$ 2.5	*7.0 $\pm$ 0.4	*13.5 $\pm$ 0.5	*33.5 $\pm$ 4.5	...	...	...	...
144	*44.0 $\pm$ 1.2	*8.4 $\pm$ 0.4	*15.6 $\pm$ 0.5	*43.3 $\pm$ 1.8	...	...	...	...

See footnotes to table II.

HGB = haemoglobin.

N = 8–14 for samples taken at 12–48 h; N = 2–4 for samples taken at 60–144 h.

* Significantly different from the control group by ANOVA ($p < 0.001$) and Newman Keul's test ($p < 0.05$).

† Control group: N = 13 for PMN, LYM, MON and EOS; N = 112–116 for WBC, RBC, HGB, and HCT.

granules" (+ reaction); a "soft gel" (++) reaction) was formed by the 8–36-h samples.

Discussion

A. hydrophila infection in mice caused extensive necrosis at the site of intramuscular inoculation,

followed by septicaemia, endotoxic shock and death (Brenden, 1984). Hypotension, chills and fever are associated with septic shock in man (Trust and Chipman, 1979; Hardaway, 1980) and animals (Kabir *et al.*, 1978). Conjunctivitis has been reported in *A. hydrophila* septicaemia in man (Feaster *et al.*, 1978; Ramsay *et al.*, 1978).

Histopathological effects were mainly confined to the lungs and liver, but all organs showed signs of congestion. Corrin (1980) observed that the lungs of human bacterial-shock victims are the most frequently damaged organs. The liver of infected mice showed focal parenchymal necrosis and swelling of Kupffer cells, both of which can be caused by bacterial endotoxin (Levy and Ruebner, 1967). Filkins (1969) suggested that long and severe endotoxaemia impairs liver function by damaging the vasculature. However, parenchymal damage may also be caused by tissue anoxia resulting from vasoconstriction or by extracellular bacterial toxin.

Periodic hyperglycaemia occurred 1–6 h after infection, and was followed by a period of declining plasma glucose concentrations. The lowest mean glucose concentration of 81.5 mg/dl was reached in 24 h, but glycogen stores in the liver were not depleted. Shands *et al.* (1969) reported glucose concentrations of 78 mg/dl at the time of death in endotoxin-treated mice.

The liver enzymes ASAT and ALAT increased, beginning at 2 h and reaching their maxima at 24 h. The ASAT and ALAT increases may have been due to changes in cell membrane permeability during the first 11 h. The high concentrations recorded at 11, 12, 24 and 36 h may reflect the release of enzymes from tissue damaged by anoxia. Focal necrosis was observed in liver as early as 8 h.

BUN concentrations rose significantly from 11 to 24 h and began to decline at 36 h. Increased BUN levels suggest suppressed kidney function. Retention of urea without obvious kidney damage has been reported by Berry and Smythe (1961) in endotoxin-treated mice, and by Walker and Chesbro (1975) in mice infected with *Klebsiella pneumoniae*. The lack of urine production at 5, 6, 10, and 11 h suggested reduced filtering ability of the kidney. Renal tissue was observed to be oedematous and congested in histological sections. Oedema alters the membrane permeability and would account for the increased excretion of protein in the urine. The presence of blood in some urine samples indicated haemorrhage.

Increased intestinal bilirubin concentrations, recorded from 5 to 36 h, reached their maxima at 24 h. Eddington and Kampschmidt (1968) noted a doubling of the bilirubin production in rats treated with endotoxin. They attributed this to lysis of RBCs or skeletal muscle cells, or to cytochromes or catalase. In the present study, the elevated bilirubin

concentrations (intestinal and urinary), positive iron stains in liver and spleen, and decreased RBC, HGB and HCT suggest RBC lysis.

Total sodium and protein concentrations changed little. The only increases in potassium concentrations that occurred were attributed to the lysis of RBCs. Hambleton *et al.* (1977) reported no changes in sodium or potassium in rabbits infected with *Francisella tularensis*.

Oedema at the injection site, biochemical changes, and haemoconcentration 2–8 h after inoculation suggested an increase in vascular permeability caused by a toxin. Oedema and haemoconcentration have been reported in mice infected with *Vibrio vulnificus* (Bowdre *et al.*, 1981).

Haemolysis of RBCs occurred in the period during which the majority of deaths occurred (24–36 h). In an account of fish infected with *A. hydrophila* Roberts (1978) reported blood-cell changes resembling those described here. Three mechanisms may account for RBC lysis: (1) activation of the alternate complement pathway by lipopolysaccharide; (2) increased phagocytosis of endotoxin-coated RBCs; and (3) the action of a haemolysin or other bacterial factor, such as phospholipase. RBCs damaged directly or indirectly would be removed from the circulation by the spleen. Iron deposits were present in spleen sections, and enlarged spleens were observed in surviving animals.

Endotoxaemia was present as early as 2 h after inoculation and persisted for at least 36 h. Thus it coincided with the period during which most deaths occurred and was undoubtedly an important factor in the pathogenesis of the infection. Other bacterial products may also have been concerned. Thus Rigney *et al.* (1978) found that red leg disease of frogs was induced only when endotoxin and partially purified haemolysin of *A. hydrophila* were administered simultaneously. Further studies should concentrate on the effects of mutant strains of *A. hydrophila* deficient in certain extracellular toxins.

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