HEMOLYSIN PRODUCTION BY *LEPTOSPIRA INTERROGANS* SEROVAR CANICOLA IN A PROTEIN-FREE MEDIUM WITH HEMIN

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INTRODUCTION

The production of hemolysin by saprophytic leptospires was first reported in 1938 (Sugimoto 1938). It was not until 1956 that hemolysin was demonstrated in cultures of pathogenic leptospires (Alexander *et al.*, 1956: Russel, 1956). Previously reported leptospiral culture media used for hemolysin production contained serum. In 1979 it was shown that hemolysin was produced by pathogenic leptospires grown in oleatepalmitate albumin medium (Stamm and Charon 1979). Leptospiral hemolysin production in protein free media or nearly protein free media has also been reported (Yanagihara *et al.*, 1982; Bernheimer and Bey 1986). In this study we describe a possible mutant of *Leptospira interrogans* serovar canicola strain moulton that would not produce hemolysin when continuously grown in protein free medium but regained it's ability for hemolysin production after the addition of hemin to the protein free medium.

MATERIALS AND METHODS

Leptospira interrogans serovar canicola strain moulton was maintained in Tween 80-albumin semisolid medium (Ellinghausen and McCullough 1965) until transferred to Tween 80-albumin broth medium and finally to leptospiral protein-free (LPF) medium. It was then passed continuously in LPF medium (50–60 passes).

The LPF medium was prepared from solutions 1 and 2 using acid washed glassware and double distilled water. The stock solutions for solution 1 (expressed in grams per 100 ml) were prepared as follows: Na_2HPO_4 (10.0), KH_2PO_4 (3.0), NaCl (10.0), NH_4Cl (25.0), thiamine (0.5), and glycerol (4.0). These stock solutions were stored at 4 °C until used. Stock solutions were added to 944 ml of distilled water in the following quantities: 10ml Na_2HPO_4, 10 ml KH_2PO_4, 10 ml NaCl, 1 ml NH_4Cl, 1 ml thiamine and 10 ml glycerol. The solution was mixed well and adjusted to pH 7.6 with 1 N NaOH. Fifty ml of solution 1 was dispensed into 125 ml screwcapped Erlenmayer flasks and autoclaved for 15 min. at 121 °C.

The stock solutions for solution 2 (expressed in grams per 100 ml) were prepared as follows: $CaCl_2$ (1.0), $MgCl_2\ 6H_2O$ (1.0), $ZnSO_4\ 7H_2O$ (0.4), cyanocobalamine (0.02), Tween 80 (10.0), and $FeSO_4\ 5H_2O$ (0.5). The $FeSO_4$ was prepared just prior to use. All solutions with the exception of $FeSO_4$ were stored at −20 °C. Stock solutions were added to 735 ml of distilled water as follows: 10 ml $CaCl_2$, 10 ml of $MgCl_2\ 6H_2O$, 10 ml of $ZnSO_4\ 7H_2O$, 100 ml of $FeSO_4\ 5H_2O$, 10 ml cyanocobalamine, and 12.5 ml of Tween 80. Solution 2 was

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mixed well and filtered through a 0.22 um millipore filter (Millipore Corp., Bedford, Mass.). The final LPF medium was prepared by adding 5 ml of solution 2 to 50 ml of solution 1.

At various times, the following filter sterilized compounds were added to 50 ml of LPF medium: 5 ml of 10% solution of bovine albumin Fr V (Pentex Corp., Kankakee, Ill.), 0.5 ml of a 0.5% solution of hemin, hematin, hematoporphyrin, protoporphyrin IX or cytochrome C (Sigma Chem. Corp., St., Louis, Mo.). Stuart's medium (Difco Inc., Detroit, Mich.) containing 10% inactivated (56 °C, 30 min.) rabbit serum was prepared in 50 ml quantities. All culture flasks were incubated at 28 °C on a rotary shaker for 12 days. Leptospiral growth curves were determined by direct cell counts using a Petroff–Hauser counting chamber (C.A. Hauser and Son Co., Philadelphia, Pa.). Corresponding nephelometer readings were done with a Coleman–Nephocolorimeter Model 9 (Coleman Inst., Oak Brook, III.)

Hemolysin titrations (Bauer and Morse 1958) were done on the supernatant of cell cultures centrifuged at 97,500 x g in a B–35 centrifuge (International Equip. Co., Needham Hts., Mass.) for 30 min. Sheep erythrocytes preserved in Alsever's solution for not longer than 1 week were used for the titration of hemolysin.

Erthrocytes were washed three times in 0.85% NaCl. A 1.0% suspension of washed erythrocytes in 0.85% NaCl was prepared for use in the hemolysin test. Serial 2–fold dilutions of the culture supernatant ranging from 1:2 through 1:1024 were made in 0.85% NaCl. An equal volume of 1.0% concentration of erythrocytes was added to each tube. A standard curve of hemolytic activity on erythrocytes using saponin ranging from 25% to 100% hemolysis was prepared with each test. A saline control consisting of equal volumes of a 1.0% erythrocyte suspension and 0.85% NaCl was also included with each test. An uninoculated medium control consisting of 2-fold dilutions ranging from a 1:2 to 1:16 was done with each test. All tubes were mixed thoroughly and incubated at 37 °C for 2 to 4 h followed by refrigeration at 4 °C for 16 h. Tubes were centrifuged at 500 × g for 10 min, and the optical density of the supernatant was determined in a Coleman Jr. model 20 spectrophotometer (Perkin–Elmer Corp., Maywood, Ill.) at 550 nm. Hemolytic activity was determined from the standard curve and expressed in units. A hemolytic unit was defined as the reciprocal of the highest initial dilution that lysed at least 50% of a 1.0% suspension of erythrocytes in a 2.0 ml volume.

RESULTS

There was a comparable rate of leptospiral growth in LPF medium with or without albumin to approximately 10^9 organisms per ml in 8 days (Fig. 1). Thereafter the count decreased sharply in LPF medium but not in LPF-albumin medium. This sharp decrease in LPF medium was probably due to the build-

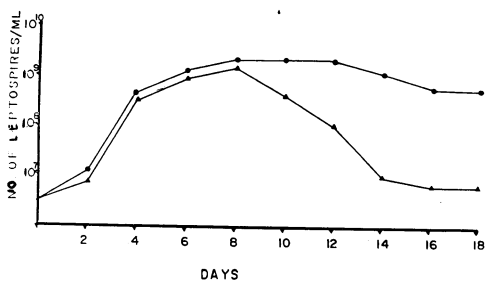

Fig. 1–Growth of *Leptospira interrogans* serovar canicola strain moulton in LPF medium with (●——●) and without (▲——▲) 1.0% bovine albumin.

up of fatty acids that are toxic for the leptospires. This phenomenon necessitated the transfer of the culture in LPF medium every 4 to 5 days before the accumulation of toxic substances detrimental to initiation of the subculture. Continuous transfer, resulting in good growth of *Leptospira interrogans* serovar canicola strain moulton in LPF medium probably represents a mutant strain relatively resistant to lysis by fatty acids. (Yanagawa and Wilson 1962).

Although hemolysin was produced by *Leptospira interrogans* serovar canicola strain moulton in Stuart's medium with 10% rabbit serum, none was produced in LPF medium, after continuous passage, with or without bovine albumin. No significance was attached to the 1:2 hemolysin titer, obtained with LPF medium since Tween 80 in the medium lysed sheep erythrocytes to that titer. Hemin at a concentration of 0.25 mg in 50 ml of LPF medium stimulated hemolysin production by the organism to a maximum average of 23 units in 12 days (Fig. 2). No increased hemolytic activity was observed in uninoculated LPF medium with hemin or when hemin was added to the supernatant of a 10 day culture grown in LPF medium. Leptospiral viability was extended 3 additional days in LPF medium containing hemin beyond that found in LPF medium alone.

Hematin, hematoporphyrin, protoporphyrin IX and cytochrome C added to LPF medium at the same concentration as hemin in LPF medium supported growth of *Leptospira interrogans* serovar canicola strain moulton comparable to the hemin containing medium except for cytochrome C which did not support good growth of this organism. None of these porphyrin compounds or cytochrome C resulted in the production of hemolysin in LPF medium (Table 1).

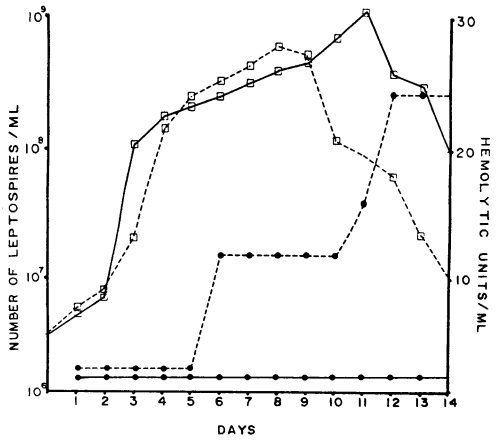

Fig. 2–Comparison of growth and hemolysin production of *Leptospira interrogans* serovar canicola strain moulton of LPF medium with and without hemin. Growth with hemin (□——□); Growth without hemin (□----□); Hemolysin production with hemin (●----●); Hemolysin production without hemin (●——●). The bar shown for hemolytic units indicates the standard error of the mean obtained with three experiments.

Table 1
Hemolysin production by *Leptospira interrogans* serovar canicola strain moulton in LPF medium with hemin or other porphyrin compounds including cytochrome C.

Compound added to LPF medium*	Hemolytic activity** (H.U./ml)
Hemin	32
Protoporphyrin IX	0
Hematin	0
Hematoporphyrin	0
Cytochrome C	0

*0.5 ml of a 0.5% solution was added to 50 ml of LPF medium.

**Determined from 12 day culture containing 10^9 leptospires/ml except for cytochrome C which contained 10^7 organisms/ml.

DISCUSSION

The demonstration that the addition of hemin results in hemolysin production by this strain of *Leptospira interrogans* suggests that it may be responsible for hemolysin production in serum containing media since serum usually contains some hemin as a result of lysed erythrocytes. It is likely that the entire hemin molecule is necessary for hemolysin production since other related compounds were not capable of producing hemolysin.

Since there are indications that hemolysin is a protein (Bernheimer and Bey 1986; Bauer *et al.*, 1961), it's production in a protein-free medium should facilitate it's purification and characterization, which could eventually lead to determining the mode of action of leptospiral hemolysin.

SUMMARY

Leptospira interrogans serovar canicola strain moulton was grown to a high cell density in a protein-free medium. When hemin was added to this medium, hemolysin was produced. Hemolysin was not detected when other porphyrins or cytochrome C were substituted for hemin in the medium.

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