MICROCOCCUS IN THE BLOOD

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BACTERAEMIA secondary to endocarditis as a complication of cardiac surgery occurs after some 0.9% of operations (Denton *et al.*, 1957; Lord *et al.*, 1961). The group of organisms most frequently implicated in infections on artificial valves are the coagulase-negative cocci (Brambridge, 1969), usually *Staphylococcus epidermidis* (Speller and Mitchell, 1973). While some cases have been ascribed to micrococci (Keys and Hewitt, 1973; Rubin, Lyons and Murcia, 1978), there are few records of bacteraemia caused by organisms that would now be accepted as members of the genus *Micrococcus*. One such case was reported by Goodman *et al.* (1968) in which a 68-year-old male treated prophylactically with oxacillin developed endocarditis 8 weeks after operation due to an oxacillin-resistant organism identified as *Micrococcus flavus*. We report the occurrence of *Micrococcus* strains in the blood in six patients.

MATERIALS AND METHODS

Sources of strains

The clinical details of the patients are listed in table I, which also shows the origins of the strains studied; the clinical and bacteriological features of case 6 have already been described by Old and McNeill (1979). Reference strains from the departmental collection (McKay-Ferguson, 1978) were as follows: *M. lylae*, strain CRF101 from Dr W. E. Kloos = ATCC27566 = CCM2693 (holotype); *M. varians*, strain CRF222 = NCTC7564 = *S. lactis* (holotype); *M. luteus*, strain CRF226 = NCTC2665 = *M. lysodeikticus* (neotype); *M. sedentarius*, strain CRF96 from Dr W. E. Kloos = ATCC14392 = CCM314 (holotype); *S. aureus*, strain CRF166 = NCTC6571 = Oxford staphylococcus.

Biochemical and physiological tests

The methods for the tests were those of Baird-Parker (1963) except where stated. Each strain was subcultured twice on nutrient agar at 30°C; a tube of nutrient broth was inoculated from the second subculture and incubated overnight at 30°C. Solid media were inoculated from this broth with a multiple-point inoculator and liquid media were seeded with 2-3 drops of the broth culture by pasteur pipette.

Mode of attack on glucose (O/F test). The medium and method recommended by the International Committee on Bacterial Nomenclature (1965) were used. Acid production under anaerobic conditions was recorded as fermentative (F), under aerobic conditions as oxidative (O) and the absence of acid production in both tubes as no change (-/-).

Acid production from "sugars". Plates were prepared from Baird-Parker's basal medium containing 0.5% (w/v) of arabinose, fructose, lactose, maltose, mannitol, ribose, sucrose, trehalose, xylitol or xylose, with brom-cresol purple 0.004% (w/v) as indicator. After inoculation, the plates were incubated at 30°C and examined daily for 7 days. A change in the indicator from purple to yellow around the inoculum was recorded as ++ (strong) or + (weak).

Terminal pH and acetoin production. Glucose broth, 5 ml in 28-ml screw-capped bottles, was incubated for 14 days at 30°C. The pH was measured with a Pye Dynacap pH meter and acetoin was detected by Barritt's method (Cowan, 1974).

Hydrolysis of arginine. Two methods were used. Arginine broth was incubated for 7 days

Received 2 August 1979; accepted 25 Aug. 1979.

TABLE I
Sources of strains

No.	Patient		Operation (date)	Complication	Specimen (date)	Number of positive samples	Organism isolated (ref. no. of strain)
	Sex	Age (years)					
1	F	10	Valvotomy for congenital heart disease (Sept. 1974)	Fever	Blood (Mar. 1975)	2	<i>Staphylococcus epidermidis</i>
				Mycotic aneurysm	Blood (Aug. 1975)	1	<i>Micrococcus</i> (75/223)
					Aneurysm wall (Sept. 1975)	1	<i>Micrococcus</i> (75/224)
2	M	51	Aortic valve replacement (June 1975)	Fever; died	Blood (Dec. 1975)	10	<i>Micrococcus</i> (76/51)
3	F	17	Patch ventricular septal defect (Oct. 1975)	Endocarditis	Blood (Mar. 1976)	7	<i>Micrococcus</i> (76/82)
4	F	Not known	Mitral valve replacement (Jan. 1977)	Fever	Blood (Feb. 1977)	4	<i>Micrococcus</i> (77/202)
5	M	44	Mitral valve replacement (not known)	Fever	Blood (Aug. 1977)	"Several"	<i>Micrococcus</i> (77/368)
6	F	16	For Fallot's tetralogy (Oct. 1976)	Dental treatment; fever	Blood (July 1977)	13	<i>Micrococcus</i> (77/3119)
				Fever	Blood (Oct. 1977)	1	<i>Micrococcus</i> (77/471)

at 37°C and the presence of ammonia was detected by the development of a brown colour on the addition of Nessler's reagent. Møller's decarboxylase medium for arginine was incubated at 37°C for 4 days; a purple colour indicated hydrolysis (Cowan, 1974).

Growth on media containing NaCl. Nutrient-agar plates containing NaCl 5%, 10%, 15% and 20% (w/v) were incubated for 7 days at 30°C. Growth was scored on a scale of 0–4 in comparison with the growth on nutrient agar.

DNAase. DNase Test Agar (Oxoid) was incubated for 5 days at 30°C. Each plate was flooded with 1N HCl to precipitate residual DNA. Clearing around the inoculum indicated a positive result.

Gelatinase. Nutrient gelatin was incubated at 37°C for 2 weeks. Every second day the tubes were placed at 4°C for 30 min. and the degree of liquefaction was recorded on a scale of 0–4 where 4 indicated complete liquefaction.

Oxidase test. A drop of tetramethyl p-phenylene diamine dihydrochloride 1% (w/v) in water was placed on the growth on nutrient agar. The rapid development of a blue colour was taken to indicate a positive result.

Phosphatase. Baird-Parker phosphatase agar was incubated for 5 days at 30°C. Free phenolphthalein was detected by placing the plate over a filter paper moistened with aqueous ammonia 0.88 sp. gr. when a pink colour developed quickly.

CAMP reaction. Sheep-blood agar was streaked diametrically with a culture of a β -lysine-producing strain of *S. aureus*. An inoculum from the broth culture of each organism was streaked up to the *S. aureus* inoculum and the plates were incubated at 37°C for 24 h. A CAMP-factor-like substance was deemed to be present when a zone of complete haemolysis surrounded the test strain in the area of altered cells adjacent to the growth of *S. aureus* but no haemolysis was evident in the unaltered RBC.

Hydrolysis of Tweens. Tweens 20, 40, 60 and 80 were incorporated into Sierra's medium (Cowan, 1974) and incubated for 7 days at 30°C. The density of any precipitate was recorded on a scale of 0–4 arbitrary units.

Reduction of nitrate. Nitrate broth was incubated for 14 days at 30°C. Nitrite was detected by the addition first of sulphanilic acid 0.8% (w/v) in 5N acetic acid and then of a saturated solution of Cleve's acid (1 naphthylamine-7-sulphonic acid) in 5N acetic acid. The rapid development of a red colour indicated reduction.

Minimum inhibitory concentration (MIC) of antibiotics and lysozyme. Plates of Diagnostic Sensitivity Test Agar (Oxoid CM261) were prepared containing doubling dilutions of: penicillin G, 0.032–8192 units/ml (0.02–5460 mg/litre); methicillin 0.032–200 mg/litre; streptomycin 0.032–4096 mg/litre; erythromycin 0.032–200 mg/litre; lincomycin 0.032–200 mg/litre, and lysozyme 0.19–1600 mg/litre. A total of 25 strains were inoculated on each plate and the plates were incubated for 24 h at 37°C. Growth was recorded on the scale: ++ = good growth; + = growth reduced; $\pm$ = growth only just perceptible, and – = no growth.

DNA-base composition. One strain was examined in the National Collection of Type Cultures by equilibrium centrifugation in a neutral caesium chloride density gradient (Meselson, Stahl and Vinograd, 1957).

RESULTS

Growth and microscopy

The eight strains studied in detail grew well at 37°C to form small- to medium-sized, entire, matt, lenticular colonies of a grey to off-white colour. Microscopically, the cells were large gram-positive cocci with a tendency to packet formation. The cells were rather easily decolourised. *M. sedentarius* and *M. lylae* resembled the strains from patients in colonial characters. *M. luteus* and *M. varians* showed characteristic lemon-yellow pigmentation while *S. aureus* showed pastel orange pigmentation.

Biochemical results

The eight study strains behaved similarly in most tests (table II). None produced acid in the

TABLE II
Biochemical reactions of the micrococci from patients and of reference strains

Test	Results obtained in the stated test with													
	indicated strains from patients							reference strain of						
	75/223	75/224	75/225	76/51	76/82	77/202	77/368	77/3119	77/471	<i>M. lylae</i>	<i>M. varians</i>	<i>M. luteus</i>	<i>M. sedentarius</i>	<i>S. aureus</i> (Oxford)
O/F test*	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	F
Acid† from	-	-	-	-	-	-	-	-	-	-	-	-	-	-
arabinose	-	-	-	-	-	-	-	-	-	-	-	-	-	+
lactose	-	-	-	-	-	-	-	-	-	-	-	-	-	+
maltose	-	-	-	-	-	-	-	-	-	-	-	-	-	+
mannitol	-	-	-	-	-	-	-	-	-	-	-	-	-	+
fructose	-	-	-	-	-	-	-	-	-	-	-	-	-	+
ribose	-	-	-	-	-	-	-	-	-	-	-	-	-	+
sucrose	-	-	-	-	-	-	-	-	-	-	-	-	-	+
trehalose	-	-	-	-	-	-	-	-	-	-	-	-	-	+
xylitol	-	-	-	-	-	-	-	-	-	-	-	-	-	+
xylose	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Acetoin‡	-	-	-	-	-	-	-	-	-	-	-	-	-	+
Terminal pH	6.5	6.7	7.1	6.6	6.7	6.7	6.7	6.7	6.6	6.4	4.6	6.2	6.3	4.6
Arginine hydrolysis:														
Nesslerisation, 7 days†	+	+	+	+	+	+	+	+	+	-	-	-	-	+
Møller's reaction, 4 days†	+	+	+	+	+	+	+	+	+	-	-	-	-	+
Growth on 5% NaCl§	4	4	4	4	4	4	4	4	4	4	4	4	4	4
10%	4	4	4	2	4	2	4	4	4	1	1	2	2	4
15%	<1	0	0	0	0	<1	0	0	0	0	0	0	0	4
20%	+	+	+	+	+	+	+	+	+	+	+	+	+	3
DNAase†	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Liquefaction of gelatin§	+	+	+	+	+	+	+	+	+	+	+	+	+	+
(6 days)	4	4	4	4	4	3	4	4	4	1	0	0	0	3
Oxidase†	+	+	+	+	+	+	+	+	+	+	+	+	+	-
Phosphatase†	+	+	+	+	+	+	+	+	+	+	+	+	+	+
CAMP factor†	+	+	+	+	+	+	+	+	+	+	+	+	+	-
Hydrolysis of:														
Tween 20	2	2	1	2	3	2	3	3	2	1	0	2	0	2
Tween 40	1	1	1	1	1	1	1	1	1	1	0	1	0	3
Tween 60	1	1	1	1	1	1	1	1	1	1	0	0	0	3
Tween 80	2	2	2	1	1	3	2	2	2	2	0	1	0	1
Reduction of nitrate†	-	-	-	-	-	-	-	-	-	-	+	-	-	+

* -/- = No acid aerobically or anaerobically; F = fermentation.
† + + + = Strong reaction; + = weak reaction; - = negative reaction.
‡ + = Positive reaction; - = negative reaction.
§ = Strength of reaction on an arbitrary scale 0-4.

TABLE III
Sensitivity of the strains to antibiotics and to lysozyme

Antimicrobial agent	Minimum inhibitory concentrations (mg/litre*) of the stated agent for												
	micrococcal strain no.						reference strain of						
	75/223	75/224	76/51	76/82	77/202	77/368	77/3119	77/471	<i>M. lylae</i>	<i>M. varians</i>	<i>M. luteus</i>	<i>M. sedentarius</i>	<i>S. aureus</i> (Oxford)
Penicillin*	16	16	8	16	16	8	8	8	0.125	0.125	0.5	8	0.125
Methicillin	>200	>200	>200	>200	>200	200	200	200	4	0.5	8	>200	2
Streptomycin	>4000	>4000	16	256	128	128	128	8	16	8	16	4	32
Erythromycin	16	16	1	16	16	1	1	1	1	2	2	1	2
Lincomycin	8	8	8	8	8	4	2	8	2	8	8	2	2
Lysozyme	>1600	>1600	>1600	>1600	>1600	>1600	>1600	>1600	>1600	>1600	6.25	1.6	>1600

* MICs of penicillin are in international units.

O/F test nor from any of the ten 'sugars'. Acetoin was not produced, the pH in glucose broth was more than 6.5 and nitrate was not reduced. All strains produced ammonia in arginine broth, liquefied gelatin and gave positive tests for oxidase, phosphatase and CAMP factor. Tweens 20 and 80 were split. Variable results were recorded for DNAase, growth on salt agar and, to an extent, in the strength of Tween-splitting activity.

Of the reference strains, *M. lylae* differed from the patients' strains only in failing to hydrolyse arginine and in causing less liquefaction of gelatin. *M. varians* differed from the patients' strains in producing acid from glucose and fructose, in reducing nitrate, in failing to hydrolyse arginine, gelatin and Tweens and in giving negative results in tests for phosphatase and CAMP factor. *M. luteus* could be distinguished from the strains from patients by failing to hydrolyse arginine or gelatin or to produce a CAMP-factor-like substance, while *M. sedentarius* differed from the patients' strains in giving negative results in tests for hydrolysis of arginine, gelatin and Tweens, for CAMP factor and oxidase. The reactions of the named strains were similar to those described (Kloos, Tornabene and Schleifer, 1974) except for the fermentative attack on glucose found for *M. varians*.

Susceptibility to antibiotics and lysozyme

Table III lists the minimum inhibitory concentrations of antibiotics for the strains. The strains from patients were resistant to moderate levels of penicillin, and to high levels of methicillin and lysozyme; resistance to streptomycin and erythromycin was variable. *M. sedentarius* was resistant to penicillin and methicillin only, while *M. lylae* and *M. varians* were resistant only to lysozyme. *M. luteus* was susceptible to all the agents tested.

The values found for the MIC of erythromycin and streptomycin for *M. lylae* were higher than those reported by Kloos *et al.*, (1974) for the same strain.

The base-composition of the DNA was assessed for strain 76/82 as G + C = 71.8%.

DISCUSSION

There seems little doubt that the strains were multiplying within the body in each patient. Similar organisms were repeatedly isolated from the blood or from blood and surgical specimens, and the patients had all been subjected to extensive cardiac surgery, often including the insertion of prosthetic material.

Although the six patients were in hospital at different times over a period of years, and were in five hospitals, the strains were remarkably similar in their properties. The strains were identified as *Micrococcus* on the basis of morphological and colonial appearance, lack of acid production from 'sugars', lack of acetoin production but positive oxidase reaction. The one strain examined for guanine-cytosine content showed this to be in the *Micrococcus* range.

Some characters were unusual for *Micrococcus*. The ability to produce ammonia from arginine has been proposed as a means of differentiating *Staphylococcus* from *Micrococcus* (Peny and Buisnière, 1970), but the eight strains from the six patients were positive in arginine broth and when tested by Møller's method. The reference strains of *Micrococcus* gave negative reactions.

The study strains from patients could be distinguished from *M. kristinae* because the latter produces acid from glucose and by colonial appearance from *M. nishinomiyaensis*, which has small, bright orange, slow-growing colonies (Kloos *et al.* 1974). They differed from *M. luteus*, *M. varians* and *M. sedentarius* in several tests and from *M. lylae* in arginine and gelatin hydrolysis only. Antibiotic-susceptibility tests showed that the strains from patients were all resistant to methicillin with MIC values of 200 mg/litre or greater; this was true only for *M. sedentarius* among the reference strains. Kloos *et al.*, (1974) state that methicillin resistance appears to be species specific for *M. sedentarius*. The strains from patients differed slightly from *M. lylae* and to a greater degree from the other reference strains in biochemical and in antibiotic-resistance characters. Whether the differences are of taxonomic value or are selected by growing in the heart is uncertain.

The frequency of septicaemia due to species of *Micrococcus* is difficult to ascertain. Over the

3-year period during which the six patients studied here presented, this laboratory received 221 strains of coagulase-negative cocci isolated from the blood of 81 patients. The majority (171 isolates from 66 patients) were identified as *Staphylococcus epidermidis*, 12 isolates from six patients were identified as *S. saprophyticus*, and *Micrococcus* was found in only eight patients, from two of whom there were single isolations, probably contaminants. This finding, however, probably overestimates the general frequency of micrococcal septicaemia. In a study of infections in a cardiac-surgery unit, no blood cultures obtained after 200 operations in which a prosthetic valve was implanted were positive for *Micrococcus* (Marples *et al.*, 1978). The case reported by Goodman *et al.* (1968) shows close resemblances to those reported here. A recent report implicated *Micrococcus mucilaginosus incertae sedis* in a patient with chronic valvular disease after cardiac catheterisation (Rubin *et al.*, 1978). The organism was catalase negative, grew on solid media only in an atmosphere with added CO₂ and was sensitive to the antibiotics tested. A source in the oral cavity was suspected.

SUMMARY

Eight isolates of micrococci from the bloodstream of six patients obtained under circumstances suggesting a pathogenic role were studied in detail. The organisms were remarkably uniform in cultural, biochemical and antibiotic-susceptibility characters. All strains showed high resistance to methicillin and hydrolysed arginine. The characters found did not correspond with those of any hitherto described species, but were closest to *Micrococcus lylae*.

We thank Mrs J. A. Crees-Morris for technical assistance and Dr L. R. Hill and the staff of the National Collection of Type Cultures for determination of the DNA-base composition.

REFERENCES

- BAIRD-PARKER, A. C. 1963. A classification of micrococci and staphylococci based on physiological and biochemical tests. *J. gen. Microbiol.*, **30**, 409.
- BRAIMBRIDGE, M. V. 1969. Cardiac surgery and bacterial endocarditis. *Lancet*, **1**, 1307.
- COWAN, S. T. 1974. Cowan and Steel's Manual for the identification of medical bacteria, 2nd ed. Cambridge University Press: Cambridge.
- DENTON, C., PAPPAS, E. G., URICCHIO, J. F., GOLDBERG, H. AND LIKOFF, W. 1957. Bacterial endocarditis following cardiac surgery. *Circulation.*, **15**, 525.
- GOODMAN, J. S., SCHAFFNER, W., COLLINS, H. A., BATTERSBY, E. J. AND KOENIG, M. G. 1968. Infection after cardiovascular surgery. *New Engl. J. Med.* **278**, 117.
- INTERNATIONAL COMMITTEE ON BACTERIAL NOMENCLATURE. 1965. Subcommittee on Taxonomy of Staphylococci and Micrococci; recommendations. *Int. J. syst. Bact.*, **15**, 109.
- KEYS, T. F. AND HEWITT, W. L. 1973. Endocarditis due to micrococci and *Staphylococcus epidermidis*. *Archs intern. Med.*, **132**, 216.
- KLOOS, W. E., TORNABENE, T. G. AND SCHLEIFER, K. H. 1974. Isolation and characterisation of micrococci from human skin including two new species: *Micrococcus lylae* and *Micrococcus kristinae*. *Int. J. syst. Bact.*, **24**, 79.
- LORD, J. W., IMPERATO, A. M., HACKEL, A. AND DOYLE, E. F. 1961. Endocarditis complicating open-heart surgery. *Circulation*, **23**, 489.
- McKAY-FERGUSON, E. 1978. Organic acids as substrates in microbiology. MD Thesis. University of London.
- MARPLES, R. R., HONE, R., NOTLEY, C. M., RICHARDSON, J. F. AND CREES-MORRIS, J. A. 1978. Investigation of coagulase-negative staphylococci from infections in surgical patients. *Zentbl. Bakt. ParasitKde.*, **I. Abt. Orig.**, **241**, 140.
- MESELSON, M., STAHL, F. W. AND VINOGRAD, J. 1957. Equilibrium sedimentation of macromolecules in density gradients. *Proc. natn. Acad. Sci. U.S.A.*, **43**, 581.
- OLD, D. C. AND McNEILL, G. P. 1979. Endocarditis due to *Micrococcus sedentarius incertae sedis*. *J. clin. Path.*, **32**, 951.

- PENY, J. AND BUISSIÈRE, J. 1970. Microméthode d'identification des bactéries. II. Identification du genre *Staphylococcus*. *Annls. Inst. Pasteur, Paris*, **118**, 10.
- RUBIN, S. J., LYONS, R. W. AND MURCIA, A. J. 1978. Endocarditis associated with cardiac catheterization due to a gram-positive coccus designated *Micrococcus mucilaginosus incertae sedis*. *J. clin. Microbiol.*, **7**, 546.
- SPELLER, D. C. E. AND MITCHELL, R. G. 1973. Coagulase-negative staphylococci causing endocarditis after cardiac surgery. *J. clin. Path.*, **26**, 517.