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GROUP R STREPTOCOCCI PATHOGENIC FOR MAN

Two Cases of Meningitis and one Fatal Case of Sepsis

By

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In 1963 *de Moor* (2) reported studies on streptococci isolated from septicaemic infections in pigs. These streptococci grew on horse blood agar with colonies surrounded by a partial zone of haemolysis, resembling the haemolysis characteristic for Group B streptococci. A few strains showed no, or very weak, haemolysis on horse blood agar. On sheep blood agar all strains were found to be non-haemolytic. *De Moor* has not reported any investigations dealing with the ability to form a soluble haemolysin. Biochemically, these streptococci were especially characteristic by their ability to split inulin and their resistance to 40 per cent bile. Serologically, the strains examined did not belong to the known Lancefield Groups A to Q. However, by precipitation with formamide extracts, antigens were demonstrated which permitted differentiation into three serological groups. *De Moor* designated these serological groups R, S and T. A few strains were precipitated by antisera of both Group R and Group S (RS streptococci). The antisera employed also precipitated extracts of Group D. These overlapping reactions were, however, removed by absorption with *Streptococcus faecalis* bacteria.

Human infections with streptococci belonging to *de Moor*'s Groups R, S and T are not reported in the literature. *De Moor* suggests that these streptococci apparently are confined to pigs. He did not succeed in finding streptococci of Groups R, S and T in a material of about 2,000 strains isolated from monkeys, cattle, sheep, horses, dogs, cats, rabbits, mice and guinea pigs, nor have they been found in man.

During the period 1960 to 1966 we have diagnosed Group R streptococci in three cases of severe human infection, *viz.* two cases of meningitis in which streptococci were isolated in pure culture from the spinal fluid (in one of the cases also from blood), and one fatal case of sepsis in which streptococci were isolated in pure culture from the spinal

Thanks are due to Dr. *Erna Lund*, who kindly put her Pneumococcus sera at our disposal.

fluid and from blood. An account is given here of the case histories and the bacteriological analysis of the Group R streptococci isolated.

CASE HISTORIES

Case B

J. no. B 1893/62–63: 61-year-old man, works manager at a sawmill. Previously generally healthy. Six days before admission to hospital the patient sustained a slight burn on the right side of the face from a spurt of flame from the central heating oven. Slight redness developed but disappeared rapidly. Two days before admission again redness and oedema of the right cheek with slight blister formation.

Four days before admission severe attacks of shivering with temperature elevated to 39.5° C. During the subsequent days septic temperature variations and increasing, severe, diffuse headache. One day before admission the patient developed stiffness of the neck and the sensorium became affected. Penicillin was prescribed by the patient's own doctor and administered for two days.

At admission the patient was exhausted, febrile, in pain, and with pronounced stiffness of the neck.

Lumbal puncture was performed at once. The spinal fluid was turbid and yellowish. Microscopy revealed 19,776/3 leucocytes, mainly granulocytes with polymorphous nuclei, protein content 491 mg per cent. No bacteria were demonstrated by direct microscopy.

Moderate oedema, redness and warmth of the right half of the face were also observed at admission, and one or two small bullae containing clear fluid were situated in the middle of the cheek. The processes spread during the first days but disappeared after five to six days. Culture from the skin was not made.

Treatment with penicillin, streptomycin and sulphadiazine was instituted primarily.

On the second day after admission it was notified from the Statens Seruminstitut that culture from the spinal fluid had shown growth of streptococci. These were later identified as haemolytic streptococci Group R. The bacteria were sensitive to all the antibiotics examined, with the exception of the sulpha preparations. Treatment with these was consequently discontinued. During the first six days in hospital the patient was deeply unconscious and had several attacks of tonic and clonic convulsions lasting for a minute or two.

The temperature gradually became normal and the patient slowly regained consciousness. During the first period he was fumbling, confused and restless. He complained of deafness and an audiogram showed severe impairment of hearing of the perception type. It was considered that this impairment was due to the meningitis and not to the streptomycin treatment, since only a total of 10 g of streptomycin had been administered. After just over one month and a half in hospital the patient was discharged. No definite psychic reduction could be demonstrated and the hearing was somewhat better.

Case L

J. no. F 1574/66–67: 38-year-old slaughterhouse worker. Apart from tendency to migraine, previously mainly healthy.

A few days before admission to hospital the patient sustained a slight injury to the right wrist during his work, having scratched himself on the rib of a slaughtered pig. One or two days later he developed pain in the right upper extremity, redness and tenderness around the site of injury, and a little purulent secretion from the wound.

Four days before admission the patient suddenly developed severe attacks of shivering and rise in temperature to about 40° C. During the subsequent days there were severe temperature variations, together with nausea, vomiting, and severe, diffuse headache.

Treatment with penicillin was prescribed by the patient's own doctor.

At admission the patient was highly febrile, confused, fumbling and motorically extremely restless. There was pronounced stiffness of the neck and positive Kernig's sign.

Lumbal puncture gave turbid, milky spinal fluid, and microscopy showed 4,964/3 leucocytes, 90 per cent of which were granulocytes, protein content 426 mg per cent. Direct microscopy revealed numerous bacteria thought to be pneumococci.

The wound on the wrist was dirty, with a little purulent secretion, and there was redness of the area around it. Culture from the wound was not performed. The lesion healed in the course of a few days.

Treatment was instituted primarily with penicillin, streptomycin and sulphadiazine. On the third day notification was received from the Statens Seruminstitut that culture from the spinal fluid and blood gave growth of non-haemolytic streptococci belonging to Group R. These were sensitive to all the antibiotics examined, with the exception of the sulpha preparations, treatment with which was then discontinued.

During the first days of the stay in hospital the patient was extremely ill with hyperthermia. This condition was treated for a time with cold blankets and truxal, with good effect. Furthermore, the treatment was supplemented by actocortin and, for a time, by chloramphenicol and erythromycin.

X-ray of the thorax at admission showed no abnormality. Four days later there were pronounced, bilateral pneumonic processes. These decreased considerably in the course of a few days.

Ophthalmoscopy showed slight blurring of the papillae without venestasis.

The temperature became normal after 7 days and the patient began to recover slowly. During the first days he was somewhat confused and had hallucinations, but these disappeared quickly. He complained of impaired hearing and dizziness. The latter symptom disappeared spontaneously but the hearing remained impaired. The patient stated that before the present illness he did not hear well (noisy work).

After one month in hospital the patient was discharged in a good state of health.

Case N[1]

J. no. 160217/60: 44-year-old man, admitted with symptoms of septicaemia or toxic shock. Death occurred a few hours after admission. Information is available that the patient had had an infected wound and that he had been ill for some time before admission. *Post-mortem* examination revealed pronounced cadaverosis such as that found in patients with septicaemia. In addition there were small yellow infiltrates in the liver (probably small abscesses). Culture from spinal fluid and blood gave growth of bacteria and the probable diagnosis is therefore fulminant sepsis emanating from the infected wound.

Besides the pathogenesis, there are several similarities in the case histories of the two patients, B and L. The courses of the disease were almost identical. Furthermore, it is possible that the origin of the infection in Cases B and L as well as in Cace N was the minor skin lesion sustained a few days before the onset of the disease.

MATERIAL

In all seven strains were studied: one strain from Case B (spinal fluid), three strains from Case L (one from spinal fluid, two from blood, taken at different times on the same day), and three strains from Case N (one from spinal fluid, two from blood, taken at different times on the same day). As reference strain, Strain 735 isolated from pig (2) was employed.

METHODS

The following *biochemical* and *cultural* procedures were used:

1) Growth on 1.8 per cent beef-broth agar containing 5 per cent defibrinated horse blood.

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[1] The writers are grateful to Dr. *Michael Schwarts*, Chief Physician, Medical Department, County Hospital, Glostrup, for permission to report this case.

2) Growth on 1.4 per cent beef-broth agar containing 8 per cent defibrinated horse blood and 40 per cent ox bile.
3) Growth on 1.8 per cent ox agar containing 10 per cent horse blood (mixed at 80° C for about 5 minutes) and 0.04 per cent potassium tellurite.
4) Growth in broth containing 6.5 per cent sodium chloride.
5) Growth in broth at 45° and 10° C.
6) Final pH after growth for 8 days in 1 per cent glucose broth.
7) Production of ammonia from L-arginine hydrochloric acid demonstrated by Nessler's reagent.
8) Hydrolysis of sodium hippurate shown by the addition of 50 per cent sulphuric acid to two parts of the supernatant broth culture.
9) Mucus formation on growth on 1.8 per cent beef-broth agar containing 5 per cent sucrose.
10) Production of acid during growth for 8 days in broth containing the drugs recorded in Table 1, using Difco broth base and phenol red as indicator.
11) Hydrolysis of esculin and glycogen, using Difco broth base, and of starch, using oxoid nutrient agar No. 2 containing 0.2 per cent soluble starch.
12) Camp reaction according to *Christie et al.* (1).
13) Test for soluble haemolysin, using rabbit erythrocytes (5 per cent).
14) Sensitivity to sulphathiazole and the antibiotics given in Table 1, measured according to *Thomsen* (6).

Serological Methods

The streptococci were harvested from growth in trypsin-digested broth for 18 hours at 36° C.

Antisera were prepared in rabbits by repeated intravenous injections of a saline suspension of heat-killed (60° C for 30 minutes) and three times washed bacteria. They were stored at 4° C in a dense suspension (1/100 of the original volume) and preserved by adding 0.5 per cent formalin.

Antigen for *absorption* was prepared in the same way as the antigen used for immunization and centrifuged at 12,000 r.p.m. About 1/3 to 1/2 volume of packed cells was added to undiluted serum and allowed to bind for one to two hours at 36° C, in some cases followed by further binding at 4° C overnight.

Agglutination tests were carried out in tubes with culture (a) killed by 0.2 per cent formalin, and (b) killed by 127° C for two hours, and read after 20 hours in water-bath at 50° C.

Neufeld reaction was carried out on slides by mixing a loopful of a 6 and an 18-hour culture with a loopful of serum. Reading was made immediately after the mixing.

Acid extracts were made according to *Lancefield* (5) (using 0.1 n hydrochloric acid), *formamide extracts* according to *Fuller* (4), and *saline extracts* according to *Elliott* (3).

Precipitin tests were carried out in microtubes with a diameter of 1.5–2.0 mm.

RESULTS

Biochemical and Cultural Procedures

Morphological examination of gram-stained smears from fluid media showed cocci arranged in pairs and short chains. Uniform turbidity was obtained in fluid media. On horse blood agar, the colonies had a glistening surface and a narrow zone of β-haemolysis. On prolonged incubation they resembled Group B haemolytic streptococci, as already observed by *de Moor* (2), except for the α-haemolytic strains isolated from one of the patients (Case L). All the strains that formed β-haemolysis on blood agar produced a soluble haemolysin.

Table 1 shows the cultural and biochemical reactions of Group R streptococci isolated from three patients (B, L, N) and the reference

TABLE 1

Biochemical and Cultural Behaviour of Human Group R Streptococci Compared with the Animal Reference Group R Strain 735.

	De Moor R 735	Case N strains N_1 N_2 N_3	Case L strains L_1 L_2 L_3	Case B strain
Acid formation in:				
Glucose, sucrose, lactose, maltose, salicin, trehalose, raffinose, inulin, melibiose	+	+	+	+
Arabinose, mannitol, sorbitol glycerol, melezitose	—	—	—	—
Hydrolysis of:				
Esculin, glycogen, starch	+	+	+	+
Arginin	+	+	+	+
Sodium hippurate	—	—	—	—
Resistance to:				
Ox bile	+	+	+	+
Tellurite	—	—	—	—
NaCl	—	—	—	—
45° C	—	—	—	—
Mucus formation	—	—	—	—
Horse blood agar	β	β	α	β
Soluble haemolysin	+*	+*	—	+*
Camp reaction*	(+)	(+)	(+)	(+)
Sensitivity to sulphathiazole	—	—	—	—
Sensitivity to:				
Penicillin	+++	+++	+++	+++
Streptomycin	++	+	++	++
Tetracycline	+++	+++	+++	+++
Chloramphenicol	+++	+++	+++	+++
Erythromycin	+++	+++	+++	+++
Bacitracin	+	0	+	0

* Varies from — to + in different tests.

Group R Strain 735 isolated from a pig. It will be seen that the findings were the same both with the animal strain and the human strains.

Serological Examinations

One strain from each patient was included in the examinations. Antisera were prepared from strains isolated from two of the three patients, *viz.* representatives of an α-haemolytic and a β-haemolytic strain (L_2 and B), and the animal reference Strain 735.

It will be seen from the results recorded in Table 2 that the human α and β haemolytic streptococci, which reacted in antisera against the animal reference strain of Group R, are apparently serologically identical, and identical with the animal Group R Strain 735.

The reactions of all strains in unabsorbed and absorbed sera were equally strong and negative, respectively. In cross absorption experi-

TABLE 2

Serological Examinations with Unabsorbed and Absorbed Immune Sera of the Present Group R Streptococci.

Capsular titre	Antisera against			Undiluted antisera of absorbed with						Rabbit pre-immune serum
	R 735	B	L_2	B	R 735	L_2	R 735	B	N_2	
R 735	20	20	40	—	—	—	—		—	< 2
B	20	20	40	—	—			—		< 2
L_2	20	20	40			—	—	—		< 2
N_2	20	20	40						—	< 2
Tube agglutination titre using formalin killed or heat (127° C) killed antigens:										
R 735	80	160	160	—	—	—	—		—	<10
B	80	160	320	—	—			—		<10
L_2	80	160	160			—	—	—		<10
N_2	80	160	160						—	<10
Precipitation with acid extracts:										
R 735	+++	+++	+++	—	—	—	—		—	—
B	+++	+++	+++	—	—			—		—
L_2	+++	+++	+++			—	—	—		—
N_2	+++	+++	+++						—	—
Precipitation with formamide extracts:										
R 735	(+++)	(+++)	(+++)	—	—	—	—		—	—
B	(+++)	(+++)	(+++)	—	—			—		—
L_2	(+++)	(+++)	(+++)			—	—	—		—
N_2	(+++)	(+++)	(+++)						—	—
Precipitation with saline extract using living (2 hours 36° C) or heated (2 hours 127° C) bacteria:										
R 735	d	d	d	—	—	—	—		—	—
B	d	d	d	—	—			—		—
L_2	d	d	d			—	—	—		—
N_2	d	d	d						—	—

+++ = prompt precipitation.
(+++) = prompt but weak precipitation.
d = firm disk-shaped precipitate.
— = no reaction.

ments with undiluted sera, all precipitins and agglutinins were removed from sera prepared against the animal strain and the human strains.

Non-concentrated filtrates of an 18-hour glucose broth culture gave a prompt precipitation reaction with homologous and heterologous antisera. More concentrated preparations obtained by saline extraction of living organisms according to the method indicated by *Elliott* (3) for capsulated PM streptococci, and by extraction at 127° C, gave a firm disk-shaped precipitate.

The swelling reaction in the presence of type-specific immune sera was comparable with that observed with *Streptococcus pneumoniae*. The reaction was equally strong when 6 and 18-hour cultures were used.

In accordance with the findings obtained by *de Moor* (2), acid extracts using 0.2 n hydrochloric acid could not be precipitated. The formamide extracts gave weak reactions independent of the temperature used (100°, 120°, 140° and 160° C). The reason may be that the carbohydrate antigen is partly precipitated by the precipitation with acid ethanol. This will be examined in details elsewhere.

DISCUSSION

Group R streptococci are reported here for the first time as the cause of meningitis and sepsis in man.

The isolated strains were culturally, biochemically and serologically identical with the Group R streptococci isolated from porcine infections in The Netherlands by *de Moor* (2). The animal strain and the human strains from two of the three cases were β-haemolytic, capsulated streptococci, while the strains from the third case were α-haemolytic, capsulated streptococci. All strains gave a positive Camp reaction. However, the demonstration of soluble haemolysin and the Camp reaction failed in some tests. Neither the animal nor the human strains gave signs of being pathogenic or toxic for white mice, as indicated from several passages in mice. No relationship between the capsule of the present strains and *Streptococcus pneumoniae* was found when the capsular antigen of the present Group R streptococci in sera of *Streptococcus pneumoniae* Types 1 to 48 were tested by the Neufeld reaction.

Recently *Elliot* (3) reported capsulated β-haemolytic streptococci containing the Group D antigen as the cause of neonatal infection in piglets in England. These streptococci "appear to be identical with those isolated from piglet infections by *de Moor*, who designated his strains Group 'S'" (cited *Elliott*). *De Moor* (2) had already drawn attention to overlapping reactions between Groups R, S and T streptococci and *Streptococcus faecalis*.

In the present examinations no evidence was found of the existence of Group D antigen in Group R streptococci, either by precipitin tests with acid extracts, in antisera prepared by prolonged immunization with the present strains, or in Group D sera from The Wellcome Laboratories, Beckenham, England.

In single antisera of Group B Strain O 90 and Group R streptococci, common antibodies for B O 90 and Group R could be demonstrated. Cross absorptions indicated, however, that the group antigens in Groups B and R were not identical.

The finding of streptococci of Lancefield Group R in cases of human infection in Denmark seems to be particularly remarkable since, as far as is known, such streptococci have not been demonstrated in animals in this country.

SUMMARY

Case histories are given of two cases of meningitis and one case of sepsis in man caused by streptococci of Lancefield Group R.

A common feature of the three cases of human infection by Group R streptococci is the presence of a minor skin lesion which might represent the origin of the infection.

Evidence is given for cultural, biochemical and serological identity with *de Moor*'s Group R Strain 735 isolated from porcine infections in The Netherlands.

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PREPARATION OF *TOXOPLASMA GONDII* ANTIGEN FOR THE COMPLEMENT FIXATION TEST

By

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With a view to the extraction of a soluble complement fixing antigen from Toxoplasma gondii the first method to be described was that of repeated freezing and thawing of the parasites in physiological salt solution (*Warren & Sabin* 1942). This method of extraction has been adopted in most laboratories. It has, however, been pointed out that repeated freezing and thawing reduced the litre of the antigen (*Kåss* 1953). Furthermore, the extraction procedure has been found to be somewhat timeconsuming, and the present investigation was, therefore, undertaken in order to find a more suitable method for the extraction of the antigen. A preliminary investigation has been reported (*Pettersen* 1967).

MATERIALS AND METHODS

Peritoneal exudate was collected from four-week-old white mice which had been infected intraperitoneally five days previously with 20,000 Toxoplasma gondii parasites of the RH strain. (The mice used were of the Institute's own breed weighing 18–20 g at the time of infection). The exudate was centrifuged at 500 × g for 40 minutes. The sediment, containing the parasites together with a vast number of mouse cells, was washed once with 0.15 M sodium chloride solution, and stored at —18° C. The average sediment contained 3×10^9 parasites, and this constituted the least amount used for any of the extractions described below.

Measured amounts of 0.15 M sodium chloride solution were added to the parasite sediments, and the mixtures were treated briefly in a Potter-Elvehjem tissue grinder.

1. *Freezing and thawing:* The parasite suspension was subjected three times to altenate freezing in a solid carbon dioxide-ethanol mixture, and thawing in water of 20–30° C, followed by centrifugation. Consecutive extractions were performed on each batch of parasite suspension.
2. *Bicarbonate extraction:* To the parasite suspension were added 25.2 mg of sodium bicarbonate per ml. The suspension was stirred for 10 minutes, and then diluted with two volumes of demineralized water. The bicarbonate was neutralized by the careful addition of N HCl, and the suspension was centrifuged. The sediment was subjected to a second bicarbonate extraction.

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