Mucin-Like Enhancement of Microbial Virulence for Mice by Bile Salts and Certain Surfactants. (24956)

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While attempting to elucidate the mechanism by which bile salts exert their variable effect upon *in vitro* activity of a number of antimicrobial agents(1,2), it was observed that simultaneous intraperitoneal injection into mice of 1% mixture of equal parts of sodium taurocholate and sodium glycocholate and an inoculum of a strain of *Staphylococcus aureus* resulted in death of nearly all animals, whereas administration of same concentration of each alone *via* same route, proved innocuous. In light of this observation it was believed of interest to ascertain whether virulence of other microbial species known to be increased with the aid of gastric mucin(3) could be similarly enhanced by bile salts mixture and by other surfactants as well, both anionic and non-ionic. Because of their marked antimicrobial action even in high dilution, cationic surfactants could not be tested in this regard.

Materials and methods. Anionic compounds arbitrarily selected for investigation were di isopropyl naphthalene sodium sulfonate (Naccosol A) and di hexyl sodium sulfosuccinate (Aerosol MA); non-ionic compounds were alkyl aryl polyether alcohol (Triton X-100) and polyoxyethylene sorbitan monooleate (Tween 80). Indicated concentrations of the above, with exception of Naccosol A, and of bile salts were dissolved in distilled water and sterilized by autoclaving at 15 lb for 15 minutes. Naccosol A was added aseptically to sterile distilled water as heat sterilization causes this solution to become turbid, and then controlled for sterility before use. Five % gastric mucin (Type 1701-Wilson) was prepared according to Miller(4). A freshly isolated sample strain of each microbial species tested was grown in appropriate media for 18 hours at 37°C and then centrifuged at 2500 rpm for 45 minutes. Supernates were then replaced by equal amount of physiological saline to remove potentially lethal toxin formed during growth. After determining maximum non-lethal dilution of each strain and of each of the 4 surface active agents investigated upon test mice, 0.25 ml of appropriate dilution of each strain (Table I), was injected intraperitoneally into each of 7 groups of 10 albino male Swiss mice weighing between 15-20 g immediately after they had received by same route 0.5 ml of: 1) physiological saline; 2) 5% hog gastric mucin; 3) 1% bile salts mixture containing equal parts of sodium taurocholate and sodium glycocholate; maximum tolerated concentration of different surfactants, namely: 4) 1% Naccosol A; 5) 0.75% Aerosol MA; 6) 0.5% Triton X-100; and 7) 1% Tween 80. Deaths occurring in each group within 48 hours were noted. In addition, heart blood cultures were placed on media selective for the injected strain immediately after expiration if death occurred during the day and early morning, if the animals died during the night and positive results tabulated. Hydrogen ion concentration was measured electrometrically. Relative viscosity was computed by determining efflux time from calibrated tube of uniform bore at constant temperature as compared to distilled water. Surface tension was measured with Cenco Du Noüy tensiometer at 25°C.

Results. Number of fatal bacteremias produced in each group with the aid of different adjuvants investigated is noted in Table I. Except for Tween 80, all proved capable of enhancing virulence of a wide variety of microorganisms, converting ordinarily innocuous infections in animals, into a high proportion of fatalities associated with septicemia. 1% bile salts failed to raise virulence of our *Neisseria meningitidis* and *Salmonella choleraesuis* strains, but increasing the concentration to 2% (which was well tolerated), did result in mortality of 4/10 and 6/10 respectively. *Candida albicans* virulence was also increased by adjuvants although heart blood cultures were

TABLE I. Mouse Mortality/10 and Positive Post-Mortem Heart Blood Cultures Elicited by a Variety of Microorganisms with Different Surface-Active Agents as Compared to Physiological Saline and 5% Gastric Mucin.

Organism	Inoculum dilution, 18 hr culture (.25 ml)	Phys. saline	Gastric mucin 5%	Bile salts mixture 1%	Naccosol A 1% (.5 ml)	Aerosol MA .75%	Triton X-100 .5%	Tween 80 1%
Candida albicans	Undil.	0	4 (0)*	8 (4)	8 (0)	6 (0)	10 (0)	0
Hemophilus influenzae	10–1	0	10 (10)	2 (0)	2 (2)	8 (8)	8 (4)	0
Neisseria meningitidis	Undil.	0	10 ,,	0 †	10 (6)	10 ,,	5 (1)	0
,,	,,	0	9 (7)	10 (6)	8 ,,	9 (5)	10 (3)	1 (0)
Staphylococcus aureus	10–1	0	9 (9)	6 ,,	7 (7)	9 (9)	8 (8)	0
Aerobacter aerogenes	10–3	0	10 (10)	9 (9)	10 (10)	8 (8)	8 ,,	0
Escherichia coli	10–4	0	10 ,,	10 (10)	6 (6)	8 ,,	8 ,,	0
Klebsiella pneumoniae	10–1	0	8 (8)	6 (6)	8 (8)	10 ,,	8 (6)	0
Paracolobactrum intermedium	10–1	1 (1)	10 (10)	8 (8)	10 ,,	10 ,,	10 (10)	3 (3)
Proteus vulgaris	10–4	0	10 ,,	6 (6)	8 ,,	8 ,,	8 (8)	0
Pseudomonas aeruginosa	10–1	0	9 (9)	0 †	4 (4)	6 (6)	4 (4)	0
Salmonella choleraesuis ,, *typhi*	10–2	1 (1)	9 ,,	8 (8)	10 (10)	10 (10)	10 (10)	1 (1)

* No. of positive post-mortem heart cultures in parentheses.

† Increasing bile salts mixture concentration to 2% resulted in mortality of 4 (0) with *N. meningitidis* and 6 (4) with *Salm. choleraesuis*.

consistently negative for the fungus except in the group that received bile salts. Four of the 8 dead mice in this group yielded positive cardiac cultures.

Since Tween 80's failure in this regard might conceivably have been due to its possible bactericidal action against the injected microorganism, 0.25 ml of the dilution of each strain used in *in vivo* tests was treated with 0.5 ml of 1% Tween 80 in test tubes for 6 hours at 37°C in shaker water bath oscillating at approximately 120/minute and then subcultured upon appropriate solid media. With exception of the meningococcus strain, all proved viable after such exposure, so that Tween 80's ineffectiveness cannot be attributed to a sterilizing effect upon the injected organisms, especially since the number of colonies obtained with the same cultures treated in the same way with physiological saline was roughly equal to that observed with Tween 80.

Hydrogen ion concentration, relative viscosity and surface tension of Tween 80 were compared with that of effective adjuvants and with saline solution to ascertain whether any physicochemical basis could be established for the former's inability to enhance mouse virulence (Table II). pH of Tween 80 solution

TABLE II. Physicochemical Characteristics of Various Adjuvants.

Adjuvant	Reaction (pH)	Relative viscosity	Surface tension (dynes/cm at 25°C)
Saline	6.05	1	76.4
Gastric mucin 5%	7.2	1.5	52.5
Bile salts mixture 1%	7.1	1	41.6
Aerosol MA .75%	6.0	1	31.4
Naccosol A 1%	6.4	1	39.0
Triton X-100 .5%	6.4	1	29.9
Tween 80 1%	5.8	1	42.0

was slightly but not significantly lower than that of the effective surfactants. With exception of 5% gastric mucin solution, whose relative viscosity was 1.5, the rest, including Tween 80, were all approximately 1, thereby confirming McLeod's observation(5) that pathogenizing activity and viscosity of adjuvants are not correlated. As for Tween 80's surface tension lowering capability, its action in this regard corresponds closely to that of bile salts mixture and is significantly greater

than mucin, both of which were highly effective in enhancing microbial virulence for mice.

Discussion. The mode of action by which hog gastric mucin exerts its action in enhancing bacterial virulence has not as yet been fully delineated(3). Among reasons ascribed for failure of many strains to produce bacteremia and fatal outcome, when injected intraperitoneally without mucin, is elicitation by their presence of a strong protective peritoneal leukocytic response which tends to destroy and localize the invading organisms thereby preventing dissemination into the blood stream. In addition, peritoneal fluid possesses bactericidal properties for certain species of microorganisms(6).

Although surface tension lowering capacity *per se* is probably not the sole determinant of an adjuvant's virulence enhancement capability as evidenced by Tween 80's failure, all agents tested that proved successful in this regard did possess this property. It is conceivable that they could interfere with contact between peritoneal phagocytes and bacteria by altering surface tension so that the former would no longer be able to envelop, engulf and destroy the latter. In addition, they may very well increase permeability of the peritoneal barrier between abdominal cavity and blood by virtue of the same property thereby permitting injected organisms to escape promptly into the blood stream and thus circumvent the protective effect of peritoneal phagocytosis and exposure to bactericidal action of peritoneal fluid.

Use of surfactants in place of gastric mucin for enhancement of microbial virulence, *e.g.*, in chemotherapeutic assays, offers a number of advantages. The former are easier to prepare, the problem of batch variation does not present itself with them and their chemical compositions are clearly defined, as is not the case with mucin. Furthermore, smaller gauge needles could be employed since they are less viscous and loss of intraperitoneally injected test material through needle puncture would be minimized.

Summary. Intraperitoneal injection of bile salts and certain surfactants into mice followed by adjusted, non-lethal inocula of a variety of different microbes results in significant enhancement of latter's virulence for mice. With few exceptions, mortality induced with these adjuvants is associated with high incidence of bacteremia involving intraperitoneally injected microorganisms.

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Received April 2, 1959. P.S.E.B.M., 1959, v101.