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Copurification of *Leptospira interrogans* Serovar *pomona* Hemolysin and Sphingomyelinase C

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The hemolytic and sphingomyelinase C activities of supernatants of cultures of *Leptospira interrogans* serovar *pomona* tended to copurify when isoelectric fractionation was carried out. Both activities focused primarily at pH 8.1. Considered in conjunction with other circumstantial evidence, the results led to the conclusion that sphingomyelinase C is responsible for hemolysis.

Supernatants obtained from cultures of some pathogenic leptospires are known to contain a hemolysin (1, 20). As long ago as 1959 it was suggested that the hemolysin might be a phospholipase (18). Much circumstantial evidence consistent with this suggestion has since accumulated, including repeated demonstration of phospholipase(s) in leptospiral culture supernatants (3, 4, 9, 10, 14–16). The combined information, although strongly suggestive, does not provide proof that phospholipase is responsible for hemolysis. The present study addresses this question.

Leptospira interrogans serovar *pomona* H10-414 was maintained at 30°C in bovine serum albumin-Tween 80 medium (8). Virulence was maintained by hamster back passage as previously described (7). One volume of inoculum culture was added to 200 volumes of protein-free culture medium prepared as previously described (8), allowed to stand at 30°C for 3 days, and then aerated on a gyratory shaker at 30°C for 3 to 4 days. Final cell densities of 8×10^8 to 9×10^8 leptospires per ml were obtained. Cultures were spun at 30,000 × *g* for 50 min in a Beckman J2-21 centrifuge at 4°C. The supernatants were carefully decanted and frozen at −70°C.

We estimated hemolytic activity by adding decreasing quantities of test solution to a constant volume of washed sheep erythrocytes and incubating the mixture at 37°C for 30 min; this was followed by chilling, centrifugation, and estimation of hemoglobin in the supernatants by colorimetric comparison with standards (6). One unit of hemolysin (HU) is that amount of test material needed to produce 50% hemolysis.

We assayed sphingomyelinase C by using the chromogenic analog of sphingomyelin, 2-hexadecanoylamino-4-nitrophenylphosphorylcholine (12, 13). The substrate was purchased from Sigma Chemical Co. (St. Louis, Mo.), and the method was as previously described (12) except that the reaction mixture was modified by inclusion of 0.025% Triton X-100 and 0.04 M $MgCl_2$.

Enzyme-grade ammonium sulfate (200 g) was dissolved in 400 ml of thawed culture supernatant (Table 1, stage 1). After standing for 16 h at 4°C, the mixture was centrifuged at 13,200 × *g* for 30 min, and the resulting precipitate was dissolved in 8 ml of distilled water and dialyzed for 2.5 h against 1.5 liters of distilled water. The dialyzed solution (stage 2) was subjected to electrofocusing (23) carried out in a linear density gradient prepared from (i) a less dense solution consisting of 4 ml of 8% (wt/vol) ampholine (pH 3.5 to 10) (LKB Instruments, Inc., Rockville, Md.), dialyzed protein solution, and sufficient water to bring the volume to 55 ml and (ii) a more dense solution of 32 ml of water, 8.5 ml of 8% (wt/vol) ampholine of the same pH range, and 25 g of sucrose. Focusing was done at about 4°C for 22 h in a 110-ml electrophoresis column (LKB) with a final potential difference of 1,000 V. Fractions of 4 ml each were collected. The results are shown in Fig. 1 and Table 1 (stage 3). It was evident that sphingomyelinase and hemolytic activities focused at about pH 8.1, a pI value significantly lower than that of staphylococcal sphingomyelinase, which is about 9.5 (19). Fractions 23 and 24 were combined and dialyzed against 20% glycerol for 16 h at 4°C (stage 4). The overall recoveries of hemolytic activity and sphingomyelinase were 27 and 19%, respectively.

Earlier work has shown that the hemolysin of pathogenic leptospires (i) acts preferentially on erythrocytes from sheep and other ruminants (1, 18, 20), (ii) is a hot-cold hemolysin (1, 3, 24), and (iii) requires Mg^{2+} for maximal activity (3, 24). This set of properties distinguishes the lysin from those of most other hemolysins but is identical with that of staphylococcal beta-hemolysin, suggesting that the two agents may be congeneric. Because it is firmly established that staphylococcal beta-hemolysin is a sphingomyelinase C (11, 19), it seemed probable that the leptospiral hemolysin is a similar enzyme. Further evidence in support of this idea derives from the fact that phospholipase activity in pathogenic leptospiral culture filtrates or supernatants has been demonstrated repeatedly (4, 10, 14–16, 18, 22) and also from the fact that hemolytic activity is inhibited by phospholipids (9, 15, 18).

Our experimental data, considered in conjunction with the information just cited, provided further evidence that the leptospiral hemolysin studied is a sphingomyelinase C. In short, electrofocusing showed that the lytic and enzymic activities copurified. An apparent exception to this generalization was found in fractions 21 and 22 (Fig. 1; Table 1), in which the ratios of sphingomyelinase to hemolysin deviated from that usually seen. Among several possible explanations, the most likely seems to be the presence of a second sphingomyelinase which is not hemolytic and which electrofocuses at a slightly lower pH than that of the sphingomyelinase associated with lytic activity. There is clear precedence for this possibility, a well-established example of which involves the phospholipases C of *Clostridium perfringens* and *Bacillus cereus*. These two enzymes are function-

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TABLE 1. Partition of sphingomyelinase and hemolytic activity

Stage	Preparation	Vol (ml)	HU/ml	Total HU	Sphingomyelinase U/ml	Total sphingomyelinase U	Sphingomyelinase U/HU ratio
1	Culture supernatant	400	36	14,400	140	56,000	3.9
2	Dialyzed precipitate	20.4	640	13,056	1,120	22,800	1.8
3	Fraction no. from electrofocusing						
	19	4	45		130		2.9
	20	4	120		340		2.9
	21	4	165		960		5.8
	22	4	270		940		3.5
	23	4	440	4,960	1,225	13,300	2.8
	24	4	800		2,100		2.6
	25	4	190		550		2.9
	26	4			232		
	27	4	56		160		2.9
4	Fractions 23 plus 24 after dialysis against 20% glycerol	9.7	400	3,900	1,100	10,700	2.7

ally identical in that both catalyze the cleavage of pure phosphatidylcholine in solution, yet the former is biologically cytotoxic whereas the latter is not (17).

Corynebacterial phospholipase D treatment of sheep erythrocytes renders the cells completely resistant to lysis by staphylococcal sphingomyelinase C (21). Because the leptospiral sphingomyelinase closely resembles the staphylococcal enzyme, it was reasoned that corynebacterial phospholipase D should also make sheep erythrocytes resistant to leptospiral sphingomyelinase. Using conditions previously described (5), we found that 0.2 μg of phospholipase D per ml rendered sheep erythrocytes completely resistant to lysis by 3 HU of leptospiral hemolysin.

Kasarov (14) found that parasitic strains of leptospires belonged to either of two classes: (i) those that were capable of degrading both phosphatidylcholine and sphingomyelin and were hemolytic for erythrocytes of sheep, humans, rabbits, and rats and (ii) those that did not degrade the

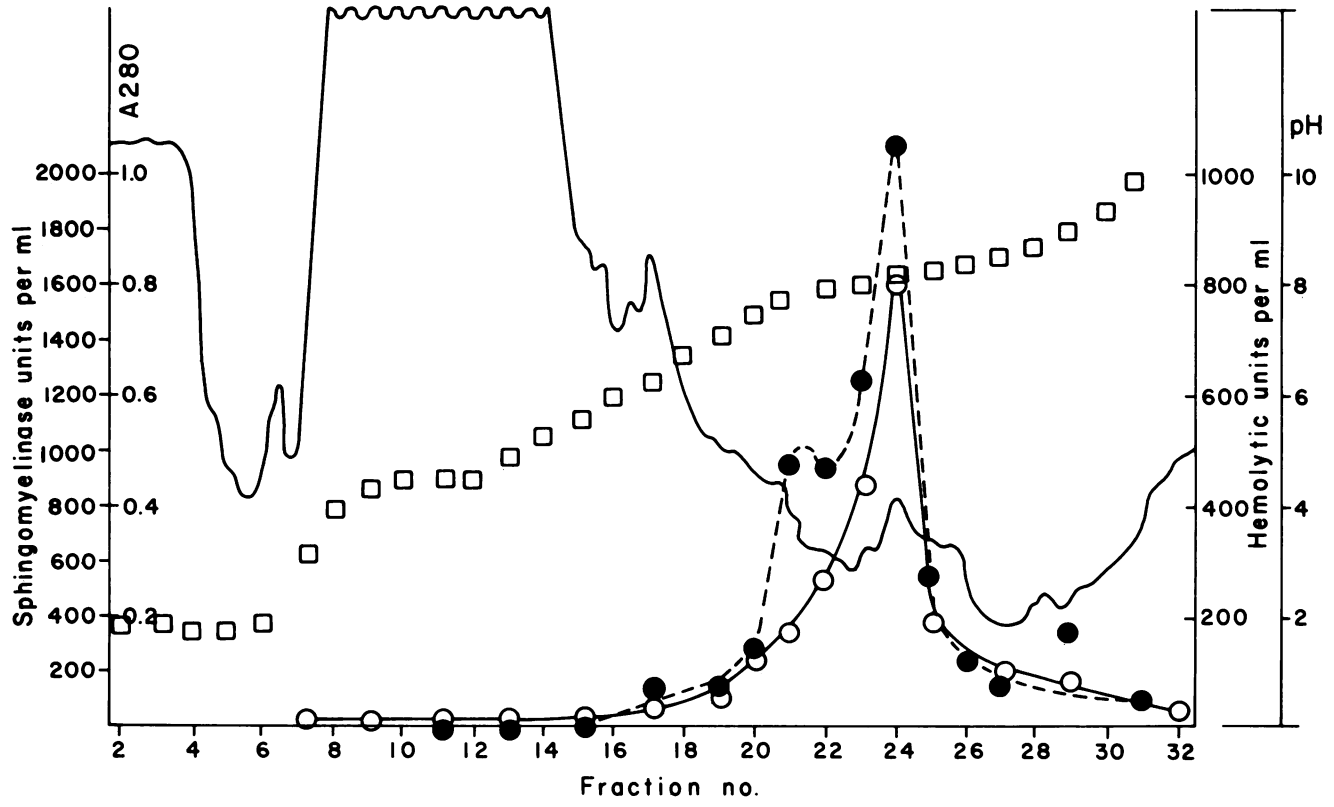

FIG. 1. Distribution of hemolytic activity (○), sphingomyelinase (●), A_{280} (——), and pH (□) after electrofocusing. Both hemolytic and sphingomyelinase activities focused primarily at pH 8.1 and coincided with a peak of A_{280}.

choline-containing phospholipids and were not hemolytic. In contrast, saprophytic leptospires degrade phosphatidylcholine but not sphingomyelin (14), are hemolytic, produce phospholipase A, and require Ca^{2+} for activation of both phospholipase and hemolytic activities (9). The observations of Bazovska (3) are consistent with these findings. Although a completely concordant synthesis of these and various other relevant observations is hard to achieve, it appears that production of sphingomyelinase C is characteristic of many but not all strains of pathogenic leptospires, whereas phospholipase A is associated with saprophytic strains and at least some pathogenic strains.

On the basis of existing knowledge, the role of phospholipases and hemolysins in the pathogenesis of leptospiral diseases is difficult to assess (see, for example, reference 2). As pointed out by Kasarov (14), phospholipases that are clearly involved in the alteration of erythrocyte membranes may also function to degrade the phospholipids of other kinds of cells and thus contribute to pathological changes seen in infections.

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