JOURNAL OF BACTERIOLOGY, Feb., 1967, p. 531-537
Vol. 93, No. 2

Mechanisms of Pathogenesis in *Listeria monocytogenes* Infection

II. Characterization of Listeriosis in the CD-1 Mouse and Survey of Biochemical Lesions

MARTIN S. WILDER[1] AND C. P. SWORD

Department of Microbiology, The University of Kansas, Lawrence, Kansas

Received for publication 29 September 1966

ABSTRACT

Several physiological and biochemical changes which occur in CD-1 pathogen-free mice during the course of infection with *Listeria monocytogenes* strain A4413 have been examined. Mice injected with 10^4 to 10^6 organisms by the intraperitoneal route displayed a significant depression in weight gain. In contrast, at 24 hr after infection an increment in total liver weight averaging 0.1 g was observed. The ratios of liver to body weight increased throughout the observation period. As the severity of the infection increased, food intake, as well as total liver protein and nitrogen, showed a corresponding decrease, with the diminution being most evident immediately prior to the death of the animals. Blood urea nitrogen remained relatively constant for 24 hr and then increased continuously as the infection progressed to the acute stage. Total liver lipid increased until the death of the animals. At 72 hr postinfection, a significant decrease in oxidative phosphorylation was observed. Xanthine dehydrogenase activity increased, with maximal values obtained 72 hr after infection. Uric acid levels remained constant for 24 hr, diminished at 48 hr, and then increased until the death of the animals. After 24 hr, uricase activity showed a slight increase. This activity returned to within normal ranges at 48 hr and decreased as the infection progressed to the acute stage at 72 hr. The results support the hypothesis that at least a part of the cause of death is a derangement in hepatic purine and carbohydrate metabolism. The data are also consistent with the possibility of changes in iron transport in the infected mice.

The gross and microscopic pathological changes which occur in murine listeriosis are well documented (6, 16). Listeriosis in mice is an acute disease, with death usually occurring between 72 and 96 hr after intraperitoneal injection of 10^4 to 10^6 organisms. *Listeria*-infected mice characteristically develop multiple necrotic lesions in organs such as the liver and spleen, and usually die early of an overwhelming disseminated infection. Denk (4) described alterations in the subchorial sinuses of the placenta of pregnant mice at 24 hr after infection. The organisms were shown to penetrate the fetal circulation by initiating extensive destruction of placental blood vessels. Experimental listeriosis in mice resembles naturally occurring listeric septicemia in rodents as well as other animal species, including man, but is unlike the encephalitic or meningitic forms often occurring in ruminants and in some human cases (6).

[1] Present address: U.S. Army Biological Laboratories, Fort Detrick, Frederick, Md.

Although several critical histopathological and morphological studies have been reported, there is still a paucity of precise information concerning the biochemical and physiological changes that attend necrosis during *Listeria* infection. McIlwain et al. (12) described several biochemical aberrations in rabbits inoculated with a toxic proteinaceous component of *Listeria*. Recent studies on ultrastructural changes in *Listeria*-infected mouse splenic tissue describe disruption of the phagosome with subsequent release of its contents (1). These studies suggest that the lysosomal membrane may possibly break down by the action of the lecithinase activity of *Listeria* hemolysin. Sword (17) established that virulence as well as in vitro growth of *L. monocytogenes* is greatly enhanced when cells are grown in the presence of iron salts. After finding increased levels of transferrin in the serum of mice infected with *Listeria*, Sword (18) suggested the possibility of changes in host iron transport.

The present study was undertaken to character-

ize the disease in the CD-1 mouse and to survey some of the physiological and biochemical changes occurring during the infection. Previous work with these animals indicated that they are susceptible and respond in a uniform manner to experimental listeriosis; thus, they seem to lend themselves to use as a model for studies on the mechanism of pathogenesis. It was hoped that this investigation would provide additional clues regarding specific biochemical lesions attributable to listeric infection.

MATERIALS AND METHODS

Bacteria. L. monocytogenes strain A4413, serotype 4b, a strain of high virulence, was employed for these studies. This strain was originally isolated at the Communicable Disease Center, Atlanta, Ga., from a child with a fatal infection, and was obtained from the U.S. Army Biological Laboratories, Fort Detrick, Frederick, Md.

Cultures were preserved on frozen Tryptose Agar (Difco) slants. Infecting inocula were prepared by inoculating Tryptose Broth (Difco) from a thawed slant and incubating the cultures at 37 C for 24 hr. Suitable dilutions were made in the above medium, and the bacterial count of the inoculum was determined by quantitative plating on Tryptose Agar.

Animals. White female Swiss Webster mice (CD-1 strain, pathogen-free, from the Charles River Mouse Farms, Inc., North Wilmington, Mass.) weighing 16 to 18 g were used for all experiments.

Infection and sacrifice. Mice were infected by the intraperitoneal route with 10^4 to 10^6 organisms suspended in 0.2 ml of Tryptose Broth. This dose range kills the first animals at about 60 hr, and 90 to 95% of the mice succumb within 3 to 4 days. At intervals of 0, 24, 48, and 72 hr, the mice, both normal and infected, were sacrificed by either a severe blow on the head or by CO_2 anesthesia. The animals were then bled, and the livers were quickly excised, freed from adhering blood with filter paper, and transferred to a petri dish chilled in ice. Pooled blood samples were obtained by cardiac puncture.

Preparation of liver homogenates. Pooled tissues were homogenized in a glass or Teflon grinder with 0.25 M sucrose. The cellular debris was removed by low-speed centrifugation at 480 $\times$ *g* for 10 min.

Animal and liver weights. Mice were randomly divided into groups of 10 animals. Several groups were infected, and others were retained as normal controls. The animals were individually numbered, and daily weights were kept for all animals. Three animals from each of two infected groups were sacrificed at 24-hr intervals, and their livers were quickly excised and weighed. Additional infected groups were retained only for total weight determinations.

Food and water intake. The mice were maintained on Purina laboratory chow and water. Water intakes were measured daily by weighing the bottles before and after consumption. Food intake was similarly measured by weighing the food in the cages every 24 hr. Food and water were provided *ad libidum*.

Protein determination. Protein was determined according to the method described by Lowry et al. (11).

Liver nitrogen. Liver nitrogen was determined colorimetrically by the addition of Nessler's reagent (10).

Liver lipid. Liver lipid was extracted with a 2:1 chloroform-methanol mixture according to the method of Folch et al. (5). Quantitative determinations were carried out by placing 0.2 ml of the extract in preweighed dried aluminum tares.

Blood urea nitrogen. Urea nitrogen was determined colorimetrically at 410 mμ after hydrolysis with urease and addition of mercuric iodide color reagent, as described in Sigma Technical Bulletin No. 14 (Sigma Chemical Co., St. Louis, Mo.).

Oxidative phosphorylation. Oxidative phosphorylation was measured by the method outlined by Hunter (8) employing a Warburg apparatus. Sodium succinate was employed as substrate. Phosphate was determined by the method of Bruemmer and O'Dell (3).

Xanthine dehydrogenase. Xanthine dehydrogenase activity was assayed colorimetrically by following the reduction of the redox dye, 2,6-dichlorophenolindophenol, at 600 mμ in a spectrophotometer (15).

Uricase. Uricase activity was determined spectrophotometrically at 290 mμ, by use of a molar absorbtivity of 1.22×10^4 $mole^{-1}cm^{-1}$ according to the method of Kalckar (9).

Uric acid. Uric acid was determined colorimetrically by reduction of the phosphotungstate reagent, as described in Sigma Technical Bulletin No. 680 (Sigma Chemical Co., St. Louis, Mo.).

RESULTS

Weight changes and dietary intake. The results, expressed as percentages of the control group weights, are presented in Fig. 1. Infection with *L. monocytogenes* caused a significant weight loss in these animals. The mean weight of the infected mice continued to decrease as the infection progressed to the acute stage. Liver weights were determined throughout the infection. Weights were approximately 1 g for both normal and infected animals. At 24 hr after infection, an increment in total liver weight averaging 0.1 g was observed for the infected animals, whereas no changes were noted in the normal controls. The ratios of liver to body weight are presented in Fig. 2. These ratios increased throughout the observation period. Food and water intake were measured to determine whether metabolic changes could be due to inanition, since this has been suggested as an influencing factor for changes in mice during *Salmonella typhimurium* infection (2). Food intake (Fig. 3) decreased to 77 and 27% of normal values at 24 and 48 hr, respectively. Similarly, the water intake dropped 29% at 24 hr and 66% at 48 hr. Essentially all intake of food and water ceased after 48 hr. It is of interest that intake did not stop abruptly after the animals were infected.

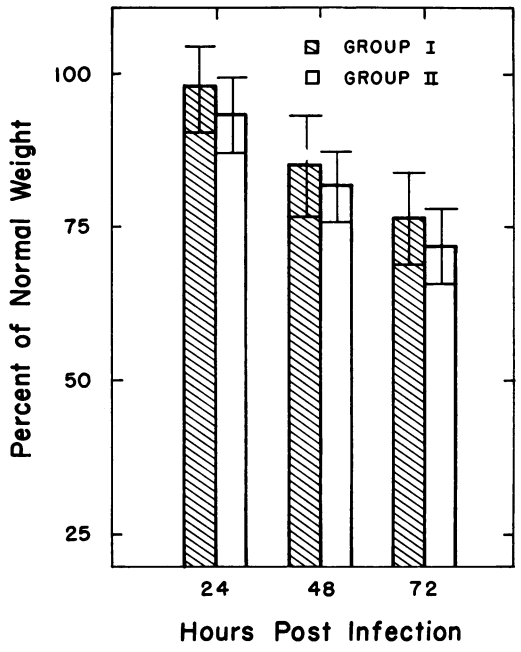

FIG. 1. *Weights of Listeria monocytogenes-infected mice expressed as percentage of control weights. Each bar represents the mean percentage of 10 animals ± SE of the mean.*

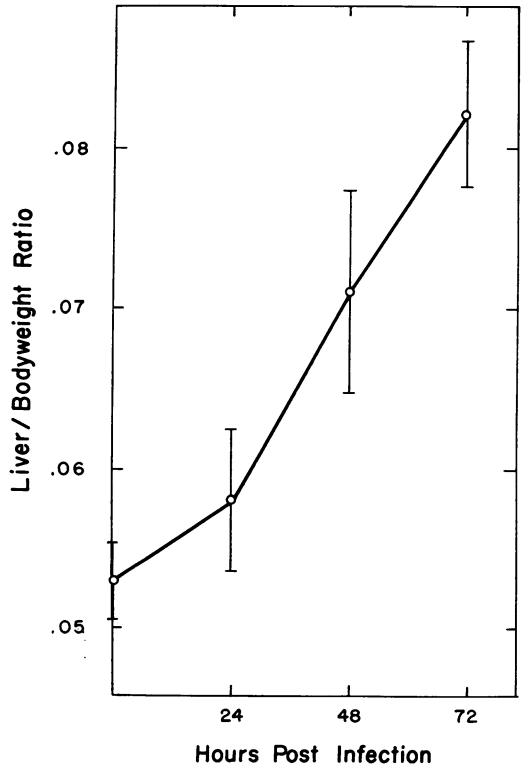

FIG. 2. *Liver-body weight ratios of Listeria-infected mice. Each point indicates the mean liver weight to body weight ratios ± SE of the mean for groups consisting of 10 mice.*

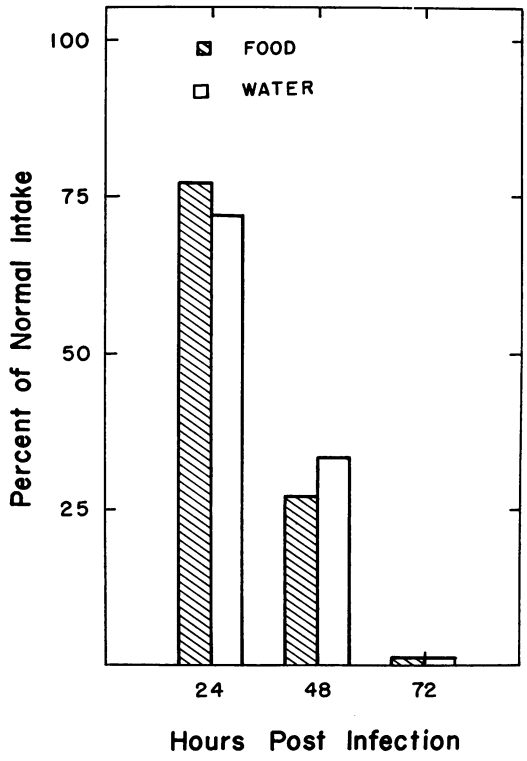

FIG. 3. *Effect of Listeria monocytogenes infection on food and water intake. Each bar represents the average intake of two groups of 10 mice, each provided with food and water ad libitum.*

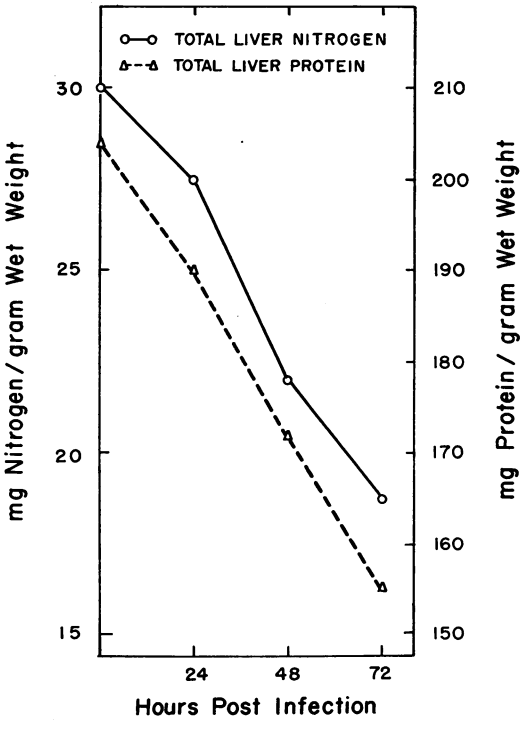

FIG. 4. *Total liver nitrogen and protein content of mice infected with Listeria monocytogenes. The results obtained represent average values of determinations performed in duplicate on pooled liver homogenates from three mice.*

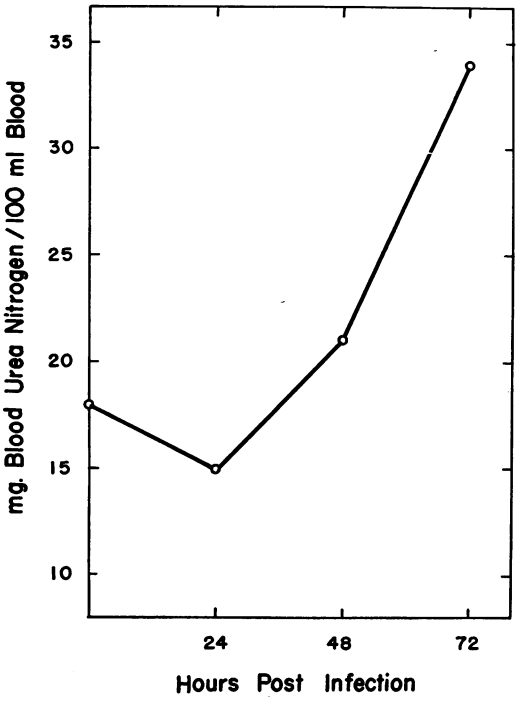

FIG. 5. *Blood urea nitrogen levels in Listeria-infected mice. The results represent mean values performed in duplicate on blood pooled from 8 to 12 mice.*

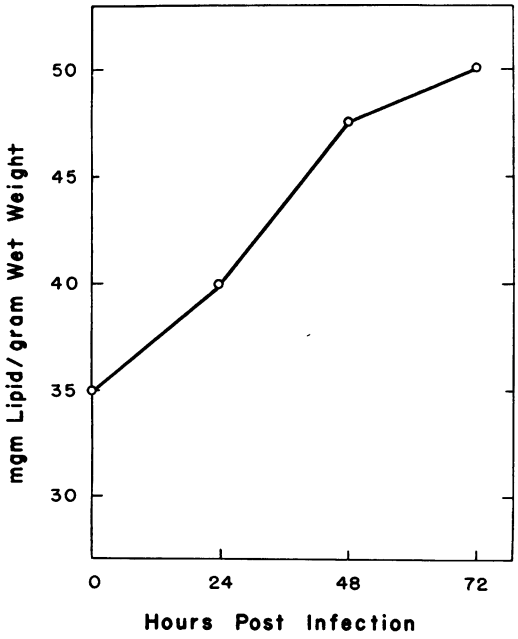

FIG. 6. *Effect of Listeria monocytogenes infection on total liver lipid content in mice. The results represent mean values from analyses performed in duplicate, each employing pooled liver extracts from three mice.*

TABLE 1. *P/O ratios of liver homogenates from normal and Listeria-infected mice*

Expt	Substrate	Normal[a]	Infected[a]	Theoretical
1	Succinate	1.99	1.00	2.00
2	Succinate	1.70	1.10	2.00

[a] The values represent the mean of four determinations, each employing pooled liver homogenates from three mice. The main reaction compartment contained, in a total volume of 2 ml, liver homogenate in 1 ml of 0.25 M sucrose (approximately 50 mg of protein); 30 μmoles of $MgCl_2$; 30 μmoles of Na succinate; 5 μmoles of adenosine triphosphate; and 0.05 ml of 1% cytochrome *c* in 0.1 M phosphate buffer (*p*H 7.4). One side arm contained 60 μmoles of glucose and 460 units of type V hexokinase (Sigma Chemical Co., St. Louis, Mo.); the other side arm contained 0.2 ml of 40% trichloroacetic acid. The center well contained 0.20 ml of 20% KOH plus filter paper.

TABLE 2. *Xanthine dehydrogenase activity in mice infected with Listeria monocytogenes*

Time post-infection	Percentage of normal activity[a]	
	Expt 1	Expt 2
hr		
0	100	100
24	159	163
48	401	363
72	465	337
96	205	—

[a] The results represent mean values for determinations performed in triplicate employing 10% suspensions of individual livers. Mean activity of control = 1.01 μmole of hypoxanthine destroyed per min per mg of protein. The reaction mixture contained 18 × 10^{-5} mmoles of 2,6-dichlorophenol-indophenol, 225 μmoles of phosphate buffer (*p*H 7.2), 0.6 ml of 1:100 liver homogenate (in 0.25 M sucrose), and 2.5 μmoles of hypoxanthine.

Total liver protein and nitrogen. Figure 4 shows that total liver protein and nitrogen decreased as the severity of the infection increased, with the diminution being most evident immediately prior to death of the animals.

Blood urea nitrogen. Figure 5 reveals that levels of blood urea nitrogen remained relatively constant for 24 hr and then increased continuously as the infection progressed to the acute state.

Total liver lipid. Figure 6 shows that the concentration of liver lipid in *Listeria*-infected mice increased until the death of the animals.

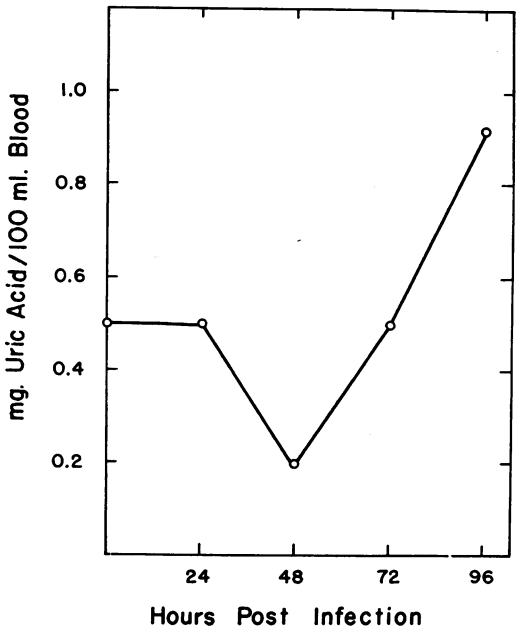

FIG. 7. *Levels of blood uric acid in Listeria-infected mice. The results represent mean values from analyses performed in duplicate on blood pooled from 8 to 12 mice.*

TABLE 3. *Uricase activity in mice infected with Listeria monocytogenes*

Time post-infection	Percentage of normal activity[a]	
	Expt 1	Expt 2
hr		
0	100	100
24	128	116
48	106	106
72	78	86

[a] The results represent mean values from determinations performed in duplicate, each employing liver homogenates pooled from three mice. Mean activity of control = 1.05 μmoles of uric acid oxidized per mg of protein per 20 min. The reaction mixture contained 0.12 mmole of uric acid, 0.5 ml of deionized water, 0.5 ml of 1:100 liver homogenate (in 0.25 M sucrose), and 0.2 mmole of borate buffer (*p*H 8.5) in a final volume of 3 ml. The reaction mixture was incubated for 20 min at 25 C.

Oxidative phosphorylation. P/O values of 1.99 and 1.70 were obtained for liver homogenates from normal animals, and values of 1.00 and 1.10 were obtained for homogenates from infected mice (Table 1). The results shown represent data obtained on livers excised from animals 72 hr after infection. P/O determinations during earlier stages of the infection often yielded variable results.

Xanthine dehydrogenase. Table 2 indicates that infection caused an increase in enzyme activity which paralleled progress of the infection.

Uric acid. The results of serum uric acid determinations are shown in Fig. 7. The levels remained constant for 24 hr, diminished at 48 hr, and then increased as the infection progressed to the acute stage at 72 hr.

Uricase. Table 3 summarizes the activities of this enzyme in infected animals. After 24 hr, liver from infected animals showed a slight increase in uricase activity. This activity returned to within the normal range at 48 hr after infection. Later in the course of infection (72 hr), a significant decrease in activity was noted.

DISCUSSION

Although many organs and tissues are injured in infected mice, the results obtained are consistent with the hypothesis that hepatic biochemical aberrations are at least partly responsible for death of the animals. The liver becomes studded with numerous lesions after intraperitoneal injection of 10^4 to 10^6 organisms. In addition, the livers become extremely friable, a result indicative of gross changes in structural constituents. Extensive liver damage is evident from increases in ratios of liver weight to body weight. Although a slight increment was observed in the weights of the infected livers, there was a significant loss of total animal weight during infection. Alteration in protein and fat content appear to correspond to weight changes observed during *Listeria* infection in mice. Inasmuch as there is a large increase in blood urea nitrogen, the possibility exists that the weight decrease may be attributable to breakdown of hepatic as well as muscle protein. Diminution in food and water intake, defecation, and absorption of food remaining in the gastrointestinal tract must also be considered as factors contributing to weight loss. The diminished levels of hepatic protein and nitrogen suggest that infection may also bring about an altered capacity of the host for endogenous protein synthesis. Altered patterns of de novo enzyme synthesis would result if derangements occurred at either the transcription or translation level.

The exact mechanism responsible for the decrease in oxidative phosphorylation after *Listeria* infection is unknown. The pathological accumulation of liver lipid during experimental listeriosis in the mouse appears to be closely associated with impaired carbohydrate metabolism. The decrease in oxidative phosphorylation during in-

fection would create a demand for alternative energy sources as well as new processes necessary for the generation of adenosine triphosphate. Thus, during infection, it is possible that large quantities of fatty acids are mobilized from adipose tissue and transferred to the liver, where they would be oxidized to provide for hepatic energy requirements. The transport of large quantities of nonesterified fatty acids from the depots to the liver may also result in added triglyceride synthesis. The decrease in food intake observed in the infected mice should also serve to increase the influx of lipid material into the liver cells.

Since it has been established that *L. monocytogenes* exhibits a decreased LD_{50} under experimental conditions of iron excess in mice (17), it is also quite possible that the pathogen is using host iron reserves and interfering with the regulatory mechanisms operative in the transport of iron from the liver to the serum.

Iron transport is dependent on hepatic xanthine dehydrogenase activity as well as energy produced during oxidative metabolic reactions (13, 14). Xanthine dehydrogenase has been shown by Mazur et al. (13, 14) to participate in the in vivo liberation of hepatic ferritin iron to the plasma. The reduced flavoprotein enzyme converts the ferric ferritin to the ferrous form, which can be removed from the protein in the presence of the plasma iron-binding globulin, transferrin. The increase in hepatic xanthine dehydrogenase during infection may affect the in vivo liberation of tissue iron. This process could enhance virulence by enabling the free iron concentration to be much more readily available to the invading pathogen.

In addition to the dehydrogenase activity of xanthine oxidase, uric acid, produced in large quantities by this enzyme, similarly brings about the reduction of ferritin iron (7). The alterations in uric acid levels are also consistent with the possibility of changes in iron metabolism during experimentally induced listeriosis in mice. In subprimate mammals, uricase catalyzes the oxidation of uric acid to allantoin, the principal end product of purine metabolism in such animals. At 24 hr postinfection, uricase activity in the liver is maximal, and during the next 24 hr blood uric acid levels reach a minimum. Perhaps, this denotes a critical period when the organisms firmly establish themselves in the host's liver in the presence of accessible iron.

ACKNOWLEDGMENTS

This investigation was supported by Public Health Service grant AI-04343 from the National Institute of Allergy and Infectious Disease. Martin S. Wilder was supported by Public Health Service Training Grant 5 T1 GM-703 and Public Health Service Predoctoral Fellowship 1-F1-GM-29, 701-01.

The excellent technical assistance of Sondra Frye on certain aspects of this study is gratefully acknowledged.

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