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COMPARATIVE STUDIES ON CORYNEBACTERIUM PYOGENES TOXIN FORMATION IN MONOCULTURES AND MIXED CULTURES

THE DEMONSTRATION OF A STIMULATING EFFECT OF PEPTOCOCCUS INDOLICUS AND STUART-SCHWAN COCCI

By

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HØI SØRENSEN, GUNNER: *Comparative studies on Corynebacterium pyogenes toxin formation in monocultures and mixed cultures. The demonstration of a stimulating effect of Peptococcus indolicus and Stuart-Schwan cocci.* Acta vet. scand. 1980, *21*, 438—447. — The growth and the toxin (i.e. hemolysin) producing capacity of Corynebacterium pyogenes were studied in monocultures and in co-cultures with 1 or more of the organisms frequently accompanying it in summer mastitis in cattle (Peptococcus indolicus, Stuart-Schwan cocci, Bacteroides melaninogenicus subsp. levii, Fusobacterium necrophorum and Streptococcus dysgalactiae) or with organisms seldom associated with summer mastitis (Streptococcus uberis, Streptococcus agalactiae, non-toxic staphylococci and Escherichia coli).

Pc. indolicus, and to some extent also Stuart-Schwan cocci, stimulated the growth as well as the hemolysin producing capacity of Cb. pyogenes (Table 1) while Str. dysgalactiae, Str. uberis, Str. agalactiae, E. coli and the majority of the staphylococci reduced these activities. Most F. necrophorum strains stimulated the growth, but not the hemolytic activity. With B. melaninogenicus the results were inconclusive.

The effect of Pc. indolicus appeared to be associated with the production of a filterable factor (Tables 2 and 3).

Mouse toxicity and hemolytic activity of culture filtrates were closely correlated (Table 4).

Corynebacterium pyogenes; toxin formation; Peptococcus indolicus; "peptococcus factor"; Stuart-Schwan cocci.

In summer mastitis in cattle Corynebacterium pyogenes often occurs together with a multiplicity of other microorganisms, in particular Peptococcus indolicus, unclassified microaerophilic

cocci (Stuart-Schwan cocci) described by *Stuart et al.* (1951) and *Schwan et al.* (see *Schwan* 1979), but also Bacteroides melaninogenicus, Fusobacterium necrophorum, Streptococcus dysgalactiae, and others (see recent works by *Høi Sørensen* 1974, 1978, *Steiner* 1975, *Shinjo et al.* 1977, *Aalbæk* 1978, and *Schwan & Holmberg* 1978). *Leth Jørgensen* (1937) presumed a synergism between Cb. pyogenes and Pc. indolicus to be of etiological significance, and this has later been confirmed experimentally (*Stuart et al.* 1951, *Høi Sørensen* 1972). Recently it was suggested that summer mastitis is in fact caused by the pathogenic effect of a complex microbial ecosystem (*Høi Sørensen* 1978, 1979). However, little is known about the possible interaction between the individual organisms.

In the present study Cb. pyogenes was examined with respect to growth and hemolysin (i.e. toxin) formation in monoculture and in mixed cultures representing combinations of organisms typically or atypically found in summer mastitis secretions.

MATERIAL AND METHODS

Ten strains of Cb. pyogenes (P1—P10) and 10 strains of each of the following organisms (in the present work referred to as "associate organisms") were used for the experiments: Non-hemolytic Pc. indolicus (Pc1—Pc10, including Type Strains A—F; *Høi Sørensen* 1973), Stuart-Schwan cocci (X1—X10), B. melaninogenicus subsp. levii (Bm1—Bm10), F. necrophorum (Fn1—Fn10), Str. dysgalactiae (D1—D10), Str. uberis (U1—U10), non-hemolytic Str. agalactiae (B1—B10), non-toxic staphylococci (S1—S10), and non-hemolytic E. coli (Ec1—Ec10). Type Strain F of Pc. indolicus is of porcine origin, while the rest of the strains originate from bovine mastitis (mostly summer mastitis) except Strains P6—P10, Pc7—Pc10 (Serotypes B, C or E), and Ec6—Ec10, which originate from various other affections in bovines*.

The strains were maintained by cultivation on blood agar media with the following basic ingredients: meat infusion broth, 1.2 % Davis Agar and 5 % citrate-stabilized calf blood (pH 7.2—7.4). For Cb. pyogenes, streptococci, staphylococci and E. coli 1 % Bacto Peptone was added to the medium, for the rest of the organisms 1 % BBL Trypticase Peptone, and for bacteroids additionally 0.5 μg menadione/ml, 5 μg hemin/ml, and (for F. necrophorum) 0.04% cysteine-HCl. Plates with streptococci, staphylococci and E. coli were incubated aerobically, plates with Cb. pyogenes in jars with 10 % CO_2, and plates with peptococci, Stuart-Schwan cocci and bacteroids in anaerobic jars with H_2 and 10 % CO_2. Bacteroids were subcultured on pre-

* Strains Ec6—Ec10 were kindly supplied by Dr. A. Dam, the State Veterinary Serum Laboratory, Copenhagen.

reduced media (freshly poured or stored anaerobically) at 7-day intervals, the rest of the organisms on aerobically stored media at intervals of 2—3 days.

The test medium (SKM medium) was skim milk with hemin, menadione and cysteine-HCl as indicated for blood agar; natural pH approx. 6.6. Prior to inoculation the tubes were placed in a boiling water bath for 15 min and thereafter cooled to 37°C.

Series of 10-ml volumes of SKM medium were inoculated with 0.5 ml of 24-h Cb. pyogenes culture in W-S medium (*Jayne-Williams & Skerman* 1966). Tubes for mixed cultures were superinoculated with 24- or 48-h blood agar cultures of the "associate organisms" in question. The tubes were incubated aerobically at 37°C for 48 h.

The test cultures were controlled for contamination and for the possible presence of "associate organisms" by cultivation on blood agar plates. The growth of Cb. pyogenes was determined by viable counts (pour plate method), and its hemolytic activity was examined as follows: The cultures were centrifuged, and 0.1, 0.05 and 0.02 ml of, respectively, undiluted supernatant (hemolysin) and dilution 1:10, 1:100 and 1:1000 in saline were mixed with 1.0-ml volumes of a 0.5 % suspension of washed calf r.b.c. in saline, prepared from citrate-stabilized blood. The tubes were read after incubation in a water bath at 37°C for 60 min. One hemolytic unit (h.u.) was defined as the least amount of hemolysin producing 100 % hemolysis, and the titer was expressed in h.u. per ml. In each test the results were controlled by titration of a standard hemolysin, stored in 1.0-ml amounts at —18°C. The suitability for the test of blood stored at 5°C for different periods of time was examined.

Cultures investigated

Initial experiments. Monocultures of all "associate organisms" were tested for hemolytic activity. Experiment 1: The individual Cb. pyogenes strains (P1—P10) were tested in monoculture and in mixed cultures with each of the strains Pc1, X1, Bm1, Fn1, D1, U1, B1, S1 and Ec1. Experiment 2: Strain P1 was tested in monoculture and in mixed cultures with each of the strains Pc1—Pc10, X1—X10, Bm1—Bm10, Fn1—Fn10, D1—D10, U1—U10, B1—B10, S1—S10 and Ec1—Ec10. Experiment 3: In 5 trials Strain P1 was tested in monoculture and in mixed cultures with Strains Pc1+X1, Pc1+Bm1, X1+Bm1, Pc1+X1+Bm1 and Pc1+X1+Bm1+Fn1. Experiment 4: Strain P1 was tested in monoculture in SKM medium and in SKM medium with admixture of increasing amounts (from 1—50 %) of sterile filtrate (Millipore 0.22 μm) of a 48-h culture of Strain Pc1 in MHT medium (meat infusion with 1 % BBL Trypticase Peptone, 0.3 % Bacto Yeast Extract, 0.5 μg menadione/ml and 5 μg hemin/ml, pH 7.2—7.4) or the corresponding amounts of uninoculated MHT medium. Experiment 5: In 2 trials Strain P1 was tested in monoculture in SKM medium with admixture of 20 % uninoculated MHT medium or 20 % filtrate of 48-h cultures of Strains Pc1, X1, Bm1, Pc1+X1, Pc1+Bm1, Bm1+X1 or Pc1+X1+Bm1 in MHT medium.

Toxicity tests

The hemolytic activity of freeze-stored, pooled culture filtrates (Millipore 0.22 μm) from Experiments 1, 2 and 3 was titrated, and groups of 3 white mice (16—19 g) from a laboratory colony were inoculated intravenously with 0.5-ml volumes of undiluted or diluted filtrate (serial doubling dilutions in saline). The mice were observed for 48 h.

Table 1. Growth and hemolysin formation of Cb. pyogenes in monocultures and mixed cultures. Experiments 1, 2 and 3.

	Cultures	Number of cultures	Viable counts*	Hemolysin titres	
				min./max.	average
Experiment 1	P1 — P10	10	18×10^7	50/200	175
	P1+Pc1 — P10+Pc1	10	118×10^7	1000/5000	2500
	P1+X1 — P10+X1	10	27×10^7	500/2000	1100
	P1+Bm1 — P10+Bm1	10	40×10^7	200/1000	690
	P1+Fn1 — P10+Fn1	10	67×10^7	100/500	180
	P1+D1 — P10+D1	10	6×10^7	0/100	35
	P1+U1 — P10+U1	10	4×10^6	0/20	3
	P1+B1 — P10+B1	10	18×10^6	0/500	138
	P1+S1 — P10+S1	10	45×10^7	200/2000	720
	P1+Ec1 — P10+Ec1	10	13×10^5	0/20	2
Experiment 2	P1	10	19×10^7	100/200	178
	P1+Pc1 — P1+Pc10	10	113×10^7	1000/10.000	2600
	P1+X1 — P1+X10	10	41×10^7	1000/2000	1300
	P1+Bm1 — P1+Bm10	10	23×10^7	200/1000	560
	P1+Fn1 — P1+Fn10	10	121×10^7	50/500	150
	P1+D1 — P1+D10	10	1×10^7	10/100	45
	P1+U1 — P1+U10	10	10×10^7	0/100	32
	P1+B1 — P1+B10	10	$< 10^5$	0/0	0
	P1+S1 — P1+S10	10	18×10^7	10/1000	263
	P1+Ec1 — P1+Ec10	10	$< 10^5$	0/50	10
Experiment 3	P1	5	16×10^7	100/200	140
	P1+Pc1+X1	5	113×10^7	1000/5000	2200
	P1+Pc1+Bm1	5	133×10^7	500/2000	1200
	P1+X1+Bm1	5	27×10^7	1000/2000	1200
	P1+Pc1+X1+Bm1	5	36×10^7	500/2000	1100
	P1+Pc1+X1+Bm1+Fn1	5	147×10^7	50/200	110

* Average numbers of colony forming units of Cb. pyogenes per ml.

P = Cb. pyogenes
Pc = Pc. indolicus
X = Stuart-Schwan cocci
Bm = B. melaninogenicus
Fn = F. necrophorum
D = Str. dysgalactiae
U = Str. uberis
B = Str. agalactiae
S = Non-toxic staphylococci
Ec = E. coli

RESULTS

All the organisms except B. melaninogenicus grew well in the SKM medium, which was clotted and digested by Cb. pyogenes, Str. dysgalactiae, E. coli and B. melaninogenicus; the latter, however, grew very sparsely on the control plates, if at all. None of the "associate organisms" produced demonstrable hemolysin in monoculture, while all Cb. pyogenes strains did. The results of Experiments 1—5 are shown in Tables 1, 2 and 3, the results

Table 2. Growth and hemolysin formation of Cb. pyogenes (Strain P1) in SKM medium with admixture of increasing amounts of uninoculated MHT medium or sterile filtrate from 48-h cultures of Pc. indolicus (Strain Pc1). Experiment 4.

% MHT medium or cult. filtrate	Viable counts*		Hemolysin titres	
	MHT med.	cult. filtr.	MHT med.	cult. filtr.
0	30	45	50	50
1	15	20	100	200
5	54	16	100	500
10	41	40	100	1000
20	9	50	100	2000
30	28	200	200	2000
40	7	30	100	1000
50	1	140	100	1000

* Colony forming units per ml/10^7.

Table 3. Growth and hemolysin formation of Cb. pyogenes (Strain P1) in SKM medium with admixture of 20 % uninoculated MHT medium or 20 % sterile filtrate from 48-h monocultures or mixed cultures of different organisms in MHT medium. Experiment 5.

SKM medium with 20 % of		Average values from 2 trials	
		viable counts	hemolysin titres
MHT medium		35×10^7	150
Filtr. from cult. of Strain/Strains	Pc1	58×10^7	2000
—	X1	25×10^7	200
—	Bm1	60×10^7	350
—	Pc1+X1	250×10^7	1000
—	Pc1+Bm1	125×10^7	1000
—	Bm1+X1	84×10^7	200
—	Pc1+Bm1+X1	126×10^7	1000

Footnotes as for Table 1.

of the toxicity tests in Table 4. Intravenous injection of 500—1000 h.u. into mice caused immediate paralysis and death, while 100—250 h.u. caused heavy convulsions and death within a few minutes. Mice receiving 50—62.5 h.u. showed convulsions of varying severity, and the majority of them died after 5—10 min; some of them, however, made a quick and apparently complete recovery.

Table 4. Mouse toxicity tests.

Filtrate		Number of deaths among 3 mice after intravenous injection of 0.5 ml of undiluted culture filtrate (u) or of dilutions 1:2, 1:4 etc.						
culture type	h.u./ml	u	1:2	1:4	1:8	1:16	1:32	1:64
P	100	2	0	0	0	0	0	0
P+Pc	2000	3	3	3	3	2	0	0
P+X	1000	3	3	3	2	0	0	0
P+Bm	200	3	2	0	0	0	0	0
P+Fn	100	3	0	0	0	0	0	0
P+Pc+X+Bm+Fn	100	2	0	0	0	0	0	0

P, Pc, X, Bm and Fn: See footnotes to Table 1.
h.u./ml = Hemolytic units per ml (= hemolysin titres).

No fall in the hemolytic activity of the standard hemolysin was observed during the investigation period (5 months), and uniform results were obtained with 16 different batches of blood. It was necessary, however, to use freshly drawn and washed blood, since storage for more than 3—4 days of unwashed or washed blood might increase, respectively reduce, the resistance of the r.b.c. to hemolysin.

DISCUSSION

Cb. pyogenes hemotoxin was described by *Lovell* (1937) and further characterized by *Roberts* (1968) and *Katsaras & Zeller* (1978). In the present study skim milk was employed for hemotoxin production, as described by *Lovell* in 1944. Since B. melaninogenicus and F. necrophorum require, respectively, hemin/menadione and cysteine-HCl for growth (see *Barnes* 1969 and *Holdeman et al.* 1977) these substances were added to the milk. Still, however, the SKM medium was insufficient for B. melaninogenicus, and the results with this organism were therefore inconclusive.

The stimulating effect of Pc. indolicus, and also, though less marked, of Stuart-Schwan cocci, on the growth and hemolysin producing capacity of Cb. pyogenes is most interesting (Table 1), the more so because organisms seldom met with in the summer mastitis flora (Str. uberis, Str. agalactiae, staphylococci and E. coli) would often depress the one or both of these activities. On the other hand, it should be noted that also Str. dysgalactiae had a depressing effect and that high hemolysin titres were obtained with certain combinations of Cb. pyogenes and Staphylococcus strains. It is also noteworthy that the majority of F. necrophorum strains would stimulate the growth of Cb. pyogenes, but not the hemolysin formation. Indeed, in studies of infective bulbar necrosis in sheep a synergism has been demonstrated between Cb. pyogenes and F. necrophorum (*Roberts* 1967a, b).

Co-cultivation of Cb. pyogenes with more than 1 of the organisms that would to a greater or lesser degree enhance its growth or hemolytic activity had no further stimulating effect (Table 1). On the contrary, lower hemolytic titres were generally obtained in such cultures than in Cb. pyogenes—Pc. indolicus cultures, and Strain Fn1 was even found to eliminate the positive influence of peptococci and Stuart-Schwan cocci.

The effect of Pc. indolicus, unlike that of Stuart-Schwan cocci, appeared to be associated with the production of a filterable factor (Tables 2 and 3). Further studies on the mode of action of the 2 organisms, and in particular a characterization of the "peptococcus factor", would be of interest.

The toxicity of mixed-culture hemolysin for mice was closely correlated with its hemolytic activity, and equivalent to the toxicity of Cb. pyogenes monoculture hemolysin (Table 4). Probably the same factor is responsible for both phenomena.

Since a quantitative relationship exists between the hemolysin titre of Cb. pyogenes cultures and their immunogenicity for mice (*Michael-Meese & Gürtler* 1973) the above results may be of interest from the point of view of immuno-prophylaxis. However, discouraging reports about vaccination against summer mastitis with Cb. pyogenes toxoid are numerous (e.g. *Lovell et al.* 1950, *Holtkamp-Endemann* 1977, *Zeller* 1978), and the virulence of the combinations of organisms associated with this disease may depend upon many factors other than Cb. pyogenes toxin, such as coagulase and hyaluronidase, formed by, respectively, Pc. indolicus and Stuart-Schwan cocci (*Høi Sørensen* 1972, *Swi-*

talski et al. 1978, and *Schwan et al.*, see *Schwan* 1979). Antibody response to Pc. indolicus and Stuart-Schwan cocci has been demonstrated in both natural and experimental mastitis (*Schwan* and *Schwan & Smyth*, see *Schwan* 1979). In any case, the stimulating effect of these 2 organisms on the activity of Cb. pyogenes would seem to reflect an important etiological detail.

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SAMMENDRAG

Komparative undersøgelser over Corynebacterium pyogenes' toksindannelse i mono- og blandingskulturer. Påvisning af en stimulerende effekt af Peptococcus indolicus og Stuart-Schwan kokker.

Corynebacterium pyogenes' vækst og toksindannelse undersøgtes i monokulturer og i blandingskulturer med én eller flere af de mikroorganismer, som typisk optræder sammen med den ved sommermastitis hos kvæg (Peptococcus indolicus, Stuart-Schwan kokker, Bacteroides melaninogenicus subsp. levii, Fusobacterium necrophorum og Streptococcus dysgalactiae) samt med sådanne, som sjældent påvises i sommermastitis-sekreter (Streptococcus uberis, Streptococcus agalactiae, ikke-toksiske stafylokokker og Escherichia coli).

Specielt Pc. indolicus, men også Stuart-Schwan kokker, stimulerede såvel vækst som toksinproduktion (Tabel 1), medens Str. dysgalactiae, Str. uberis, Str. agalactiae, E. coli og flertallet af de undersøgte stafylokok-stammer hæmmede disse aktiviteter. Hovedparten af F. necrophorum stammerne stimulerede væksten, men ikke toksindannelsen. Forsøgene med B. melaninogenicus gav usikre resultater.

Effekten af Pc. indolicus viste sig at være associeret med dannelse af en filtrerbar faktor ("peptococcus-faktor") (Tabel 2 og 3).

Kulturfiltraters toksicitet for mus var snævert korreleret med hæmolysinindholdet (Tabel 4).

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